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Metabolomic Endotype of Asthma

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Metabolomics, the quantification of small biochemicals in plasma and tissues, can provide insight into complex biochemical processes and enable the identification of biomarkers that may serve as therapeutic targets. We hypothesized that the plasma metabolome of asthma would reveal metabolic consequences of the specific immune and inflammatory responses unique to endotypes of asthma. The plasma metabolomic profiles of 20 asthmatic subjects and 10 healthy controls were examined using an untargeted global and focused metabolomic analysis. Individuals were classified based on clinical definitions of asthma severity or by levels of fraction of exhaled NO (F_ENO), a biomarker of airway inflammation. Of the 293 biochemicals identified in the plasma, 25 were significantly different among asthma and healthy controls ($p < 0.05$). Plasma levels of taurine, lathosterol, bile acids (taurocholate and glycodeoxycholate), nicotinamide, and adenosine-5-phosphate were significantly higher in asthmatics compared with healthy controls. Severe asthmatics had biochemical changes related to steroid and amino acid/protein metabolism. Asthmatics with high F_ENO, compared with those with low F_ENO, had higher levels of plasma branched-chain amino acids and bile acids. Asthmatics have a unique plasma metabolome that distinguishes them from healthy controls and points to activation of inflammatory and immune pathways. The severe asthmatic and high F_ENO asthmatic have unique endotypes that suggest changes in NO-associated taurine transport and bile acid metabolism. ***The Journal of Immunology*****, 2015, 195: 643–650.**

Asthma is a common chronic disorder of the airways, characterized by the presence of inflammation and airway remodeling. The pathogenesis of asthma involves complex interactions of various cell types (immune, airway epithelial, and smooth muscle, as well as inflammatory cells) and numerous biologically active proinflammatory mediators (1–3). Among these, there is evidence to suggest that cytokines (TNF-α, IFN-γ, IL-13, IL-4), endogenous reactive oxygen species (ROS), and reactive nitrogen species (RNS) such as hydrogen peroxide, superoxide, and NO are responsible for the inflammation and tissue damage of asthma (1–3). The causes of asthma remain incompletely understood; however, based on clinical, functional, and biochemical profiles, it is apparent that asthma is the result of a broad range of immunological, inflammatory, and biochemical perturbations.

NO and Th2 responses are recognized as fundamental to the pathophysiology of asthma. Patients have been categorized into phenotypes of low Th2 and high Th2, with the latter having been proposed as useful to apply biologic therapies (anti–IL-5 or anti–IL-13) targeting Th2 pathways (4–8). In general, the fraction of NO in expired air (F_ENO) is high in asthmatic populations as compared with healthy controls (6, 9), and it indicates a Th2 eosinphilic inflammation and predicts clinical response to inhaled or oral glucocorticoids (6). NO is produced in the airways by inducible NO synthase, which is induced by the high levels of cytokines in the inflamed airways. However, asthmatics also may have low or normal levels of F_ENO, and little is known of this asthma phenotype.

The diagnosis of asthma is presently based on assessment of symptoms and physiological tests of airway reactivity (7). These measurements do not precisely reveal the biology of inflammation and bronchoconstriction. Metabolomics is a high-throughput method of rapidly assessing the impact of a disease state on tissue/organ and on the whole body (10–13). By quantifying small molecules that are the products of disparate metabolic pathways, metabolomics can reveal the relative activity of each pathway (10–13). We postulated that the plasma metabolome of asthma would reveal metabolic consequences of the specific immune and inflammatory response. In this pilot study we report the metabolomic profile of subjects with asthma and compare it with healthy controls. We subgroup asthmatics clinically by standardized definitions of severity of asthma or by levels of F_ENO to examine whether specific metabolomic endotypes can identify the altered biochemical pathways in these defined groups.

Materials and Methods

Study population

The study population included 20 asthmatics (10 nonsevere and 10 severe) and 10 healthy controls. The severity of asthma, that is, severe and nonsevere, was defined as per the proceedings of the American Thoracic Society Workshop on Refractory Asthma, with major and minor characteristics (14). Briefly, major characteristics for severe asthma include 1) treatment with continuous or near continuous oral corticosteroids and/or 2) high-dose inhaled corticosteroids. The minor criteria are as follows: 1) daily treatment with other medication in addition to inhaled corticosteroids; 2) use of short-acting β-agonist on a daily or near daily basis; 3) persistent airway obstruction (forced expiratory volume in 1 s [FEV_1] > 80% predicted and diurnal peak expiratory flow variability > 20%); 4) one or more urgent care visits for asthma per year; 5) three or more oral corticosteroid bursts

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Abbreviations used in this article: BCAA, branched-chain amino acid; BMI, body mass index; ccK18, caspase-cleaved keratin-18; F_ENO, fraction of exhaled NO; FEV_1, forced expiratory volume in 1 s; GC/MS, gas chromatography/mass spectrometry; MS/MS, tandem mass spectrometry.

