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Severity of allergic rhinitis assessed by using urine metabolomic profiling: Proof of concept



To the Editor:

The ability to monitor the severity of respiratory diseases is difficult in the typical clinical setting, particularly for diseases of the upper airway, such as allergic rhinitis (AR). Patients often do not recognize how severe their symptoms are because of progressive “tolerizing” to symptoms. Chronic nasal congestion becomes the new normal. Clinical trials of AR therapies are associated with high placebo response rates to the degree that the US Food and Drug Administration has indicated AR as one of the few conditions in which including a placebo arm is not only ethically justified but also mandatory.¹ As such, self-reported symptoms used by clinicians to guide therapy are likely often flawed. In most outpatient settings, physicians rely simply on the patient’s history before administering trials of therapy. Potentially, this can lead to unnecessary implementation of additional treatments with potential side effects.

Metabolomics is the study of metabolic pathways and the unique biochemical molecules created in a living system.^{2,3} By measuring changes in metabolite concentrations, the range of biochemical effects induced by a disease or its therapeutic intervention can be determined. Previously, we demonstrated that metabolomic profiling of urine can be used to differentiate healthy children from those with asthma,⁴ children with unstable asthma in the emergency department from those with stable asthma in an outpatient clinic,⁴ and adults with asthma versus those with chronic obstructive pulmonary disease (COPD).⁵

We hypothesized that the metabolic activity of adults with AR would change in accordance with increased nasal airway dysfunction after allergen challenge. We recruited participants with proved ragweed-induced AR and similar age, sex, and body

mass index for ragweed challenge (see [Table E1](#) in this article’s Online Repository at www.jacionline.org; for complete methodology, see the [Methods](#) section in this article’s Online Repository at www.jacionline.org). Participants scored their symptoms before and after allergen challenge by using the accepted Total Nasal Symptom Score (TNSS). The TNSS is the sum of 4 individual symptom scores for rhinorrhea, nasal congestion, nasal itching, and sneezing, resulting in a possible score of 0 to 12 (see [Table E2](#) in this article’s Online Repository at www.jacionline.org).

A urine sample was collected from each participant before and after challenge and stored in a -80°C freezer. Metabolites were measured and analyzed for differences between participants’ values before and after allergen challenge in relation to TNSSs.

Initially, we compared the metabolites of one cohort of participants before and after ragweed challenge. We saw that the TNSS severity of each participant was not uniform, even though they were all obtained before allergen challenge (TNSS, 0-5/12). In addition, the reaction to the challenge was not uniform (TNSS, 6-12/12). Based on this, we hypothesized that the metabolome of these subjects also might not be as uniform. To better define a clear metabolomic difference, we needed to better define those who were quite well versus those who had experienced a clear significant allergen challenge response. The TNSS data were used to sort the subject’s prechallenge and postchallenge values. We created a metabolomic model using the subjects with the lowest (0) versus the highest (12) TNSS.

Partial least-squares discriminant analysis (PLS-DA) was performed (SIMCA-P 11; Umetrics, Umea, Sweden). This process identifies metabolites with concentrations that differed significantly between groups of participants expressed as a coefficient of variation plot. Metabolites with consistently greater differences in concentration between groups are displayed by the software as variables of importance to the projection plot (VIP). 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 showed the model the blinded values of metabolites from the remaining participants in group 1 with TNSSs from 1 to 11. The PLS-DA-based model generates a prediction score (0-1) of unclassified blinded data not part of the model. The model blindly assigned scores to these participants (ie, scores >0.5 would be predicted to be TNSS 0, whereas scores <0.5 were predicted to be TNSS 12). We then removed metabolites from the model based on correct blinded analysis scores (scores, 1-11). Using this approach, we created a robust metabolomic model.

The final list of metabolites used to separate mild AR scores from severe scores used 2 components consisting of 11 metabolites, resulting in an R^2 value of 0.85 and Q^2 value of 0.77 ([Fig 1, A](#)). Differences in concentrations of these metabolites before and after challenge are shown ([Fig 1, B](#)). The final metabolites chosen and their concentrations are shown in [Table E3](#) in this article’s Online Repository at www.jacionline.org.

