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Urinary metabolomic changes as a predictive biomarker of asthma exacerbation

To the Editor:

Exacerbations requiring hospitalization represent a high risk in patients with severe asthma. Predictive biomarkers of exacerbations are needed but are not available. Oxidative stress resulting from pulmonary reactive oxygen species formation is involved in asthma, leading to physiologic damage.[1] Asthma exacerbation is associated with increased oxidative stress,[2] with increased levels of carbon monoxide, nitric oxide, nitrotyrosine, and H_2O_2 found in breath condensate. Aldehydes and alkanes, the end products of lipid peroxidation, are also known to be involved in asthma-related oxidative stress.[3]

Urine is a stable, noninvasively collectable body fluid with a complex metabolic composition, the profiling of which might have a role in asthma diagnosis and monitoring. The 2 techniques used in this study, comprehensive bidimensional gas chromatography coupled to mass spectrometry (GC × GC-ToFMS) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, allow the analysis of volatile and nonvolatile compounds, thus providing complementary information on the urinary metabolome.

The aim of this pilot study was to assess the urinary metabolic changes linked to asthma exacerbation. Aldehydes and alkanes were studied by using GC × GC-ToFMS analysis, and NMR was used to provide an overview of changes in major metabolites involved in central metabolic pathways.

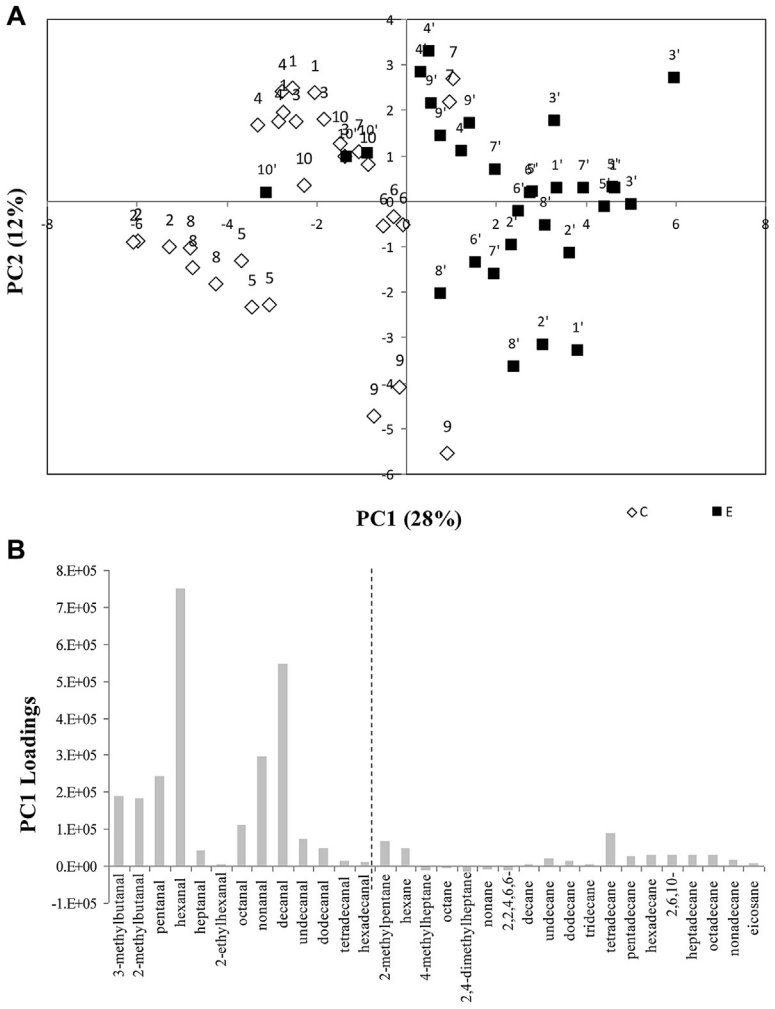

FIG 1. PCA applied to GC × GC peak areas of aliphatic aldehydes and alkanes detected in the urine of subjects in the stable state and under exacerbation conditions. **A,** PC1 versus PC2 score scatter plot in which *c* corresponds to the control condition *(open symbols)* and *e* corresponds to exacerbation *(solid symbols)*. The numbers included close to the symbols correspond to the sample ID. **B,** PC1 loadings plot explaining the separation observed in the score map (positive loadings = compounds increased in exacerbated condition).

