

Metabolomic Biomarkers in a Model of Asthma Exacerbation

Urine Nuclear Magnetic Resonance

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Rationale: Airway obstruction in patients with asthma is associated with airway dysfunction and inflammation. Objective measurements including sputum analysis can guide therapy, but this is often not possible in typical clinical settings. Metabolomics is the study of molecules generated by metabolic pathways. We hypothesize that airway dysfunction and inflammation in an animal model of asthma would produce unique patterns of urine metabolites measured by multivariate statistical analysis of high-resolution proton nuclear magnetic resonance (¹H NMR) spectroscopy data.

Objectives: To develop a noninvasive means of monitoring asthma status by metabolomics and urine sampling.

Methods: Five groups of guinea pigs were studied: control, control treated with dexamethasone, sensitized (ovalbumin, administered intraperitoneally), sensitized and challenged (ovalbumin, administered intraperitoneally, plus ovalbumin aerosol), and sensitized-challenged with dexamethasone. Airway hyperreactivity (AHR) to histamine (administered intravenously) and inflammation were measured. Multivariate statistical analysis of NMR spectra based on a library of known urine metabolites was performed by partial least-squares discriminant analysis. In addition, the raw NMR spectra exported as xy-trace data underwent linear discriminant analysis.

Measurements and Main Results: Challenged guinea pigs developed AHR and increased inflammation compared with sensitized or control animals. Dexamethasone significantly improved AHR. Using concentration differences in metabolites, partial least-squares discriminant analysis could discriminate challenged animals with 90% accuracy. Using only three or four regions of the NMR spectra, linear discriminant analysis-based classification demonstrated 80–90% separation of the animal groups.

Conclusions: Urine metabolites correlate with airway dysfunction in an asthma model. Urine NMR analysis is a promising, noninvasive technique for monitoring asthma in humans.

Keywords: asthma; biomarkers; metabolomics; nuclear magnetic resonance; urine

Asthma is characterized by shortness of breath due to reversible airway obstruction and abnormal airway reactivity to various stimuli. The airway pathology found in patients with asthma is

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Objective measurements of airway inflammation can improve therapy. Development of improved noninvasive diagnostics is considered a valuable area of research.

What This Study Adds to the Field

In an animal model, ¹H NMR spectroscopic analysis of urine metabolites differentiates animals with or without airway inflammation, using a panel of biomarkers. These data suggest that urine NMR analysis could be applied to humans with asthma.

a unique mix of abnormal structural cells (1, 2) and inflammatory cells (3), which are not commonly described in other airway diseases. The severity of asthma and the degree of airway hyperreactivity (AHR) correlate with the presence and magnitude of airway inflammation in the airways (4). Thus, asthma management has relied on the control of inflammation (5). Improving the ability to accurately monitor airway dysfunction and inflammation through noninvasive means is a key goal in managing asthma therapy (6).

Metabolomics is the study of metabolic pathways and the measurement of unique biochemical molecules generated in a living system (7). Metabolites are small, nonpeptide molecules with molecular masses less than 1 kD (8) and are the end products of cellular activity. Detecting changes in metabolite concentrations reveals the range of biochemical effects induced by a disease condition or its therapeutic intervention. Proton nuclear magnetic resonance (¹H NMR) spectroscopy allows for the characterization and quantification of these metabolites in biological fluids. The main advantage of using NMR is its ability to provide a rapid and accurate metabolic picture with minimal sample pretreatment (9, 10). The advantages of urine include its noninvasive collection and wide availability, its low protein and cellular levels, and its richness in metabolites. Overall, urine is an ideal biofluid for NMR-based metabolomic investigations.

Guinea pigs are a reliable animal model of asthma as their airway physiology is uniquely similar to that of humans (11, 12). We hypothesized that in this animal model of allergic asthma, the effect of airway inflammatory cells on the airways would produce in the body a unique pattern of metabolites, which would be excreted in the urine. These urine metabolites could be measured by NMR spectroscopy and used as a biomarker panel to discriminate the subtypes of animals. This concept of urine NMR analysis in an animal model of asthma was previously presented as an abstract (13).

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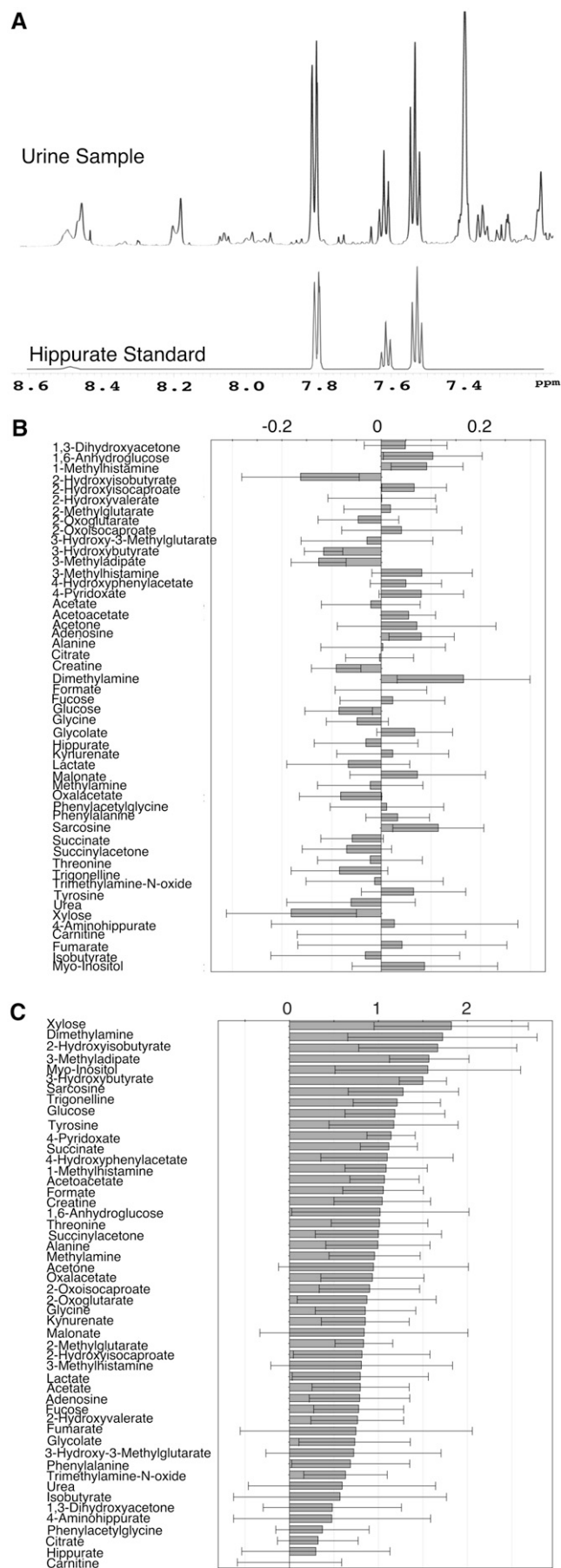