Graphic presentation of the separation between groups in the model (TNSS, 0 or 12) or test subjects with intermediate TNSSs (1-11) are shown by their respective PLS-DA scores ([Fig 1, C](#)). There is a stepwise change in prediction score as the severity of the nasal symptoms increase.

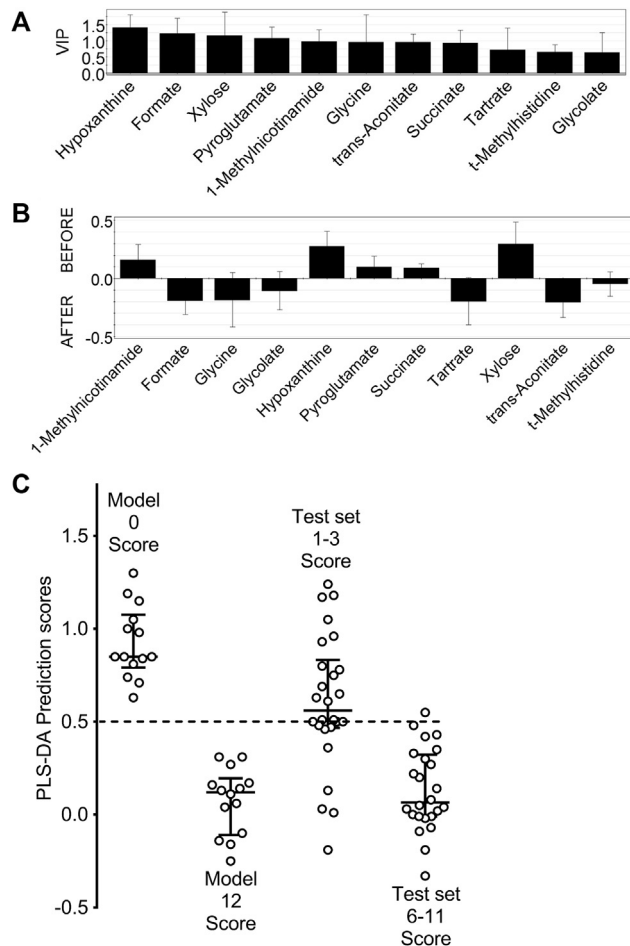


FIG 1. Metabolomic model differentiating adults before and after allergen challenge. Urine metabolite levels were measured in subjects before and after allergen challenge. Values were sorted based on TNSSs (TNSS = 0, 1-5, 6-11, and 12). PLS-DA analysis of metabolites created a model of separation for those with scores of 0 or 12 ($R^2 = 0.85$, $Q^2 = 0.77$). By using subjects with scores of 1 to 11 as a test set, metabolites of lower significance were removed. **A**, VIP plot ranking metabolites according to their significance. **B**, Scaled and centered metabolite differences between groups 0 and 12 are shown before and after challenge. **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 participant has a low TNSS (0), whereas a value of less than 0.5 indicates a greater TNSS (12).

To validate the proposed model and test its applicability as a tool for rhinitis severity, we presented the concentrations of metabolites from another group of subjects from a separate visit with similar ragweed sensitization and allergen challenge ($n = 28$). The subjects' urine metabolite values were entered for analysis by using the PLS-DA model without a diagnosis, and thus the computer was blinded for the TNSS (rhinitis severity). Individual PLS-DA prediction scores are shown (Fig 2, A), with error bars representing medians and interquartile ranges. A threshold value of 0.5 was used to separate groups. Again, a stepwise prediction score is seen as the severity of the rhinitis score increases. The accuracy of the metabolomic model is shown as a receiver operating characteristic (ROC) with an area under the curve of 0.92 (Fig 2, B). Values of the key metabolites for each subject are shown in Table E3.

