

Metabolomic profiling of asthma and chronic obstructive pulmonary disease: A pilot study differentiating diseases

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Background: Differentiating asthma from other causes of chronic airflow limitation, such as chronic obstructive pulmonary disease (COPD), can be difficult in a typical outpatient setting. The inflammation of asthma typically is different than that of COPD, and the degree of inflammation and cellular damage varies with asthma severity. Metabolomics is the study of molecules created by cellular metabolic pathways.

Objectives: We hypothesized that the metabolic activity of adults with asthma would differ from that of adults with COPD. Furthermore, we hypothesized that nuclear magnetic resonance spectroscopy (NMR) would measure such differences in urine samples.

Methods: Clinical and urine-based NMR data were collected on adults meeting the criteria of asthma and COPD before and after an exacerbation ($n = 133$ and 38 , respectively) and from patients with stable asthma or COPD ($n = 54$ and 23 , respectively). Partial least-squares discriminant analysis was performed on the NMR data to create models of separation (86 metabolites were measured per urine sample). Some subjects' metabolomic data were withheld from modeling to be run blindly to determine diagnostic accuracy.

Results: Partial least-squares discriminant analysis of the urine NMR data found unique differences in select metabolites between patients with asthma and those with COPD seen in the emergency department and even in follow-up after

exacerbation. By using these select metabolomic profiles, the model could correctly diagnose blinded asthma and COPD with greater than 90% accuracy.

Conclusion: This is the first report showing that metabolomic analysis of human urine samples could become a useful clinical tool to differentiate asthma from COPD. (*J Allergy Clin Immunol* 2015;136:571-80.)

Key words: Metabolomics, asthma, chronic obstructive pulmonary disease, biomarkers, urine, nuclear magnetic resonance spectroscopy

Using objective measurements of airway inflammation to guide asthma therapy can produce superior therapeutic results compared with traditional measures alone (eg, symptoms and lung function).¹⁻³ For clinicians in a typical outpatient setting, detecting airway inflammation is often difficult. In addition, differentiating asthma from other respiratory diseases, such as chronic obstructive pulmonary disease (COPD), can be difficult because both have somewhat similar clinical presentations.⁴ Most clinicians continue to rely on patient history and examination before administering trials of therapy.

Metabolomics is the study of metabolic pathways and biochemical molecules created in a living system.^{5,6} By measuring changes in metabolites, biochemical effects induced

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Abbreviations used

ADMA:	Asymmetric dimethyl-arginine
COPD:	Chronic obstructive pulmonary disease
COV:	Coefficient of variation
ED:	Emergency department
FVC:	Forced vital capacity
NMR:	Nuclear magnetic resonance spectroscopy
NO:	Nitric oxide
ROC:	Receiver operating characteristic
PLS-DA:	Partial least-squares discriminant analysis
VIP:	Variables of importance plot

by a disease or its therapeutic intervention can be determined. ¹H-nuclear magnetic resonance spectroscopy (NMR) can be used to quantify metabolites within a biofluid.⁷ NMR is an attractive technology because it provides both qualitative and quantitative measurements while simultaneously studying a number of compounds in the same biological fluid. Urine is an excellent biological fluid because of its ease of collection in patients of all ages, low cell and protein content, and rich chemical composition.⁸

The pathology of asthma is different from that of COPD,⁹ and the degree of inflammation and cellular damage varies with disease severity. We hypothesized that the metabolic activity of asthmatic adults would differ from that of patients with COPD. We hypothesized that NMR would measure such differences in urine samples.

We previously reported that metabolomic profiling of urine could differentiate asthmatic patients from healthy subjects.^{10,11} In this report we demonstrate for the first time that analysis of metabolites in urine has the ability to differentiate adults with asthma from those with COPD both in the emergency department (ED) at the time of exacerbation and in follow-up after exacerbation.

METHODS

For details, see the [Methods](#) section in this article's Online Repository [et al.](#)^{10,11}

Patients' characteristics

Patients were enrolled after obtaining written informed consent, as approved by the University of Alberta and St Joseph's Healthcare Hamilton Health Research Ethics Boards.

Patients with asthma or COPD with exacerbation

Adults with asthma seen at the time of an exacerbation through either the University of Alberta (n = 110) or McMaster University (n = 23) were selected based on 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) bronchodilator response measured based on peak expiratory flow or significant clinical improvement; and/or (3) a previous history of physician-diagnosed asthma. Those from McMaster also had previously demonstrated greater than 12% reversibility to salbutamol or a positive methacholine challenge test result (PC₂₀ < 8 mg/mL). Adults with COPD seen in the ED at the University of Alberta (n = 38) were selected based on 1 or more of the following: (1) increasing symptoms (eg, cough, wheeze, shortness of breath, or chest tightness) requiring assessment and a history of similar episodes and/or (2) a previous history of physician-diagnosed COPD. All patients had to

present primarily for acute asthma or COPD exacerbation (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. Some subjects consented to return for a follow-up survey and urine collection. Available clinical data are shown in [Table I](#).

Patients with asthma or COPD who are stable in an outpatient setting. Adults with asthma (n = 58) or COPD (n = 24) were recruited in an outpatient setting at the University of Alberta. Asthma diagnosis was based on the following: (1) a previous history of physician-diagnosed asthma; (2) previously demonstrated greater than 12% reversibility to salbutamol or a positive methacholine challenge test result (PC₂₀ < 8 mg/mL); (3) no current smoking; and (4) a less than 10 pack-year smoking history. COPD diagnosis was based on 1 or more of the following: (1) a previous history of physician-diagnosed COPD and/or (2) a postbronchodilator FEV₁/forced vital capacity (FVC) ratio of less than 70%. Patients were excluded if they were taking systemic corticosteroids or prescription medications for diabetes or hypothyroidism. Available clinical data are shown in [Table I](#).

Statistical analysis of clinical data

GraphPad Prism software (version 6; GraphPad Software, San Diego, Calif) was used for statistical analysis of the clinical data. A nonparametric *t* test was used to compare 2 group experiments. Repeated-measures ANOVA with the Bonferroni multiple comparison test was used to compare multiple groups. Data are presented as means ± 1 SEM or medians with interquartile ranges. A *P* value of less than .05 was considered significant.

Urine sample collection and preparation

A urine sample was collected from each patient and promptly placed in a freezer (−20°C). Within 3 hours of collection, the urine samples were stored in a −80°C freezer. 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 addition of 70 μL of a reference buffer solution.