Figure 1. (A) An example of a 600-MHz one-dimensional proton nuclear magnetic resonance (^1H NMR) spectrum from a urine sample (the aromatic region is displayed, 6.7–8.7 ppm) above the trace of the NMR spectrum for hippurate to illustrate the methodology of Chemomx NMR Suite software in identifying and quantifying metabolites. (B) Partial least-squares discriminant analysis (PLS-DA) determines the scaled and centered metabolite differences between groups; illustrated is an example of the coefficients of variation plot between challenged and sensitized animals for all metabolites (horizontal lines represent 95% confidence intervals). (C) PLS-DA analysis (SIMCA P-11) ranks the metabolites according to their significance in the model; illustrated is an example of the variables of importance plot for challenged versus sensitized guinea pigs (horizontal lines represent 95% confidence intervals).

METHODS

Details of all methods are available in the online supplement.

Animals and Generation of Model of Asthma

Female Dunkin-Hartley guinea pigs were used in accordance with standards established by the University of Alberta (Edmonton, AB, Canada) Health Sciences Animal Policy. On the basis of previous work in this asthma exacerbation model (14, 15), guinea pigs were divided into five groups: (1) control (untreated, nonsensitized), (2) “controldex” (control guinea pigs administered dexamethasone intraperitoneally; Sigma-Aldrich, Oakville, ON, Canada), (3) sensitized (guinea pigs sensitized to intraperitoneal ovalbumin [OVA] alone; Sigma-Aldrich), (4) challenged (after 21 d, sensitized guinea pigs were challenged with aerosolized OVA), and (5) “challengeddex” (challenged animals treated with dexamethasone [6.5 $\mu\text{g/kg}$], administered intraperitoneally). Airway function and inflammation were assessed 4 days after OVA challenge or 21 days after OVA sensitization alone.

Assessment of Airway Hyperreactivity

Guinea pigs were anesthetized with urethane, tracheotomized, and ventilated after paralysis with succinylcholine chloride (Sigma-Aldrich). Pulmonary inflation pressure (Ppi) was measured with a PowerLab system (ADInstruments, Colorado Springs, CO) as previously described (16). To assess airway reactivity, histamine (Sigma-Aldrich) was administered at 6-minute intervals (1–20 $\mu\text{g/kg}$, intravenous). The resulting bronchoconstriction was recorded as increases in Ppi.

Assessment of Airway Inflammation

Animals were killed with an overdose of urethane (3 g/kg, intravenous). Cytosmears for total and differential cell counts were produced from lung lavage specimens and stained with Diff-Quik (Baxter Healthcare VWR, West Chester, PA) (16). The lungs were removed, inflated, and fixed in 3.7% formaldehyde for 24 hours. Airway sections were stained with 2% chromotrope 2R to determine the average number of eosinophils per 10 high-power fields as previously described (16, 17).

Statistical Analyses of Airway Reactivity and Inflammation

The bronchial reactivity measurements (histamine responses) were analyzed by two-way analysis of variance for repeated measures and histological measurements and lung lavage data were analyzed by analysis of variance (StatView 4.5; Abacus Concepts, Inc., Berkeley, CA). The results are expressed as mean and standard error of the mean (SEM) and standard deviation (SD), respectively. A P value of 0.05 was considered significant.

Collection of Urine Sample and Collection of One-dimensional ^1H NMR Spectra

Before performing airway inflammatory measurements, urine samples (1.0–2.0 cm^3) were collected by transabdominal cystocentesis. Samples were cataloged and stored (-80°C) at the National High Field Nuclear Magnetic Resonance Centre (Edmonton, AB, Canada). All ^1H NMR spectra were acquired on a 600-MHz INOVA spectrometer (Varian, Inc., Palo Alto, CA) equipped with a 5-mm triple-resonance (HCN) probe with Z axis gradient coil.

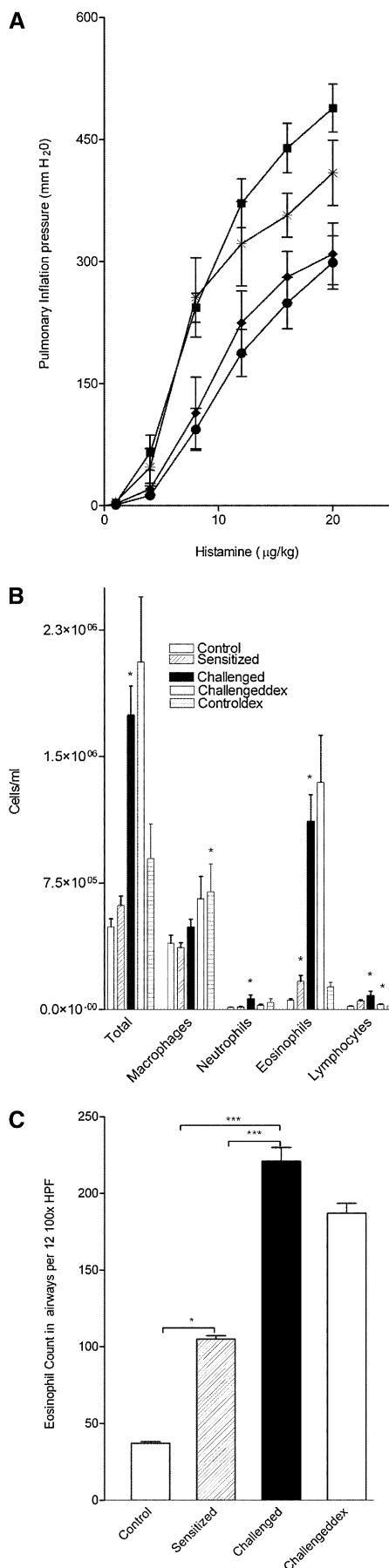


Figure 2. (A) Histamine (1–20 μg/kg, intravenous) produced dose-dependent bronchoconstriction, measured as an increase in pulmonary inflation pressure. Challenged guinea pigs (solid squares, $n = 10$) showed increased response to histamine compared with controls (solid circles, $n = 12$, $P < 0.0009$) and sensitized animals (solid diamonds, $n = 8$, $P < 0.008$), whereas challengeddex animals (challenged animals treated with dexamethasone, 6.5 μg/kg intraperitoneal; stars, $n = 6$) showed a diminished response compared with challenged animals ($P < 0.05$). (B) Total and differential leukocyte counts were performed on the lung lavage of control (open columns), sensitized (hatched columns), challenged (solid columns), challengeddex (gray columns), and controldex animals (the latter being control animals administered dexamethasone, intraperitoneal; lined columns) ($n = 9$ each). (C) Eosinophils were counted in the cartilaginous airways of control (open columns), sensitized (hatched columns), challenged (solid columns), and challengeddex (gray columns) animals ($n = 12$ each). Each point or column represents the mean, with the SEM shown by error bars. * $P < 0.05$; *** $P < 0.01$. HPF = high-power field.