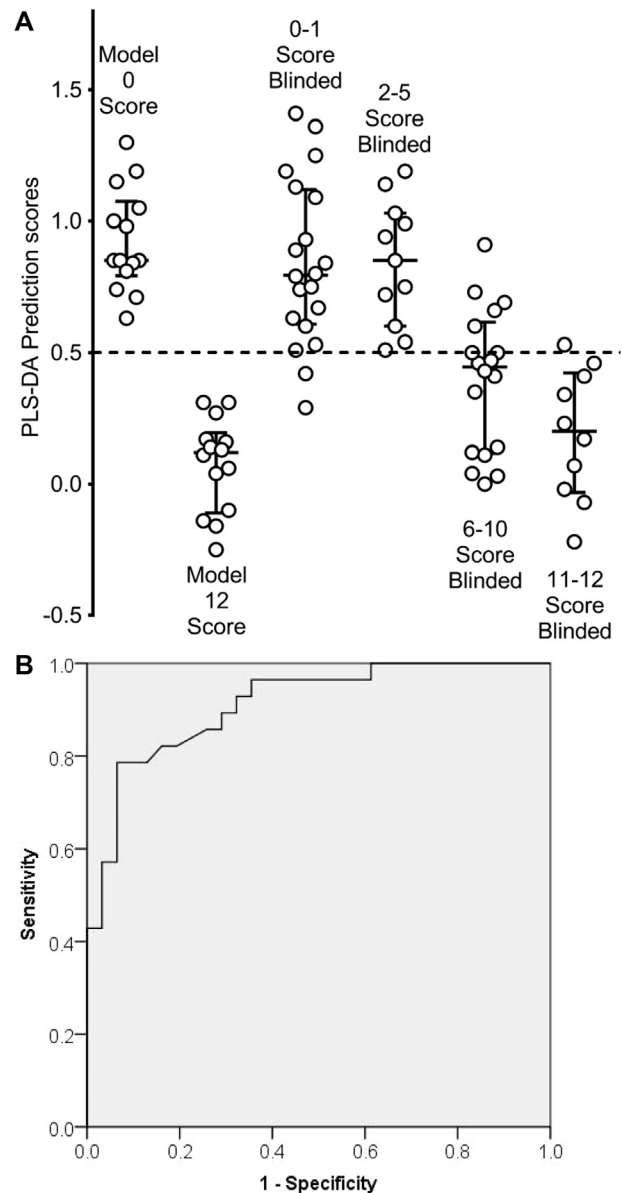


FIG 2. The metabolomic model correctly identifies a blinded test. Urine metabolite levels were measured from a separate cohort of allergen-challenged subjects. **A**, PLS-DA analysis of selected metabolites shows similar stratification of subjects in relation to TNSSs, as shown by PLS-DA prediction scores. **B**, Using the second group of participants ($n = 28$), we assessed the blind accuracy of the model. The ROC 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 (IBM SPSS Statistics for Windows, Version 20.0). TNSSs of 6 or greater were defined as the disease state, and smaller predictive scores indicate a more positive test result.

There remain limitations of this study. Although our sample size is small, we believe this strengthens the findings given that we were still able to detect an AR metabolomics signal. We modeled on participants with the most severe symptom scores. It would be useful to evaluate those with moderate responses to allergen challenge, but it is likely that this would require a larger sample size.

In conclusion, we provide proof-of-concept evidence that the metabolomic analysis of excreted urine metabolites can differentiate prechallenge and postchallenge values and grade

severity in patients with AR. We suggest that having such an objective test could assist primary care physicians, allergists, or researchers to determine actual disease severity and, as such, potential response to therapy.

Metabolomic technology could easily be incorporated into a standard laboratory, especially with the advent of enhancements (ie, robotics) that decrease operational costs. The ability to use urine as a diagnostic tool makes the methodology readily applicable to most clinical settings. This metabolic approach could also provide new insights into the pathophysiology of airway dysfunction, suggesting novel pathways for drug discovery.

Finally, we believe this work can be complementary to allergic airway diseases, such as asthma, and provide an objective measure of severity.

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Disclosure of potential conflict of interest: D. J. Adamko is CEO of a startup company that someday hopes to see this type of work used in clinical practice but does not have any patents for the data in this letter. A. K. Ellis reports personal fees from AstraZeneca, Aralez, Boehringer Ingelheim, Padiapharm, Novartis, Pfizer, ALK-Abelló, and Merck and also reports grants from Merck, Novartis, and Sanofi-Genzyme outside the submitted work. The rest of authors declare that they have no relevant conflicts of interest.

