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Longitudinal urinary metabolomic profiling reveals metabolites for asthma development in early childhood

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Abstract

Background: Several metabolites and altered metabolic pathways have been reported to be associated with asthma. However, longitudinal analysis of the dynamics of metabolites contributing to the development of asthma has not yet been fully clarified.

Methods: We sought to identify the metabolic mechanisms underlying asthma development in early childhood. Thirty children with asthma and paired healthy controls from a prospective birth cohort were enrolled. Time series analysis of urinary metabolites collected at ages 1, 2, 3, and 4 years was assessed using ¹H nuclear magnetic resonance (NMR) spectroscopy coupled with partial least squares discriminant analysis (PLS-DA). Metabolites identified were studied in relation to changes over time in a linear mixed model for repeated measures.

Results: A total of 172 urine samples collected from the enrolled children were analyzed. Urinary metabolomics identified four metabolites significantly associated with childhood asthma development, with longitudinal analysis. Among them, dimethylamine, a metabolite produced by intestinal bacteria, appeared to shift from higher to lower level during asthma development. A persistent lower level of 1-methylnicotinamide and allantoin was found in children with asthma, with a peak difference at age 3 years ($P = .032$ and $P = .021$, respectively). Furthermore, a significant inverse correlation was found between allantoin and house dust mite sensitization (Spearman's $r = -.297$ $P = .035$).

Conclusions: Longitudinal urinary metabolomic profiling provides a link of microbe-environment interactions in the development of childhood asthma. 1-Methylnicotinamide and allantoin may participate in allergic reactions in response to allergen exposure, potentially serving as specific biomarkers for asthma.

KEYWORDS

asthma, metabolomics, microbe-environment interactions, urine

1 | INTRODUCTION

Asthma is a heterogeneous condition caused by the combined effects of multiple genetic factors interacting with the environment.¹ Numerous risk factors for asthma development including gender, family atopic history, allergen exposure, infections, tobacco smoke exposure, obesity, and perinatal factors have been identified.^{2,3} Potential biomarkers such as vitamin D levels, allergen sensitizations, and serum IgE levels for asthma prediction have also been studied widely.^{4,5} In young children, the incidence of asthma varies with age, and reaching the diagnosis of asthma and its management however are still challenging. A solid understanding of the complexities of age-related changes in asthma development may provide a potential strategy for prevention and treatment in early life.

Asthma is a chronic inflammatory disease of the airways characterized by a wide variety of cellular and molecular mediators resulting in a broad spectrum of possible metabolic products.⁶ Metabolomics enables the discovery of small molecule metabolites by revealing specific biomarkers in human disease or certain changes in the metabolism pattern after genetic and environmental intervention.^{7,8} Several studies have revealed altered metabolic pathways that are associated with asthma pathogenesis.⁹ However, a longitudinal analysis of the dynamic metabolic processes governing asthma development is still lacking.

Metabolic profiles from urine provide a fingerprint for each individual, containing significant amount of information on age, gender, lifestyle, dietary intake, and disease history.¹⁰ High-resolution ¹H nuclear magnetic resonance (NMR) spectroscopy is widely used to measure dynamic changes in metabolite concentrations. The major aim of this study was to identify the longitudinal trajectory of urinary metabolic profiles using ¹H NMR spectroscopy from 1 to 4 years of age in children from a birth cohort in the Prediction of Allergies in Taiwanese Children (PATCH) study. The changes in urinary metabolites with respect to age were assessed, and their relationships with asthma development were also examined.

2 | METHODS

2.1 | Study population

A time series study was designed to investigate the urinary metabolic profiles of children recruited into a birth cohort (the PATCH study) from 1 to 4 years of age. The subject flow diagram of this birth cohort study was reported previously, with only 182 of 258 (70.5%) children were regularly followed at the clinic for 4 years.² At 4 years of age, thirty children were physician-diagnosed as asthma with the occurrence of recurrent wheeze in the last 12 months or current use of asthma medication.¹¹ Healthy children without personal history of asthma or other atopic conditions were diagnosed in fifty-eight children. Among them, ten children with significant infectious illnesses or other systemic conditions were excluded. Thirty of the forty-eight healthy children having

urine samples taken over at least 3 time-points during a 4-year follow-up were enrolled as healthy controls and paired.¹² Ninety-four children with other atopic diseases such as allergic rhinitis or eczema were excluded. Details of information regarding demographic data, family atopy history, and healthcare history including breastfeeding and medical conditions of enrolled children were collected. This study was approved by the Ethics Committee of Chang Gung Memory Hospital (No. 104-9469B). Written Informed consent was obtained from the parents or guardians of all study subjects.

