

Metabolomic profiling of asthma: Diagnostic utility of urine nuclear magnetic resonance spectroscopy

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Background: The ability to diagnose and monitor asthma on the basis of noninvasive measurements of airway cellular dysfunction is difficult in the typical clinical setting.

Objective: Metabolomics is the study of molecules created by cellular metabolic pathways. We hypothesized that the metabolic activity of children with asthma would differ from healthy children without asthma. Furthermore, children having an asthma exacerbation would be different compared with children with stable asthma in outpatient clinics. Finally, we hypothesized that ¹H-nuclear magnetic resonance (NMR) would measure such differences using urine samples, one of the least invasive forms of biofluid sampling.

Methods: Children (135 total, ages 4–16 years) were enrolled, having met the criteria of healthy controls (C), stable asthma in the outpatient clinic (AO), or unstable asthma in the emergency department (AED). Partial least squares discriminant analysis was performed on the NMR data to create models of separation (70 metabolites were measured/urine sample). Some NMR data were withheld from modeling to be run blindly to determine possible diagnostic accuracy.

Results: On the basis of the model of AO versus C, 31 of 33 AO samples were correctly diagnosed with asthma (94% accuracy). Only 1 of 20 C samples was incorrectly labeled as asthma (5%

misclassification). On the basis of the AO versus AED model, 31 of the 33 AO samples were correctly diagnosed as outpatient asthma (94% accurate).

Conclusion: This is the first report suggesting that ¹H-NMR analysis of human urine samples has the potential to be a useful clinical tool for physicians treating asthma. (*J Allergy Clin Immunol* 2011;127:757–64.)

Key words: Asthma, inflammation, biomarkers, urine, NMR, metabolomics

Asthma is a complex syndrome characterized by airflow obstruction. The symptoms and changes in lung function, used by clinicians to guide therapy, are largely the consequence of abnormal airway inflammation^{1,2} and its effects on structural airway cells, including epithelium, smooth muscle, and nerves.^{3,4} Thus, international guidelines suggest that regardless of the classification of asthma, the desired goal is to adjust therapy on the basis of inflammation.⁵

Using objective measurements of airway inflammation to guide asthma therapy was shown to produce superior therapeutic results compared with using traditional measures alone (eg, symptoms and lung function).^{6,7} However, for clinicians in a typical outpatient setting, detecting airway inflammation is often difficult. Physiological (eg, spirometry, peak flow measures) or functional tests (eg, symptoms, quality of life) are insensitive to small changes in inflammatory status that might be important clinically relevant.⁷ Performing direct measures (eg, bronchoscopy) is not feasible because it is expensive and invasive. To address these issues, noninvasive measures of inflammation such as induced sputum or exhaled nitric oxide have been developed. Although induced sputum is a valid method of measuring airway inflammation,^{8,9} there remain significant barriers to its use, including limited availability outside a tertiary care setting and the inability of children (and many adults) to expectorate sufficient or valid samples. Although exhaled nitric oxide values correlate with asthma inflammation and are commercially available, the test is invalid in children younger than 4 years, and it appears to lack the sensitivity and specificity of induced sputum for improving patient outcomes.¹⁰ Overall, a simple, noninvasive test for patients with asthma is not widely available. In most outpatient settings, especially those outside a tertiary care facility, physicians simply rely on patient history before administering trials of therapy.

Metabolomics is the study of metabolic pathways and the unique biochemical molecules created in a living system.^{11,12} By measuring changes in metabolite concentrations, the range of biochemical effects induced by a disease or its therapeutic intervention can be determined. ¹H-nuclear magnetic resonance (NMR) spectroscopy can be used to quantify specific chemical

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Abbreviations used

ED: Emergency department
 EPX: Eosinophil protein X
 NMR: Nuclear magnetic resonance spectroscopy
 PLS-DA: Partial least squares discriminate analysis
 Q2: A measure of the predictive strength of the model
 R2: A measure of the model's fit of the data
 VIP: Variables of importance plot

constituents within a body fluid.¹³ NMR is an attractive technology because of its ability to provide both qualitative and quantitative measurements while simultaneously studying a number of compounds in the same biologic fluid. Furthermore, unlike mass spectrophotometry, NMR requires little pretreatment of samples.¹⁴ Urine is an excellent biological fluid for various medical studies because of its ease of collection in patients of all ages, low cell and protein content, and rich chemical composition, with over 1000 metabolites already identified by NMR.¹⁵

We hypothesized that the metabolic activity of patients with an acute exacerbation of asthma or with chronic stable asthma would produce a unique pattern of metabolic molecules compared with those without asthma. We further hypothesized that this metabolic profile would change as the severity of the disease changed. Although the abnormal cellular metabolic activity of asthma is primarily localized in the lung, impaired lung function influences systemic metabolism. Thus, we hypothesized that urine would see the entire effect of airways disease and could serve as a useful biofluid for asthma through analysis by high-resolution 1-dimensional ¹H-NMR spectroscopy. In this report, we demonstrate for the first time that analysis of metabolites in urine by using NMR technology has the potential to become a noninvasive diagnostic technique for clinicians managing asthma in a typical outpatient setting, especially for those treating children.

Some of the results of this study have been previously reported in abstract form.¹⁶

METHODS

For detailed Methods, please see this article's Online Repository at www.jacionline.org.

Patient characteristics

Patients were enrolled after their parents provided written informed consent as approved by the respective health research ethics boards of the University of Alberta and the University of Manitoba.

Stable asthma in the outpatient clinic. Children with asthma (n = 73) were recruited from the Stollery Children's Hospital outpatient clinic and emergency department (ED; see this article's Table E1 in the Online Repository at www.jacionline.org). Children with stable asthma were initially referred to a pediatric pulmonary or allergy subspecialist clinic and were enrolled after at least 1 clinic visit, having met diagnostic criteria for atopic asthma as described in the Canadian Consensus guidelines.¹⁷ All children had airway hyperresponsiveness as determined by a methacholine challenge test (PC₂₀ <8 mg/mL) or a positive response to bronchodilator (>15% improvement in FEV₁). As might be expected, most of the children selected were also atopic on the basis of at least 1 positive skin test response to a panel of common aeroallergens.

Unstable asthma in the ED. Children in the ED with acute asthma (n = 20) were selected on the basis of 1 or more of the following: (1) increasing asthma symptoms (eg, cough, wheeze, shortness of breath, or chest tightness) requiring assessment and a history of similar episodes, (2) clinical

or symptomatic response to inhaled bronchodilator therapy, and/or (3) a previous history of physician-diagnosed asthma. Patients had to present primarily for acute asthma (not a simple prescription refill) and were excluded if they had acute pneumonia, needed immediate resuscitation (status asthmaticus), had cognitive impairment, or had a known immunodeficiency.

Controls. Healthy age-matched and sex-matched controls (n = 42) were recruited from the community and from the University of Manitoba, SAGE birth cohort (Study of Asthma, Genes and the Environment).¹⁸ Healthy controls were excluded if they had any known underlying lung disease (eg, chronic cough or wheeze, cystic fibrosis, asthma, or oral steroid use). Subjects were excluded if they had a known nonrespiratory chronic inflammatory/infective disorder (eg, malignancy, cardiac disease, immunodeficiency, or neonatal lung disease associated with prematurity). The children from the SAGE birth cohort (n = 10) were also known to have negative results for aeroallergen skin testing and methacholine responsiveness.

Urine sample collection

A single urine sample was collected from each child and promptly placed in a freezer (−20°C). Within 3 hours of collection, the urine samples were stored in a −80°C freezer. Although most samples were analyzed within 1 month of collection, we have previously reported that such samples can be stable in the freezer for up to a year.^{19,20} We were aware of the variability in urine metabolomic data that can occur within each person for diet and time of day^{21,22}; however, our objective was to design a test that could be used in a typical clinical setting, especially for young children. Thus, we did not stipulate a single time of day for each urine collection, nor did we mandate dietary restrictions. We hypothesized that the metabolites of interest would be altered sufficiently between disease and nondisease groups that such intrapersonal variability would be superseded.

Sample preparation

Urine samples were thawed only once in a biosafety fume hood, and a 630-μL aliquot was removed and placed in a 1.5-mL microcentrifuge tube, followed by the addition of 70 μL of a reference buffer solution (see the Methods section in this article's Online Repository at www.jacionline.org).