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A novel human mast cell activation test for peanut allergy



To the Editor:

Peanut allergy (PA) has a significant effect on patients' lives, and therefore an accurate diagnosis is extremely important. Peanut-specific IgE (P-sIgE) is associated with false-positive results and overdiagnosis.¹ Measurement of Ara h 2-specific IgE is more accurate but is associated with false-negative results. Thus a considerable proportion of patients need to undergo an oral food challenge (OFC), the current gold standard to diagnose food allergy.² OFCs carry the risk of causing allergic reactions, including anaphylaxis. With the advent of new treatments for PA, use of reliable *in vitro* tests rather than OFCs to identify eligible patients and monitor clinical response to treatment is desired.

Previously, we showed that the basophil activation test (BAT) is highly discriminative between children with PA and children with peanut sensitization but not allergy (PS children) and can reduce the number of OFCs.³ Because the BAT requires fresh blood and 10% to 15% of individuals have uninterpretable BAT results caused by nonresponding basophils (ie, basophils that do not respond to IgE-mediated but only non-IgE-mediated stimulants),^{4,5} we investigated whether the ability to elicit peanut-induced cell activation could be transferred by passive sensitization of LAD2 mast cells⁶ with patients' plasma.

Children being assessed for PA (n = 174), including 73 children with PA, 60 PS children and 41 nonsensitized nonallergic (NA) children, underwent clinical assessment, skin prick tests, blood collection for immunoglobulin measurement (by using ImmunoCAP; Thermo Fisher Scientific, Waltham, Mass), and OFCs to peanut, as previously described.^{3,7} Participants were grouped as patients with PA, PS patients, or NA subjects. The allergic reaction severity was classified according to the method of Ewan and Clark,⁸ and the threshold dose was determined as the total amount of peanut protein ingested during the OFC. The study was approved by the South East London Research Ethics Committee 2. Whole blood BATs and mast cell activation tests (MATs) to peanut were performed, as previously described.^{3,9}

Statistical analyses were performed with SAS 9.4 software (SAS Institute, Cary, NC) and JMP Pro software, Version 13.2.1. Depending on data distribution, nonparametric Wilcoxon tests or normality-based *t* tests were used, where specified. Optimal cut points were estimated from receiver operating characteristic analyses based on logistic regression models. Relationships between mechanistic outcomes were analyzed by using stratified linear models; cubic splines were used to allow for more linear curve relationships between variables. When relationships appeared linear, Pearson correlation coefficients were reported and visualized with simple linear models and 95% CIs.

LAD2 cells expressed FcεRI and CD32 on their surfaces (see Fig E1 in this article's Online Repository at www.jacionline.org). After addition of patients' plasma, IgE was detected on the cell surface. Stimulation index (SI) IgE phycoerythrin-Cy7 was strongly correlated with plasma total IgE levels ($R_s = 0.914$, $P < .001$; see Fig E2, A, in this article's Online Repository at www.jacionline.org) and comparable between children with PA

METHODS

Patient enrollment

We recruited men and women with proved ragweed sensitization for ragweed challenge. All participants had a positive skin prick test response to short ragweed (defined as a wheal ≥ 3 mm compared with the diluent control) and a minimum 2-year history of symptoms consistent with allergic rhinoconjunctivitis. Participants with signs/symptoms of active perennial rhinitis were excluded. Participants were not allowed to take any concomitant medications, with the exception of vitamins and oral contraceptive pills for women of childbearing potential if that was their pre-existing method of contraception at the time of screening. Patients were enrolled after providing written informed consent approved by Queens University Health Science and Affiliated Teaching Hospitals Research Ethics Board and registered at clinicaltrials.gov (NCT02230475).

Ragweed challenge

We had 2 groups ($n = 41$ and 28). Each group scored their symptoms before and after allergen challenge by using the accepted TNSS. The TNSS is the sum of 4 individual symptom scores for rhinorrhea, nasal congestion, nasal itching, and sneezing, resulting in a possible score of 0 to 12 (Table E2). The groups were challenged on different days but exposed to identical levels of ragweed pollen. The environmental exposure unit (EEU), which is located in Kingston General Hospital, allows for reliable exposure of up to 120 participants to pre-determined levels of allergen that remain consistent regardless of outside weather conditions to determine the efficacy and onset of action of new therapies for AR. A custom-engineered computer-aided system disperses allergen at a single point of delivery. Specifically placed and aimed groups of directional fans propel the allergen over the participants' seating area.