NMR spectral and statistical analysis

Quantification of 86 metabolites was performed with the Chenomx NMR Suite Professional software package (version 4.6; Chenomx, Edmonton, Alberta, Canada). The software contains a database of known metabolites, with their referenced spectral resonant frequencies or signatures enabling the qualitative and quantitative analysis of metabolites in urine. Metabolites were referenced to creatinine and the values were log transformed to account for the hydration status of the subjects. Partial least-squares discriminant analysis (PLS-DA) was performed (SIMCA-P 11; Umetrics, Umea, Sweden). This process identifies the metabolites with concentrations that differed significantly between groups of patients. As might be expected, most metabolites do not differ greatly between groups, and including metabolites of low significance is detrimental to creating an accurate diagnostic model of separation. Metabolites with consistently greater differences in concentration between groups are displayed by the software as a coefficient of variation (COV) plot and a variables of importance plot (VIP). To choose an accurate list of metabolites, we removed metabolites until we were satisfied that the model could correctly diagnose blinded samples not part of the model. We set a false-positive rate of 5% to 10% as an acceptable limit. We previously reported using this technique in an animal model of asthma¹⁰ and children with asthma. The PLS-DA-based model was then used to generate a prediction score (0-1) of unclassified blinded data not part of the model (ie, scores <0.5 would be predicted to be asthmatic patients versus patients with COPD). The receiver operating characteristic (ROC) curve was generated to evaluate the relationship between the sensitivity and specificity of the prediction score at various cut points and to consider the score's overall function as a predictor.

TABLE I. Subjects' characteristics

	Alberta asthma model (n = 110)	Alberta COPD model (n = 38)	McMaster asthma test set (n = 23)	Alberta outpatient asthma test set (n = 58)	Alberta outpatient COPD test set (n = 23)
Mean $\pm$ SD age (y)	32.7 $\pm$ 11.3	69.4 $\pm$ 12.2	49.9 $\pm$ 15.7	33.9 $\pm$ 13.2	59.2 $\pm$ 13.1
Female sex, no. (% of cohort)	70 (62)	23 (52)	14 (61)	34 (59)	10 (43)
Current smoker, no. (%)	32 (28.3)	19 (43.2)	0	0	7 (32)
Pack years of smoking, median (IQR)	5 (2-8)	33.3 (25-43)	Unknown	0 (0-2.75)	30 (15-44)
FEV ₁ (L), median (IQR)	Unknown	0.81 (0.58-1.22)	1.81 (1.12-2.30)	3.31 (2.87-3.62)	1.96 (1.42-2.41)
FEV ₁ (%), median (IQR)	Unknown	35.0 (25.0-50.8)	66.5 (37.0-82.0)	91.5 (81.8-98.0)	69.0 (54.0-77.3)
Inhaled corticosteroids, no. (%)	78 (68)	32 (80)	Unknown	46 (85)	21 (95)

IQR, Interquartile range.

RESULTS

Baseline patients' characteristics for the metabolomic model of asthma versus COPD

The populations with COPD were both significantly older than the asthma groups, although the asthma group from McMaster was significantly older than the other asthma cohorts in Edmonton. The sex difference among these groups was similar. Subjects having an asthma exacerbation at the University of Alberta were not excluded if they currently smoked; however, the number of pack years of smoking was much lower in the asthma group compared with the COPD exacerbation group (a median of 5 compared with 33 pack years, respectively). None of the subjects recruited with asthma at McMaster were current smokers, and all had a smoking history of less than 10 pack years. All the patients with COPD used to create the models of asthma versus COPD in the ED or in follow-up had smoked at one time. Their pack years of smoking were also substantially higher compared with those of the asthma groups. As such, the models are largely based on COPD related to prior smoking. There were 4 patients with COPD in the Alberta COPD outpatient test set who denied ever smoking. That being said, there were also a number of asthmatic patients included in the models who were current smokers.

Regarding lung function data, FEV₁ was significantly lower in the COPD cohort compared with the blinded asthma cohort from McMaster ($P < .003$). Spirometric data were not available from the asthma model subjects from Alberta. Only the McMaster cohort had skin tests performed; 13 (56%) of the 23 subjects had positive skin test responses for at least 1 aeroallergen.

Baseline patient characteristics for the test set of outpatient asthma versus COPD

Adults with a diagnosis of asthma or COPD were recruited for urine sampling in an outpatient setting (Table I). The COPD population was significantly older than the asthma group. The sex difference between these groups was similar. Asthmatic patients were excluded based on smoking history, and as such, they had a lower pack-year history of smoking compared with the COPD group. Regarding lung function data, FEV₁ percent predicted and the FEV₁/FVC ratio were both significantly lower in the COPD cohort compared with the blinded asthma cohort from McMaster ($P < .0001$ each).

Urine NMR metabolomic profiling can differentiate asthma versus COPD exacerbation

We compared the metabolites of patients with asthma and those with COPD. Most of the metabolites excreted in the urine did not

differ greatly between groups, and adding metabolites of low importance rendered the PLS-DA-based model less accurate. To remove irrelevant metabolites, we randomly withheld urine samples from 19 asthmatic patients to be used as a test set. The model used 91 subjects with asthma and 38 with COPD. Using the test set, we removed metabolites that allowed the model to correctly classify these blinded asthmatic patients with 95% accuracy (18/19 with a blinded PLS-DA prediction score >0.5 , data not shown). The final list of remaining metabolites used one component consisting of 22 metabolites in the VIP list, resulting in an R^2 value of 0.72 and a Q^2 value of 0.69. The importance of the metabolites used for separation of these 2 groups is shown as a VIP (Fig 1, A). The differences in concentration of these metabolites between groups are shown as the COV plot (Fig 1, B). Metabolites with higher levels in patients with COPD than in asthmatic patients are shown with bars increasing above zero, whereas those with higher levels in asthmatic patients than in patients with COPD decrease below zero. The graphic presentation of the quality of separation between groups in the model is shown by their respective PLS-DA scores (Fig 1, C). The final metabolites chosen and their concentrations are shown in Table II.

Validity assessment (asthma vs COPD during exacerbation)

To validate the proposed metabolomic model and its applicability as a diagnostic tool for asthma in the ED setting, we entered the concentrations of metabolites from adults ($n = 23$) with asthma exacerbations from McMaster. The urine metabolite values for these patients were entered into the PLS-DA model without a diagnosis, and thus the computer was blinded for the PLS-DA-derived model of asthma versus COPD. The individual PLS-DA prediction scores are shown in Fig 1, C. A threshold PLS-DA score of greater than 0.5 was used to designate asthma. The model was able to correctly diagnose the blinded asthma samples in 20 of 23 samples (87% accuracy). We had insufficient ED patients with COPD to use as a blinded test set, and thus we could not create an ROC curve for diagnostic accuracy.