Quantification of Known Metabolites

Quantification of 50 easily identifiable metabolites involved in various relevant metabolic pathways was performed with Chenomx NMR Suite Professional software package version 4.6 (Chenomx, Inc., Edmonton, AB, Canada) (18, 19). The software contains a database of known metabolites with their referenced spectral resonant frequencies or signatures. These known resonant frequencies are matched to the observed resonant frequencies of the collected spectra, enabling the qualitative and quantitative analysis of metabolites in urine NMR spectra (Figure 1A). This method is capable of providing metabolite concentration accuracies in excess of 90% (18, 20). To correct for dilution, metabolite concentrations were referenced against urinary creatinine (21). There is daily variation in the excretion of metabolites, although we have not identified in humans any variation that was specifically attributed to diet (19, 21).

Separation of Animal Groups on the Basis of Known Metabolites

As seen in our human data (21), the concentrations (millimoles per millimole of creatinine) varied greatly depending on the metabolite measured. For example, the amount of urea in the urine is orders of magnitude greater compared with lactate. To appreciate the impact of a lower concentration metabolite in the model, the statistical analysis must be designed to detect the relative change in concentration of a metabolite. Thus, metabolite concentrations were mean-centered followed by unit variance scaling (or z scoring). Thereafter, partial least-squares discriminant analysis (PLS-DA) was performed (SIMCA-P 11; Umetrics, Umeå, Sweden). PLS-DA determines the relationship between the response vector Y (animal group) and the matrix X (concentration of each metabolite) by simultaneous projections of both Y and X spaces to a plane. Sevenfold internal cross-validation was performed. This process identifies the metabolites whose concentrations differed significantly between groups of animals, seen as a coefficients of variation plot (Figure 1B). Initially, a model with all metabolites was created. The greater the consistent difference in metabolite concentration between groups, the more important the metabolite became in creating the model, seen as a variables of importance plot (Figure 1C). Nonparametric testing was also performed on the metabolite values to cross-check the metabolites of importance generated by SIMCA (Mann-Whitney test; StatView 4.5). The final list of metabolites (see RESULTS) was chosen on the basis of the variables of importance plot and the confirmation of statistical significance by nonparametric analysis. This procedure resulted in the most accurate model, both with respect to correlation coefficients (R^2) and prediction properties (Q^2).

Separation of Animal Groups on the Basis of xy-Trace of NMR Spectral Data

A one-dimensional ^1H NMR spectrum for each animal's sample was exported by means of the Varian macro "xy-trace" (ASCII format) for multivariate statistical analysis. Results were visualized by relative

TABLE 1. CONCENTRATIONS OF METABOLITES USED TO DISCRIMINATE VARIOUS ANIMAL GROUPS

Metabolite	Control (n = 18)	Sensitized (n = 20)	Challenged (n = 32)	Control/Dex (n = 4)	Chall/Dex (n = 12)
1-Methylhistamine	0.067 (0.079)	0.073 (0.092)	0.091 (0.062)	0.015 (0.009)	0.040 (0.034)
2-OH-isobutyrate	0.045 (0.024)	0.052 (0.019)	0.032 (0.020)	0.042 (0.006)	0.044 (0.021)
2-Oxoglutarate	0.012 (0.006)	0.014 (0.010)	0.011 (0.006)	0.004 (0.001)	0.009 (0.006)
3-OH-3-methylglutarate	0.016 (0.010)	0.013 (0.009)	0.011 (0.006)	0.004 (0.001)	0.011 (0.006)
3-OH-butyrate	0.038 (0.011)	0.038 (0.017)	0.024 (0.014)	0.038 (0.013)	0.024 (0.016)
3-Methyladipate	0.027 (0.009)	0.024 (0.009)	0.016 (0.008)	0.013 (0.007)	0.013 (0.006)
4-OH-phenylacetate	0.162 (0.068)	0.127 (0.051)	0.124 (0.044)	0.112 (0.032)	0.136 (0.070)
4-Pyridoxate	0.012 (0.011)	0.008 (0.011)	0.009 (0.004)	0.004 (0.001)	0.009 (0.006)
Acetate	0.604 (0.764)	0.650 (0.969)	0.359 (0.754)	0.139 (0.050)	0.162 (0.123)
Acetoacetate	0.013 (0.009)	0.015 (0.011)	0.014 (0.006)	0.011 (0.002)	0.008 (0.005)
Acetone	0.005 (0.005)	0.006 (0.007)	0.007 (0.005)	0.004 (0.001)	0.007 (0.006)
Adenosine	0.002 (0.003)	0.001 (0.002)	0.002 (0.003)	0.002 (0.002)	0.001 (0.001)
Creatine	0.043 (0.028)	0.047 (0.039)	0.029 (0.020)	0.040 (0.008)	0.024 (0.012)
Dimethylamine	0.099 (0.024)	0.101 (0.027)	0.118 (0.027)	0.099 (0.015)	0.123 (0.031)
Glucose	0.366 (0.681)	0.192 (0.106)	0.122 (0.095)	0.127 (0.017)	0.275 (0.281)
Glycolate	0.011 (0.009)	0.006 (0.016)	0.009 (0.005)	0 (0)	0.001 (0.005)
Kynurenate	0.014 (0.012)	0.010 (0.011)	0.019 (0.007)	0.007 (0.004)	0.006 (0.003)
Malonate	0.018 (0.013)	0.017 (0.010)	0.019 (0.007)	0.015 (0.006)	0.015 (0.007)
Methylamine	0.026 (0.021)	0.036 (0.025)	0.029 (0.015)	0.012 (0.005)	0.043 (0.025)
Oxalacetate	0.240 (0.083)	0.211 (0.075)	0.169 (0.075)	0.237 (0.013)	0.259 (0.105)
Phenylacetyl glycine	0.153 (0.055)	0.121 (0.040)	0.118 (0.054)	0.024 (0.003)	0.181 (0.151)
Phenylalanine	0.056 (0.028)	0.049 (0.024)	0.048 (0.020)	0.011 (0.002)	0.029 (0.020)
Sarcosine	0.016 (0.010)	0.013 (0.011)	0.020 (0.023)	0.004 (0.001)	0.011 (0.006)
Succinate	0.043 (0.039)	0.040 (0.026)	0.026 (0.020)	0.013 (0.002)	0.007 (0.003)
Succinylacetone	0.015 (0.009)	0.017 (0.009)	0.013 (0.006)	0.004 (0.001)	0.013 (0.007)
Trigonelline	0.073 (0.024)	0.081 (0.029)	0.061 (0.027)	0.052 (0.011)	0.075 (0.018)
Trimethylamine	0 (0)	0 (0)	0 (0)	0 (0)	0.001 (0.003)
Trimethyl-N-oxide	0.052 (0.048)	0.063 (0.029)	0.054 (0.037)	0.092 (0.019)	0.054 (0.041)
Tyrosine	0.050 (0.026)	0.036 (0.016)	0.036 (0.018)	0.029 (0.016)	0.026 (0.014)
4-Aminohippurate	0.011 (0.010)	0.008 (0.010)	0.009 (0.005)	0.077 (0.056)	0.007 (0.005)
Carnitine	0.023 (0.011)	0.015 (0.008)	0.016 (0.010)	0.147 (0.039)	0.011 (0.007)
Fumarate	0.004 (0.005)	0.002 (0.004)	0.003 (0.004)	0 (0)	0 (0)
Isobutyrate	0.001 (0.003)	0 (0)	0.001 (0.003)	0.025 (0.050)	0.001 (0.003)
myo-Inositol	0.044 (0.021)	0.046 (0.022)	0.041 (0.016)	0.475 (0.153)	0.051 (0.040)