NMR acquisition

All ¹H-NMR spectra were acquired on a 600-MHz VNMRs spectrometer (Varian Inc, Palo Alto, Calif) equipped with a 5-mm inverse-proton (HX) probe with z-axis gradient coil. One-dimensional ¹H-NMR spectra were collected at 25°C by using the first increment of a 2-dimensional-proton nuclear overhauser effect spectroscopy (see Methods in the Online Repository for defined NMR acquisition parameters).

Spectral and statistical analysis

Spectral identification and quantification of 70 identifiable metabolites were performed by using the Chenomx NMR Suite Professional software package Version 4.6 (Chenomx Inc, Edmonton, Alberta, Canada). The software contains a database of known metabolites with their referenced spectral resonant frequencies or signatures. These known resonant frequencies were matched to the observed resonant frequencies of the collected spectra, enabling the qualitative and quantitative analysis of metabolites in urine (Fig 1, A). To account for hydration status of the subjects, metabolites were referenced to creatinine. Partial least squares discriminate analysis (PLS-DA) was performed (SIMCA-P 11; Umetrics, San Jose, Calif). This process identifies the metabolites whose concentrations differed significantly between groups of patients, expressed as a coefficient of variation plot (Fig 1, B). As might be expected, most metabolites do not differ greatly between groups. In contrast, the greater the consistent difference in metabolite concentration between groups, the more important a metabolite becomes in creating the final model, seen as a variables of importance plot (VIP; Fig 1, C). Metabolites of low significance can be detrimental to diagnostic accuracy. On the basis of the list of metabolites included in the algorithm, PLS-DA generated a prediction score (0-1) of unclassified, blind data not part of the model (ie, scores <0.5 would be predicted to be healthy subjects). See this article's Table E2 in the

Online Repository at www.jacionline.org for an example of how these scores are calculated. To choose the most accurate list of metabolites, we removed metabolites listed as being lower in significance on the VIP until we were satisfied that the model could correctly diagnose healthy control subjects not part of the model. We set a false-positive rate of 5% for healthy control children as an acceptable limit. We applied the same approach to the separation of stable asthma versus unstable asthma by using blind stable outpatient subjects with asthma and a 5% error rate. This resulted in the most sensitive models, both with respect to correlation coefficients (R2) and prediction properties (Q2) (see Methods in the Online Repository). We previously reported using this technique in an animal model of asthma.²³

RESULTS

Baseline patient characteristics

There were no statistically significant differences among the 3 groups of children (including comparisons of the blind vs nonblind cohorts) for any of the parameters listed in Table E1.

Urine NMR profile of asthma in outpatient clinic compared with healthy controls

We compared children with stable asthma attending an outpatient clinic with healthy age-matched and sex-match controls (Table E1). Using a library of known metabolite standards (Chenomx Inc), the concentrations of 70 metabolites were measured in the urine for each child. These values were standardized to their creatinine level. On the basis of these values for each group of children, PLS-DA created a model of separation. The importance of all the metabolites measured for these 2 groups is shown as a VIP (Fig 1, A). As would be expected, most of the metabolites excreted in the urine did not differ greatly between groups, and adding metabolites of low importance rendered the metabolite model less accurate. To remove irrelevant metabolites, we used a test set of urine samples from healthy control children (n = 20) not part of the model. The metabolites of lower importance were removed on the basis of the PLS-DA blind prediction score (<0.5). We removed metabolites that allowed the model to classify these blind healthy control children correctly with 95% accuracy (19/20), leaving a 5% false-positive rate. The final list of metabolites used to separate outpatients with asthma (n = 40) versus healthy controls (n = 22) used 1 component consisting of 23 metabolites in the VIP list (R2 = 0.72; Q2 = 0.67). The differences in concentration of these metabolites between groups are shown as the coefficient of variation plot (Fig 1, B). Graphic presentation of the quality of separation between groups in the model is shown in Figs 1, C, and 2, B. The final metabolites chosen and their concentrations are shown in this article's Table E3 in the Online Repository at www.jacionline.org.

Validity assessment (stable asthma in the outpatient clinic vs controls)

To validate the proposed model and test its applicability as a diagnostic tool for asthma in the outpatient clinic, we entered the concentrations of metabolites from children (n = 33) with stable asthma who were not originally part of the modeling exercise. These values were entered into the computer without a diagnosis; thus, the computer was blind for the PLS-DA-derived model of outpatient asthma versus control. The individual PLS-DA prediction scores are shown in Fig 2, A, with error bars representing medians and interquartile ranges. A threshold value of 0.5 was used to separate groups. The model was able to diagnose the blind asthma

samples correctly in 31 of 33 samples (94% accuracy). The receiver operating characteristics curve for the proposed model (Fig 2, B) showed that the proposed model had a sensitivity of 94% and a specificity of 95% (highlighted by the red square). In addition, the model had a positive likelihood ratio of 19 (95% CI, 2.78-127) and a negative likelihood ratio of 0.06 (95% CI, 0.02-0.25).

Reproducibility assessment

To determine a measure of reproducibility (ie, whether this approach is affected by confounders such as time of day or diet), we studied samples from many of the same children from the outpatient asthma group (n = 36) who gave repeated urine samples on follow-up clinic visits. These samples were collected months later compared to the samples used in the diagnostic model. The values of the same list of 23 metabolites examined were entered into the PLS-DA model. The software correctly predicted that these children had a metabolite profile similar to the asthma group (a value >0.5) in all except 2 subjects (34/36), giving 94% reproducibility. The PLS-DA prediction scores for these are shown in Fig 2, B.

NMR-derived urine metabolite profiles of stable outpatient asthma versus asthma exacerbation in the ED

In addition to separating asthma from healthy control subjects, the ability to predict impending asthma exacerbation in an outpatient setting would also be important. Pediatric patients presenting to the ED with an asthma exacerbation were recruited for urine sampling (E1). We compared the metabolites of children with stable asthma with those having an asthma exacerbation in the ED. Removing the least important metabolites, we determined that the best model to predict separation of asthma outpatients (n = 40) versus asthma in the ED (n = 20) used 3 components consisting of 28 metabolites in the VIP list (R2 = 0.84; Q2 = 0.74). The differences in concentration of these metabolites between groups are shown as the coefficient of variation plot (Fig 3, A). Graphic presentation of the separation model is shown in Fig 3, B. The final metabolites chosen and their concentrations are shown in Table E3. It is important to note that although many of these metabolites were also part of the outpatient asthma versus control model, new metabolites were needed for the separation of asthma in the ED.

Validity assessment (unstable asthma in the ED vs stable asthma in the outpatient clinic)

To determine the accuracy of this model and its applicability as a diagnostic tool for impending asthma exacerbation in the outpatient clinic, we entered the concentrations of metabolites from outpatient children with asthma (n = 36) not originally part of the modeling. These values were entered into the computer without a diagnosis; thus the computer was blind for the PLS-DA-derived model of outpatient asthma versus control. The model was able to diagnose the blind asthma samples correctly with 89% sensitivity (32 correct of 36 samples). We did not have enough asthma exacerbation children to perform a second blind analysis.

Three-way comparison of healthy control children versus those with asthma in the outpatient setting or ED

Although we suggest that a physician would never need to use this technology to distinguish between a child with asthma