Urine NMR metabolomic profiling can differentiate asthma versus COPD in follow-up after an exacerbation

Urine samples were studied from the patients with asthma or those with COPD 1 to 2 weeks after exacerbation to determine whether this metabolomic approach could be used to differentiate asthma and COPD in the outpatient setting (Table I). These follow-up data were also obtained to determine a measure of

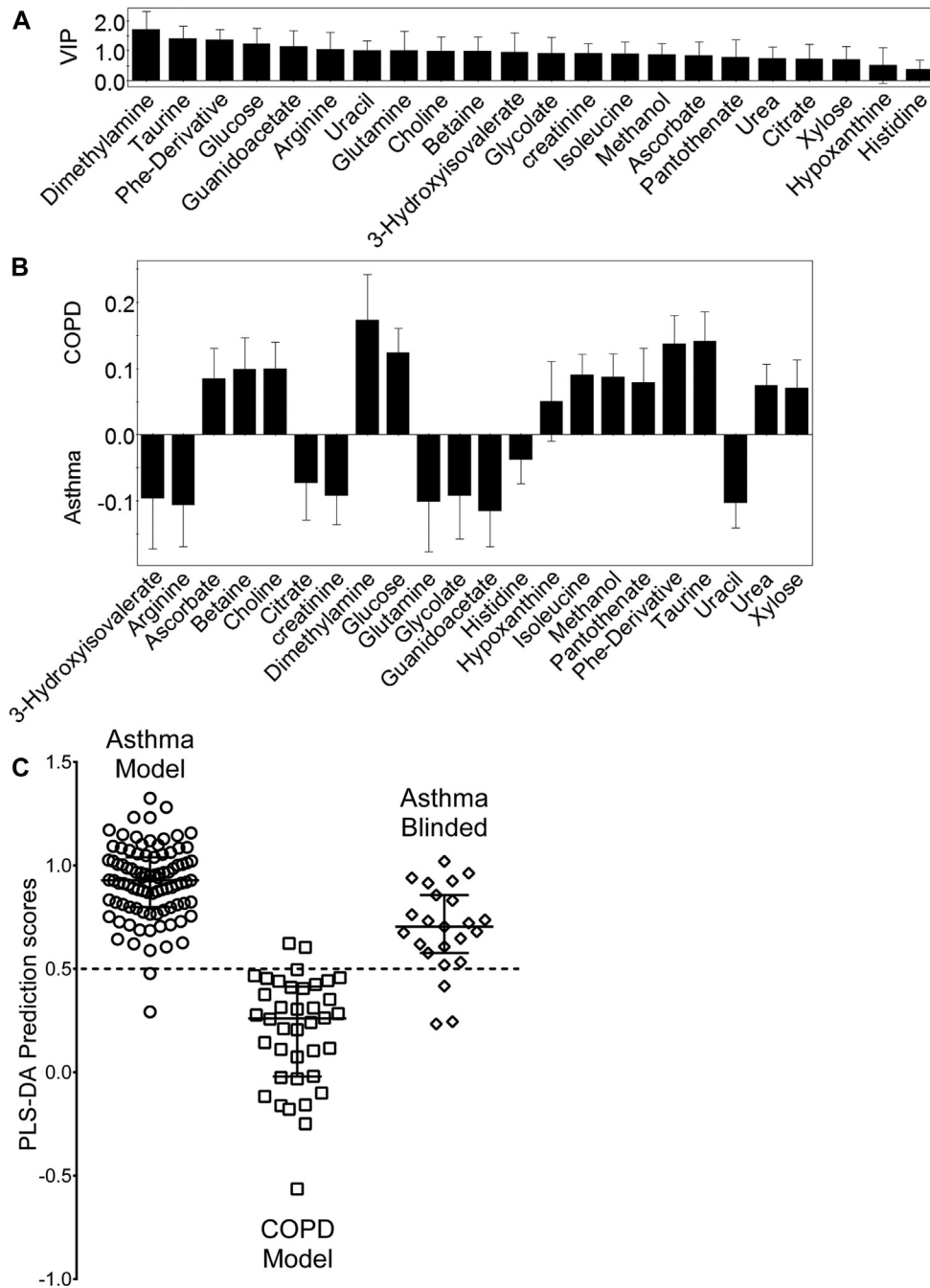


FIG 1. Metabolomic model of exacerbation of asthma versus COPD in the ED. Urine metabolite levels were measured in patients in the ED with either an exacerbation of asthma or COPD. Using a blind test set, PLS-DA (SIMCA P-11) of these metabolites created a model of separation ($R^2 = 0.72$, $Q^2 = 0.69$). **A**, VIP ranking the metabolites according to their significance in the model. **B**, Scaled and centered metabolite differences between groups shown as the COV plot. Metabolite levels that were higher in patients with COPD than in asthmatic patients are shown with bars rising above zero, whereas levels that were higher in asthmatic patients than in those with COPD are less than zero. **C**, PLS-DA prediction scores for each subject, with error bars representing medians and interquartile ranges. The PLS-DA algorithm separates groups of data based on a score of 0 to 1. In this case a value of greater than 0.5 indicates the subject has asthma, whereas a value of less than 0.5 indicates the subject has COPD.

reproducibility for this metabolomic approach (ie, if these data were simply dependent on confounders, such as time of day or diet). Unfortunately, some subjects were lost in follow-up in each cohort. We were left with 61 asthmatic patients and 23 patients with COPD to create a follow-up model. We withheld

the same returning asthmatic patients (now $n = 16$) as a test set to remove metabolites of low importance. The metabolomic model correctly classified the blinded asthma test set with 94% accuracy (16/17). The final list of metabolites used to separate postexacerbation asthma versus COPD used 2 components

TABLE II. The concentration of each metabolite for each subject group is shown as the median and IQR in millimoles of metabolite per millimole of creatinine (except for creatinine, for which actual values are shown [in millimoles])