2.2 | Measurement of serum immunoglobulin E levels

Total and allergen-specific serum IgE levels were examined as described in our previous study.⁵ Total serum IgE level was measured by ImmunoCAP (Phadia, Uppsala, Sweden), and IgE sensitization was defined as IgE levels ≥ 100 kU/L.¹³ Three food allergens (egg white, milk, and wheat) and three inhalant allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, and *Cladosporium herbarum*) were measured simultaneously.¹⁴ Allergen-specific IgE was determined using a commercial assay for IgE (ImmunoCAP Phadiatop Infant; Phadia), and allergen sensitization was defined as values ≥ 0.35 kU/L.¹⁵

2.3 | Urine sample preparation

Spot urine samples of enrolled subjects collected at 1, 2, 3, and 4 years of age were examined. All urine samples were collected in the morning and immediately stored at -80°C in aliquots until required. Urine samples prior to spectrum acquisition were prepared as described previously.¹² Briefly, 900 μL of urine was mixed with 100 μL of 1.5 M phosphate buffer (pH 7.4) in deuterium water which containing 0.04% 3-(trimethylsilyl)-propionic-2,2,3,3- d_4 acid sodium salt (TSP) as an internal chemical shift reference standard. Samples were vortexed for 20 seconds and centrifuged at 12 000 g at 4°C for 30 minutes. A 650 μL aliquot of the supernatant was transferred to a standard 5-mm NMR tube for further analysis.

2.4 | ¹H Nuclear magnetic resonance (NMR) spectroscopy

A Bruker Avance 600 MHz spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a 5-mm CPTCI ¹H cryoprobe was used for ¹H NMR spectra acquisition at Chang Gung Healthy Aging Research Center, Taiwan. For each spectrum, 64 scans were collected into 64K computer data points using a spectral width of 10 000 Hz (10 ppm) during the relaxation delay (4.0 seconds) and an acquisition time of 2.73 seconds. One-dimensional (1D) ¹H NMR spectra were applied for analysis before Fourier transformation with exponential multiplication and zero filling. Spectra were phased, baseline corrected, and referenced to TSP (δ 0.0 ppm) as a chemical shift using TopSpin 3.2 software (Bruker BioSpin, Rheinstetten, Germany).

2.5 | NMR data processing and analysis

The spectral bucket, spectral region exclusion, and spectral normalization of raw ^1H NMR spectra were performed using AMIX version 3.9.12 (Bruker BioSpin, Rheinstetten, Germany). ^1H NMR spectra were subdivided into integrated regions of 0.01 ppm corresponding to the region of δ 0–10 ppm. Signals arising from residual water (δ 4.745–4.845 ppm) and urea (δ 5.465–6.195 ppm) were excluded. Spectra were normalized to the integral of creatinine peak at δ 3.045 ppm to overcome the variation of urinary concentration. To identify metabolites contributing to the discrimination between groups, the normalized ^1H NMR bucket data were uploaded to MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>) for partial least squares discriminant analysis (PLS-DA). The spectral variables were mean-centered and scaled using Pareto scaling, and 10-fold internal cross-validation was performed to evaluate the quality of the resulting statistical models by considering the diagnostic measures R^2 and Q^2 .^{16,17} Potential metabolites were selected based on the Variable Importance in Projection (VIP) score greater than 1.5. NMR resonances of metabolites were assigned using Chenomx NMR Suite 8.1 professional software (Chenomx Inc., Edmonton AB, Canada). Metabolite pathway analysis was based on the database source of the Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>). VENNY 2.1 was used to create the Venn diagram (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

2.6 | Statistical analysis

Comparisons of baseline characteristics categorized by asthmatics and healthy controls were made with univariable parametric and nonparametric tests such as Student's *t* test, Mann-Whitney test, chi-square test, and Fisher's exact test. The fold changes in metabolites between asthmatics and healthy controls were performed with the Mann-Whitney test at different years of ages. Repeated measures two-way analysis of variance (ANOVA) with first-order autoregressive covariance structure was used to study the impact of asthma development on the metabolite profiling over time (1–4 years), adjusted for confounding variables for asthma including maternal atopy, passive smoking, household income, infant's sex, maternal age, gestational age, birth BMI, and breastfeeding. Pearson's or Spearman's correlation tests were used to determine the correlation between the asthma-related metabolites and allergen-specific IgE levels. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Statistics for Windows version 20.0; Armonk, NY, USA). All statistical hypothesis tests were 2-tailed, and a *P* value < .05 was considered significant.

3 | RESULTS

3.1 | Population characteristics

A total of 172 urine samples in 30 asthmatics and 30 healthy children from a birth cohort for a 4-year follow-up were enrolled. The

comparisons of baseline characteristics of children with asthma and healthy controls are shown in Table 1. A statistically significant difference of atopic indices was found in children with asthma compared to healthy controls at age 3 years ($P < .05$). There was no difference in sex, maternal age, gestational age, family history of atopic diseases, passive smoking, household income, and different patterns of breastfeeding.