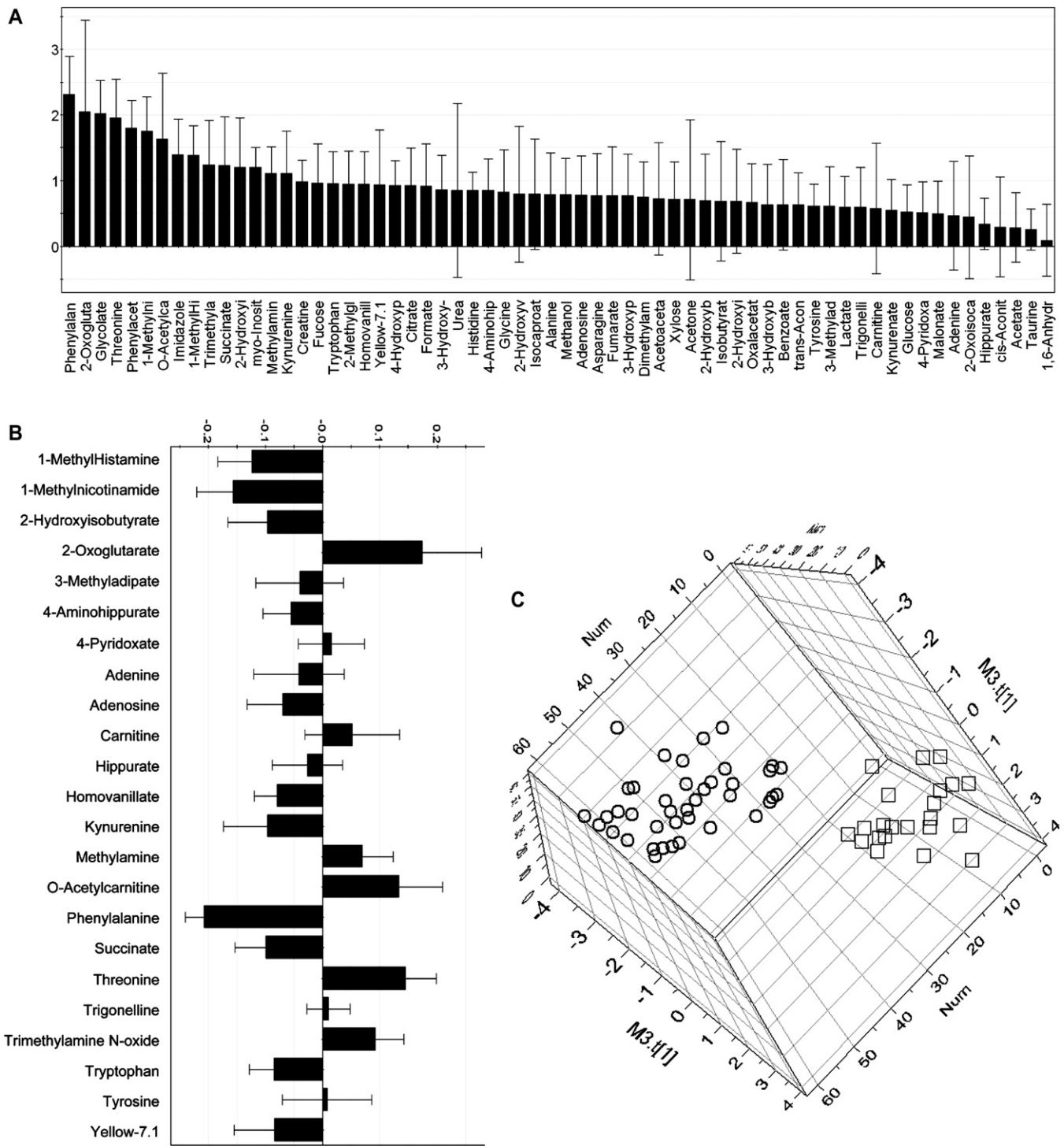


FIG 1. Creating a metabolomic model of asthma in outpatient clinic versus healthy controls. PLS-DA analysis (SIMCA-P 11) of urine metabolite levels for outpatient asthma compared with healthy control children. **A**, VIP ranking the metabolites according to their significance in the model. **B**, Scaled and centered metabolite differences between groups shown as the coefficients of variation plot. **C**, Three-dimensional scatter plot displaying the model's degree of separation between outpatient asthma (circles) and healthy controls (squares; $R^2 = 0.72$; $Q^2 = 0.67$). Error bars represent 95% CIs.

exacerbation and a healthy child, we have provided the opportunity to visualize the physical separation among all 3 groups. The best model to separate outpatient asthma, asthma in the ED, and healthy controls used 30 metabolites (listed in Table E3;

$R^2 = 0.74$; $Q^2 = 0.61$). This separation was based on differences in concentration of metabolites among groups (Fig 4, A). Graphic presentation of the 3-way comparison of groups is shown in Fig 3, B. As might be expected given the small sample size, the 3-way

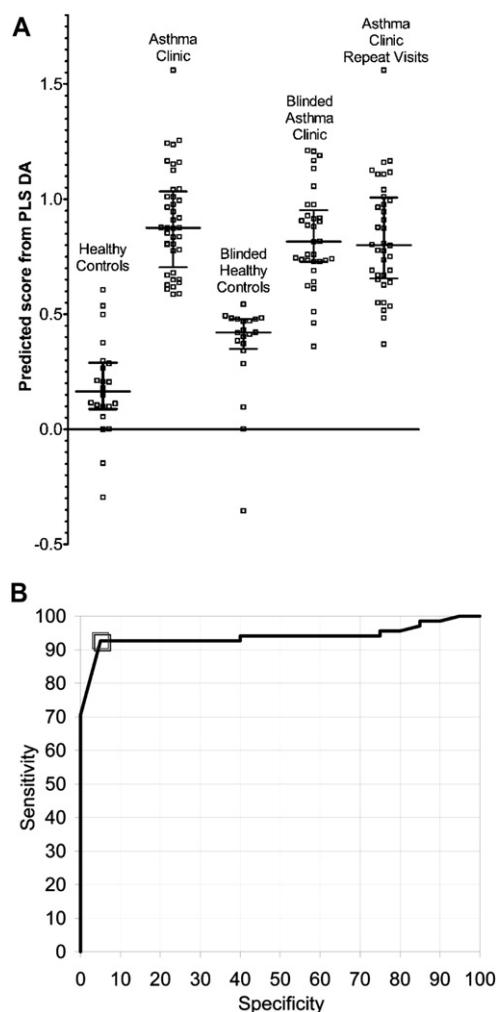


FIG 2. Differentiating asthma from healthy subjects. The PLS-DA algorithm separates groups of data on the basis of a score of 0-1; in this case a value closer to 0 indicates no asthma, and above 0.5 indicates the subject has asthma. **A**, PLS-DA prediction scores for each subject. **B**, The accuracy of predicting outpatient asthma or healthy control in the form of an ROC. Error bars represent medians and interquartile ranges.

model was unable to provide the same accuracy of the 2-way models. The blind outpatient asthma samples were correctly diagnosed in only 22 of 33 samples (66% accuracy) and healthy controls correctly in 13 of 20 samples (a 35% false-positive rate). Again, in the clinical setting, a 2-way analysis model would be used to distinguish groups; thus, we do not consider this lack of accuracy in the 3-way model as detracting from our results overall.

DISCUSSION

We hypothesized that the cellular activity associated with stable asthma should produce a unique pattern of metabolites compared with patients without asthma or those with unstable asthma seen in the ED setting. We also hypothesized that these metabolites may not arise solely from the lung. As will be discussed further, airway dysfunction with increased work of breathing and hypoxemia would cause varying degrees of cellular stress outside the lung that could also serve to discriminate