Definition of abbreviations: Chall/Dex = challenged animals treated with dexamethasone, 6.5 μ g/kg intraperitoneal; Control/Dex = control animals administered dexamethasone, intraperitoneal.

Data represent mean concentrations, expressed as millimoles per millimole of creatinine; standard deviations are shown in parentheses.

distance plane mapping (22). Six pairs of classifiers were developed: control versus challenged, sensitized versus challenged, control versus sensitized, control versus challengeddex, challenged versus challengeddex, and sensitized versus challengeddex (see Table 2). To separate each pair of classifiers, values of Y for each 0.04 section of width on the X axis were calculated and compared by a genetic algorithm-based feature selection approach (23). The feature selection was wrapper based: that is, a classifier, in this case linear discriminant analysis (LDA) with leave-one-out (LOO) internal cross-validation was used to identify optimal features. These features are the subregions within the spectra that best separate the two classes for the various pair classifiers. Having obtained the optimal feature set for each pair of classifiers or animal groups, external cross-validation (EXCV) was performed to estimate a more realistic error rate. Each data set was split randomly into a training set and a monitoring test set (50:50). Ten splits were done for each data set and the averages and standard deviations were calculated. On the basis of experience with small sample sizes, this approach was used to give a more realistic result than would be obtained using the entire data set (22, 24).

RESULTS

Airway Hyperreactivity and Inflammation

Baseline responses. There were no significant differences in weight (grams) among the groups (control, 449 ± 0.02 ; controldex, 399.5 ± 0.04 ; sensitized, 476 ± 0.03 ; challenged, 434 ± 0.02 ; and challengeddex, 389 ± 15.3). To ventilate the animals, a baseline pulmonary inflation pressure (Ppi, millimeters of

water) was measured and showed no significant difference among the groups (control, 126.7 ± 4.2 ; controldex, 105 ± 5.2 ; sensitized, 137.7 ± 8.1 ; challenged, 125.6 ± 4.8 ; and challengeddex, 120.7 ± 6.3). The amount of urethane anesthesia injected was similar and was confirmed by our urine NMR levels of urethane (millimoles per millimole of creatinine) (control, 3.5 ± 0.8 ; sensitized, 5.2 ± 1.6 ; challenged, 4.5 ± 0.9 ; and challengeddex, 3.6 ± 1.9).

Challenged animals develop airway hyperreactivity and inflammation. Histamine (1–20 μ g/kg, intravenous) induced dose-dependent bronchoconstriction as measured by increasing Ppi (Figure 2A). Although the response to histamine in sensitized guinea pigs was similar to that of control animals, the challenged group demonstrated increased reactivity with significantly higher bronchoconstriction responses compared with control animals ($P < 0.0001$). Challengeddex animals showed diminished response compared with challenged animals alone ($P < 0.05$). Whereas there was no significant difference between total cell counts of the control, sensitized, and controldex groups, total cells in the lung lavage of challenged animals were elevated ($P < 0.0001$) (Figure 2B). The increase consisted largely of lymphocytes, eosinophils, and neutrophils. Macrophage values were similar among all groups except the controldex group, which presented a higher value compared with the control group ($P = 0.03$). Total cells in challengeddex animals remained higher, similar to challenge, except for lymphocytes, which were significantly lower ($P < 0.05$). Sensi-