	COPD in the ED			Asthma in the ED			COPD in the ED: follow-up			Asthma in the ED: follow-up		
	Median	IQR		Median	IQR		Median	IQR		Median	IQR	
1-Methylnicotinamide (β)	0.0034	0.0010	0.0055	0.0024	0.0010	0.0037	0.0035	0.0010	0.0071	0.0016	0.0010	0.0034
3-Hydroxyisovalerate (α,β)	0.0050	0.0026	0.0088	0.0066	0.0047	0.0097	0.0034	0.0017	0.0044	0.0063	0.0051	0.0082
Arginine (α,β)	0.0070	0.0065	0.0245	0.0189	0.0134	0.0237	0.0082	0.0070	0.0234	0.0190	0.0146	0.0240
Ascorbate (α)	0.0005	0.0005	0.0302	0.0005	0.0005	0.0005	0.0005	0.0005	0.0131	0.0005	0.0005	0.0005
Betaine (α,β)	0.0138	0.0056	0.0278	0.0071	0.0050	0.0105	0.0095	0.0049	0.0173	0.0057	0.0034	0.0088
Choline (α,β)	0.0029	0.0006	0.0047	0.0006	0.0006	0.0025	0.0024	0.0011	0.0041	0.0013	0.0006	0.0024
Citrate (α)	0.1986	0.0819	0.3327	0.2443	0.1744	0.3673	0.2752	0.1387	0.4160	0.2417	0.1534	0.4114
Creatinine (α)	6,671	2,394	9,324	11,252	6,094	18,286	5,213	2,910	7,474	10,426	5,928	18,413
Dimethylamine (α,β)	0.0457	0.0395	0.0525	0.0340	0.0300	0.0391	0.0419	0.0369	0.0464	0.0330	0.0289	0.0375
Glucose (α,β)	0.0711	0.0337	1.6860	0.0334	0.0218	0.0473	0.0375	0.0291	0.0631	0.0271	0.0223	0.0354
Glutamine (α,β)	0.0286	0.0158	0.0466	0.0392	0.0319	0.0539	0.0196	0.0167	0.0309	0.0446	0.0298	0.0548
Glycine (β)	0.0952	0.0486	0.1594	0.1160	0.0640	0.2006	0.0759	0.0497	0.1113	0.1041	0.0672	0.2057
Glycolate (α)	0.0258	0.0176	0.0413	0.0366	0.0258	0.0552	0.0210	0.0118	0.0321	0.0347	0.0235	0.0498
Guanidinoacetate (α,β)	0.0091	0.0020	0.0186	0.0166	0.0103	0.0255	0.0143	0.0104	0.0244	0.0166	0.0092	0.0270
Histidine (α,β)	0.0300	0.0139	0.0484	0.0353	0.0202	0.0549	0.0177	0.0112	0.0257	0.0290	0.0117	0.0441
Hypoxanthine (α,β)	0.0083	0.0035	0.0131	0.0056	0.0025	0.0091	0.0060	0.0009	0.0076	0.0036	0.0009	0.0070
Isoleucine (α)	0.0017	0.0003	0.0032	0.0009	0.0003	0.0016	0.0013	0.0003	0.0020	0.0006	0.0003	0.0015
Methanol (α)	0.0029	0.0015	0.0070	0.0016	0.0011	0.0036	0.0054	0.0024	0.0072	0.0021	0.0010	0.0039
Pantothenate (α)	0.0050	0.0027	0.0075	0.0030	0.0017	0.0045	0.0024	0.0016	0.0081	0.0026	0.0018	0.0035
Phe-Derivative (α,β)	0.1044	0.0754	0.1392	0.0481	0.0279	0.0726	0.0948	0.0722	0.1442	0.0538	0.0290	0.0942
Succinate (β)	0.0056	0.0037	0.0112	0.0065	0.0030	0.0117	0.0060	0.0027	0.0111	0.0067	0.0042	0.0151
Taurine (α,β)	0.1148	0.0314	0.2133	0.0184	0.0051	0.0517	0.0568	0.0219	0.1045	0.0078	0.0042	0.0141
Uracil (α,β)	0.0010	0.0010	0.0018	0.0028	0.0010	0.0046	0.0010	0.0010	0.0039	0.0029	0.0010	0.0055
Urea (α)	26.73	17.38	36.81	19.58	14.42	27.06	27.56	19.15	39.22	19.94	14.34	28.37
Xylose (α)	0.0239	0.0020	0.0487	0.0080	0.0020	0.0163	0.0020	0.0020	0.0151	0.0072	0.0020	0.0142

The metabolites used to discriminate the different groups of subjects are labeled as follows: α, required for separation of COPD versus asthma during exacerbation; β, required for separation of COPD versus asthma in follow-up.

IQR, Interquartile range.

consisting of 16 metabolites in the VIP list producing an R^2 value of 0.76 and a Q^2 value of 0.70 (VIP; Fig 2, A). Most of the metabolites used for this follow-up visit model were the same as those used for the first-visit model, and the direction of difference of each metabolite between groups (ie, greater or lesser) was the same (see COV plot 2B). The graphic presentation of the quality of separation between groups in the model is shown by their respective PLS-DA scores (Fig 2, C). The final list of metabolites chosen and their concentrations are shown in Table II.

Validity assessment of this model studying after exacerbation

To validate the metabolomic model of asthma versus COPD after exacerbation, we again entered the metabolite concentrations from McMaster University ($n = 21$). These urine metabolite values were entered blindly for the PLS-DA model to predict a diagnosis. The individual PLS-DA prediction scores are shown in Fig 2, C, with error bars representing medians and interquartile ranges. The model was able to correctly diagnose 19 of 21 blinded asthma samples (90% accuracy).

Validity assessment of this model studying stable asthma versus COPD in an outpatient setting

To better validate the proposed metabolomic model of asthma versus COPD, we entered the metabolite concentrations from

another cohort of adults with asthma or COPD. These subjects were recruited in an outpatient setting and not specifically after exacerbation. We were interested to see whether the model would still perceive similar metabolomic differences in what should be a somewhat healthier group not recently recovering from an exacerbation. The individual PLS-DA prediction scores are shown in Fig 3, A, with error bars representing medians and interquartile ranges. The model has a sensitivity of 92.6% (95% CI, 82.1% to 97.9%) and a specificity of 79.2% (95% CI, 57.8% to 92.9%). The positive predictive value for asthma (if prediction score > 0.5) was 90.9% (95% CI, 80.0% to 97.0%), and the negative predictive value indicating COPD rather than asthma (prediction score ≤ 0.5) was 82.6% (95% CI, 61.2% to 95.0%). The ROC curve is shown (area under the curve, 0.937; Fig 3, B).