3.2 | Identification of metabolite sets between asthmatics and controls at different years of age

^1H NMR data of urine samples obtained at ages 1, 2, 3, and 4 years were collected and analyzed. Two hundred and forty-seven out of 1000 buckets varied across NMR spectra corresponding to 76 known metabolites.¹² Unsupervised principal component analysis failed to reveal clearly the separation between asthmatics and controls at different years of age. In contrast, the PLS-DA parameters and permutation test showed a clear separation between asthmatics and controls with a significant permutation test at age 1 and 2 years (Table S1). The two-dimensional graphs of PLS-DA score plots showed a separate grouping between asthmatics and healthy controls (Figure S1A). Metabolites identified in the fold change of expression level between asthmatics and controls at different years of age are shown in Table S2; the metabolites were selected using PLS-DA VIP cutoff score of >1.5 and a raw *P* value of <.05. *N*-acetyltyrosine at age 1; *D*-galactose, allantoin, and 1-methylnicotinamide at age 3; and carnitine at age 4 were found to be significantly lower in children with asthma compared with healthy controls.

3.3 | Metabolites associated with asthma development over time

Identified metabolites were studied in relation to changes over time in a linear mixed model for repeated measures. There was a significant effect of time on most of the metabolites. Metabolites identified in the interaction between asthma development and increasing age by selecting the metabolite sets using the cutoff of PLS-DA VIP score >1.5 with a *P* value < .05 are shown in Table 2. A representative 600 MHz ^1H NMR spectra of metabolite signals related to asthma is shown in Figure S1B. Among them, dimethylamine was found to be significantly involved in asthma development over time. Three metabolites including 1-methylnicotinamide, allantoin, and guanidoacetic acid were significantly associated with asthma disease. Metabolic pathway analysis revealed that dimethylamine and allantoin were responsible for microbial metabolism in diverse environments (Table 3). Comparisons and differences in the NMR intensity ratios of these metabolites to creatinine between asthmatics and healthy controls at different years of age are shown in Figure 1. A shift to lower level of dimethylamine was found in children with asthma after age 2 years. The NMR intensity ratios of 1-methylnicotinamide and allantoin were significantly lower in asthmatics compared with healthy controls at age 3 years. Figure 2 shows the metabolic pathways of metabolites associated

TABLE 1 Epidemiologic characteristics of the children investigated in this study

Characteristics	Asthma (n = 30)	Control (n = 30)	P value
Family			
Maternal atopy	15 (51.7%)	10 (33.3%)	.153
Passive smoking	14 (46.7%)	11 (36.7)	.432
Household income			
Low, ≤500 000 NTD	11 (37.9%)	12 (40.0%)	.547
Medium, 500 000-1 000 000 NTD	11 (37.9)	14 (46.7%)	
High, >1 000 000 NTD	7 (24.1%)	4 (13.3%)	
Infant			
Sex, male	9 (30.0%)	13 (43.3%)	.284
Maternal age (y)	30.3 ± 4.0	32.4 ± 4.4	.057
Gestational age (wk)	38.1 ± 1.7	37.7 ± 1.8	.394
Birth BMI (kg/m ²)	12.4 ± 1.1	12.8 ± 2.9	.570
Breastfeeding until 6 mo			
Exclusive	9 (30.0%)	13 (43.3%)	.112
Partial	10 (33.3%)	13 (43.3%)	
Formula	11 (36.7%)	4 (13.3%)	
Atopic indices at age 3			
Mite sensitization	21 (80.8%)	7 (28.0%)	<.001
Food sensitization	16 (61.5%)	7 (28.0%)	.016
IgE sensitization	14 (53.8%)	2 (8.0%)	<.001
IgE levels, kU/L	225.4 ± 240.2	29.6 ± 25.8	.001

Data shown are mean ± SD or number (%) of patients as appropriate.

BMI, body mass index; mo, month; NTD, New Taiwan Dollar; wk, week; y, year.

with asthma development. Gut microbial-derived metabolites of dimethylamine and allantoin are derived from choline and purine metabolisms, respectively.

3.4 | Identification of metabolite sets categorized by atopic indices

A cross-sectional analysis was designed and performed at age 3 years, the age at which a higher prevalence of asthma was diagnosed initially, for further investigation of the roles of metabolites in asthma pathogenesis. ¹H NMR data of urine samples collected at age 3 years categorizing by allergen sensitization to mite, food, and IgE sensitization (≥100 kU/L) were analyzed. Metabolites identified in sets with mite, food, and IgE sensitization selected using the cutoff of PLS-DA VIP score >1.0 are shown in Table S3. Venn diagram showed the distribution of the metabolites identified in each atopic index sets (Figure 3A). Dimethylamine was unique to asthma, and both 1-methylnicotinamide and allantoin were found to be only involved in mite sensitization and asthma. In the correlation analysis, a negative correlation was found between allantoin and *D. pteronyssinus* expression, whereas a positive correlation was found between guanidoacetic acid and egg white expression (Figure 3B).