per year; 6) prompt deterioration with reduction in oral or inhaled corticosteroid dose; and 7) near-fatal asthma event in the past. Subjects met criteria for severe asthma with at least one major and at least two minor criteria. Inclusion criteria for healthy control subjects were 1) lack of cardiopulmonary symptoms, 2) normal baseline spirometry, and 3) a negative methacholine challenge test (defined as <20% decline in FEV_1 with the maximum dose of methacholine). Exclusion criteria included current smoking history, or smoking history within 1 y, former smokers with >5 pack-year total history, pregnancy, and HIV infection. Separate from the subclass of nonsevere and severe asthma, we also grouped asthmatics based on high (≥35 ppb) or low (<35 ppb) F_ENO according to American Thoracic Society guidelines (4, 15, 16). Plasma samples in EDTA had been obtained from the study subjects randomly at time of study visit, and not following any specific period of fasting or any dietary preparation. The protocol was approved by the Cleveland Clinic Institutional Review Board and all participants provided informed consent.

Metabolomic analysis

Nontargeted metabolomic analysis was performed at Metabolon (Durham, NC). The global, unbiased platform was based on a combination of three separate platforms: ultra-HPLC/tandem mass spectrometry (MS/MS) optimized for basic species, ultra-HPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). The major components of the analytic process and of the analytic platform have been described in detail in previous publications (10, 12). Ultra-HPLC/MS/MS analysis used a Waters Acquity UPLC (Waters, Milford, MA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source. Two separate injections were performed on each sample: one optimized for positive ions and one for negative ions. Derivatized samples for GC/MS were analyzed on a Thermo Finnigan Trace DSQ fast-scanning single-quadrupole MS operated at unit mass resolving power. Chromatographic separation followed by full scan mass spectra was carried to record retention time, mass to charge (m/z) ratio, and MS/MS of all detectable ions present in the samples. Compounds were identified by automated comparison with Metabolon's reference library entries. Identification of known chemical entities was based on comparison with Metabolon's library entries of purified standards.

Data were normalized to correct for variation resulting from instrument inter-day tuning differences. Raw areas counts for a compound were divided by the median value, setting the medians equal for each day's run. Missing values were assumed to result from areas being below the limits of detection. Missing values for a given compound were imputed with half the observed minimum after the normalization step. Quantitative values were derived from integrated raw detector counts of the mass spectrometers. Importantly, although peak area comparisons between samples represent relative amounts of each ion detected, different compounds and ions have different ionization potentials. To preserve all of the variation, yet allow compounds of widely different raw peak areas to be compared directly on a similar graphic scale, a simple arithmetic transformation was performed in which each datum point was divided by the mean of the entire set for that compound.

Amino acid analysis

Plasma amino acid concentrations were measured using HPLC (Agilent 1100 series HPLC; Agilent Technologies, Wilmington, DE), following *ortho*-phthalaldehyde derivatization using a fluorescent detector as described previously (17)

Cytokine analyses

Plasma cytokines were measured using a human cytokines/chemokines kit from LINCOplex multiplexed biomarker immunoassays. Each sample from a formatted 96-well plate was drawn into the flow-based Bio-Plex array reader (Bio-Rad Laboratories Hercules, CA) in which analytes were identified and quantified. Bio-Plex Manager software automated the data analysis.

Apoptosis analysis

Apoptosis-associated caspase-cleaved keratin-18 (ccK18) was measured in the plasma using an M30 Apoptosense ELISA kit (Enzo Life Sciences, Farmingdale, NY)

Table I. Demographics, pulmonary function, and cytokines for all subjects

	Healthy Control (n = 10)	Asthma (n = 20)
Demographics		
Age (y)	35.1 ± 3.8	37.6 ± 2.6
Gender (male/female)	4/6	9/11
Ethnicity (non-Hispanic/Hispanic)	10/0	17/3
Race (African American/white/others)	2/7/1	9/10/1
Duration of asthma (y)	N/A	24.8 ± 2.9
BMI	25.9 ± 1.8	30.4 ± 1.4*
AQLQ	7 ± 0.0	4.53 ± 0.31*
Blood counts		
Total cell count ($\times 10^6$)	5.1 ± 0.4	6.0 ± 0.5
Eosinophils (%)	2.67 ± 0.4	4.27 ± 0.6*
Lung functions		
FEV_1 (l)	3.56 ± 0.18	2.60 ± 1.0*
FEV_1 (%)	99.4 ± 5.8	76.0 ± 4.5*
FVC (l)	4.63 ± 0.29	3.70 ± 0.3*
FVC (%)	106.5 ± 4.6	92.0 ± 4.5*
FEV_1/FVC	0.77 ± 0.02	0.70 ± 0.02
F_ENO (ppb)	18.3 ± 1.8	37.6 ± 6.4
PC_{20}	N/A	2.02 ± 0.53
Asthma phenotypes		
Severity (% severe asthmatics)	—	50
F_ENO (% with $F_ENO \geq 35$ ppb)	—	45
Cytokines		
IL-6 (pg/ml)	3.45 ± 1.1	2.49 ± 0.77
IL-8 (pg/ml)	2.27 ± 0.46	2.61 ± 0.23
TNF-α (pg/ml)	4.00 ± 0.46	4.66 ± 0.34
MCP-1 (pg/ml)	169.6 ± 15.7	145.8 ± 11.5
HGF (pg/ml)	830.9 ± 74.8	976.9 ± 95.4
NGF (pg/ml)	7.77 ± 1.49	5.91 ± 0.83
Apoptosis markers		
ccK18 (U/l)	162.1 ± 13.8	152.7 ± 7.8