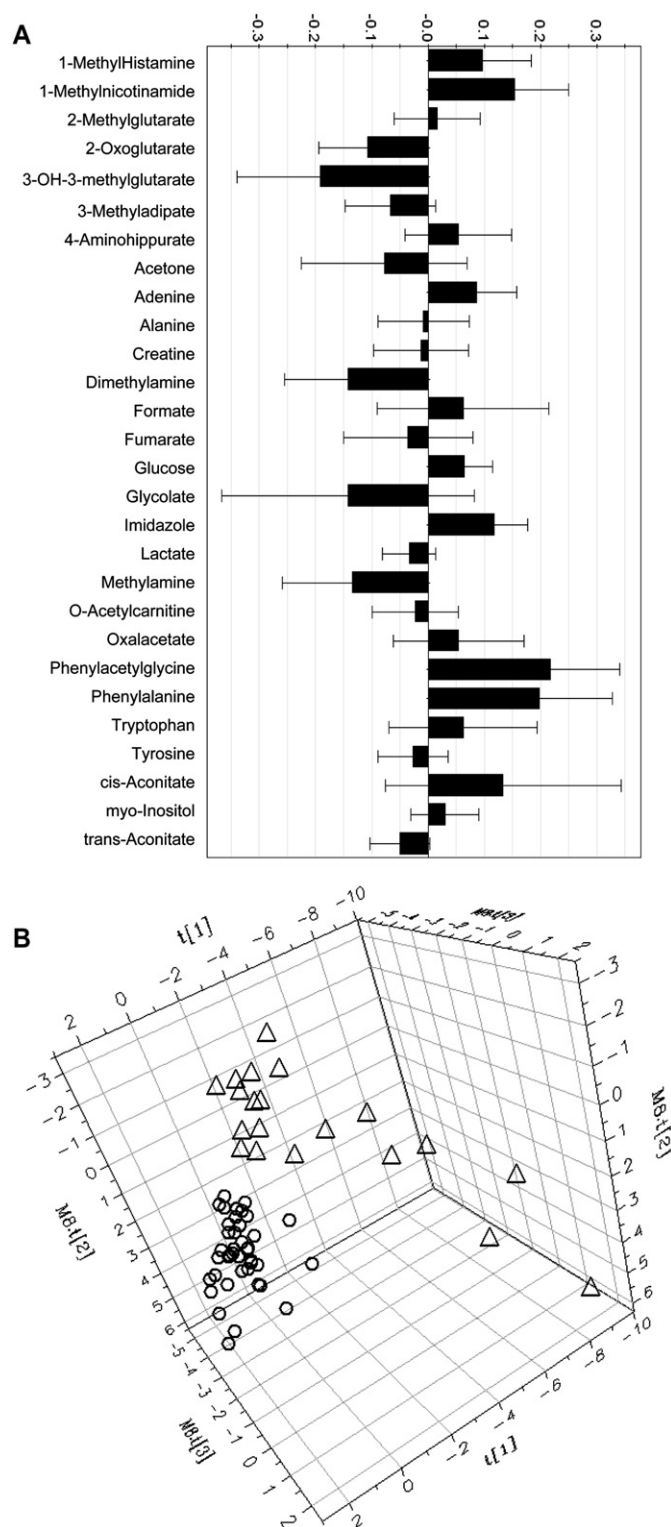


FIG 3. Stable versus unstable asthma. PLS-DA analysis of children with stable outpatient asthma compared with those having an asthma exacerbation. **A**, Scaled and centered metabolite differences between groups shown in the coefficients of variation plot. **B**, Three-dimensional representation of separation between outpatient asthma (open circles) and asthma exacerbation (triangles; $R^2 = 0.84$; $Q^2 = 0.74$). Error bars represent 95% CIs.

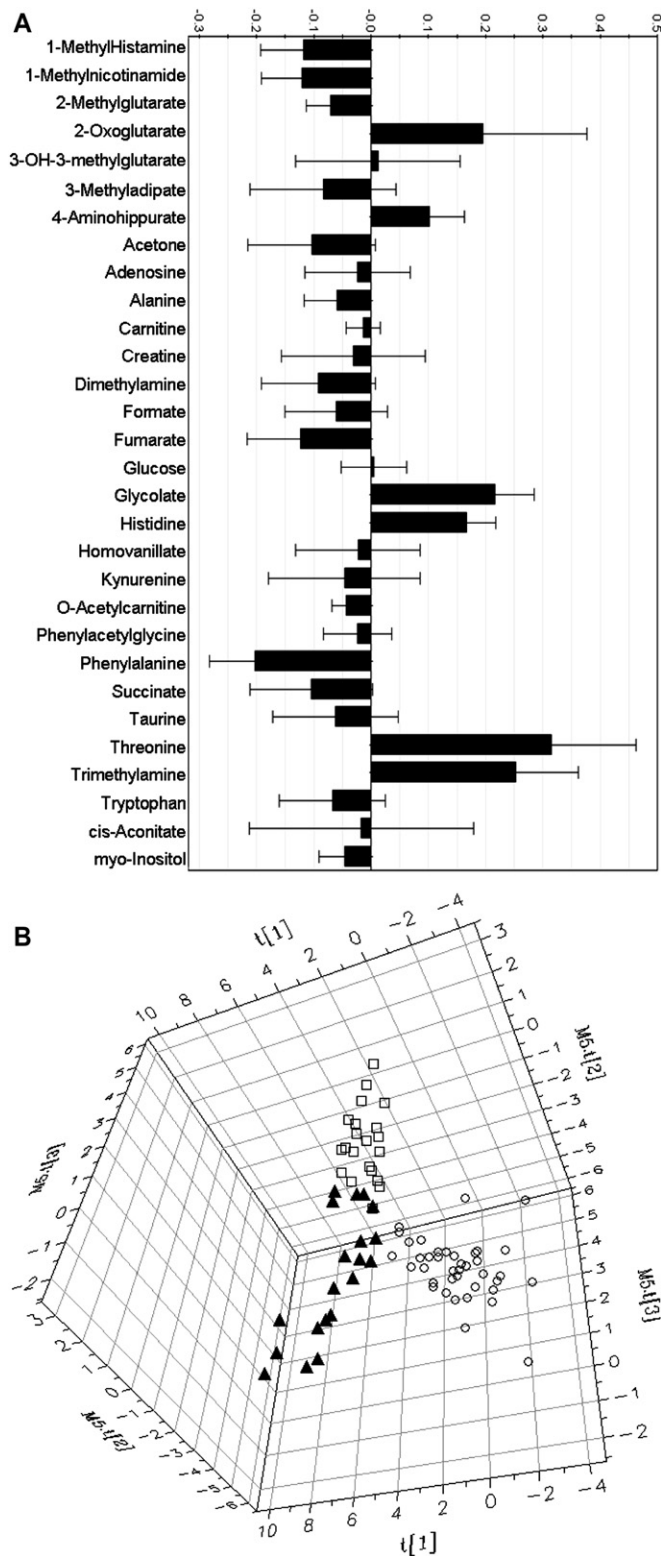


FIG 4. Stable asthma, unstable asthma, and healthy controls. PLS-DA analysis results for all 3 groups of children. **A**, Scaled and centered metabolite differences between groups shown in the coefficients of variation plot. **B**, Three-dimensional separation among healthy controls (squares), outpatient asthma (open circles), and asthma exacerbation (triangles; $R^2 = 0.74$; $Q^2 = 0.61$). Error bars represent 95% CIs.

between healthy people and patients with stable asthma or patients with stable versus unstable asthma. As such, we were not deterred from using urine as our biofluid of interest compared with using samples from the airway.

Unlike previous urine studies in asthma, which relied on a single variable (eg, EPX or leukotrienes^{24,25}), by using NMR and PLS-DA we have the advantage of studying a wide range of cellular pathways with accuracy. It should not be surprising that a combination of metabolites, rather than a single metabolite, would be required to separate the patient groups reliably. Because even healthy subjects excrete some amount of compounds found in disease (eg, leukotrienes), to separate patient groups, the differences in a single compound would need to be large. Further, it is more likely that a single variable would be affected by confounders like diet or time of collection.

We specifically placed no restrictions on time of urine collection or diet before collection because we sought to create the easiest test possible, and we believed that effects of disease would outweigh the effects of such confounders. Although it appears that we were correct, we have also addressed this issue by studying our outpatients with asthma returning for repeat visits. Each visit was quite variable in time of day, diet, and season, occurring over a period of 2 years in some cases. The fact that the model had excellent accuracy for these follow-up patients with asthma supports our test design. In our previous animal model, we also did not control for time of day but still observed separation of the groups.²³

Although this study was not designed to confirm specific metabolic pathways, we can speculate on possible metabolic pathways required to generate the more important metabolites in the models. For example, patients with asthma exacerbation have severe breathing difficulties and often exhibit a degree of hypoxemia. Even in outpatient settings, patients with asthma have some airway obstruction, which could cause a mild degree of hypoxemic stress on body tissues. Metabolites related to the citric acid cycle (2-oxaloglutarate,^{26,27} succinate,^{28,29} fumarate,³⁰ 3-hydroxy-3-methylglutarate,^{30,31} threonine, and cis-aconitate and trans-aconitate) appear to be critical in the separation/differentiation models. We previously documented similar citric acid cycle metabolites by using an animal model of asthma.²³

Another common metabolic area could relate to stress on energy metabolism. For example 2-hydroxyisobutyrate, 3-hydroxybutyrate, and 3-methyladipate are known for their roles in glucose and lipid metabolism.³² O-acetylcarnitine and carnitine are reported in oxidative metabolism in mitochondrial³⁰ and hypoxemic stress.^{33,34} Adenine is required for the production of adenosine,³⁰ an endogenous purine nucleoside important in cellular energy metabolism. In response to cellular damage, levels of adenosine typically rise. Adenosine can be both proinflammatory for mast cell stimulation and anti-inflammatory.³⁵

Some metabolites relevant only in differentiating stable versus unstable asthma could be related to prolonged exertion. For example, lactate is elevated during anaerobic exercise.³⁰ Acetone is formed when stores of glucose are low and stores of oxaloacetate have been exhausted.³⁰ Further, when lactate is produced, alanine levels are expected to rise as part of gluconeogenesis.³⁰ Creatine is phosphorylated to phosphocreatine in muscle, and a rise in concentration may indicate a physiological state that is energy depleted.^{36,37} Finally, glycolate is also important in energy production by mitochondria.³⁸

Protein and amino acid metabolism appear to be altered. 1-Methylnicotinamide is a derivative of nicotinamide and is reported as protective against asthma exacerbation.³⁹ Phenylalanine is an essential amino acid critical in the production of tyrosine and catecholamines like epinephrine.³⁰ Tyrosine may also be modified by eosinophil activity.⁴⁰ We previously reported on modified tyrosine residues in sputum samples.⁴¹ Unfortunately, the levels of 3-chloro- or 3-bromotyrosine were below the limit of NMR detection.