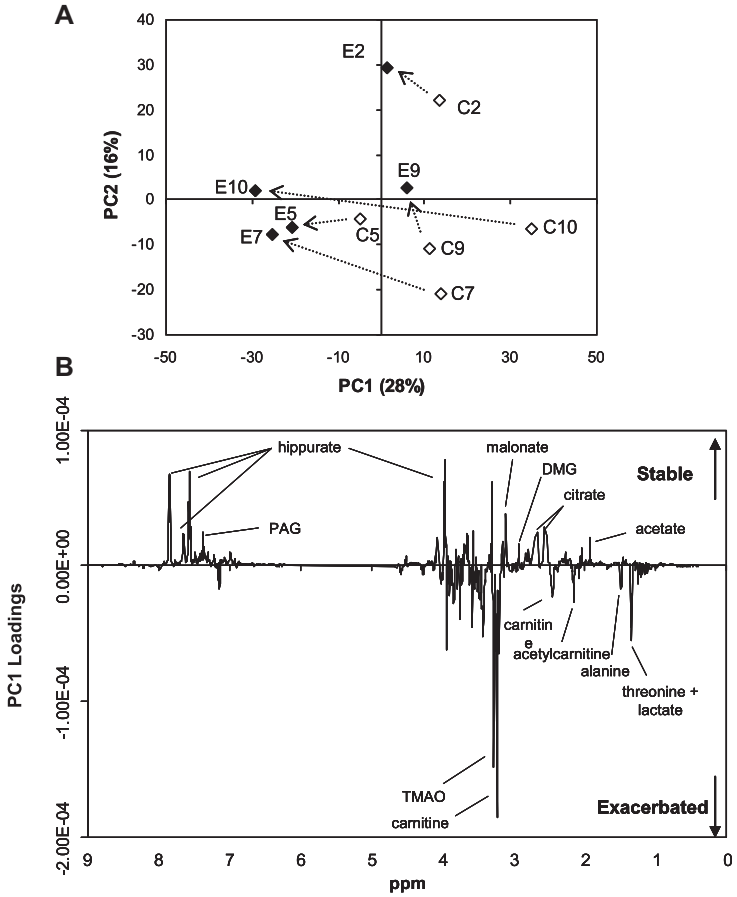

FIG 2. PCA applied to ^{1}H-NMR urine spectra of subjects in the stable state and under exacerbation conditions. **A,** PC1 versus PC2 score scatter plot. *Arrows* highlight the direction of change from stable *(open symbols)* to exacerbation *(solid symbols)* conditions for each subject. The numbers included close to the symbols correspond to the sample ID. **B,** PC1 loadings plot explaining the separation observed in the score map (positive loadings = compounds increased in stable conditions; negative loadings - compounds increased in exacerbated conditions).

A prospective cohort included 10 adult asthmatic patients. Written informed consent was provided, as was approval from the ethics board of the Hospitais da Universidade de Coimbra. All of the patients had a definite diagnosis of asthma (see the Methods section in this article's Online Repository at www.jacionline.org). All patients were studied at 2 different stages: during exacerbation (see the Methods section in this article's Online Repository at www.jacionline.org) and during a control state, as defined by results of the Asthma Control Test.

Urine was collected during exacerbation and in the stable state (see the Methods section in this article's Online Repository). All samples were analyzed by using solid-phase microextraction (SPME)/GC × GC-ToFMS,[4] and samples from 5 of 10 subjects were also analyzed by using ^{1}H-NMR spectroscopy (see the Methods section in this article's Online Repository).[5]

Exploratory principal component analysis (PCA) was applied to GC × GC peak areas, as well as to ^{1}H-NMR spectra, to extract the metabolic features of the exacerbation (see the Methods section in this article's Online Repository).