Results are presented as mean ± SE.

*$p < 0.05$ between control and asthma.

AQLQ, Asthma Quality of Life Questionnaire; FVC, forced vital capacity; HGF, hepatocyte growth factor; NGF, nerve growth factor; PC_{20}, the first provocative concentration that caused a 20% fall in FEV_1.

Statistical analysis

Statistical analysis of the metabolomic data have been described previously (12) Briefly, statistical analysis was performed using either JMP (SAS, Cary, NC) or R (http://cran.r-project.org), a freely available open-source software package. The observed relative concentration of each biochemical was log transformed and inputed with minimum observed values for that compound. A Welch two-sample *t* test was used to identify biochemicals that differed significantly between nonsevere asthma, severe asthma, and healthy control, as well as between healthy controls and high and low F_ENO asthmatics. A significance level of 0.05 was used. When $p < 0.05$, we have enough evidence to conclude that the population means are statistically significant. An estimate of the false discovery rate (*q* value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies; as *q* values were reasonable for $p \leq 0.05$, no *q* value cutoff was established for this study. Correlations were assessed using a linear model.

Results

The study included 20 asthmatics and 10 healthy control subjects. The clinical characteristics of the study subjects are shown in Table I. Age, gender, and ethnicity distribution were similar among the healthy controls and asthmatics. The body mass index (BMI) of subjects with asthma was significantly higher than that of healthy controls (Table I). None of the asthmatics had overt diabetes or were receiving any anti-diabetic medication. As anticipated, the pulmonary function of asthmatics was lower than controls. Subjects with severe asthma were older and had higher BMI and worse lung function as compared with nonsevere asthmatics (Table II). All severe asthmatics received high-dose inhaled or oral corticosteroids either singly or in combination with long-acting β agonists (Table II). Nonsevere asthmatics did not receive any corticosteroids or long-acting β agonists and received inhaled β agonist (rescue medication) infrequently but less than twice per week. Nonsevere asthmatics were well controlled (Asthma Quality of Life Questionnaire > 5) and had significantly better lung function than did severe asthmatics (Table II). Sixty-seven percent (six of nine) of nonsevere subjects and 33% (three of nine) of severe asthmatics had high F_ENO (≥35 ppm); two individuals did not undergo exhaled NO measurements.

The plasma levels of cytokines IL-6, IL-8, TNF-α, MCP-1, hepatocyte growth factor, and nerve growth factor were not significantly different between asthmatics and healthy controls (Table I). Plasma levels of ccK18, a caspase cleavage fragment released from apoptotic epithelial cells (18), was also not different among subjects with asthma and healthy controls (Table I).

Plasma amino acids

Focused metabolomics revealed that taurine was the only amino acid significantly higher in asthmatics as compared with healthy controls (Fig. 1A, Table III). Severity of asthma or F_ENO did not influence plasma taurine levels (mean [SE] micromoles/l; nonsevere, 132.3 [19.7]; severe, 145.1 [16.8], $p > 0.05$; low F_ENO, 150.3 [20.6]; high F_ENO, 122.6 [19.1], $p > 0.05$)

Asthmatics with low F_ENO had significantly lower branched chain amino acids (BCAA), that is, leucine, isoleucine, and valine, in the plasma as compared with those with high F_ENO and healthy controls (Table III). The differences remained significant even when BCAA levels were measured using the metabolomic platform (see *Metabolome of asthma based on severity*). Severity of asthma had no effect on BCAA.