4 | DISCUSSION

The incidence of asthma is increasing in most countries and is one of the most common chronic childhood diseases.¹⁸ Many children with asthma develop symptoms before the age of 5 years. Prevention and long-term control are keys to stopping asthmatic attacks. Metabolic profiling provides a new approach to explore the metabolic effects of many conditions in complex biological systems. Recently, increasing application of metabolomics in human diseases and some metabolites have been reported in association with asthma.^{19,20} However, childhood asthma is buried within adolescent and adult series. This study provides an overview of metabolic changes in asthma during early childhood (1-4 years), which potentially provides information on the prevention, diagnosis, and treatment of childhood asthma.

Amino acids have been shown to contribute to various antioxidant and immunological activities relevant to asthma pathogenesis.²¹ In the cross-sectional analysis of this study, amino acids, *N*-acetyltyrosine and carnitine, were identified to be significantly associated with early childhood asthma. *N*-Acetyltyrosine is an acetylated derivative of *L*-tyrosine, which may be modified by eosinophil activity in allergen-induced asthma.²² Carnitine, derived from an amino acid, plays a critical role in energy production by metabolizing fat. Leukotrienes are lipid mediators and are known to play

TABLE 2 Metabolites identified in the interaction between asthma development and increasing age

Metabolites	Chemical shift, ppm	VIP Score	P Interaction		P Asthma		P Age	
			Crude	Adjusted*	Crude	Adjusted	Crude	Adjusted
Dimethylamine	2.715 (s)	1.96	.057	.047	.769	.497	<.001	<.001
1-Methylnicotinamide	8.985-8.965 (d)	2.18	.692	.666	<.001	<.001	<.001	<.001
Allantoin	5.395 (s)	1.67	.953	.888	.004	.032	<.001	<.001
Guanidoacetic acid	3.805-3.795 (s)	2.24	.663	.531	.104	.040	<.001	<.001
Glutamine	2.465-2.425 (m)	3.54	.473	.427	.101	.143	<.001	<.001
Lysine	1.765-1.705 (m)	3.24	.884	.950	.281	.374	<.001	<.001
N-Acetylglucosamine	2.055-2.045 (m)	2.93	.874	.886	.468	.208	<.001	<.001
Succinic acid	2.415-2.405 (s)	2.90	.763	.925	.084	.087	<.001	<.001
Acetic acid	1.925 (s)	2.86	.130	.136	.605	.450	.032	.018
Creatine	3.935 (s)	2.81	.589	.640	.354	.367	<.001	<.001
3-Hydroxyisovaleric acid	1.275 (s)	2.46	.624	.661	.179	.085	<.001	<.001
Carnitine	3.235-3.225 (s)	2.32	.114	.093	.328	.272	<.001	<.001
Citric acid	2.575-2.515 (d)	2.04	.739	.596	.239	.323	<.001	<.001
Alanine	1.495-1.465 (d)	1.87	.836	.829	.296	.215	<.001	<.001
N-Methylhydantoin	2.935-2.925 (s)	1.80	.809	.876	.653	.546	<.001	<.001
Betaine	3.905 (m)	1.68	.883	.822	.525	.550	<.001	<.001

*P value was adjusted for maternal atopy, passive smoking, household income, infant's sex, maternal age, gestational age, birth BMI, and breastfeeding. All P values <.05, which is in bold, are significant.

d, doublet; m, multiplet; ppm, parts per million; s, singlet; VIP, Variable Importance in Projection.

TABLE 3 Functional pathway analysis of metabolites associated with asthma development in early childhood

Metabolites	Upstream metabolite	Pathway	Function
Dimethylamine	Trimethylamine N-oxide	Methane metabolism Microbial metabolism in diverse environments	Energy metabolism
1-Methylnicotinamide	Nicotinamide	Nicotinate and nicotinamide metabolism	Metabolism of cofactors and vitamins
Allantoin	Xanthine	Purine metabolism Microbial metabolism in diverse environments	Nucleotide metabolism
Guanidoacetic acid	Glycine	Glycine, serine, and threonine metabolism Arginine and proline metabolism	Amino acid metabolism

a central pathophysiological role in asthma.²³ It has been reported that L-carnitine decreases leukotriene synthesis through inhibition of lipoxygenase enzyme.²⁴

Longitudinal analysis in this study identified three metabolites, including 1-methylnicotinamide, allantoin, and guanidoacetic acid, that were significantly associated with asthma development. Guanidoacetic acid is synthesized from arginine and glycine and is a natural precursor of creatine.²⁵ Creatine has been reported to activate airway epithelium in asthma, and creatine supplementation increased airway inflammation with asthma-like symptoms in mice.²⁶ In contrast, 1-methylnicotinamide, a primary metabolite of

nicotinamide, is reported to be protective against asthma exacerbations through anti-inflammatory effects.²⁷ Allantoin, a purine metabolite like xanthine, has also been shown to provide protective effects in lung inflammation in asthma.²⁸