All patients (5 male and 5 female patients; mean age, 50 ± 18.8 years) were treated before exacerbation with inhaled corticosteroids and long-acting β-agonists. Seven patients had an allergic phenotype (see Table E1 in this article's Online Repository at www.jacionline.org).

PCA was applied to data matrices comprising aliphatic aldehydes and alkanes (Fig 1; and see Table E2 in this article's Online Repository at www.jacionline.org). The score scatter plot of the first and second principal components (PC1 and PC2; Fig 1, *A*), explaining 40% of the total variability, show the separation of exacerbation-related samples from control samples along PC1. The corresponding PC1 loadings (Fig 1, *B*) show that subjects under the exacerbated conditions were characterized by higher levels of alkanes and aldehydes. For 9 of the subjects, the aldehyde and alkane content consistently increased in the exacerbation state compared with the stable condition (see Fig E1 in this article's Online Repository at www.jacionline.org).

The ^{1}H-NMR spectrum shows signals from several amino acids and derivatives, organic acids, amines, and other metabolites, such as glucose and creatinine (see Fig E2 in this article's Online Repository at www.jacionline.org). The score scatter plot of the 2 main PCs (explaining almost 50% of the total variability) shows, for each subject, a clear trend for sample scores to shift from the positive to the negative PC1 axis between stable and exacerbated conditions (Fig 2, *A*). The PC1 loadings plot unveiled the signals

responsible for this separation: levels of threonine (and/or lactate), alanine, carnitine, acetylcarnitine, and trimethylamine-N-oxide were suggested to be increased in the exacerbated condition, whereas levels of acetate, citrate, malonate, hippurate, dimethylglycine, and phenylacetylglutamine seemed to be decreased compared with the stable condition (Fig 2, *B*).

During exacerbations, urine revealed increased levels of aldehydes and alkanes, as well as alterations in a number of nonvolatile metabolites.

As for limitations and strengths of this study, diet and current treatment might interfere with the urine metabolomic profile. No food restriction was made. Despite all patients having a similar initial dose of methylprednisolone (approximately 80 mg/d), this remains a confounding factor. Further studies with a larger population are necessary to confirm these findings.

The use of 2 high-throughput techniques used in this study provides complementary information, enhancing the current understanding of the metabolic pathways affected (see the Results and discussion, Limitations and strengths section, in this article's Online Repository at www.jacionline.org).

Alkanes and aldehydes, end products of the peroxidation of unsaturated fats, can be formed during inflammation. Their levels were found to be increased during exacerbation, suggesting a high level of oxidative stress compared with the stable state (see the Results and discussion, Data interpretation section, in this article's Online Repository at www.jacionline.org).

Carnitine and acetylcarnitine can be linked to increased oxidative burden because they play an essential role in the transport of fatty acids into mitochondria for oxidation. These metabolites are critically altered in asthmatic patients.[2] Moreover, changes in metabolites, such as citrate and alanine, suggest a disturbance of the tricarboxylic acid cycle, whereas altered levels of trimethylamine-N-oxide, hippurate, and phenylacetylglutamine might be related to diet.

Urine is an easily accessible and information-rich biofluid. Our preliminary data show that urine metabolomics might provide important information on the patients' oxidative stress status. They also show promise in asthma management. This would suggest that research on metabolomic signatures in a broader group of asthmatic patients, including different phenotypes and disease presentation, might be valuable.

In conclusion, urinary metabolic composition was highly altered during exacerbation compared with that seen in the stable state. GC × GC-TOFMS– and ^{1}H-NMR–based methodologies allowed complementary information to be retrieved. In spite of the limited number of cases considered, the present results suggest that oxidative stress is a fundamental factor in asthma exacerbation.

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Disclosure of potential conflict of interest: J. Bousquet is a board member for Actelion, Almirall, Meda, Merck, MSD, Novartis, Sanofi Aventis, Takeda, Teva, and Uriach; is a member of the board of directors for Stallergenes; and has received lecture and travel fees from Almirall, AstraZeneca, Chiesi, GlaxoSmithKline, Meda, Merck, MSD, Novartis, OM Pharma, Sanofi Aventis, Schering-Plough, Takeda, Teva, and Uriach. T. Bom has received consultancy fees from Lab Vitoria, has received lecture fees from GlaxoSmithKline and MSD, has received payment for development of educational presentations from Thermo Scientific, and has received travel fees from Boehringer Ingelheim. The rest of the authors declare that they have no relevant conflicts of interest.