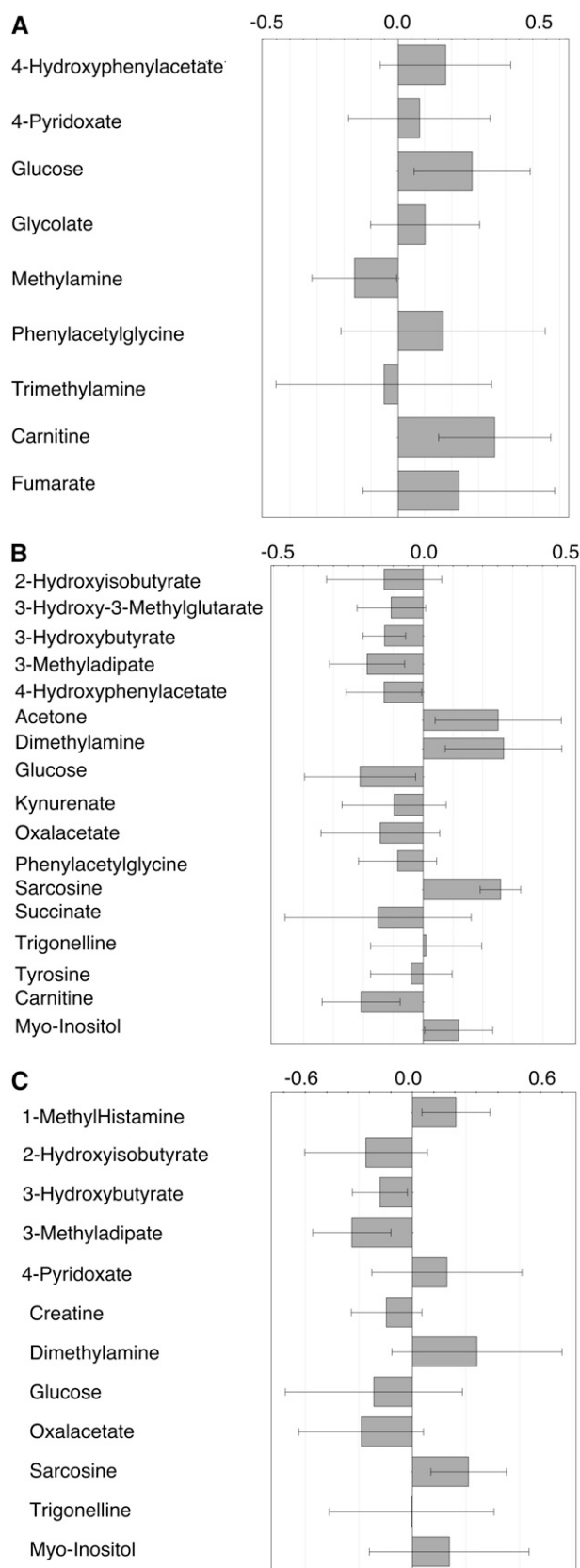


Figure 3. Coefficients of variation plots for nuclear magnetic resonance (NMR)-derived urine metabolite concentrations used by partial least-squares discriminant analysis (PLS-DA) in the final modeling of separation between the following: (A) control versus sensitized guinea pigs, (B) control versus challenged guinea pigs, and (C) sensitized versus challenged guinea pigs. Horizontal lines represent 95% confidence intervals.

tized animals showed higher eosinophil counts than did controls ($P < 0.0001$; Figure 2C). Challenge of sensitized animals further increased the eosinophil count compared with control animals ($P < 0.0006$) and sensitized animals ($P < 0.0001$). Eosinophil counts in the challengeddex group remained similar to those in the challenged group.

Metabolomic Analysis

Targeted NMR metabolite profiling can differentiate groups of animals. Using a library of known metabolite standards (Chenomx), the concentrations of 50 metabolites were measured in the urine of all animals (see Figure 1A) and each group was compared by PLS-DA. Not all metabolites were required to separate the groups and in some cases adding metabolites made the model accuracy worse. The complete list of metabolites and their concentrations (Table 1) used in at least one separation model were based on the results of PLS-DA, variables of importance ranking, and the confirmation by nonparametric analysis. The relevant metabolites for each separation model and their differences between animal groups are shown as coefficients of variation plots (Figures 3A–3C). Separation of each pair of animal groups and the three-way comparison of groups is illustrated in Figure 4 (Figure 4A: control vs. sensitized [$R^2 = 0.53$, $Q^2 = 0.29$]; Figure 4B: control vs. challenged [$R^2 = 0.74$, $Q^2 = 0.59$]; Figure 4C: sensitized vs. challenged [$R^2 = 0.63$, $Q^2 = 0.50$]; and Figure 4D: three-way analysis of animal groups [$R^2 = 0.54$, $Q^2 = 0.25$]). As expected, dexamethasone also induced an altered urine metabolite profile, as determined by PLS-DA analysis (Figures 5A and 5B, challenged vs. challengeddex [$R^2 = 0.76$, $Q^2 = 0.44$]; control vs. controldex [$R^2 = 0.83$, $Q^2 = 0.69$]; data not shown). Despite the small sample size, we sought to determine an accuracy measurement with PLS-DA-generated models. Fifteen urine samples from animals not part of the original PLS-DA modeling (sensitized, $n = 4$; challenged, $n = 7$; challengeddex, $n = 4$) were run blindly in the relevant paired models. The modeling created by PLS-DA correctly categorized challenged animals in 19 of 21 tests (challenged vs. control, sensitized, or challengeddex), giving an accuracy of 90%. Sensitized had an accuracy of 63% (five of eight tests; sensitized vs. control or challenged) and challengeddex had 75% accuracy (three of four tests, challengeddex vs. challenged).

xy-Trace of NMR spectra can differentiate guinea pig models. NMR spectra exported as xy-trace were analyzed with the feature selection component (with LDA/LOO internal cross-validation) of the statistical classification strategy (24). This produced spectral regions (features) that could separate the various animal populations (Figure 6A). To determine an estimate of separation accuracy using these regions, EXCV, using ten 50:50 random splits, was performed. EXCV confirmed the ability of xy-trace data analysis to separate, in a pair-wise fashion, the various groups of animals with the average accuracies and standard deviations presented in Table 2. For example, the control versus challenged groups could be discriminated with a minimal accuracy of $80.4 \pm 5.9\%$, in the training set and $82.6 \pm 6.8\%$ in the monitoring set. The ability to separate groups is illustrated by relative distance plan mapping (Figures 6B–6D). Overall, on the basis of xy-trace data, NMR spectral regions could discriminate between the populations with a minimal overall accuracy of 80%, with some discrimination occurring with greater than 90% accuracy.

DISCUSSION

It has become evident that the current methods used to monitor patients in most asthma clinics (i.e., history, physical examination, and spirometry) are somewhat insensitive in their ability to