The allergen was sourced from Greer Laboratories (Lenoir, NC). Before use, the allergen was independently tested for fungal and bacterial, as well as endotoxin, contamination (Paracel Laboratories, Ottawa, Ontario, Canada). Any concentration of allergen from 200 to 10,000 grains/m³ can be achieved for as long as required and limited only by the participant's tolerance, and studies have been carried out for up to 14 continuous hours of exposure. Typically, ragweed, grass, and birch studies in the EEU target 3,500 grains/m³, which is consistent with peak seasonal levels and can be delivered on consecutive days with an average variability of only 5% to 9%.

Rotorod impact samplers rotate sampling rods at 2,400 rpm, sampling 47.3 L of air per minute by collecting pollen grains on their greased surfaces, which can be microscopically counted. Samples are taken for 30 seconds at 30-minute intervals, which correspond to the participant symptom scoring times. The 7 Rotorod samplers are distributed throughout the participant's seating area, and allergen levels are monitored and recorded throughout each clinical study period. Allergen levels in the EEU have been shown to be consistent throughout the participant's seating area and over time during different study periods. The ventilation system uses 100% HEPA-filtered outdoor air, with temperature (18°C to 25°C) and humidity (40% to 60%) control, and the CO₂-laden air is exhausted directly to the outdoors. The room is kept under slightly negative pressure to minimize allergen loss to the rest of the hospital. Throughout the study, participants record their symptoms on diary cards, which are scanned into a locked database, providing real-time data in graphic format within 20 minutes of each data collection period.

Urine sample collection

A single urine sample was collected from each patient and promptly placed in a freezer (-20°C). Samples were frozen shortly after collection and stored in a -80°C freezer. Although most samples were analyzed within 1 month of collection, we have reported previously 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 for diet 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 sufficiently different between the disease

and nondisease groups that such intrapersonal variability would be superseded.

Statistical analysis of clinical data

GraphPad Prism software (V.6; GraphPad Software, La Jolla, Calif) was used for statistical analysis of the clinical data. Nonparametric *t* tests were used to compare 2-group experiments. Repeated-measures ANOVA with the Bonferroni multiple comparison test were used to compare multiple groups. Data are presented as the mean $\pm$ 1 SEM or median with interquartile range. 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 D₂O; Chenomx, 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 nuclear magnetic resonance (NMR) tube (Wilmad, Vineland, NJ).

NMR acquisition and spectral analysis

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 a 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 (free induction decay). 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.^{E5}

Spectral identification and quantification of 86 identifiable metabolites was performed by using 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. These known resonant frequencies were matched to the observed resonant frequencies of the collected spectra, enabling qualitative and quantitative analysis of metabolites in urine. To account for hydration status of the subjects, metabolites were referenced to creatinine, and values were log-transformed.

Statistical analysis of metabolite values

We used one group of subjects ($n = 41$) to create a metabolomic model while saving the second group as a test set for accuracy ($n = 28$). Samples were categorized based on rhinitis severity by using the TNSS. Subjects with extremes of severity (ie, TNSSs of 0 or 12) were used to create a model of separation. 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 using simultaneous projections of both *Y* and *X* spaces to a plane.

Seven-fold internal cross-validation was performed. This process identifies metabolites with concentrations that differed significantly between groups of patients expressed as a coefficient of variation plot. 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 VIP.

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 showed the model the blinded values of metabolites from the remaining subjects in group 1 with TNSS scores from 1 to 11. The PLS-DA-based model generates a prediction score (0-1) of unclassified blinded data that were not part of the model. The model blindly assigned scores to these subjects (ie, scores >0.5 would be predicted to be TNSS 0, whereas scores <0.5 were predicted to be 12). We then removed metabolites from the model based on correct blinded analysis scores of these test (having scores of 1-11). We set a false-positive rate of 5% to 10% as an acceptable limit. This also resulted in the most sensitive models, with respect to correlation coefficients (R^2) and prediction properties (Q^2).