DISCUSSION

We have provided proof-of-concept evidence that urine metabolites can be used to differentiate asthma from COPD. We have the metabolomic data comparing asthma and COPD before and after exacerbation, but the focus of this report was on the differentiation of asthma from COPD. A subsequent article will compare metabolites for exacerbation.

COPD and asthma involve a number of unique cellular pathways, but these pathways can still converge to produce a similar clinical outcome. Symptoms or lung function

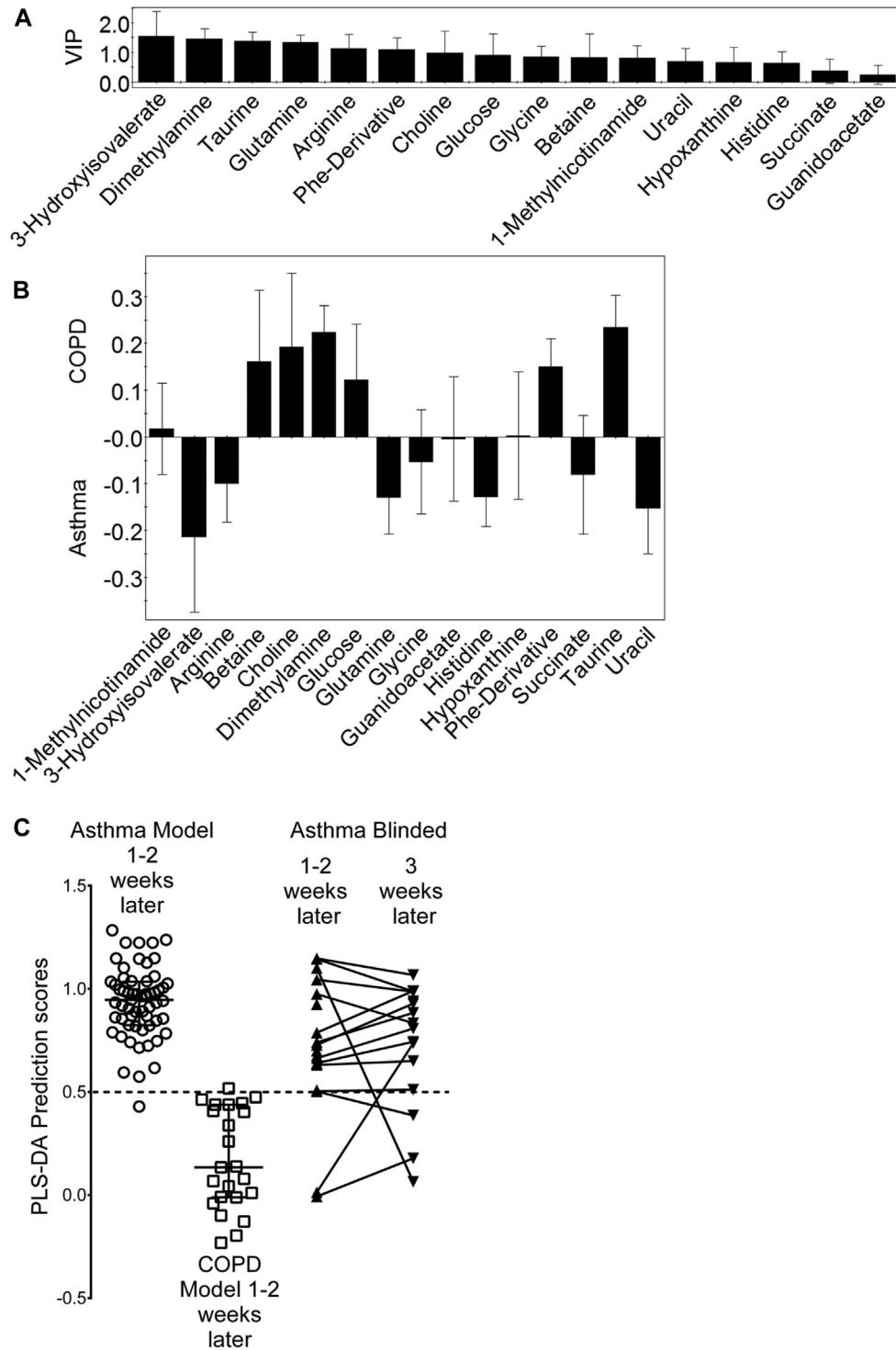


FIG 2. Metabolomic model of patients in follow-up after exacerbation of asthma versus COPD. Urine metabolite levels were measured from patients in follow-up from an ED visit for either asthma or COPD. Using a blind test set, PLS-DA (SIMCA P-11) on these metabolite levels created a model of separation ($R^2 = 0.78$, $Q^2 = 0.70$). **A**, VIP ranking the metabolites according to their significance in the model. **B**, Scaled and centered metabolite differences between groups shown as the COV plot. **C**, PLS-DA prediction scores for each subject, with error bars representing medians and interquartile ranges. The PLS-DA algorithm separates groups of data based on a score of 0 to 1. In this case a value of greater than 0.5 indicates the subject has asthma, whereas a value of less than 0.5 indicates the subject has COPD.