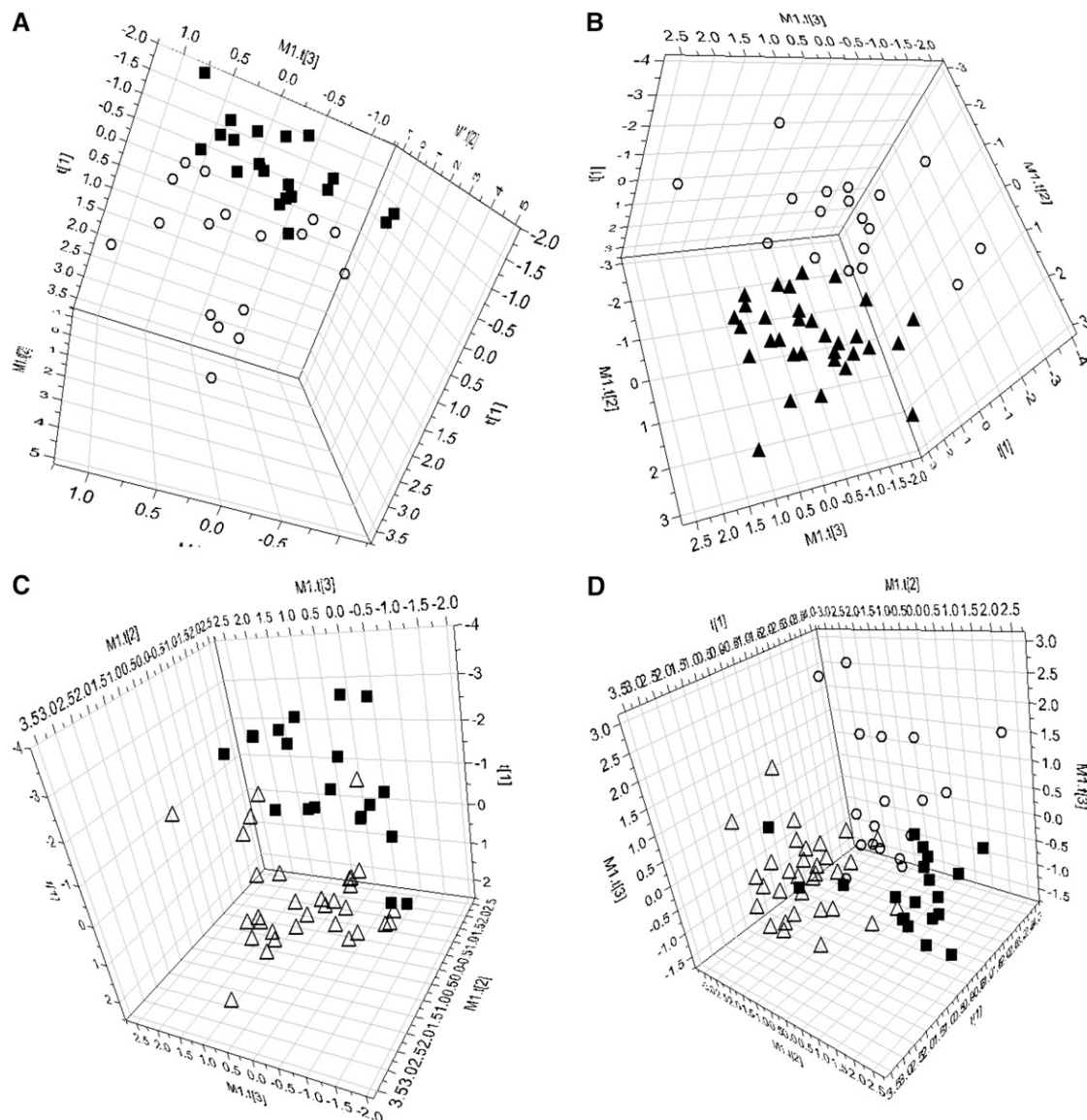


Figure 4. Three-dimensional plots illustrating partial least-squares discriminant analysis (PLS-DA) separation of animal groups on the basis of nuclear magnetic resonance-derived urine metabolite concentrations: (A) control (circles) versus sensitized (squares) ($R^2 = 0.53$; $Q^2 = 0.29$); (B) control (circles) versus challenged (triangles) ($R^2 = 0.74$; $Q^2 = 0.59$); (C) sensitized (squares) versus challenged (triangles) ($R^2 = 0.63$; $Q^2 = 0.50$); and (D) three-way analysis of all animal groups ($R^2 = 0.54$; $Q^2 = 0.25$).

reliably identify changes in airway pathophysiology necessitating therapeutic intervention (25, 26). Furthermore, spirometry, the most common objective measurement available to the majority of physicians, is not feasible for most children less than 6 years of age. A Cochrane analysis suggested that measurement of inflammatory changes improves clinical outcomes (27). Although noninvasive tests, such as sputum or exhaled NO analysis, have shown clinical utility, each has some limitations (28). Sputum is not practical for all patients, especially the very young. Exhaled NO is easier to determine, but its diagnostic utility appears less than that of sputum analysis. In addition, both tests rely on essentially one biomarker (i.e., eosinophilia or NO, respectively) to describe the heterogeneous phenotype of asthma. Experience with NMR analysis of sputum in patients with cystic fibrosis led us to consider urine as another biofluid for NMR analysis (29).

Although we acknowledge that our animal model is not a replica of the human asthma experience, it represents an established animal model of allergic airway dysfunction, which in part reflects what occurs during a human asthma exacerbation (30). Airway reactivity is commonly measured as the degree of bronchoconstriction in response to a variety of agents, including histamine (14). As expected, antigen-challenged ani-

mals developed AHR, which correlated with increased airway inflammation measured in lung fluid and histology. Airway inflammation is a complex physiological state involving the metabolism of not only the inflammatory cells, but also the cells affected by inflammation, including epithelium, smooth muscle, nerves, and connective tissue. In addition, it is important to consider that asthma is a systemic illness with cell recruitment from bone marrow, through blood to lung tissue (31). Further, the stress of airway dysfunction could affect organs outside the lung (i.e., hypoxemic brain injury). Thus, our methodology is designed to consider a wider range of biomarkers than just those associated with classic airway inflammation.

Multivariate statistical analysis of the NMR spectra, using either the metabolite concentrations or spectral data exported as an xy-trace, could discriminate between the various groups of animals in our model representing the spectrum of an asthma exacerbation. It is important to note that although most of the regions determined from xy-trace data largely represent as yet unknown metabolites, there was overlap between the methodologies. For example, of the regions shown in Figure 6A, metabolites such as oxalacetate, glucose, and tyrosine have resonant peaks in these areas and could be one of the metabolites being detected by the xy-trace/statistical method.

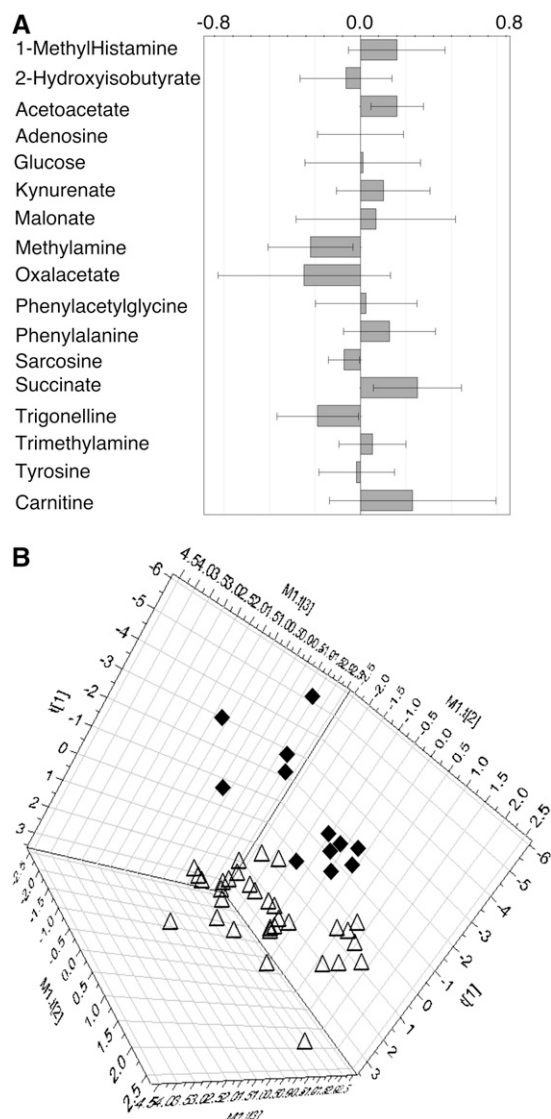


Figure 5. (A) The coefficients of variation plot of nuclear magnetic resonance-derived urine metabolite concentrations used by partial least-squares discriminant analysis in modeling of challenged versus challengeddex guinea pigs (*horizontal lines* represent 95% confidence intervals). (B) partial least-squares discriminant analysis visualization of separation between challenged animals (*triangles*) and challengeddex animals (*diamonds*) ($R^2 = 0.76$; $Q^2 = 0.44$).