Using the second group of subjects ($n = 28$), we assess the blinded accuracy of the model. The ROC 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 (IBM SPSS Statistics for Windows, Version 20.0; IBM, Armonk, NY). TNSSs of 6 or greater were defined as the disease state, and smaller predictive scores indicated a more positive test result.

DISCUSSION

This is the first report to show metabolites associated with AR. 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 model. For example, the urinary concentration of 1-methylnicotinamide decreased in the post-allergen challenge samples. 1-Methylnicotinamide is known to exhibit anti-inflammatory properties through oxygen radical scavenging.^{E6} Decreased urinary concentration levels of 1-methylnicotinamide has been also reported in patients with COPD^{E7} and lung cancer.^{E8} The observed change in 1-methylnicotinamide levels might indicate its increased consumption to combat inflammation induced by ragweed or might be due to impairment in the methylation process of nicotinamide.^{E9}

Trans-aconitate increased postallergen exposure

Trans-aconitate is a precursor for synthesis of S-adenosyl-L-homocysteine, which is the immediate precursor for all homocysteines produced in the body. S-adenosyl-L-homocysteine is also an integral component in the metabolism of several amino acids, methionine, and nicotinamide.^{E9,E10} Trans-aconitate has been identified as an oxidative stress biomarker that was significantly increased in mouse models of Alzheimer disease.^{E11} Its increase was also reported in patients with relapsing ulcerative colitis.^{E12} Its increase in this model might indicate increased oxidative and inflammatory stress after allergen exposure.

3-Methylhistidine levels increased in urine of patients after allergen exposure. 3-Methylhistidine is a component of the skeletal muscles, and its increased excretion in urine indicates myofibrillar protein breakdown.^{E13} Ubhi et al^{E14} have reported an increase in serum 3-methylhistidine levels in patients with GOLD stage IV COPD in comparison with control subjects.

Urinary formate, tartarate, and glycolate expression increased in subjects after allergen challenge, whereas succinate expression decreased post challenge. Formate, succinate, tartarate, and glycolate are intermediates in the glyoxylate and dicarboxylate metabolism pathway, a central component of carbohydrate metabolism.^{E9} Accordingly, these metabolites can indirectly

influence other biochemical processes in the body, such as the citric acid cycle, gluconeogenesis, carbon fixation, and pyruvate and thiamine metabolism.^{E9} Succinate is also a component of the citric acid cycle and is an intermediate in metabolism of several amino acids.^{E9} Its level has been reported in association with mitochondrial dysfunction and muscle.^{E15} We have previously reported increased levels of formate in patients with stable and unstable asthma.^{E16} Increased levels of formate have been also demonstrated in children with atopic dermatitis.^{E17} Alterations in tartarate and glycolate levels in patients with different respiratory diseases have not been reported previously.

Pyroglutamate levels decreased in samples after allergen exposure. Pyroglutamate is an intermediate in glutathione metabolism.^{E9,E10} Glutathione has a powerful antioxidant role and is also essential for the integrity of the immune system.^{E10} In comparison with healthy control subjects, decreased levels of pyroglutamate were reported in bronchoalveolar lavage samples collected from patients with lung cancer,^{E18} plasma samples of patients with H1N1 pneumonia,^{E19} and serum samples of patients with type 2 diabetes.^{E20} Decreased pyroglutamate levels in this study might indicate impairment in the glutathione homeostasis.