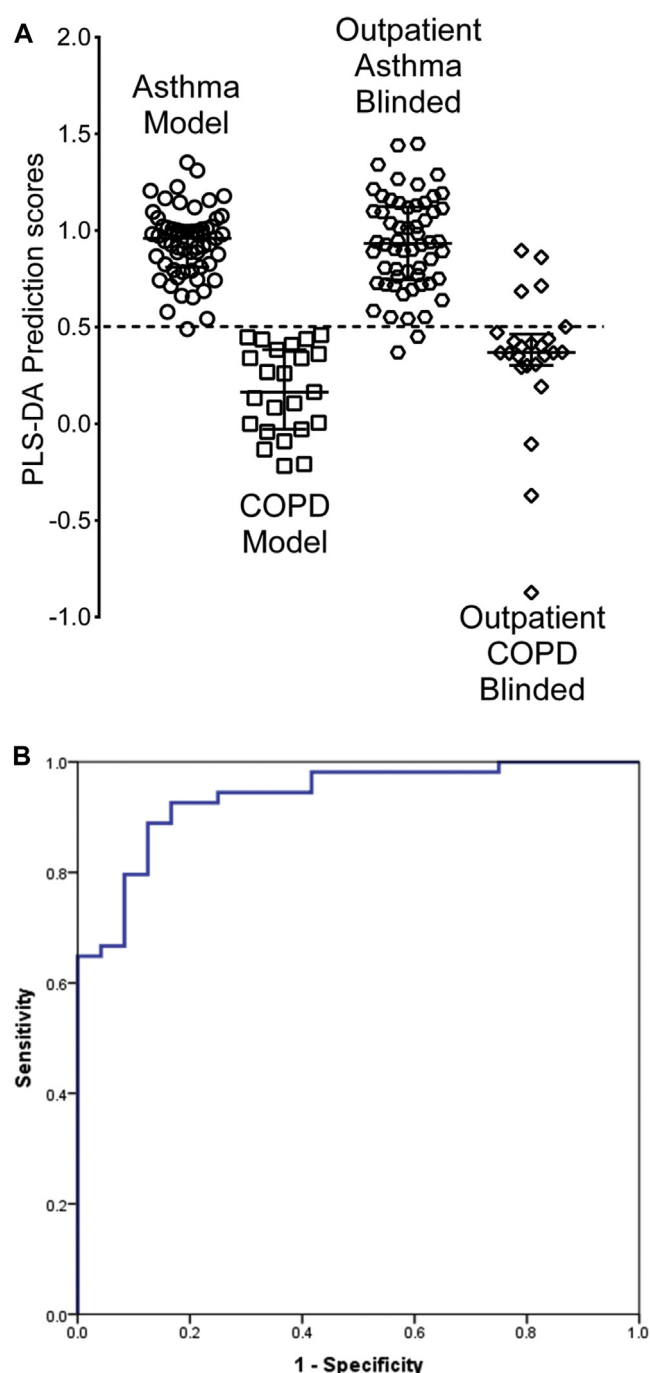


FIG 3. The metabolomic model correctly predicts asthma versus COPD in blinded outpatients. The metabolomic model of asthma versus COPD after exacerbation was used to study new patients with asthma or COPD in an outpatient setting to further determine diagnostic accuracy. **A**, PLS-DA prediction scores for each subject, with *error bars* representing medians and interquartile ranges. The PLS-DA algorithm separates groups of data based on a score of 0 to 1. In this case a value of greater than 0.5 indicates the subject has COPD, whereas a value of less than 0.5 indicates the subject has asthma. **B**, ROC curve for the prediction of asthma versus COPD. Area under the curve is 0.937.

measurements might not differentiate asthma from COPD (eg, airway remodeling in asthma provides a pattern of fixed obstruction). Thus it should not be surprising that a combination of metabolites, rather than a single metabolite, would be required

to differentiate these complex pathologies. Using only 1 factor to separate groups requires large consistent differences with minimal measurement overlap. This has not been demonstrated for previous single biomarkers (eg, exhaled nitric oxide [NO], urine leukotrienes, and eosinophil cationic protein).¹²⁻¹⁴ Airway dysfunction, increased work of breathing, and hypoxemia will cause cellular stress inside and outside the lung. As such, we were not deterred from using urine as our biofluid of interest compared with using more invasive samples from the airway. We also placed no restrictions on time of urine collection or diet before collection. We hypothesized that metabolites from disease should outweigh the influence of such confounders. The similarity of subjects returning for follow-up visits and our prior animal and human studies^{10,11} support this approach.

Some asthmatic patients can be difficult to differentiate from those with COPD if they are current smokers. We did not exclude smokers from the asthma cohort in the ED because patients with asthma still continue to smoke. There were 32 (28.3%) asthmatic patients included in the models who were current smokers. Despite this, the models still saw the smoking asthmatic patients in our test sets correctly as having asthma. As such, smoking was not the major factor in the metabolomic differentiation of asthma and COPD.

There remain some limitations of this study. For example, to get metabolomics to work in a clinic, we acknowledge that further development of the methodology will be necessary. Although NMR could serve as the platform, it is not as fast as other methodologies, including mass spectrometric analysis. We chose NMR because it allowed us to screen samples for potential metabolomic targets. This is the novel aspect of the work that needed to be established before further development could proceed. With the targets in place, we are designing larger studies with improved methods, including mass spectrometry. The increased sensitivity of mass spectrometry will also allow measurement of lower concentrations of metabolites, such as bromotyrosine,^{15,16} which would further help differentiate asthma from COPD.

Second, even with optimal clinical phenotyping, some asthma can mimic the lung function changes typically seen in patients with COPD. We used standard clinical criteria, cigarette consumption limits, and FEV₁/FVC ratios in an effort to accurately classify the groups. Despite this, we know that some of the follow-up asthma exacerbation subjects had fixed obstruction even 3 weeks after exacerbation. They were left in the category of asthma based on prior spirometry showing reversibility or hyperresponsiveness. The fact that the metabolome still identifies them as having asthma despite this fixed obstruction makes the results of this study more compelling. This would suggest that a subject might have a unique metabolomic phenotype that transcends this physiologic presentation of fixed obstruction. This approach might also help to define COPD and asthma overlap syndromes.⁴

Third, we were limited in our ability to obtain full spirometric data on all subjects having an exacerbation. To obtain such samples would have required a prospective study waiting for subjects to exacerbate. This was a pilot study to determine whether there is a metabolomic diagnostic signal that is worth pursuing further. Given our encouraging data, we are designing such a study, and this should also give us the metabolome of impending exacerbation because we will have urine samples on subjects even before they present to the ED. We will also enroll

subjects with allergen skin test data because atopy is likely another important element to define in this metabolome.

Finally, most of our patients were taking some prescription respiratory and nonrespiratory medications. It would be difficult to find a sample with asthma or COPD that did not have these confounders, and thus we did not control for the types of respiratory medications used. We did remove subjects with comorbidities, such as hypothyroidism and diabetes, because both would have known effects on many of our measured metabolites. We did not stratify based on heart failure. These populations could be incorporated into the technology but would require future larger cohorts of patients.

Although this study was not designed to mechanistically confirm specific metabolic pathways, we can speculate on possible pathways required to generate the more discriminatory metabolites. Arginine and dimethylamine are produced in relation to the NO pathway. Exhaled NO is produced from arginine and is well known for its association with asthma and asthma severity.^{17,18} Low arginine levels are associated with impaired smooth muscle relation *in vitro*.¹⁹ In addition, asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NO synthase. ADMA is produced from arginine.²⁰ ADMA is metabolized to citrulline and dimethylamine by the enzyme dimethylarginine dimethylaminohydrolase. ADMA is evolving as a marker of cardiovascular risk, and a role for ADMA was reported in an allergic asthma model,²¹ studies in obesity-induced asthma,^{22,23} and studies of obstructive sleep apnea and COPD.²⁴ This is the first report comparing patients with COPD and asthmatic patients for urine arginine and dimethylamine levels.