Some metabolites have been reported in association with allergic inflammation. 1-Methylhistamine is a downstream metabolite from histamine. Serum levels of 1-methylhistamine are higher in patients with asthma. They rise acutely after an asthma attack and are lowered by antiallergy medications.⁴²⁻⁴⁴ Kynurenine is a product of tryptophan catabolism by the enzyme indoleamine 2,3, dioxygenase.⁴⁵ Indoleamine 2,3, dioxygenase activity is an important element in immune regulation in health and disease, including allergy.⁴⁶

There remain shortcomings of this study. For example, most of our children, as expected, had atopy and were on some prescription medication. It would be difficult to find a pediatric sample with asthma that did not have these confounders. Further, we cannot say with certainty that the differentiation of children with asthma in the ED from other comparison groups is a result of increased asthma or the results of increased work of breathing. It is possibly that all airway exacerbation would look the same in the ED. Because this was our first attempt at using this approach, we are designing further studies, mostly in adults and animals, to answer these questions.

Without the benefit of a history, one would never suspect that our children with stable asthma had a chronic illness. Indeed, it is fascinating that urine NMR analysis sees anything different between the outpatient asthma group and the healthy controls. We suggest that having another objective test could assist a primary care physicians in convincing themselves and the patients/families that the breathing problem is related to asthma. Even with a good history, physicians know that there are many diseases that mimic asthma. Virus-induced cough in the acute phase could appear somewhat similar to asthma acutely. One could expect that after an acute virus-induced cough, the patient would return to normal health/metabolism. In contrast, even in between virus-induced exacerbations, the asthma patient still has asthma inflammation and likely is not "normal" in terms of metabolism. This comparison of healthy versus stable asthma was chosen as the first step in creating the diagnostic. The next steps will be to compare our stable asthma population to other chronic airway diseases. In addition, we are following patients longitudinally to determine the metabolites that predict impending exacerbation.

In conclusion, we provide proof-of-concept evidence that the analysis of excreted urine metabolites measured by ¹H-NMR can be used to differentiate stable asthma from healthy controls and patients with asthma undergoing asthma exacerbation. We believe development of urine metabolomic analysis will lead to improved diagnostic capabilities for physicians treating patients with asthma and other airway diseases. Just as magnetic resonance imaging technology has become commonplace, NMR technology could easily be incorporated into a standard laboratory, especially with the advent of enhancements (ie, robotics) that lower operational costs. The ability to use urine as a diagnostic tool makes the methodology readily applicable to standard

clinical settings. This metabolic approach could also provide new insights into the pathophysiology of airway dysfunction, suggesting novel pathways for drug discovery.

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Clinical implications: Objective measurements for diagnosis and management of asthma are not used in a typical doctor's office. We suggest that development of a metabolomic approach through urine NMR analysis as a solution.

REFERENCES

- Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008;38:872-97.
- Taylor DR, Pavord ID. Biomarkers in the assessment and management of airways diseases. *Postgrad Med J* 2008;84:628-34, quiz 33.
- Nadel JA, Murray JF, Nadel JA. Textbook of respiratory medicine. Philadelphia, PA: W.B. Saunders; 2000.
- Fixman ED, Stewart A, Martin JG. Basic mechanisms of development of airway structural changes in asthma. *Eur Respir J* 2007;29:379-89.
- Expert Panel report 3 (EPR3): guidelines for the diagnosis and management of asthma NHLBI produced publications 2007; section 3: overview and component 1: measures of assessment and monitoring. (452 K) 39.
- Brightling CE, Green RH, Pavord ID. Biomarkers predicting response to corticosteroid therapy in asthma. *Treat Respir Med* 2005;4:309-16.
- Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715-21.
- Hargreave FE. Quantitative sputum cell counts as a marker of airway inflammation in clinical practice. *Curr Opin Allergy Clin Immunol* 2007;7:102-6.
- Scichilone N, Deykin A, Pizzichini E, Bellia V, Polosa R. Monitoring response to treatment in asthma management: food for thought. *Clin Exp Allergy* 2004;34: 1168-77.
- Szeffler SJ, Mitchell H, Sorkness CA, Gergen PJ, O'Connor GT, Morgan WJ, et al. Management of asthma based on exhaled nitric oxide in addition to guideline-based treatment for inner-city adolescents and young adults: a randomised controlled trial. *Lancet* 2008;372:1065-72.
- Xu EY, Schaefer WH, Xu Q. Metabolomics in pharmaceutical research and development: metabolites, mechanisms and pathways. *Curr Opin Drug Discov Devel* 2009;12:40-52.
- Gowda GA, Zhang S, Gu H, Asiago V, Shanaiah N, Raftery D. Metabolomics-based methods for early disease diagnostics. *Expert Rev Mol Diagn* 2008;8:617-33.
- Beckwith-Hall BM, Nicholson JK, Nicholls AW, Foxall PJ, Lindon JC, Connor SC, et al. Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. *Chem Res Toxicol* 1998;11:260-72.
- Godet C, Hira M, Adoun M, Eugène M, Robert R. Rapid diagnosis of alcoholic ketoacidosis by proton NMR. *Intensive Care Med* 2001;27.
- Forsythe IJ, Wishart DS. Exploring human metabolites using the human metabolome database. *Curr Protoc Bioinformatics* 2009;Chapter 14:Unit14 8.
- Skappak Ejs C, Cook K, Rowe B, Adamko DJ. The use of nuclear magnetic resonance analysis of urine in the diagnosis of asthma. *Am J Respir Crit Care Med* 2009;179:A2531.
- Becker A, Lemiere C, Berube D, Boulet LP, Ducharme FM, FitzGerald M, et al. Summary of recommendations from the Canadian Asthma Consensus guidelines, 2003. *CMAJ* 2005;173:S3-11.
- Kozyrskyj AL, HayGlass KT, Sandford AJ, Pare PD, Chan-Yeung M, Becker AB. A novel study design to investigate the early-life origins of asthma in children (SAGE study). *Allergy* 2009;64:1185-93.
- Saude EJ, Slupsky CM, Sykes BD. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics* 2006;2:113-23.
- Saude EJ, Sykes BD. Urine stability for metabolomic studies: effects of preparation and storage. *Metabolomics* 2007;3:19-27.
- Saude EJ, Adamko D, Rowe BH, Marrie T, Sykes BD. Variation of metabolites in normal human urine. *Metabolomics* 2007;3:439-51.