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Unsuspected mild emphysema in nonsmoking patients with chronic asthma with persistent airway obstruction

To the Editor:

We have previously demonstrated that reversible loss of lung elastic recoil is a significant contributing factor in limiting maximal expiratory airflow in acute asthma.[1] We[2] and others[3-7] have also reported chronic loss of lung elastic recoil in clinically stable, nonsmoking, adult patients with moderate-to-severe asthma with persistent expiratory airflow limitation that may be partially reversible with treatment. The mechanism(s) responsible for the loss of lung elastic recoil in acute[1] and chronic asthma[2-7] remains unknown, especially with normal diffusing capacity and normal or only mild parenchymal attenuation of lung density on high-resolution thin-section lung computed tomography (CT) at full inspiration.[2] Here, in a prospective study, we investigated the potential pathophysiologic mechanism(s) limiting maximal expiratory airflow ($V\text{max}_{E}$) after optimal clinical improvement. Our hypothesis is that undiagnosed diffuse breakdown of lung tissue leading to mild, diffuse emphysema may be responsible for both the loss of lung elastic recoil and persistent expiratory airflow limitation.

METHODS

Patients' characteristics

Adults with asthma were recruited from the emergency department of Hospitais da Universidade de Coimbra, where they were treated for an asthma attack. All had a clinical asthma diagnosis based on relevant respiratory symptoms plus airway reversibility to salbutamol according to Global Initiative for Asthma guidelines, a provocative dose of methacholine resulting in a 20% reduction in FEV_1 of less than 8 μmol, or both. None of them had signs of bacterial respiratory tract infection.

Exacerbation was defined on the basis of increasing asthma symptoms or insufficient symptomatic response to inhaled bronchodilator therapy and the need for systemic corticosteroids. At discharge, they all had a similar systemic oral corticosteroid regression scheme. Each asthmatic patient was referred to an asthma specialist clinician, and the stable period was defined in a clinical setting having met clinical criteria and an appropriate score on the Asthma Control Test (≥20). None of them were receiving systemic corticosteroids at the time. Patients with skin prick test responses or specific IgE levels positive for at least 1 common aeroallergen were considered allergic by using the GA^{2}LEN battery.[E1]

Urine sample collection

Two urine samples were collected from each asthmatic patient: one sample during the exacerbation period (in the first 4 hours after emergency department presentation or systemic corticosteroid administration) and the other during the stable period (6-8 weeks after). Urine samples were collected in sterile cups, stored at −20°C for 2 hours, and then transferred to a −80°C freezer, where they were stored for up to 3 months before analysis. The time of day at which samples were collected was variable, and there were no dietary or pharmacologic restrictions because it was presumed that metabolites would be sufficiently altered between analysis and that the stable state would serve as a control reference for exacerbations. Actually, these conditions are more representative of a real-life situation because exacerbations can take place at any time of the day.

Urine analysis using SPME/GC × GC-ToFMS

Urine samples were analyzed by means of SPME, followed by comprehensive GC × GC-ToFMS. The SPME experimental parameters used to extract urine metabolites and the GC × GC-ToFMS conditions have been previously reported by Rocha et al.[E2] Each sample was analyzed in triplicate. (GC × GC-ToFMS is a highly reproducible technique with a limit of detection at the picogram per liter level using standards. Spiking experiments have not been performed for quantification purposes.) The deconvoluted total ion current GC × GC area data were used as an approach to estimate the relative content of each metabolite in the urine samples. Each total ion chromatogram was processed by using the automated data processing software ChromaTOF (LECO), which allows an area for each analyte to be obtained. No automatic alignment of the chromatograms was performed.

Urine analysis using ^{1}H-NMR

The ^{1}H-NMR methodology was applied to 5 of 10 subjects for which samples were available. For the NMR analysis, each thawed sample was buffered to pH 7.0, according to Carrola et al.[E3] Each sample was analyzed once because NMR is a highly reproducible technique. (The limit of detection with a standard [noncryogenic] probe is in the low micromolar range. Spiking experiments have not been performed for quantification purposes.)