Table II. Demographics for all asthmatics based on asthma severity

	Asthma Severity	
	Nonsevere (n = 10)	Severe (n = 10)
Demographics		
Age (y)	31.4 ± 2.6	44.5 ± 3.4*
Gender (male/female)	6/4	8/2
Duration of asthma (y)	21.6 ± 4.6	28.2 ± 3.2
Weight (kg)	84.3 ± 3.5	90.1 ± 7.8
Height (cm)	177 ± 0.03	162 ± 0.04*
BMI	26.8 ± 3.1	33.9 ± 7*
AQLQ	5.11 ± 0.30	3.75 ± 0.33*
Lung functions		
FEV_1 (l)	3.26 ± 0.32	1.92 ± 0.17*
FEV_1 (%)	87.2 ± 5.3	64.78 ± 5.2*
FVC (l)	4.63 ± 0.42	2.77 ± 0.18*
FVC (%)	104.1 ± 5.5	79.6 ± 9.9*
FEV_1/FVC	0.71 ± 0.04	0.69 ± 0.04
PC_{20}	2.2 ± 0.8	1.7 ± 0.9
F_ENO (ppb)	48.9 ± 9.9	23.2 ± 4.7*
F_ENO phenotype		
$F_ENO \geq 35$ppb (%)	67	33
Medication (n)		
Corticosteroids		
Inhaled	0	9
Oral	0	3
Injected	0	0
β-agonist: inhaled	9	7
Long-acting β-agonist	0	8
Theophylline	0	0
Leukotriene receptor agonist	0	6
Ipratropium	0	1
Immunotherapy	0	0

Results are presented as mean ± SE.

*$p < 0.05$ between healthy control and asthma.

AQLQ, Asthma Quality of Life Questionnaire; FVC, forced vital capacity; PC_{20}, the first provocative concentration that caused a 20% fall in FEV_1.

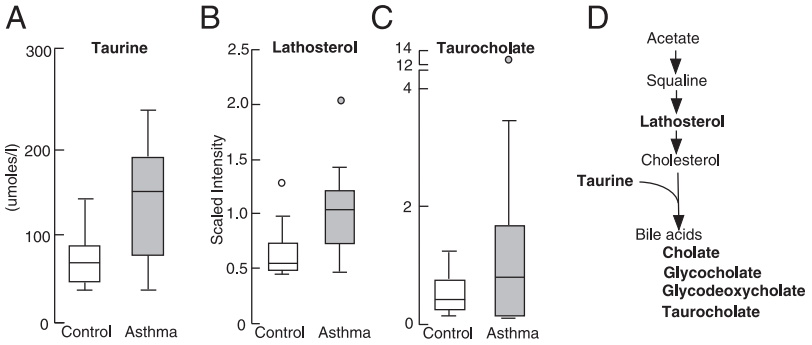

FIGURE 1. Biochemicals reflecting the impact of increased NO production in Asthma. Taurine (**A**), lathosterol (**B**), and taurocholate (**C**) levels were increased in asthmatics (n = 20) as compared with healthy controls (n = 10). Data are box plots with median value and minimal and maximal distribution. (**D**) Abbreviated scheme of bile acid production pathway. Bold type indicates that the metabolites higher in asthma. NO can cause functional impairment of the taurine transporter leading to higher taurine levels, which can be augmented by higher arachidonate levels in asthma. NO, by increasing bile acid metabolism, can result in high bile acid levels (see *Discussion* for details).

Metabolomic analysis

Using LC/MS and GC/MS analysis, 293 distinct metabolites were identified in the plasma samples. The changes in the metabolites between the groups were calculated by the ratio of their group means. The list of all the identified biochemicals with their relative change among the asthma phenotypes and healthy controls are appended in Supplemental Tables I and II. The number of named compounds, with statistically significant different levels, ranged from 13 to 25 across various comparisons (Table IV). Overall, the number of statistically different compounds between asthmatics and healthy control subjects was 25; most of the metabolites were higher in asthmatics as compared with healthy controls (Supplemental Table I, Table IV). Analysis of asthmatic cohort based on severity, that is, severe versus nonsevere asthma, identified 18 biochemicals as statistically different with 1 biochemical higher and 17 biochemicals lower in severe asthmatics as compared with nonsevere asthmatics (Supplemental Table I, Table IV). Analysis of the metabolites based on F_ENO levels revealed that 13 biochemicals were statistically different among the groups, that is, 3 higher and 10 lower in those with low F_ENO as compared with the high F_ENO group (Supplemental Table II, Table IV).