Thus, the two methodologies for spectral and metabolomic analysis become complementary by identifying expected and unexpected metabolites in NMR spectra. Ongoing research is characterizing the relevant new metabolites identified by xy-trace data and multivariate analysis.

The dexamethasone data show that although AHR is reduced relatively quickly (i.e., 4 d), the number of eosinophils was not decreased. This was not unexpected. *In vitro*, corticosteroids induce eosinophil apoptosis (32–35); however, although apoptosis inhibits the ability of eosinophils to induce airway hyperreactivity, it will still leave the structure of the cells intact for histologic staining (35–37). In addition, Uller and coworkers report that at least 3 days is required to show decreased tissue eosinophils after dexamethasone treatment, and that these cells are removed by transit into the airway lumen (37), further increasing the numbers in bronchoalveolar lavage. In this animal model similar results with dexamethasone describe

improved lung function without a decrease in eosinophil number (15). The explanation suggested is that whereas dexamethasone does not decrease eosinophils in bronchoalveolar lavage, it does decrease the number of tissue eosinophils, specifically near airway parasympathetic nerves (38). These nerves play a critical role in controlling airway smooth muscle and mucous glands (39). We have not repeated the work described in these reports as it would require focused work beyond this article's objective. Our rationale for adding dexamethasone to our model was simply to add another condition that could be clinically relevant and to demonstrate the ability of a potentially noninvasive technique to monitor another phenotype of asthma.

This pilot study was not designed to confirm the importance of specific metabolic pathways resulting from airway dysfunction. However, on the basis of the data, we have some speculations concerning possible relevant pathways, which will impact future study design. For example, 2-hydroxyisobutyrate, 3-hydroxybutyrate, 3-methyladipate, and glucose were all lower in challenged versus both sensitized and control animals. The difference observed in challenged guinea pigs may reflect a switch from fatty acid oxidation to glycolysis.

The antigen-challenged animals go through a period of increased respiratory effort and smooth muscle bronchoconstriction. Creatine levels declined in challenged animals compared with both sensitized and control animals. Creatine, endogenously synthesized, is transported to organs such as muscle, where it is phosphorylated to phosphocreatine (40, 41). Similarly, carnitine, a nonessential amino acid, transports fat into the mitochondria of muscle cells for oxidation (42). Sarcosine, an intermediate in the metabolism of choline to glycine, is found in muscles and plays a role in neurotransmission. The increased level of sarcosine in challenged guinea pigs decreased after treatment with steroid. Overall, these changes may reflect changes in skeletal and lung muscles.

Tyrosine was decreased in challenged animals. Modification of free tyrosine residues after eosinophil stimulation and degranulation occurs in the airways of humans with asthma (43). Previous work from our group identified the presence of modified tyrosine residues, 3-chlorotyrosine and 3-bromotyrosine, in sputum samples taken from patients with cystic fibrosis and asthma, respectively (29). Given our inflammatory cell data, we hypothesize that the reduction of urinary tyrosine could relate to increased metabolic activity of eosinophils and/or neutrophils. Unfortunately, directly measuring concentrations of 3-chlorotyrosine and 3-bromotyrosine in urine is not possible, as they are below the limit of NMR detection using our current parameters.

myo-Inositol is a second messenger in various cell types, including inflammatory cells (44, 45). In respiratory distress syndrome of prematurity, *myo*-inositol levels correlate with lung maturation and recovery from disease (46). *myo*-Inositol was lowest in challenged and highest in challengeddex animals, possibly reflecting altered inflammatory signaling or lung recovery after steroid treatment.

Phenylacetylglutamine decreased after sensitization, and declined further after challenge. The human equivalent of phenylacetylglutamine in our rodent model is phenylacetylglutamine (47). Phenylacetylglutamine levels increase in humans and rodents with syndromes of phospholipidosis (48–50), impaired amino acid absorption, and states of increased gut absorption of phenylacetate (48). In humans, gut permeability and microflora may influence the development of atopy and asthma (51). The decreased concentration of phenylacetylglutamine in the urine of sensitized and challenged guinea pigs may signify the generalized systemic effects of our asthma exacerbation model on gastrointestinal function.