Dimethylamine was the only metabolite identified to be associated with asthma over time during early childhood. Dimethylamine is a biogenic amine and mainly formed from trimethylamine N-oxide (TMAO), a compound produced by bacteria in the intestine. The production of TMAO is dependent on certain bacteria in the gut, and imbalance in gut microbiota with low diversity has been reported to precede asthma.²⁹ There was also a trend of dimethylamine being

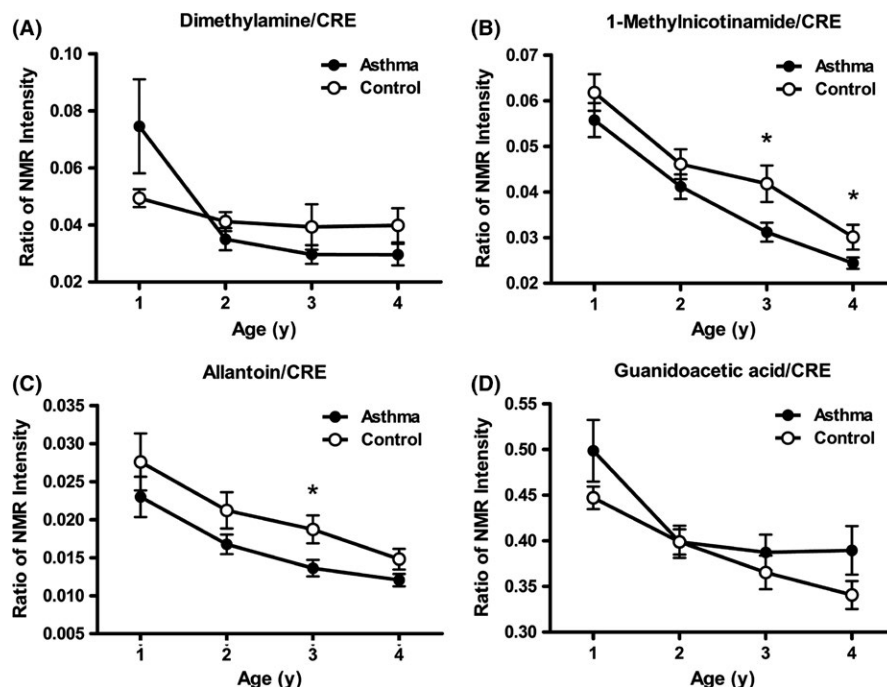
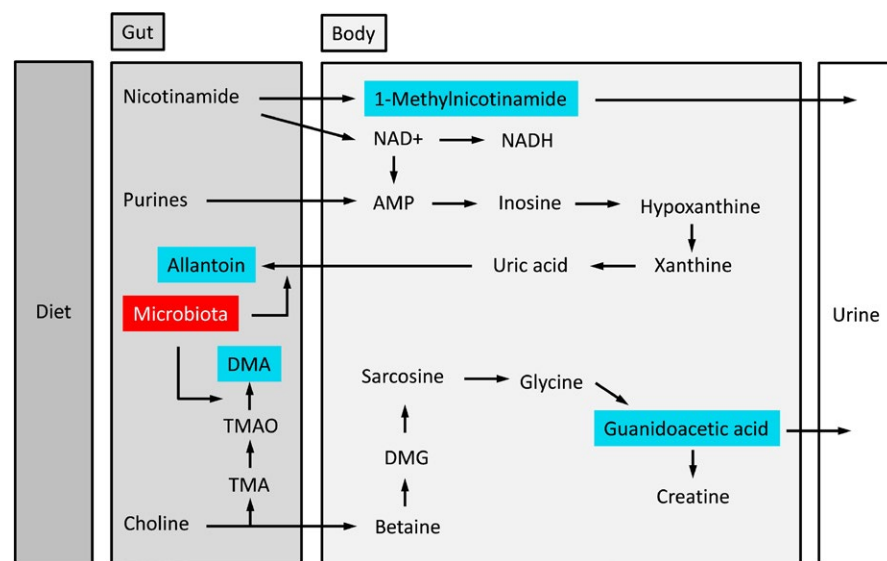


FIGURE 1 Comparisons and differences in NMR intensity ratios between asthmatics and healthy controls at different years of age. (A) Dimethylamine. (B) 1-Methylnicotinamide. (C) Allantoin. (D) Guanidoacetic acid. Data shown are mean $\pm$ SEM. P values refer to the comparisons indicated by the marker. * $P < .05$

FIGURE 2 Schematic overview of metabolic pathways of metabolites related to asthma development during early childhood. Dimethylamine (DMA) and allantoin are gut microbial-derived metabolites originated from choline and purine metabolisms, respectively. Identified metabolites significantly associated with asthma are shown in blue color. DMA, dimethylamine; DMG, dimethylglycine; NAD, nicotinamide adenine dinucleotide; TMA, trimethylamine; TMAO, trimethylamine N-oxide



lower in children with asthma after 2 years of age, the age at which the prevalence of asthma increases with a rapid upward trend.^{2,30} These findings support the evidence that environmental changes are a major factor in the development of allergies and microbial metabolism in diverse environments could particularly be key processes in asthma development.