Biochemicals related to protein, carbohydrate, lipids, xenobiotics, peptides, cofactors and vitamins, and nucleotide metabolism were significantly altered in asthmatic subjects as compared with the healthy controls (Supplemental Table I). Table V shows all biochemicals significantly different between asthmatics and healthy controls. Most of the identified biochemicals (11 of 25) were associated with lipid, steroid, and long-chain fatty acid metabolism. Of particular interest was the marked increase in bile acids (taurocholate) in the asthmatic subjects (Fig. 1C). Lathosterol levels also were higher in asthma (Fig. 1B). Asthmatics had increased levels of peptides related to glutamate cycle, glutamylphenylalanine, and glutamyltyrosine, as compared with the healthy controls. Additionally, markers of inflammation such as nicotinamide (2-fold), adenosine monophosphate (3-fold), and arachidonate (1.5-fold) were higher in asthma (Fig. 2, Table V). None of these biochemicals, except taurocholate, were significantly different when asthmatics were segregated by severity (Supplemental Table I) or by F_ENO (Supplemental Table II). Asthmatics with high F_ENO had significantly higher taurocholate as compared with healthy controls (taurocholate, scaled intensity, mean [SE]: healthy control, 0.72 [0.23]; high F_ENO, 2.69 [1.34]; p = 0.028) (Supplemental Table II).

Metabolome of asthma based on severity. Severity of asthma, based on use of high-dose corticosteroid treatment, impacted mostly the biochemicals related to steroid metabolism: 1-stearoyylglycerol, dehydroisoandrosterone sulfate, epiandrosterone sulfate, and androsterone sulfate (Fig. 3, Table VI). Additionally,

Table III. Plasma levels of amino acids in healthy and asthmatic individuals

	Healthy Controls (n = 10)	Asthma (n = 20)	Asthma Based on F_ENO	
			F_ENO < 35 ppm (n = 9)	F_ENO ≥ 35 ppm (n = 9)
Taurine	70.4 ± 15.7	138.5 ± 11.1*	150.3 ± 20.6	122.5 ± 19.1
Aspartic acid	6.8 ± 1.2	7.25 ± 0.8	7.8 ± 0.9	6.6 ± 0.7
Glutamic acid	53.9 ± 10.7	73.6 ± 7.6	72.2 ± 9.2	73.4 ± 14.6
Asparagine	52.5 ± 4.7	49.9 ± 2.3	51.8 ± 4.6	51.1 ± 6.7
Serine	99.8 ± 8.0	89.2 ± 5.6	87.4 ± 7.0	95.3 ± 8.4
Glutamine	545.2 ± 17.5	527.0 ± 12.4	550.4 ± 16.8	505.1 ± 15.8
Histidine	93.6 ± 5.5	89.9 ± 3.9	96.2 ± 5.2	88.4 ± 6.3
Glycine	240.1 ± 24.2	221.8 ± 17.1	223.4 ± 27.5	232.5 ± 30.4
Citrulline	37.5 ± 3.5	28.9 ± 2.5	28.9 ± 1.7	28.1 ± 3.0
Threonine	140.0 ± 14.4	147.1 ± 10.2	155.4 ± 14.1	144.1 ± 18.7
Alanine	391.7 ± 27.7	387.7 ± 19.6	401.9 ± 28.6	382.7 ± 27.1
Arginine	98.4 ± 11.4	101.8 ± 8.0	112.7 ± 11.9	93.7 ± 14.1
Tyrosine	64.2 ± 4.8	71.0 ± 3.4	71.2 ± 3.7	73.7 ± 4.4
Amino butyric acid	20.3 ± 1.4	16.8 ± 1.0*	16.4 ± 1.02	17.9 ± 1.8
Methionine	22.5 ± 1.6	22.7 ± 1.1	21.6 ± 0.7	24.6 ± 2.0
Valine	242.3 ± 12.6	229.3 ± 8.9	216.7 ± 10.1	244.3 ± 8.2#
Tryptophan	49.0 ± 3.1	49.5 ± 2.2	46.0 ± 3.9	54.7 ± 2.8
Phenylalanine	61.7 ± 3.2	61.2 ± 2.3	61.6 ± 2.7	63.2 ± 2.8
Isoleucine	72.5 ± 5.8	65.3 ± 4.1	60.1 ± 4.4	71.8 ± 3.1#
Leucine	138.8 ± 9.9	125.0 ± 7.0	113.8 ± 8.7	138.0 ± 5.5#
Ornithine	67.7 ± 7.1	70.5 ± 5.1	69.9 ± 8.9	70.9 ± 6.9
Lysine	180.8 ± 10.5	172.4 ± 7.4	177.4 ± 12.7	172.7 ± 10.5

All data are μmole/l and mean ± SE.
*p < 0.05 between healthy controls and asthmatics, #p < 0.05 between asthmatics with low and high F_ENO.

Table IV. Number of biochemicals that were significantly different in asthma

Welch Two-Sample *t* Test	Asthma versus Healthy Control	Severe Asthma versus Nonsevere Asthma	Low F_ENO versus High F_ENO
Total no. biochemicals with $p \leq 0.05$	25	18	13
Biochemicals (↑/↓)	20/5	1/17	3/10

Significantly ($p < 0.05$) higher ↑ and ↓ biochemicals.

significant changes were seen in biochemicals related to amino acid metabolism (Table VI). β-Alanine, a nonessential amino acid, was significantly higher in severe asthmatics as compared with nonsevere asthmatic (Supplemental Table I, Table VI).