Statistical analysis

PCA is a nonsupervised method aimed at exploring data variability and detecting intrinsic clustering and outliers. The distribution of samples in the score maps allows one to infer on the similarity between the samples' composition. Both intraindividual and interindividual variability can be assessed when including replicate samples from different subjects. Score scatter plots were used to visualize and explore the relationships among samples, whereas loadings profile plots were used to inspect the variables (metabolites) that contributed the most in the observed group distribution.

GC × GC-ToFMS data. The GC × GC peak areas data set consisted of 60 observations (3 replicate analyses from 20 urine samples, 2 from each of 10 subjects, at 2 clinical states [stable and exacerbation]) and 32 variables (peak areas of aliphatic aldehydes and alkanes). A list of the specific compounds included is provided in Table E2.

^{1}H-NMR data. Variably sized bucketing was performed in the 10.00- to 0.40-ppm region of the standard 1D spectra (excluding the subregion δ 4.55-6.05, corresponding to water and urea signals). Each spectrum was normalized to total area, and the data were scaled to unit variance (to give all variables the same weight).

Given the small sample numbers, we have applied PCA to only the data, a method in which class membership is not included in the modeling process, and we have purposely avoided supervised methods, such as partial least squares-discriminant analysis, which would carry a high risk of overfitting. Future work comprising a larger sample set will address the predictive ability and classification rate of the multivariate models.

Results and discussion

Limitations and strengths. As suggested by Reisdorph and Wechsler,[E4] studies targeting single molecules do not capture the network of asthma processes. One of the novel and key points of this study is the complementary use of these 2 high-throughput techniques because they provide information on different windows of the urinary metabolome.

Data interpretation. Alkanes and aldehydes, in the sequence of oxidation reactions, are end products that have been associated with oxidative stress and inflammatory processes. The obtained results suggest that the oxidative state is at a higher extent in exacerbation when compared with that in the stable condition.

The results of patient 9 might be explained by his clinical profile, which has led to a high and constant burden of oxidative stress. His related exacerbation was driven by persistent noncontrolled rhinitis and nasal polyposis, with no related allergen exposure or known viral infection.

A plausible confounding factor for molecular mechanisms is his hypersensitivity to aspirin. Increased urinary eicosanoids, a group of molecules produced by oxidative processes from arachidonic acid, have already been implicated in asthma exacerbation, and the arachidonic acid cycle is related to the tricarboxylic acid cycle (alkanes can arise as products of lipid peroxidation of unsaturated fats, as already stated). Furthermore, isoprostanes are bioactive prostaglandin F_2–like compounds, produced independently of the COX enzymes through the peroxidation of arachidonic acid, which has been already implicated in asthma exacerbation,[E5] and are potent constrictors of smooth muscle, which can explain the patient's persistent airflow obstruction. A recent study by Paredi et al[E6] demonstrated that concentrations of exhaled ethane (a lipid peroxidation marker) were increased in patients with more severe bronchoconstriction (FEV_1 < 60%) compared with less constricted patients ($FEV_1 \geq 60\%$).

Clinical importance of the study. Despite the small sample of the study, the authors, based on these preliminary findings, are convinced that this approach can be successfully applied to a broader investigation of asthmatic patients with different phenotypes and disease presentation.