3-Hydroxyisovalerate levels were consistently higher in asthmatic patients compared with those with COPD. Such branch chain amino acids were reported in relation to their uptake by muscle, including the diaphragm.²⁵ This could be a marker for work of breathing or muscle dysfunction in COPD. 3-Hydroxyisovalerate levels might reflect relative biotin deficiency.²⁶ Such a link to asthma or COPD has not previously been cited.

Taurine levels were higher in our patients with COPD. Levels of taurine were measured in human airways.²⁷ It is reported to have a role in airway smooth muscle relaxation²⁸ and appears to correlate with airway neutrophilia.²⁹ There is also a reported link between increased inflammation, oxidative stress, and high taurine levels.³⁰ This is the first comparison of taurine levels in asthmatic patients and those with COPD.

Betaine and choline are 2 related metabolites found in the diet, and levels of both were increased relatively in the patients with COPD compared with those with asthma. Choline is required for synthesis of membrane phospholipids but is also converted to betaine.³¹ This conversion can occur through bacterial enzymes, which might be a unique feature in the airways of patients with COPD. Betaine is an osmolyte that is also important for methylation, including that of DNA and homocysteine production.³¹ A previous link of these metabolites to asthma has been reported but not to COPD.³²⁻³⁴

Ascorbate (vitamin C) is known for its role as an antioxidant. Ascorbate levels have been measured in the airways of asthmatic patients.³⁵ There is a report for ascorbate enhancing β -adrenergic-mediated smooth muscle relaxation *ex vivo*³⁶ and improving muscle fatigue in patients with COPD.³⁷ There are no reports comparing asthma and COPD levels.

Glutamine is the most abundant amino acid in the serum and is found largely in muscles but also in the lungs. Levels were lower in our patients with COPD. Glutamine becomes conditionally essential after severe stress, such as a burn injury,³⁸ and supplements with glutamine are reported to improve outcomes related to infection.³⁹ Glutamine levels are reported as low in patients with COPD compared with those in healthy subjects⁴⁰ and higher in asthmatic patients compared with healthy control subjects.³⁴ No previous group has directly compared asthma with COPD.

Histidine is an essential amino acid critical in many enzymatic reactions. One such reaction is the production of histamine, which is well known for its role in asthma.⁴¹ 1-Methylhistamine is a downstream metabolite from histamine. Serum levels of 1-methylhistamine are higher in asthmatic patients; they increase acutely after an asthma attack and are decreased by antiallergy medications.⁴²⁻⁴⁴ Although histidine levels have been reported as increased in asthmatic patients compared with those in healthy control subjects,³⁴ they have not been reported in comparison with levels in patients with COPD.

Hyperglycemia is well known in patients with both asthma and COPD exacerbation.⁴⁵ Even after exacerbation, many of our patients with COPD, although not known as diabetic, had higher urine glucose levels compared with those in asthmatic patients. This might not be surprising because patients with COPD are known to have many comorbidities, including glucose intolerance,⁴⁶ and this is also reported in an animal model of COPD.⁴⁷ There are no reports comparing urine glucose levels in asthmatic patients and those with COPD.

Succinate levels appear to remain lower in patients with COPD. As part of the citric acid cycle, succinate levels change with prolonged exertion, energy metabolism, or hypoxemic stress on body tissues.^{48,49} There is also awareness of mitochondrial abnormalities in the muscles of patients with COPD, which alter succinate levels.⁵⁰ Succinate levels have not been previously compared in asthmatic patients and those with COPD.

Metabolites of niacin were important.⁵¹ We found that 1-methylnicotinamide levels were increased in our patients with COPD versus our patients with asthma. 1-Methylnicotinamide was reported as an anti-inflammatory molecule in asthma exacerbation,⁵² possibly through its ability to induce release of prostacyclin.⁵³ 1-Methylnicotinamide levels in patients with COPD have been compared with those in healthy control subjects previously⁵⁴ but not with those in asthmatic patients. Trigonelline is found in a number of plant beans. It is a product of nicotinamide metabolism after oxidative stress.⁵⁵ Its levels are also reported in relation to tobacco smoking.⁵⁶ Its levels were reported to correlate with lung function in patients with COPD,⁵⁷ but this has not been measured in relation to levels in asthmatic patients.

Uracil and pantothenate could both be linked based on their role in the coenzyme A biosynthesis pathway, which is critical in energy metabolism. Pantothenate levels are increased by cigarette smoke in epithelial cell cultures.⁵⁸ This pathway was cited as relevant for asthma⁵⁹; however, neither metabolite has been reported in relation to COPD.

Overall, physicians know that better objective tests of asthma and COPD are needed. We believe development of urine metabolomic analysis will lead to improved diagnostic capabilities. Metabolomic technology could be incorporated into a standard laboratory once the methods are fully refined.

A personalized metabolomic profile would be provided as a score to aid the clinician. The ability to use urine makes the methodology readily applicable to most clinical settings, especially for children. These data could also provide new insights into airway dysfunction, suggesting novel pathways for drug discovery.

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Clinical implications: Objective measurements for differentiation of asthma and COPD are not used in a typical doctor's office. We suggest that a metabolomic approach could become a diagnostic solution in the future.

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METHODS

Patients' characteristics

Patients were enrolled after obtaining written informed consent, as approved by the University of Alberta and St Joseph's Healthcare Hamilton Health Research Ethics Boards.

Patients with asthma or COPD with exacerbations

Adults with asthma seen at the time of an exacerbation through either the University of Alberta ($n = 110$) or McMaster University ($n = 23$) were selected based on 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 bronchodilator response measured based on peak expiratory flow symptomatic or significant clinical improvement response to inhaled bronchodilator therapy; and/or (3) a previous history of physician-diagnosed asthma. Those from McMaster also had previously demonstrated greater than 12% reversibility to salbutamol or a positive methacholine challenge test result ($PC_{20} < 8$ mg/mL). Adults with COPD seen in the ED at the University of Alberta ($n = 38$) were selected based on 1 or more of the following: (1) increasing symptoms (eg, cough, wheeze, shortness of breath, or chest tightness) requiring assessment and a history of similar episodes and/or (2) a previous history of physician-diagnosed COPD. All patients had to present primarily for acute asthma or COPD exacerbation (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. All data for ED patients are shown in [Table I](#).