Metabolome of asthma based on F_ENO. High or low F_ENO was discriminated mostly by differences in amino acid, lipids, and bile acid–related metabolites (Table VII). As with focused metabolomics (Table III), BCAA were significantly lower in asthmatics with low F_ENO as compared with those high F_ENO (Table VII). Bile acids glycocholate and cholate are significantly higher in asthmatics with high F_ENO (Table VII).

Correlations. Plasma taurine levels were positively correlated with plasma arachidonate ($R = 0.50$, $p = 0.004$).

Discussion

In the present study, we performed untargeted and focused metabolomic analysis to identify the plasma metabolomic endotypes of asthma. By using these techniques, we have identified differences in plasma biochemicals of asthmatics as compared with healthy controls. Asthmatics had significantly higher plasma levels of taurine, bile acids, nicotinamide, arachidonate, and adenosine-5-phosphate as compared with healthy controls. Severe asthmatics had significantly lower biochemicals related to steroid metabolism as compared with nonsevere asthmatics. High F_ENO in asthmatics was associated with significantly higher bile acids as compared with asthmatics with low F_ENO.

The concentration of plasma taurine was higher in asthmatics as compared with healthy controls irrespective of severity or F_ENO phenotype. Taurine, a β amino acid, is not incorporated into proteins and is present in high concentrations in the intracellular compartment in most tissues. Although the physiological roles of taurine continue to be explored, it is now accepted that taurine can function as an intracellular osmolyte, an antioxidant, and a critical compound in the development of brain for certain animals (19). The mechanism of increase in plasma taurine can only be speculated because of lack of any data in the literature in relation to asthma. Taurine levels have been reported to be higher in the bronchoalveolar lavage fluid of individuals with asthma (20, 21). Whether the increase in levels of taurine in bronchoalveolar lavage fluid is related to higher NO or other cytokines in asthma or is a result of release from damaged cells has not been examined. NO has been shown to functionally impair the taurine transporter

Table V. Metabolites with significant fold change between asthma and healthy control subjects

Pathway		Fold Change*: Asthma/Healthy Controls
Amino acid		
Urea	Urea	0.8
BCAA	α-Hydroxyisocaproate	0.74
Carbohydrate		
Glycolysis, gluconeogenesis	3-Phosphoglycerate	1.7
Glycogen	Maltose	3.0
	Maltotriose	3.59
Lipid		
Sterol/steroid	7-α-Hydroxy-3-oxo-4-cholestenoate	1.31
	Androsterone sulfate	0.7
	Epiandrosterone sulfate	0.7
Glycerolipid	Glycerophosphorylcholine	1.65
	Phosphoethanolamine	1.82
Long-chain fatty acid	Arachidonate (20:4n6)	1.37
Fatty acid, amide	Oleamide	0.57
Sphingolipid	Sphingosine	2.27
Bile acid and cholesterol		
	Glycodeoxycholate	2.18
	Taurocholate	3.22
	Lathosterol	1.64
Xenobiotics		
Xanthines	Caffeine	3.89
	Paraxanthine	2.14
	Theophylline	2.35
Benzoate	Catechol sulfate	1.54
Peptide		
γ-Glutamyl	γ-Glutamylphenylalanine	1.22
	γ-Glutamyltyrosine	1.33
Dipeptide	Cyclo(leu-pro)	1.79
Cofactors and vitamins		
Nicotinate and nicotinamide	Nicotinamide	2.32
Nucleotide		
Purines	AMP	2.84

*$p < 0.05$.

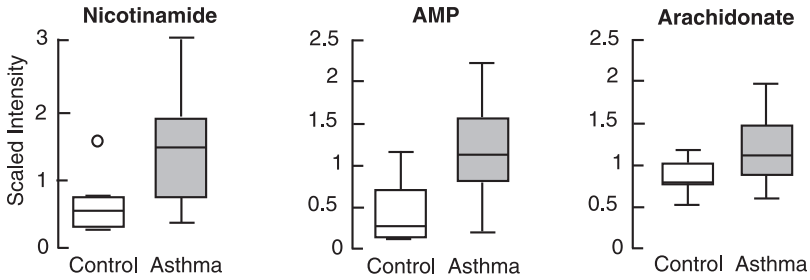

FIGURE 2. Biochemicals indicative of augmented immune and inflammatory responses. Untargeted metabolomics revealed significantly higher levels of nicotinamide, adenosine monophosphate, and arachidonate in asthmatics ($n = 20$) as compared with healthy control subjects ($n = 10$). Data are box plots with median value and minimal and maximal distribution.