22. Lenz EM, Bright J, Wilson ID, Hughes A, Morrisson J, Lindberg H, et al. Metabonomics, dietary influences and cultural differences: a ¹H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J Pharm Biomed Anal* 2004;36:841-9.
23. Saude EJ, Obiefuna IP, Somorjai RL, Ajamian F, Skappak C, Ahmad T, et al. Metabolomic biomarkers in a model of asthma exacerbation: urine nuclear magnetic resonance. *Am J Respir Crit Care Med* 2009;179:25-34.
24. Wojnarowski C, Roithner B, Koller DY, Halmerbauer G, Gartner C, Tauber E, et al. Lack of relationship between eosinophil cationic protein and eosinophil protein X in nasal lavage and urine and the severity of childhood asthma in a 6-month follow-up study. *Clin Exp Allergy* 1999;29:926-32.
25. Severien C, Artlich A, Jonas S, Becher G. Urinary excretion of leukotriene E4 and eosinophil protein X in children with atopic asthma. *Eur Respir J* 2000;16:588-92.
26. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003;3:721-32.
27. Aragonés J, Fraisl P, Baes M, Carmeliet P. Oxygen sensors at the crossroad of metabolism. *Cell Metab* 2009;9:11-22.
28. Shockcor JP, Holmes E. Metabonomic applications in toxicity screening and disease diagnosis. *Curr Top Med Chem* 2002;2:35-51.
29. Azmi J, Connelly J, Holmes E, Nicholson JK, Shore RF, Griffin JL. Characterization of the biochemical effects of 1-nitronaphthalene in rats using global metabolic profiling by NMR spectroscopy and pattern recognition. *Biomarkers* 2005;10:401-16.
30. Nelson DL, Lehninger AL, Cox MM. *Lehninger principles of biochemistry*. 5th ed. New York: W.H. Freeman; 2008.
31. Leipnitz G, Seminotti B, Haubrich J, Dalcin MB, Dalcin KB, Solano A, et al. Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. *J Neurosci Res* 2008;86:683-93.
32. Kumps A, Duez P, Mardens Y. Metabolic, nutritional, iatrogenic, and artifactual sources of urinary organic acids: a comprehensive table. *Clin Chem* 2002;48:708-17.
33. Pauly DF, Pepine CJ. The role of carnitine in myocardial dysfunction. *Am J Kidney Dis* 2003;41:S35-43.
34. Oka T, Itoi T, Terada N, Nakanishi H, Taguchi R, Hamaoka K. Change in the membranous lipid composition accelerates lipid peroxidation in young rat hearts subjected to 2 weeks of hypoxia followed by hyperoxia. *Circ J* 2008;72:1359-66.
35. Brown RA, Spina D, Page CP. Adenosine receptors and asthma. *Br J Pharmacol* 2008;153:S446-56.
36. Benedict JD, Kalinsky HJ, Scarrone LA, Wertbeim AR, Stetten D, Jr. The origin of urinary creatine in progressive muscular dystrophy. *J Clin Invest* 1955;34:141-5.
37. Fitch C, Sinton D. A study of creatine metabolism in diseases causing muscle wasting. *J Clin Invest* 1964;43:444-52.
38. Baker PR, Cramer SD, Kennedy M, Assimios DG, Holmes RP. Glycolate and glyoxylate metabolism in HepG2 cells. *Am J Physiol Cell Physiol* 2004;287:C1359-65.
39. Bekier E, Wyczolkowska J, Szyk H, Maslinski C. The inhibitory effect of nicotinamide on asthma-like symptoms and eosinophilia in guinea pigs, anaphylactic mast cell degranulation in mice, and histamine release from rat isolated peritoneal mast cells by compound 48-80. *Int Arch Allergy Appl Immunol* 1974;47:737-48.
40. Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, et al. Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest* 2000;105:1455-63.
41. Saude EJ, Lacy P, Musat-Marcu S, Mayes DC, Bagu J, Man SF, et al. NMR analysis of neutrophil activation in sputum samples from patients with cystic fibrosis. *Magn Reson Med* 2004;52:807-14.
42. Nishiwaki F, Kuroda K, Inoue Y, Endo G. Determination of histamine, 1-methylhistamine and N-methylhistamine by capillary electrophoresis with micelles. *Biomed Chromatogr* 2000;14:184-7.
43. Takei S, Shimago A, Iwashita M, Kumamoto T, Kamuro K, Miyata K. Urinary N-methylhistamine in asthmatic children receiving azelastine hydrochloride. *Ann Allergy Asthma Immunol* 1997;78:492-6.
44. Stephan V, Zimmermann A, Kuhr J, Urbanek R. Determination of N-methylhistamine in urine as an indicator of histamine release in immediate allergic reactions. *J Allergy Clin Immunol* 1990;86:862-8.
45. Tan P, Bharath A. Manipulation of indoleamine 2,3 dioxygenase; a novel therapeutic target for treatment of diseases. *Expert Opin Ther Targets* 2009.
46. Odemuyiwa SO, Ghahary A, Li Y, Puttagunta L, Lee JE, Musat-Marcu S, et al. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. *J Immunol* 2004;173:5909-13.

METHODS

Patient characteristics

Stable asthma in the outpatient clinic. Children with asthma ($n = 73$) were recruited from the Stollery Children's Hospital outpatient clinic and ED (Table E1). Children with stable asthma were initially referred to a pediatric pulmonary or allergy subspecialist clinic and enrolled after at least 1 clinic visit, having met diagnostic criteria for atopic asthma as described in the Canadian Consensus guidelines.^{E1} All children had airway hyperresponsiveness as determined by a methacholine challenge test ($PC_{20} < 8$ mg/mL) or a positive response to bronchodilator ($>15\%$ improvement in FEV_1). As might be expected, most of the children selected were also atopic on the basis of at least 1 positive skin test response to a panel of common aeroallergens.

Unstable asthma in the ED. Children in the ED with acute asthma ($n = 20$) were selected on the basis of 1 or more of the following: (1) increasing asthma symptoms (eg, cough, wheeze, shortness of breath, or chest tightness) requiring assessment and a history of similar episodes, (2) clinical or symptomatic response to inhaled bronchodilator therapy, and/or (3) had a previous history of physician-diagnosed asthma. Patients had to present primarily for acute asthma (not a simple prescription refill) and were excluded if they had acute pneumonia, needed immediate resuscitation (status asthmaticus), had cognitive impairment, or had a known immunodeficiency.

Controls. (C): Healthy age and sex-matched controls ($n = 42$) were recruited from the community and from the University of Manitoba, SAGE birth cohort.^{E2} Healthy controls were excluded if they had any known underlying lung disease (eg, chronic cough or wheeze, CF, asthma, or oral steroid use). Subjects were excluded if they had a known nonrespiratory chronic inflammatory/infective disorder (eg, malignancy, cardiac disease, immunodeficiency, or neonatal lung disease associated with prematurity). The children from the SAGE birth cohort ($n = 10$) were also known to have negative results for aeroallergen skin testing and methacholine responsiveness. Patients were enrolled after their parents provided written informed consent as approved by the respective health research ethics boards of the University of Alberta and the University of Manitoba, in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Urine sample collection

A single urine sample was collected from each child at each visit in a standard orange-topped 50-mL specimen container and promptly placed in a freezer at the outpatient clinic or ED (-20°C). Within 3 hours of collection, the urine samples were stored in a -80°C freezer. Each sample was coded on freezing for future analyses. Although most samples were analyzed within 1 month of collection, we have previously reported that such samples can be stable in the freezer for up to a year.^{E2-E4} We were aware of the variability in urine metabolomic data that can occur within each person for diet and time of day^{E4,E5}; however, our objective was to design a test that could be used in a typical clinical setting, especially for young children. Thus, we did not stipulate a single time of day for each urine collection, nor did we mandate dietary restrictions. We hypothesized that the metabolites of interest would be altered sufficiently between disease and nondisease groups that such intrapersonal variability would be superseded.

Sample preparation

Urine samples were thawed only once in a biosafety fume hood, and a 630- μL aliquot was removed and placed in a 1.5-mL Eppendorf tube followed by the addition of 70 μL of a reference buffer solution (4.9 mmol/L disodium-2, 2-dimethyl 2-silapentane-5-sulfonate and 100 mmol/L imidazole in deuterium oxide; Chenomx Inc, Edmonton, Alberta, Canada). The pH of each sample was adjusted to 6.8 ± 0.1 by using HCl and NaOH before transferring an aliquot of 600 μL to a standard 5-mm glass NMR tube (Wilmad LabGlass, Wilmad, NJ). We have previously reported on these techniques using adult urine and have found no significant effects of such preprocessing on

the samples because all sample groups are handled similarly in a timely fashion.^{E2}

NMR acquisition

All ^1H -NMR spectra were acquired on a 600-MHz VNMRS spectrometer (Varian Inc, Palo Alto, Calif) equipped with a 5-mm inverse-proton (HX) probe with z-axis gradient coil. One-dimensional ^1H -NMR spectra were collected at 25°C by using the first increment of a 2-dimensional- ^1H , ^1H -NOESY (1-dimensional, 3-pulse NOESY, with a transmitter presaturation delay of 900 ms for water suppression during the preacquisition delay and 100 ms mixing time), and a spectral width of 7200 Hz (phase cycle available on request). The time-domain data points were 64 kilo complex points, acquisition time was 4 seconds, the 90° pulse was 6.8 microseconds, repetition time was 5 seconds, there were 4 steady-state scans, and the number of acquired scans was 32 per free induction decay. The data were apodized with an exponential window function corresponding to a line broadening of 0.5 Hz, 0-filled to 128k complex points, and Fourier transformed.^{E6}