Urinary hypoxanthine levels decreased after ragweed challenge. Hypoxanthine is an integral intermediate in the synthesis of adenosine and purine nucleic acid by the salvage pathway.^{E9,E10} It is further metabolized by xanthine oxidase into xanthine and finally uric acid.^{E21} We have previously reported lower levels of hypoxanthine in asthmatic patients in comparison with levels seen in those with COPD.^{E7} Hypoxanthine levels also showed a significant decrease in human airway epithelial cells collected from patients with cystic fibrosis.^{E22} Decreased hypoxanthine levels might indicate its increased metabolism after allergen exposure, with concomitant production of oxygen radical superoxide anion that further contributes to the developing oxidative stress.^{E21}

Xylose levels decreased in postchallenge samples. Through the pentose and glucuronate interconversion pathway, xylose is converted into pyruvic acid, which is a central metabolite in several biochemical pathways, such as the citric acid cycle.^{E9,E10} The cellulolytic microflora of the human colon also breaks down cellulose and hemicellulose into xylose.^{E23} Patients with ulcerative colitis had lower urinary xylose levels in comparison with those given a diagnosis of Crohn disease.^{E24} Xylose levels have been also reported to be decreased in asthmatic patients in comparison with those in patients with COPD.^{E7}

Glycine levels increased in postchallenge samples. Glycine is an intermediate metabolite in numerous biochemical pathways, most importantly glutathione metabolism.^{E9,E10} Higher levels of glycine were detected in the sera of asthmatic patients^{E25} and patients with lung cancer.^{E26} Urine samples collected from patients with inflammatory bowel disease also showed increased glycine levels.^{E27}

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TABLE E1. Subjects' characteristics

	Group 1 (n = 41)	Group 2 (n = 28)	Group 1 model (n = 28)
Age (y), mean $\pm$ SD	41.4 $\pm$ 10.2	42.5 $\pm$ 9.3	42.0 $\pm$ 10.7
Female sex, no. (% of the cohort)	24 (59)	14 (50)	14 (50)
Body mass index, mean (SD)	29.9 (6.9)	26.5 (4.7)	29.9 (6.9)

TABLE E2. TNSS: The sum of 4 individual symptom scores for rhinorrhea, nasal congestion, nasal itching, and sneezing given a possible score of 0 to 12

Severity	Definition
None (0)	Symptom is completely absent
Mild (1)	Symptom is present but not bothersome
Moderate (2)	Symptom is bothersome but tolerable
Severe (3)	Symptom is hard to tolerate, with desired

TABLE E3. Concentrations of each metabolite for each subject group in millimoles of metabolite per millimole of creatinine

	TNSS 0, group 1			TNSS 12, group 1			TNSS 0-1, group 2			TNSS 11-12, group 2		
	Median	IQR		Median	IQR		Median	IQR		Median	IQR	
Hypoxanthine	0.0056	0.0030	0.0156	0.0005	0.0005	0.0016	0.0031	0.0050	0.0082	0.0005	0.0005	0.0005
Formate	0.0137	0.0094	0.0182	0.0274	0.0197	0.0380	0.0071	0.0125	0.0181	0.0315	0.0249	0.0422
Xylose	0.0069	0.0030	0.0181	0.0030	0.0030	0.0030	0.0036	0.0060	0.0113	0.0030	0.0030	0.0030
Pyroglutamate	0.0220	0.0188	0.0272	0.0169	0.0050	0.0214	0.0175	0.0189	0.0209	0.0207	0.0050	0.0274
1-Methylnicotinamide	0.0035	0.0027	0.0048	0.0010	0.0010	0.0029	0.0019	0.0051	0.0071	0.0010	0.0010	0.0010
Glycine	0.0691	0.0596	0.1164	0.1288	0.1000	0.1592	0.0700	0.0915	0.1461	0.2372	0.0861	0.2900
Trans-aconitate	0.0041	0.0029	0.0050	0.0062	0.0010	0.0093	0.0023	0.0030	0.0042	0.0010	0.0010	0.0010
Succinate	0.0040	0.0025	0.0052	0.0026	0.0011	0.0033	0.0015	0.0025	0.0037	0.0043	0.0002	0.0051
Tartarate	0.0005	0.0005	0.0201	0.0081	0.0022	0.0214	0.0012	0.0015	0.0084	0.0023	0.0005	0.0763
3-Methylhistidine	0.0193	0.0140	0.0223	0.0178	0.0030	0.0221	0.0129	0.0144	0.0182	0.0030	0.0030	0.0030
Glycolate	0.0348	0.0269	0.0451	0.0420	0.0325	0.0582	0.0213	0.0314	0.0455	0.0300	0.0040	0.0458

IQR, Interquartile range.