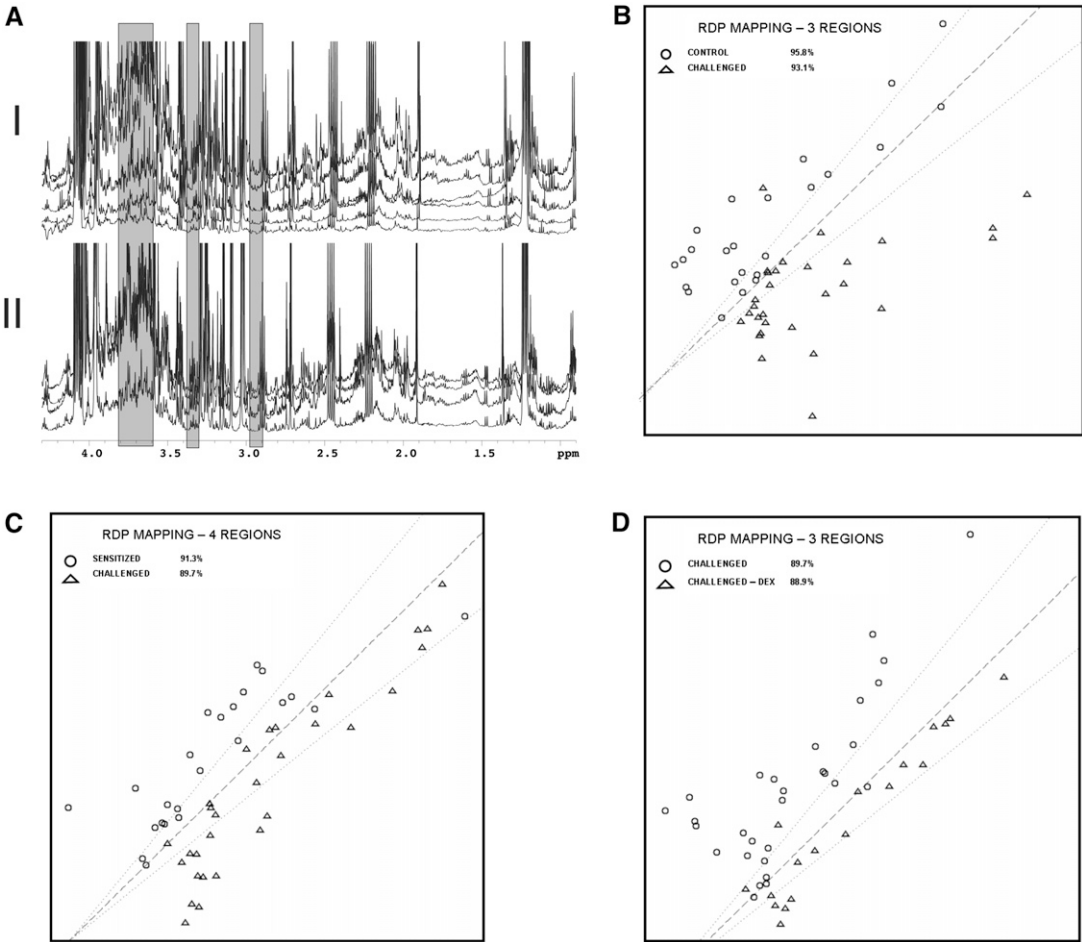


Figure 6. The multivariate statistical classification strategy was applied to xy-trace data exported from urine nuclear magnetic resonance (NMR) spectra. (A) An example of one focused area within the one-dimensional ¹H NMR spectrum from some challenged (I) and control (II) guinea pigs, illustrated as a stack of individual results (n = 6 each). Regions differentiating challenged from control animals are highlighted (gray). The separation of each pair of animal groups (classifiers) is illustrated by relative distance plane (RDP) mapping: (B) control (circles) versus challenged (triangles); (C) sensitized (circles) versus challenged (triangles); and (D) challenged (circles) versus challengeddex (triangles).

Succinate excretion in urine was low after both sensitization and sensitization challenge. Exposure to various drugs or toxins, which affect liver, renal proximal tubules, and lung, are reported to produce a reduction in urine levels of succinate (52, 53), although the mechanism remains unclear.

As expected, dexamethasone administration altered urinary measurements of metabolism in both control and challenged guinea pigs. Dexamethasone-treated controls showed significantly lower levels of phenylacetylglycine compared with controls. However, challenged animals appeared to have even

TABLE 2. GROUP SEPARATION ACCURACY BY OPTIMAL NUCLEAR MAGNETIC RESONANCE SPECTRAL REGION ANALYSIS

Pair Classifiers	Optimal Regions (PPM)	Acc(1)	Training Sets Acc(2)	Acc(O)	Acc(1)	Test Sets Acc(2)	Acc(O)
Control vs. sensitized	3.93–3.92 3.71–3.72 1.10–1.12	82.5 ± 7.3	88.2 ± 8.6	85.2 ± 6.6	85.8 ± 9.7	88.4 ± 11.0	87.1 ± 6.0
Control vs. challenged	3.56–3.58 3.33–3.34 2.91–2.94	76.7 ± 12.0	83.6 ± 9.6	80.4 ± 5.9	81.7 ± 14.0	83.3 ± 14.0	82.6 ± 6.8
Sensitized vs. challenged	3.92–3.94 3.20–3.22 2.25–2.27	83.6 ± 15.0	87.2 ± 9.4	85.6 ± 6.3	81.2 ± 21.0	88.7 ± 12.0	85.6 ± 7.5
Challenged vs. challengeddex	2.11–2.14 3.88–3.92 3.44–3.48	87.2 ± 8.8	85.6 ± 5.4	86.6 ± 4.8	87.9 ± 12.0	80.0 ± 19.0	84.6 ± 6.2
Control vs. challengeddex	3.16–3.20 4.18–4.21 2.69–2.72	91.7 ± 7.9	87.8 ± 8.2	90.0 ± 2.7	96.7 ± 5.8	91.1 ± 13.0	94.3 ± 4.9
Sensitized vs. challengeddex	2.39–2.41 2.91–2.93 1.80–1.80 1.65–1.65	88.2 ± 6.1	94.5 ± 7.9	91.0 ± 5.2	93.3 ± 9.5	90.0 ± 11.0	91.9 ± 6.7

Optimal nuclear magnetic resonance (NMR) spectral regions for separation of the guinea pig populations were determined by linear discriminant analysis and leave-one-out internal cross-validation. Training sets show the average and standard deviations of the classification result after external cross-validation, using 10 random splits of the data. Test sets show the predictive discriminative accuracy for these regions determined after NMR data from animals not part of the original training set were blindly tested using the model. Acc(1), Acc(2), and Acc(O) refer to class 1, class 2, and overall percent classification accuracies, respectively.

lower phenylacetylglycine levels, which returned to control levels after dexamethasone treatment. This suggests that although dexamethasone may disrupt normal amino acid and phospholipid homeostasis in control animals, it may be counteracting a pathological pathway in the challenged animals (48, 50). Dexamethasone appeared to reverse the effects of challenge on urinary excretion of 2-hydroxyisobutyrate and glucose, as levels rose in challenged animals. However, future studies targeted at defining the specific metabolic pathways are required to confirm their relevance both in this animal model and ultimately in humans with asthma.

Given the complexity of asthma pathophysiology, it may not be surprising that a combination rather than a single metabolite or NMR spectral region would be required to reliably separate the animal models. Our report is the first to demonstrate that airway dysfunction in an animal model of asthma correlates with an altered urinary NMR metabolite profile. The results of our study show that ^1H NMR spectroscopic analysis of urine in an animal model of asthma could differentiate animals with or without airway inflammation and AHR. It appears that the candidate factors in differentiating animals are a combination of more than one metabolite, that is, a “panel” or profile of biomarkers. There is increasing interest in the use of metabolomics-based techniques in human disease (54). In the field of asthma, groups have studied single metabolites in a variety of biofluids including urine with variable success in clinical practice (55–58). Our approach, applying multivariate statistical analysis to a combination of factors in urine, is unique in the field of asthma research. We believe it is a more insightful approach given the heterogeneity of humans with asthma. In conclusion, we suggest that urine NMR analysis may become a useful diagnostic tool in monitoring humans with asthma, especially children, for whom most objective measures of airway function are often difficult.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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