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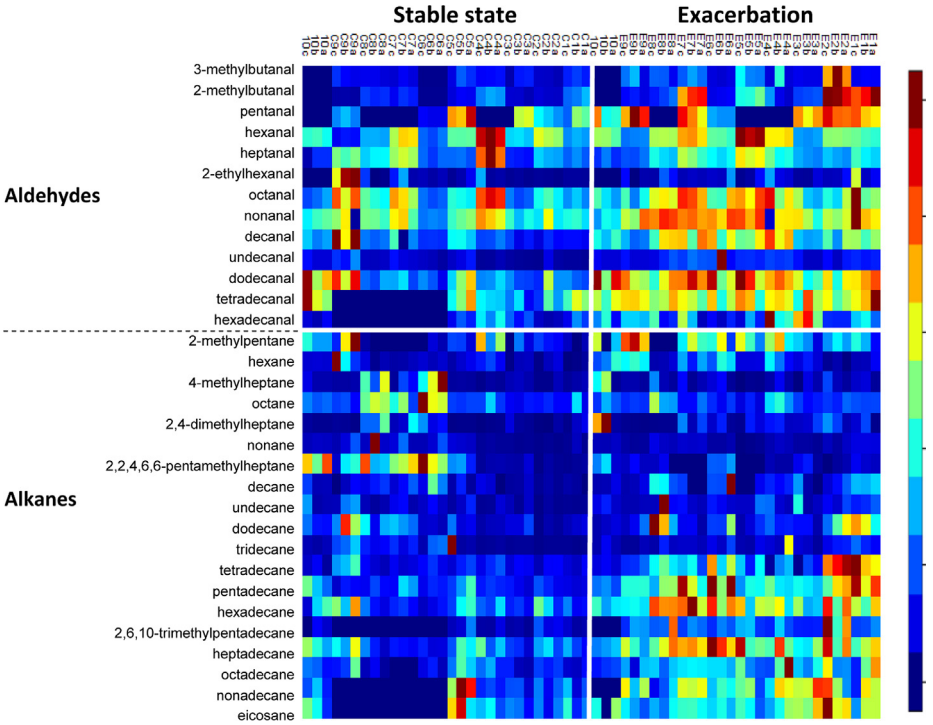

FIG E1. Each *line* corresponds to 1 metabolite (organized by aldehydes and alkanes chemical families), and each *column* corresponds to each subject (organized by stable state and exacerbation conditions).

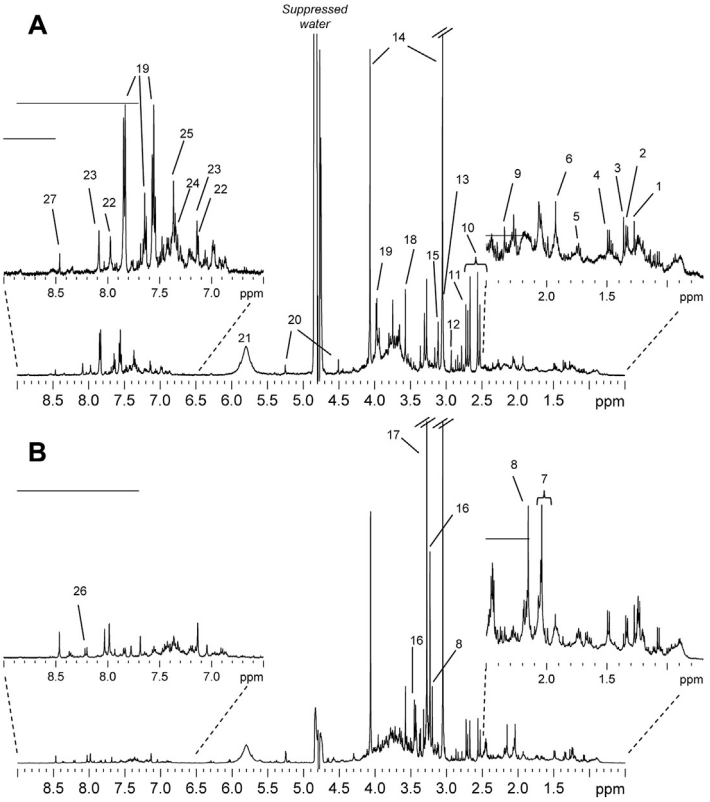

FIG E2. ^{1}H-NMR spectra (500 MHz) of urine from patient 10 in the stable state **(A)** and in exacerbation conditions (**B;** each spectrum is normalized to total area). Vertical expansions (6×) of aliphatic and aromatic subregions are shown. Signal assignment: 1, 3-hydroxy-isovalerate; 2, threonine and lactate; 3, 2-hydroxyisobutyrate; 4, alanine; 5, lysine; 6, acetate; 7, N-acetylated amino acids; 8, acetylcarnitine; 9, p-cresol sulphate; 10, citrate; 11, dimethylamine (DMA); 12, dimethylglycine (DMG); 13, creatine; 14, creatinine; 15, malonate; 16, carnitine; 17, trimethylamine-N-oxide (TMAO); 18, glycine; 19, hippurate; 20, glucose; 21, urea; 22, histidine; 23, 3-methylhistidine; 24, phenylacetylglutamine (PAG); 25, indoxylsulfate; 26, hypoxanthine; 27, formate.