in rat renal brush border membrane vesicles (22) and in oocytes (23). The impairment of taurine transport was mediated via the formation of peroxynitrite from the reaction of NO with superoxide (23). Asthmatic airways have increased formation of peroxynitrite due to increased production of NO and decreased antioxidant activities, suggesting that a similar effect on taurine transporter may occur in asthma (24, 25). However, the interactions of NO with taurine transporter are more complex and possibly depend on the tissue examined because NO also transcriptionally upregulates expression of taurine transporter in cultured human retinal epithelial cells (22). NO regulates extracellular taurine levels in other tissues, such as the hippocampus, where the effects are related to specific NO synthase enzymes (26). Arachidonate, a precursor of leukotrienes, was higher in asthmatics, regardless of phenotype. The mobilization and oxidation of arachidonic acid via 5-lipoxygenase to leukotrienes are an important pathway of inflammation in asthma, as well as in the swelling-induced activation of the taurine-releasing pathways leading to an efflux of taurine from the intracellular compartment (27, 28). In the present study, there was a positive correlation between plasma levels of taurine and those of arachidonate in asthma, suggesting that taurine may serve as a biomarker of this specific inflammatory pathway in asthma. The levels of β alanine were higher in severe asthma compared with the nonsevere asthma. β Alanine competes for the transporter of taurine, and it could also contribute to the higher levels of taurine in asthma.

The major metabolic route of elimination of taurine is in the form of bile acids. In the present study, nontargeted metabolomics revealed higher levels of taurocholate in asthma, with the highest levels in asthmatics with high F_ENO compare with the healthy controls. Previous studies in vivo and in vitro have demonstrated that NO modulates bile acid metabolism and bile production. Schonhoff et al. (29) showed that NO inhibits taurocholate uptake by inducing nitrosylation of the sodium-taurocholate transporter. Hepatic perfusion with NO donors increased bile acid out flow (30). In isolated hepatocytes, NO inhibited bile acid uptake, and inhibition of NO synthase with L-N^G-nitroarginine decreased the synthesis of bile acids and increased cholesterol levels (31) Transgenic mice overexpressing endothelial NO synthase have increased levels of primary and secondary bile acids as well as conjugated bile acids (32). Based on these data, Sansbury et al. (32) proposed that the mechanism of increase in bile acids by NO may be due to increased hepatic synthesis, higher bacterial dehydroxylation in the gut, and increased conjugation in the liver. The higher levels of lathosterol in asthma in our study suggest a higher rate of synthesis of cholesterol, a precursor of bile acids (33, 34). Overall, asthmatics have higher plasma taurine, likely a result of cellular efflux, and higher lathosterol and bile acids, likely a result of increased synthesis, which all may be related to greater NO and inflammation in asthma (Fig. 1D).

The plasma concentration of nicotinamide was markedly increased (3-fold) in asthmatics irrespective of the severity of the disease or the level of F_ENO. There was no associated change in tryptophan levels or in the levels of the biochemical intermediates of tryptophan metabolism. This would suggest that the increase in nicotinamide may not be related to an increase in production of NAD^+ but rather to an increase in its consumption. NAD^+ is a substrate for three classes of enzymes that cleave NAD to produce nicotinamide (35). These are ADP-ribose transferase, cADP-ribose synthase, and sirtuins (protein lysine deacetylases). Of these, cADP-ribose synthase, also known as the lymphocyte Ag CD38 and CD157, is of particular interest because of its described role in regulation of immune responses and airway hyperresponsiveness in asthma. CD38 is a multifunctional cell surface protein expressed in airway smooth muscle cells and on immune cells (36). Additionally, CD38 is an ectoenzyme and its major enzyme activity is hydrolysis of NAD^+ (36, 37). Its expression is augmented by inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, and IL-13 (38, 39). Increased CD38 expression resulted in high rate of production of cADP-ribose by airway smooth muscle cells (40), whereas inhibition of its activity by small interfering RNA–mediated knockdown or by gene deletion ($CD38^{-/-}$ mice) resulted in lower levels of cADP-ribose and increased intracellular levels of NAD^+ (37). We postulate that the higher levels of nicotinamide in asthmatics are related to increased rate of production via CD38 in response to ongoing immunologic activation in asthma.