Spectral and statistical analysis

Spectral identification and quantification of 70 identifiable metabolites was performed by using the Chenomx NMR Suite Professional software package Version 4.6 (Chenomx Inc). The software contains a database of known metabolites with their referenced spectral resonant frequencies or signatures. These known resonant frequencies were matched to the observed resonant frequencies of the collected spectra, enabling the qualitative and quantitative analysis of metabolites in urine (Fig 1, A). This procedure provides metabolite concentration accuracies in excess of 90%.^{E2} To account for hydration status of the subjects, metabolites were referenced to creatinine. As an example, we have plotted the value of a critical metabolite, 1-methylhistamine, for healthy control subjects versus those with stable asthma (Fig E1). As can be seen, although there is variability between individuals, this variability is small compared with the magnitude of change comparing the groups. In addition, it is important to note that there remains a significant degree of overlap between the groups; thus, we could not rely solely on 1 metabolite like 1-methylhistamine to be the diagnostic variable. Metabolite concentrations were mean-centered followed by unit variance scaling (or z scoring). PLS-DA was performed (SIMCA-P 11, Umetrics), which determines the relationship between the response vector Y (patient group) and the matrix X (concentration of each metabolite) by simultaneous projections of both Y and X spaces to a plane. Seven-fold internal cross-validation was performed. This process identifies the metabolites whose concentrations differed significantly between groups of patients, expressed as a coefficient of variation plot (Fig 1, B). As might be expected, most metabolites do not differ greatly between groups. In contrast, the greater the consistent difference in metabolite concentration between groups, the more important a metabolite becomes in creating the final model, seen as a VIP (Fig 1, C). Metabolites of low significance can be detrimental to diagnostic accuracy. On the basis of the list of metabolites included in the algorithm, PLS-DA generated a prediction score (0-1) of unclassified, blind data not part of the model (ie, scores <0.5 would be predicted to be healthy subjects).

To see how scores are calculated for each individual, consider the following example with 2 individuals, 1 with no asthma and 1 with asthma. The metabolite profile for each person is listed in Table E2. To compute the score for each individual, the following steps are performed. First, creatinine is removed from the analysis because it was used to standardize all other values to account for hydration (and hence cannot be used to distinguish among individuals). Second, each metabolite score for each individual is scaled to according to the following formula:

$$M_{i,j,\text{scaled}} = \frac{M_{i,j,\text{unscaled}} - A_j}{S_j},$$

where $M_{i,j,\text{scaled}}$ and $M_{i,j,\text{unscaled}}$ are the j^{th} metabolite for the i^{th} individual after and before scaling, respectively, A_j is the average for the j^{th} metabolite over all individuals, and S_j is the standard deviation of j^{th} metabolite for all individuals.

Thus, for example, the scaled 3-methylapitate score for NM06692_233NA (individual 1) is given by

$$M_{1,4,scaled} = \frac{0.0009 - 0.0026}{0.0019} = -0.8947.$$

Next, a score is computed for each metabolite by multiplying the scaled score by the β -value for that metabolite. This product for NM06692_233NA would be

$$-0.8947 * -0.0194 = 0.0174.$$

To get individual scores, the sum of all metabolite scores is computed. The average score for the training set (0.3548) is added to each individual score to get the final score for each individual. Using this algorithm, the score for NM06692_233NA is 0.96, whereas the score for NM04428P is 0.22. A value of more than 0.5 indicates that the individual belongs to the no asthma group, whereas a value of less than 0.5 indicates that an individual belongs to the patients with asthma class. A score of exactly 0.5 would indicate that the person has equal probability of belonging to each class. However, no individual had a score of exactly 0.5.

The proposed approach uses a large number of metabolites to compute an aggregate score. Metabolites with large positive or negative β -value classification (eg, phenylalanine) are deemed important by the algorithm for classification. Metabolites with low positive or negative scores (eg, tyrosine) are deemed by the algorithm to have little diagnostic value. The sign associated with each β -value can be used to identify how individual metabolite concentrations vary between classes. Thus, for example, tyrosine has a negative score, which means individuals with higher tyrosine levels will tend to have lower total scores and are therefore more likely to belong to the group of patients with asthma.

To choose the most accurate list of metabolites, we removed metabolites listed as lower in significance on the VIP until we were satisfied that the model

could correctly diagnose healthy control subjects not part of the model. We set a false-positive rate of 5% for healthy control children as an acceptable limit. We applied the same approach to the separation of stable asthma versus unstable asthma by using blind stable outpatient subjects with asthma and a 5% error rate. This resulted in the most sensitive models with respect to both R2 and Q2. R2 represents a measure of the models fit of the data. Q2 represents a measure of the models predictive ability. Values closer to 1 are more robust for each. We previously reported using this technique in an animal model of asthma.^{E7}

REFERENCES

- E1. Becker A, Lemiere C, Berube D, Boulet LP, Ducharme FM, FitzGerald M, et al. Summary of recommendations from the Canadian Asthma Consensus guidelines, 2003. *CMAJ* 2005;173:S3-11.
- E2. Saude EJ, Slupsky CM, Sykes BD. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics* 2006;2:113-23.
- E3. Saude EJ, Sykes BD. Urine stability for metabolomic studies: effects of preparation and storage. *Metabolomics* 2007;3:19-27.
- E4. Saude EJ, Adamko D, Rowe BH, Marrie T, Sykes BD. Variation of metabolites in normal human urine. *Metabolomics* 2007;3:439-51.
- E5. Lenz EM, Bright J, Wilson ID, Hughes A, Morrisson J, Lindberg H, et al. Metabolomics, dietary influences and cultural differences: a 1H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J Pharm Biomed Anal* 2004;36:841-9.
- E6. Kumar A, Ernst RR, Wuthrich K. A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. *Biochem Biophys Res Commun* 1980;95:1-6.
- E7. Saude EJ, Obiefuna IP, Somorjai RL, Ajamian F, Skappak C, Ahmad T, et al. Metabolomic biomarkers in a model of asthma exacerbation: urine nuclear magnetic resonance. *Am J Respir Crit Care Med* 2009;179:25-34.

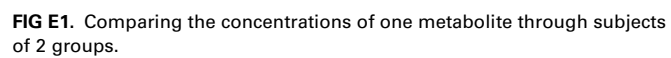


TABLE E1. Characteristics of children enrolled in the study

	Outpatients with asthma (n = 40)	Asthma ED (n = 20)	Healthy controls (n = 32)	Blind healthy controls (n = 10)	Blind outpatients with asthma (n = 33)
Median age (y)	9.0	5.0	8.4	14.0	10.0
Range	3-13	2-14	4-13	13-14	4-13
Sex (male/female), n	28/12	10/10	18/14	8/2	22/11
Atopy status (yes/no), n	32/8	13/7	NA	0/10	23/10
FEV ₁ , mean, SD (% predicted)	87.6, 16.0	NA	NA	91.4, 12.3	86.9, 17.1
ICS use (yes/no), n	27/13	9/11	NA	NA	24/9

ICS, Inhaled corticosteroid; NA, not applicable/available.

TABLE E2. List of metabolites for 2 individuals

Metabolite/patient	Average for all individuals	SD for all individuals	NM06692_23 2NA (No asthma)	NM04428P (Asthma positive)	β values
1-MethylHistamine	0.0221	0.0296	0.000	0.0265	−0.0598
1-Methylnicotinamide	0.0105	0.0072	0.0174	0.0049	−0.0745
2-Hydroxyisobutyrate	0.0071	0.0025	0.0037	0.0059	−0.0522
2-Oxoglutarate	0.0113	0.0102	0.0073	0.0157	0.0829
3-Methyladipate	0.0026	0.0019	0.0009	0.0020	−0.0194
4-Aminohippurate	0.0014	0.0012	0.0009	0.0010	−0.0261
4-Pyridoxate	0.0013	0.0014	0.0027	0.0000	0.0075
Adenine	0.0001	0.0004	0.0000	0.0010	−0.0193
Adenosine	0.0006	0.0016	0.0000	0.0010	−0.0307
Carnitine	0.0105	0.0090	0.0018	0.0029	0.0212
Creatinine	1	0	1.0000	1.0000	0
Hippurate	0.2130	0.2594	0.1136	0.5295	−0.0149
Homovanillate	0.0033	0.0033	0.0009	0.0049	−0.0349
Kynurenine	0.0019	0.0020	0.0000	0.0000	−0.0465
Methylamine	0.0022	0.0019	0.0073	0.0010	0.0448
O-Acetylcarnitine	0.0050	0.0030	0.0073	0.0020	0.0669
Phenylalanine	0.0083	0.0047	0.0055	0.0069	−0.1019
Succinate	0.0283	0.0266	0.0119	0.0118	−0.0483
Threonine	0.0094	0.0055	0.0101	0.0059	0.0666
Trigonelline	0.0060	0.0053	0.0037	0.0079	0.0075
Trimethylamine Noxide	0.0481	0.0383	0.2005	0.0216	0.0413
Tryptophan	0.0064	0.0072	0.0009	0.0098	−0.0425
Tyrosine	0.0114	0.0051	0.0082	0.0088	0.0046
Yellow-7.1	0.0078	0.0111	0.0000	0.0255	−0.0390

TABLE E3. The concentration of each metabolite used to discriminate the different groups of children. (α) - required for separation of Outpatient Asthma vs Control; (β) - required for separation of Outpatient Asthma vs. ED Asthma; (γ) - required for separation of Outpatient Asthma vs ED Asthma vs Control. Shown are median and interquartile ranges (IQR) in mmol of metabolite/mmol creatinine.