Several studies have emphasized that an altered microbiota is associated with a higher prevalence of atopic diseases and asthma.³¹ Sensitization to allergens has been regarded as the most important risk factors for the development of allergic airway diseases.² In this study, the metabolites, 1-methylnicotinamide and allantoin, which were significantly associated with asthma, also appeared to be mite-sensitized. Furthermore, a strong correlation was observed between allantoin and *D. pteronyssinus* expression. It has been suggested that

some enterobacterial populations produce uricase, an enzyme which degrades uric acid into allantoin.³² Using urinary metabolomics, our findings provide a link between gut microbiota and mite sensitization for early childhood asthma development.

The small sample size and relatively low sensitivity of NMR-based metabolomic analysis, resulting in limited statistical power, are the major limitation of this study. As shown in this study, urinary creatinine is commonly used to adjust the concentrations of urine analytes for hydrate state, although it is known to differ among different age groups. However, an age-matched comparison of children with asthma and healthy controls makes our results valid and potentially important. The overall comparability of urine samples between groups may have resulted in the low Q^2 scores observed in this study. Therefore, a larger sample size with subgroup analysis

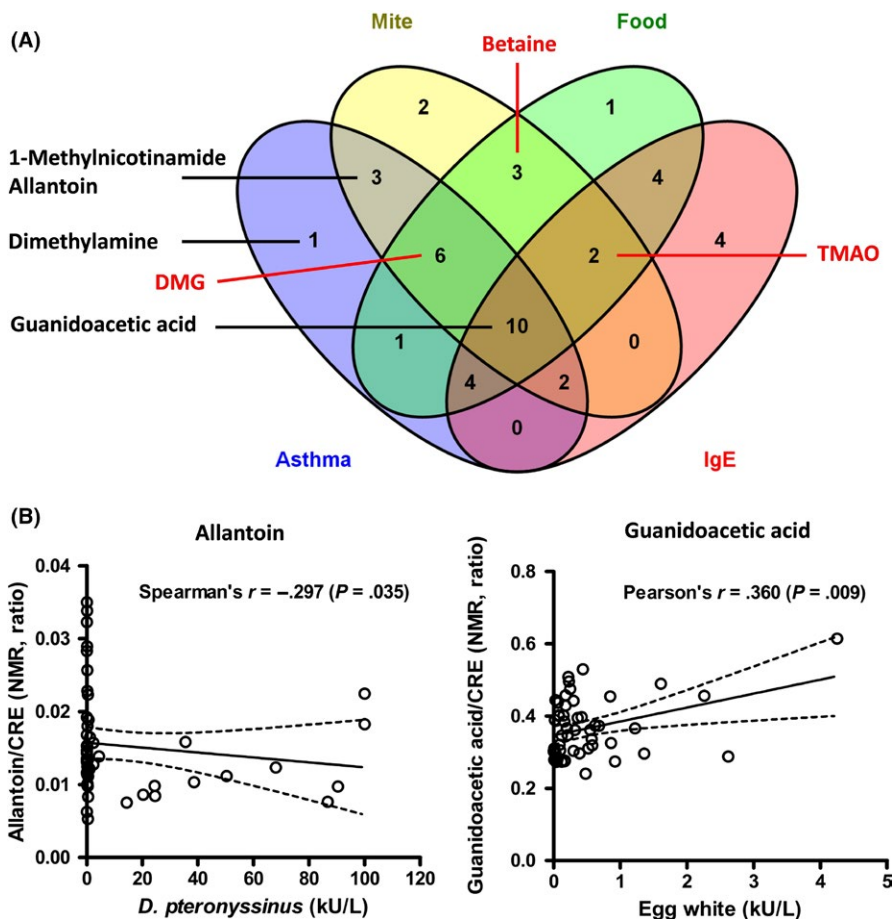


FIGURE 3 Venn diagram of the three atopic index sets affecting asthma at age 3. A, The total metabolite number in each set and the overlapping areas are indicated. Dimethylamine is unique to the asthma, and 1-methylnicotinamide and allantoin both are only involved in mite sensitization and asthma. Betaine, DMG, guanidoacetic acid, and TMAO involved in choline metabolism for dimethylamine are related to both mite and food sensitization. DMG, dimethylglycine; TMAO, trimethylamine. B, Correlation between asthma-related metabolites and allergen-specific IgE levels (kU/L). Significant correlations were observed between allantoin expression and *D. pteronyssinus*-specific IgE levels (Spearman's $r = -.297$, $P = .035$) and between guanidoacetic acid expression and egg white-specific IgE levels (Pearson's $r = .360$, $P = .009$) [Colour figure can be viewed at wileyonlinelibrary.com]

to reduce the within-group variability may make our findings more generalized. Nevertheless, the significant strengths of this study are the use of a longitudinal analysis and the long-term follow-up at very close intervals, which allows a systematic observation and measurement of changes in urinary metabolite levels to be made.