TABLE E1. Individual patients' characteristics

Patient no.	Age (y)	Sex	FEV_1 (% predicted),* before BD	ACT	Atopy	NMR analysis
1	57	M	35	24	Pollens	No
2	64	F	148	20	Mites	Yes
3	39	F	65	22	Mites, pollens	Yes
4	71	M	83	24	No	Yes
5	23	F	102	25	Mites, pollens	Yes
6	69	F	37	20	No	No
7	22	M	103	25	Mites	No
8	40	M	92	23	Mites	No
9	59	M	78	20	No	Yes
10	58	F	132	20	Mites	No

ACT, Asthma Control Test; *BD*, bronchodilator; *F*, female; *M*, male.
*Percent predicted FEV_1/forced vital capacity after bronchodilation.

TABLE E2. Urinary aliphatic alkanes and aldehydes identified by using HS-SPME/GC × GC-ToFMS and selected for multivariate analysis

^1t_R* (s)	^2t_R* (s)	Compounds	CAS no.	RI_{calc}†	RI_{lit}‡
Alkanes					
90	0.34	2-Methylpentane	107-83-5	587	558
102	0.34	Hexane§	110-54-3	600	600
222	0.43	4-Methylheptane	589-53-7	738	765
276	0.41	Octane§	111-65-9	800	800
324	0.45	2,4-Dimethylheptane	2213-23-2	819	822
498	0.43	Nonane§	111-84-2	900	900
666	0.41	2,2,4,6,6-Pentamethylheptane	13475-82-6	991	997
684	0.42	Decane§	124-18-5	1000	1000
840	0.46	Undecane§	1120-21-4	1105	1100
972	0.43	Dodecane§	112-40-3	1200	1200
1098	0.47	Tridecane§	629-50-5	1300	1300
1212	0.48	Tetradecane§	629-59-4	1401	1400
1314	0.45	Pentadecane§	629-62-9	1500	1500
1416	0.46	Hexadecane§	544-76-3	1601	1600
1464	0.48	2,6,10-Trimethylpentadecane	3892-00-0	1651	1649
1512	0.51	Heptadecane§	629-78-7	1701	1700
1602	0.46	Octadecane§	593-45-3	1808	1800
1686	0.50	Nonadecane§	629-92-5	1901	1900
1770	0.49	Eicosane§	112-95-8	2001	2000
Aldehydes					
126	0.55	3-Methylbutanal	590-86-3	628	646
132	0.53	2-Methylbutanal	96-17-3	635	646
150	0.62	Pentanal§	110-62-3	656	697
276	0.97	Hexanal§	66-25-1	800	800
504	0.98	Heptanal§	111-71-7	904	899
606	0.76	2-Ethylhexanal	123-05-7	958	955
690	0.81	Octanal§	124-13-0	1004	1001
846	0.82	Nonanal§	124-19-6	1105	1098
978	0.80	Decanal§	112-31-2	1205	1204
1104	0.73	Undecanal§	112-44-7	1306	1291
1218	0.73	Dodecanal§	112-54-9	1407	1407
1428	0.72	Tetradecanal	124-25-4	1613	1611
1614	0.76	Hexadecanal	629-80-1	1815	1819

CAS, Chemical Abstracts Service.

*Retention times in seconds for first (^1t_R) and second (^2t_R) dimensions.

†Retention index obtained through the modulated chromatogram.

‡Retention index reported in the literature for 1-dimensional GC with a 5%-phenyl-methylpolysiloxane GC column or equivalent and for a comprehensive GC × GC system with HP-5 for the first dimension.

§Compound confirmed by means of coinjection of chemical standards.