Metabolites (mmol/mmol creatinine)	Healthy controls	Outpatient asthma	Asthma in ED	Outpatient asthma blinded
1-MethylHistamine (α,β,γ)	0.003 (0.000, 0.006)	0.017 (0.008, 0.049)	0.007 (0.002, 0.010)	0.018 (0.009, 0.038)
1-Methylnicotinamide (α,β,γ)	0.005 (0.003, 0.007)	0.012 (0.008, 0.017)	0.005 (0.004, 0.008)	0.011 (0.005, 0.016)
2-Hydroxybutyrate	0.001 (0.001, 0.002)	0.002 (0.001, 0.002)	0.003 (0.002, 0.004)	0.003 (0.001, 0.004)
2-Hydroxyisobutyrate (α)	0.006 (0.005, 0.007)	0.007 (0.006, 0.008)	0.010 (0.010, 0.010)	0.007 (0.007, 0.010)
2-Methylglutarate (β,γ)	0.010 (0.009, 0.012)	0.011 (0.009, 0.015)	0.015 (0.010, 0.040)	0.012 (0.011, 0.014)
2-Oxoglutarate (α,β,γ)	0.015 (0.011, 0.024)	0.005 (0.004, 0.009)	0.018 (0.012, 0.029)	0.012 (0.005, 0.021)
3-OH-3-Methylglutarate (β,γ)	0.004 (0.003, 0.005)	0.003 (0.003, 0.005)	0.005 (0.004, 0.007)	0.004 (0.004, 0.006)
3-Methyladipate (α, β,γ)	0.002 (0.001, 0.003)	0.002 (0.002, 0.003)	0.005 (0.003, 0.008)	0.003 (0.002, 0.005)
4-Aminohippurate (α,β,γ)	0.001 (0.000, 0.002)	0.001 (0.001, 0.002)	0.000 (0.000, 0.002)	0.001 (0.001, 0.003)
4-Pyridoxate (α)	0.001 (0.001, 0.002)	0.001 (0.000, 0.002)	0.003 (0.002, 0.004)	0.002 (0.001, 0.003)
Acetone (β,γ)	0.003 (0.002, 0.003)	0.019 (0.015, 0.023)	0.007 (0.003, 0.043)	0.003 (0.002, 0.004)
Adenine (α,β)	0.000 (0.000, 0.000)	0.000 (0.000, 0.000)	0.000 (0.000, 0.000)	0.000 (0.000, 0.002)
Adenosine (α,γ)	0.000 (0.000, 0.000)	0.000 (0.000, 0.001)	0.001 (0.000, 0.001)	0.000 (0.000, 0.001)
Alanine (β,γ)	0.030 (0.024, 0.043)	0.035 (0.028, 0.044)	0.059 (0.041, 0.124)	0.043 (0.032, 0.058)
Carnitine (α,γ)	0.011 (0.006, 0.017)	0.007 (0.002, 0.016)	0.015 (0.008, 0.059)	0.008 (0.004, 0.014)
Creatine (β,γ)	0.104 (0.074, 0.141)	0.203 (0.050, 0.417)	0.219 (0.132, 0.460)	0.183 (0.075, 0.323)
Dimethylamine (β,γ)	0.034 (0.032, 0.038)	0.039 (0.032, 0.043)	0.054 (0.045, 0.066)	0.040 (0.034, 0.048)
Formate (β,γ)	0.029 (0.021, 0.049)	0.043 (0.027, 0.057)	0.045 (0.029, 0.055)	0.041 (0.026, 0.052)
Fumarate (β,γ)	0.000 (0.000, 0.001)	0.000 (0.000, 0.001)	0.002 (0.001, 0.007)	0.001 (0.000, 0.002)
Glucose (β,γ)	0.039 (0.036, 0.047)	0.041 (0.032, 0.053)	0.092 (0.035, 0.590)	0.048 (0.031, 0.073)
Glycolate (β,γ)	0.053 (0.050, 0.064)	0.024 (0.016, 0.042)	0.077, (0.064, 0.106)	0.057 (0.033, 0.083)
Hippurate (α)	0.158 (0.065, 0.241)	0.126 (0.083, 0.239)	0.200 (0.091, 0.348)	0.227 (0.087, 0.365)
Histidine (γ)	0.048 (0.036, 0.078)	0.028 (0.000, 0.062)	0.000 (0.000, 0.010)	0.009 (0.000, 0.043)
Homovanillate (α,γ)	0.002 (0.002, 0.003)	0.003 (0.002, 0.005)	0.017 (0.000, 0.085)	0.004 (0.002, 0.007)
Kynurenine (α,γ)	0.001 (0.000, 0.002)	0.002 (0.001, 0.003)	0.002, (0.000, 0.003)	0.002 (0.000, 0.003)
Lactate (β)	0.024 (0.021, 0.034)	0.026 (0.021, 0.037)	0.034 (0.027, 0.047)	0.028 (0.020, 0.044)
Methylamine (α,β)	0.003 (0.001, 0.003)	0.002 (0.001, 0.002)	0.002 (0.001, 0.003)	0.002 (0.001, 0.003)
Myo-Inositol (β,γ)	0.009 (0.005, 0.009)	0.010 (0.009, 0.015)	0.032 (0.013, 0.049)	0.013 (0.009, 0.022)
O-Acetylcarnitine (α,β,γ)	0.006 (0.004, 0.008)	0.004 (0.002, 0.005)	0.019 (0.009, 0.046)	0.005 (0.003, 0.007)
Oxaloacetate (β)	0.068 (0.057, 0.098)	0.069 (0.042, 0.084)	0.078 (0.069, 0.144)	0.071 (0.059, 0.095)
Phenylacetylglutamine (β,γ)	0.027 (0.022, 0.042)	0.073 (0.042, 0.104)	0.008 (0.005, 0.010)	0.031 (0.020, 0.080)
Phenylalanine (α,β,γ)	0.004 (0.003, 0.005)	0.010 (0.007, 0.013)	0.005 (0.004, 0.007)	0.010 (0.008, 0.016)
Succinate (α,γ)	0.015 (0.007, 0.018)	0.026 (0.016, 0.041)	0.039 (0.020, 0.065)	0.025 (0.019, 0.040)
Taurine (γ)	0.059 (0.032, 0.109)	0.076 (0.029, 0.113)	0.072 (0.036, 0.123)	0.056 (0.026, 0.108)
Threonine (α,γ)	0.012 (0.008, 0.015)	0.006 (0.005, 0.007)	0.006 (0.005, 0.011)	0.008 (0.005, 0.011)
Trigonelline (α)	0.004 (0.003, 0.007)	0.005 (0.002, 0.009)	0.005 (0.002, 0.012)	0.005 (0.002, 0.008)
Trimethylamine N-oxide (α,γ)	0.050 (0.037, 0.075)	0.032 (0.020, 0.045)	0.029 (0.019, 0.051)	0.040 (0.020, 0.065)
Tryptophan (α,β,γ)	0.004 (0.003, 0.006)	0.005 (0.003, 0.009)	0.004 (0.003, 0.007)	0.007 (0.003, 0.010)
Tyrosine (α,β)	0.010 (0.008, 0.015)	0.010 (0.008, 0.014)	0.015 (0.010, 0.019)	0.012 (0.009, 0.017)
Yellow-7.1 (α)	0.000 (0.000, 0.005)	0.004 (0.002, 0.016)	0.000 (0.000, 0.019)	0.000 (0.000, 0.006)
cis-Aconitate (β,γ)	0.000 (0.000, 0.000)	0.000 (0.000, 0.000)	0.006 (0.003, 0.009)	0.000 (0.000, 0.007)
trans-Aconitate (β)	0.004 (0.003, 0.005)	0.005 (0.003, 0.006)	0.004 (0.003, 0.007)	0.004 (0.003, 0.006)