In conclusion, urinary metabolomics revealed that microbial-derived metabolites are associated with early childhood asthma. Dimethylamine, a compound produced by bacteria in the intestine, appears to shift to a lower level at the age at which the prevalence of asthma development shows an upward trend; this supports the evidence of microbe-environment interactions in the development of asthma. Asthma-associated metabolites, 1-methylnicotinamide and allantoin, could potentially be therapeutic targets to prevent childhood asthma. A significant inverse correlation between house dust mite sensitization and allantoin suggests that an alteration of metabolic switching, through dietary or other environmental influences, may represent an opportunity to reduce the prevalence of childhood asthma. However, further studies are required to understand the role of these metabolites in asthma in greater detail.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supporting Information

Longitudinal urinary metabolomic profiling reveals metabolites for asthma development in early childhood

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Supplementary Figures and Tables

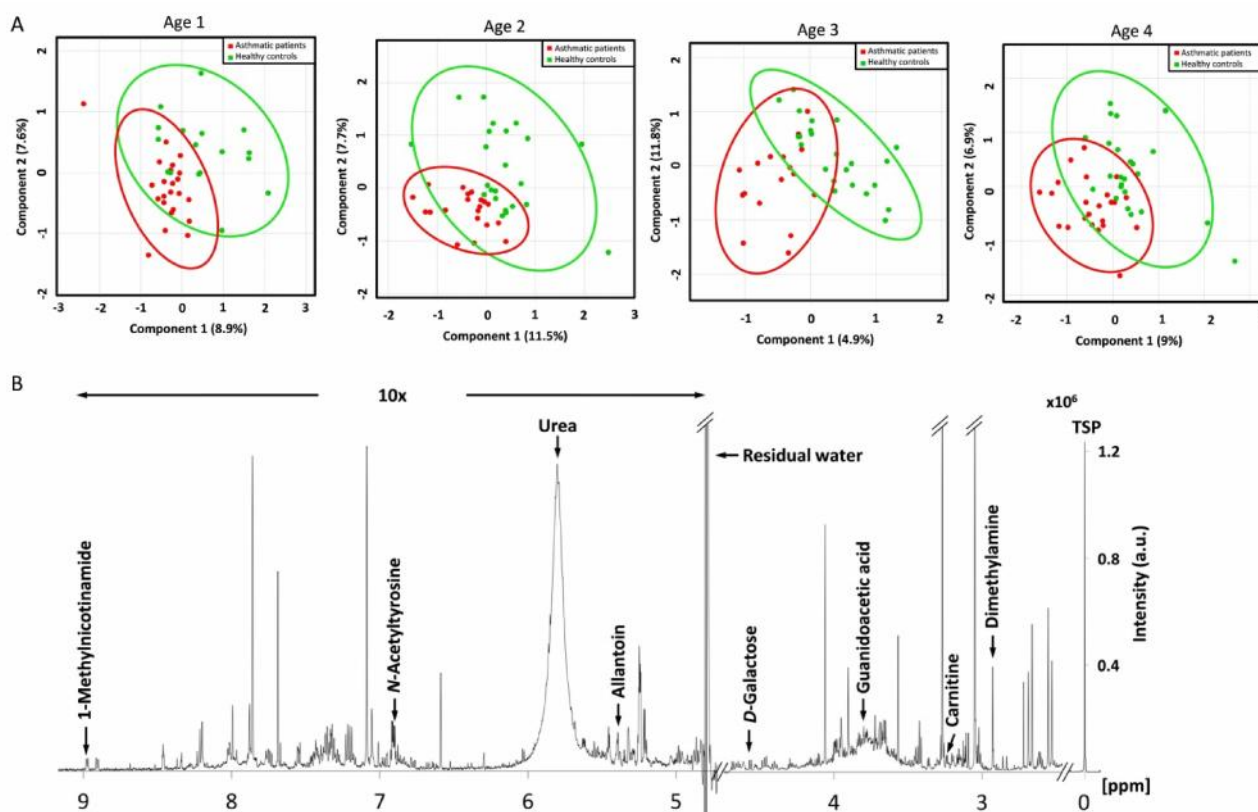


FIGURE S1 PLS-DA score plots from the analysis of ¹H-NMR spectra using urine samples. Panel A shows the two-dimensional graphs of PLS-DA score plot displaying the model's degree of separation between asthmatic patients and healthy controls at age 1, 2, 3 and 4. x axis, component 1 (% of total variance); y axis, component 2 (% of total variance). Panel B is the representative 600 MHz ¹H-NMR spectra of urine showing metabolite signals related to asthma. x axis, parts per million (ppm); y axis, intensity (a.u.).