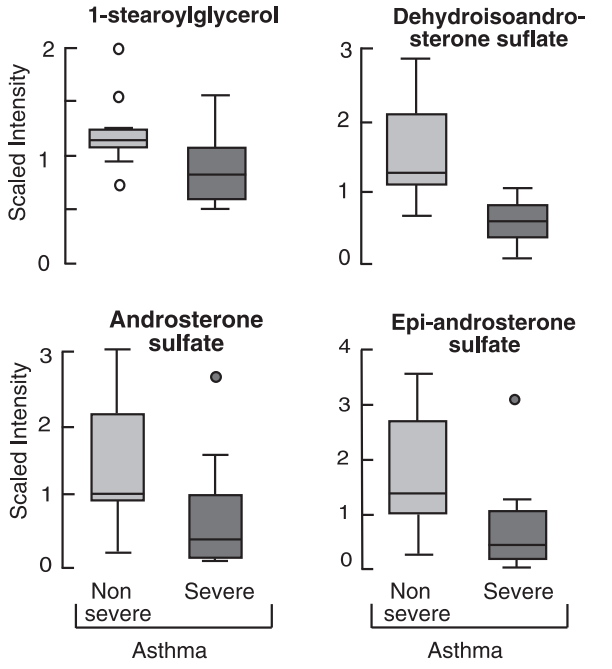

FIGURE 3. Plasma steroids levels in relationship to the severity of asthma. Steroid metabolites were significantly lower in severe asthmatics ($n = 10$) as compared with nonsevere asthmatics ($n = 10$). Data are box plots with median value and minimal and maximal distribution.

Table VI. Biochemicals significantly different between severe and nonsevere asthmatic phenotype

Pathway		Fold Change*: Severe/Nonsevere
Amino acid		
BCAA	β-Hydroxyisovalerate	0.59
	α-Hydroxyisovalerate	0.51
Lysine	Glutarylcarnitine	0.76
Tryptophan	Indolepropionate	0.65
Alanine and aspartate	β-Alanine	2.28
Carbohydrate		
Glycolysis, gluconeogenesis	1,5-Anhydroglucitol	0.76
Lipid		
Monoacylglycerol	1-Stearoylglycerol	0.75
Sterol/steroid	Dehydroisoandrosterone sulfate	0.35
	Epiandrosterone sulfate	0.48
	Androsterone sulfate	0.54
	4-Androsten-3β,17β-diol disulfate 1	0.25
	4-Androsten-3β,17β-diol disulfate 2	0.45
	5α-Androsten-3β,17β-diol disulfate	0.34
	Pregnen-diol disulfate*	0.36
	Andro steroid monosulfate 2	0.42
Peptide		
γ-Glutamyl	γ−Glutamylvaline	0.75

*$p < 0.05$.

The low levels of steroids in the severe asthmatics are consistent with suppression of adrenal steroids as a consequence of therapeutic use of corticosteroids. However, the severe group shared many of the same metabolomic signatures as did nonsevere asthmatics, who were not on corticosteroids. The ability to detect changes in steroid metabolism, yet observe similar metabolomic profile in severe and nonsevere asthma, indicates that corticosteroid therapy is unlikely to be the cause of the differences seen among asthma and healthy controls. Alternatively, the low F_ENO group of asthmatics had lower levels of BCAA in the presence of unchanged other essential amino acids (e.g., phenyalanine, methionine), suggesting alteration in the metabolism of BCAA in this group. The mechanism of this change remains unclear.

The following limitations of our study should be recognized. 1) Importantly, note that the asthmatic subjects in our study were clinically stable and well controlled on medications. The clinical management of asthma may have attenuated some of the biochemical pathways related to the disease and therefore impacted the observed endotype. 2) Our study is a pilot exploratory study and our sample size is small. 3) The blood samples were obtained randomly. Although important, it may not have been critical for the present study because the biochemicals affected by asthma are not known to be significantly impacted by feeding and fasting. 4) Our measurements in the blood mostly reflect systemic responses to airway disease and are not pulmonary responses to asthma. These confounders could impact the data obtained and their interpretation. Asthma is a heterogeneous disorder and larger samples of carefully characterized individuals are required to confirm the metabolomic endotypes observed in this cohort (41, 42).

In summary, the metabolomics analysis of asthma revealed a characteristic metabolome dominated by biochemical/metabolic responses related to NO, Th2 immunity, and inflammation. The definition of asthma by metabolomic endotype may offer opportunities to develop biomarkers of disease activity, adherence to medication, and targeted therapeutics.

Disclosures

The authors have no financial conflicts of interest.

Table VII. Biochemicals significantly different between F_ENO phenotype

Pathway		Fold Change*: Low F_ENO/ High F_ENO
Amino acid		
BCAA	Isoleucine	0.84
	Valine	0.88
	3-Hydroxyisobutyrate	0.65
Phenyalanine and tyrosine	*p*-Cresol sulfate	1.84
Tryptophan	Tryptophan	0.82
	C-Glycosyl tryptophan	1.3
Histidine	Trans-Urocanate	2.08
Carbohydrate		
Glycolysis, gluconeogenesis	Glycerate	0.55
Lipid		
Fatty acid	Isovalerate	0.67
Long-chain fatty acid	Pentadecanoate	0.79
Bile acid		
	Glycocholate	0.32
	Cholate	0.32
Nucleotide		
Ascorbate and alderate	Threonate	0.62

*$p < 0.05$.

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