Patients with asthma or COPD who were stable in an outpatient setting

Adults with asthma ($n = 53$) or COPD ($n = 24$) were recruited in an outpatient setting at the University of Alberta. Asthma diagnosis was based on the following: (1) a previous history of physician-diagnosed asthma; (2) previously demonstrated greater than 12% reversibility to salbutamol or a positive methacholine challenge test result ($PC_{20} < 8$ mg/mL); (3) no current smoking; and (4) a less than 10 pack-year history of smoking. COPD diagnosis was based on 1 or more of the following: (1) a previous history of physician-diagnosed COPD and/or (2) having an FEV_1/FVC ratio of less than 70%. Patients were excluded if they were taking systemic corticosteroids or prescription medications for diabetes, gastroesophageal reflux disease, or hypothyroidism. All data are shown in [Table I](#).

Urine sample collection

A single urine sample was collected from each patient 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.^{E1,E2} We were aware of the variability in urine metabolomic data that can occur within each person based on dietary intake and time of day^{E3,E4}; however, our objective was to design a test that could be used in a typical clinical setting. 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 the disease and nondisease groups that such intrapersonal variability would be superseded.

Statistical analysis of clinical data

GraphPad Prism software (version 6) was used for statistical analysis of the clinical data. The nonparametric t test was used to compare 2 group experiments. Repeated-measures ANOVA with the Bonferroni multiple comparison test was used to compare multiple groups. Data are presented as means $\pm$ 1 SEM or medians with interquartile ranges. A P value of less than .05 was considered significant.

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 addition of 70 μL of a reference buffer solution (4.9 mmol/L disodium-2, 2-dimethyl 2-silapentane-5-sulphonate and 100 mmol/L imidazole in D_2O ; Chenomx). 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, Vineland, 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, 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 a 100-ms mixing time) and a spectral width of 7200 Hz (phase cycle available on request). The time-domain data points were 64,000 complex points, acquisition time was 4 seconds, the 90° pulse was 6.8 μs , repetition time was 5 seconds, there were 4 steady-state scans, and the number of acquired scans was 32 per FID. Data were apodized with an exponential window function corresponding to a line broadening of 0.5 Hz, zero-filled to 128,000 complex points, and Fourier transformed.

Spectral and statistical analysis

Spectral identification and quantification of 86 identifiable metabolites was performed with the Chenomx NMR Suite Professional software package (version 4.6, Chenomx). 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 qualitative and quantitative analysis of metabolites in urine. Metabolites were referenced to creatinine and values were log transformed to account for the hydration status of the subjects. PLS-DA was performed (SIMCA-P 11, Umetrics), which determined the relationship between the response vector Y (patient group) and the matrix X (concentration of each metabolite) by means of simultaneous projections of both Y and X spaces to a plane. Seven-fold internal cross-validation was performed. This process identifies the metabolites with concentrations that differed significantly between groups of patients expressed as a COV plot ([Fig E1, B](#)). As an example, we have plotted the value of a critical metabolite, dimethylamine, for patients with COPD versus those with asthma in the ED ([Fig E1](#)). As can be seen, although there is variability between subjects, this variability is small compared with the magnitude of change when comparing the groups. In addition, it is important to note that there remains a significant degree of overlap between the groups, and thus we could not rely solely on one metabolite, such as dimethylamine, to be the diagnostic variable. As might be expected, levels of most metabolites do not differ greatly between groups, and including metabolites of low significance is detrimental to creating an accurate diagnostic model of separation. Metabolites with consistently greater differences in concentration between groups are displayed by the software as a VIP ([Fig E1, A](#)). 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 blinded samples not part of the model. We set a false-positive rate of 5% to 10% as an acceptable limit. This resulted in the most sensitive models both with respect to correlation coefficients (R^2) and prediction properties (Q^2). We previously reported using this technique in an animal model of asthma^{E5} and in children with asthma. The PLS-DA-based model was then used to generate a prediction score (0-1) of unclassified blinded data that were not part of the model (ie, scores <0.5 would be predicted to be asthmatic patients vs those with COPD subjects). The ROC curve was generated to evaluate the

relationship between the sensitivity and specificity of the prediction score at various cut points and to consider the score's overall function as a predictor. A diagnosis of asthma was arbitrarily considered the disease state for curve generation, which was undertaken with SPSS software (IBM SPSS Statistics for Windows, Version 20.0; IBM, Armonk, NY).

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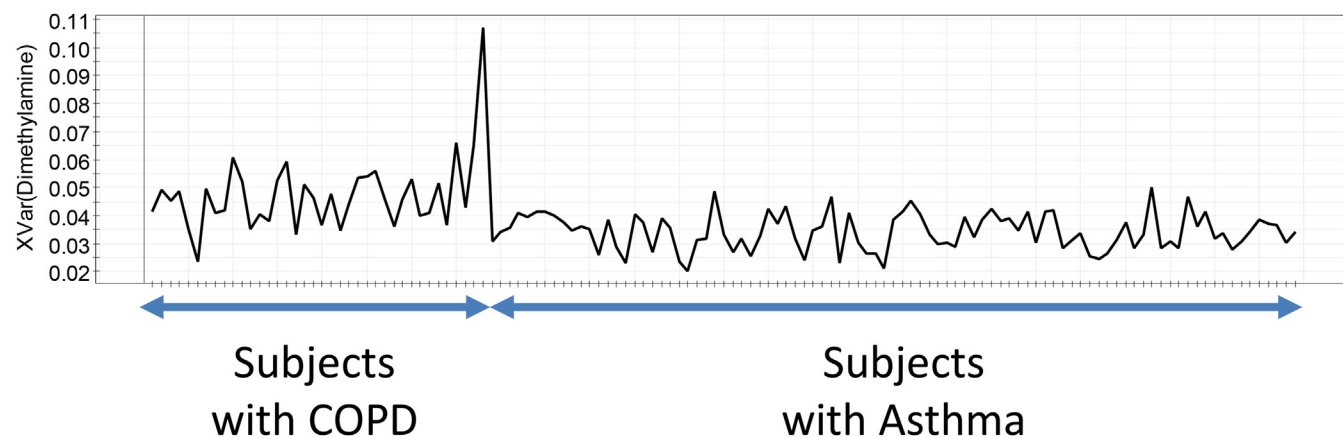


FIG E1. The values of dimethylamine are plotted for each subject with COPD versus those with asthma in the ED.