TABLE S1 PLS-DA parameters and permutation test for distinguishing between asthmatic patients and healthy controls at different years of age

Age (yr)	Asthma (n)	Control (n)	PLS-DA parameters			$P_{\text{permutation}}^{\dagger}$
			Components*	Q^2	R^2	
1	17	23	1	-0.445	0.303	0.045
2	19	24	2	-1.163	0.553	0.045
3	17	24	1	-0.581	0.481	0.178
4	25	23	1	-0.176	0.334	0.137

*The number of components based on Q^2 indicates the best classifier of PLS-DA using a 10-fold cross-validation method.

$\dagger$ 1000 random permutations were performed.

PLS-DA, partial least squares-discriminant analysis; yr, year; Q^2 , predictive capability; R^2 , correlation coefficients.

TABLE S2 The VIP score and fold change of metabolites significantly differentially expressed between asthmatic patients and healthy controls at different years of age

Metabolites	Chemical shift, ppm	Age 1			Age 2			Age 3			Age 4		
		VIP score*	Fold change†	<i>P</i> ‡	VIP score	Fold change	<i>P</i>	VIP score	Fold change	<i>P</i>	VIP score	Fold change	<i>P</i>
<i>N</i> -Acetyltyrosine	6.865-6.875 (d)	2.81	0.74	0.039	–	1.00	0.990	–	0.96	0.745	–	1.08	0.052
<i>D</i> -Galactose	4.605-4.595 (d)	–	0.95	0.935	1.59	1.01	0.745	2.72	0.80	0.032	–	0.95	0.277
Allantoin	5.395 (s)	–	0.83	0.481	–	0.80	0.186	2.06	0.71	0.025	–	0.81	0.162
1-Methylnicotinamide	8.985-8.965 (d)	–	0.84	0.232	–	0.89	0.231	1.50	0.65	0.020	–	0.68	0.007
Carnitine	3.235-3.225 (s)	3.42	1.14	0.265	1.54	1.05	0.745	–	0.98	0.386	5.90	0.70	0.026

*VIP scores were obtained from PLS-DA model and a VIP score < 1.5 was shown as “–”.

†Fold change was calculated by dividing the value of metabolites in asthmatic patients by healthy controls.

‡All raw *P* values < 0.05, which is in bold, are significant. All FDR-adjusted *P*-values were > 0.5.

VIP, Variable Importance in Projection; ppm, parts per million; d, doublet; s, singlet.

TABLE S3 The VIP score and fold change of metabolites significantly differentially expressed between with and without mite, food and IgE sensitization at 3 years of age

Metabolites	Chemical shift, ppm	Asthma			Mite sensitization			Food sensitization			IgE $\geq$ 100 kU/L		
		VIP score*	Fold change†	<i>P</i> ‡	VIP score	Fold change	<i>P</i>	VIP score	Fold change	<i>P</i>	VIP score	Fold change	<i>P</i>
Acetic acid	1.925(s)	5.20	1.50	0.787	3.70	1.38	0.918	2.72	1.14	0.757	4.21	1.44	0.349
Guanidoacetic acid	3.805-3.795(s)	3.72	1.08	0.335	1.51	1.03	0.659	2.23	0.97	0.447	2.76	1.07	0.500
Galactitol	3.705-3.685(m)	3.71	1.06	0.569	1.13	1.02	0.888	2.61	0.99	0.800	5.15	1.11	0.392
Taurine	3.445-3.415(t)	3.69	1.09	0.326	-	1.01	0.844	1.82	1.03	0.978	-	1.03	0.513
Creatine	3.055(s)	3.66	0.90	0.427	8.81	0.77	0.039	5.33	0.83	0.166	6.09	0.81	0.238
<i>N</i> -Phenylacetylglycine	7.445-7.415(s)	3.64	1.22	0.647	4.44	1.32	0.177	3.65	1.29	0.352	2.81	1.20	0.254
Lactate	1.345-1.315(d)	3.55	1.16	0.569	1.89	0.92	0.714	2.53	0.89	0.403	2.63	1.14	0.090
Pi-Methylhistidine	3.735(s)	3.16	0.86	0.903	-	0.98	0.800	3.05	0.82	0.517	2.28	0.88	0.896
Hippuric acid	7.575-7.545(n)	3.10	0.82	0.978	-	1.06	0.728	3.35	0.77	0.529	1.24	0.91	0.802
Carnitine	3.235-3.225(s)	2.95	0.86	0.108	1.38	0.92	0.333	1.23	0.96	0.673	-	1.03	0.179
Lactose	4.465-4.455(d)	2.52	0.84	0.053	2.21	0.84	0.080	-	0.95	0.903	1.35	0.89	0.222
Galactose	4.605-4.595(d)	2.33	0.85	0.075	2.93	0.80	0.128	2.00	0.84	0.372	1.67	0.86	0.319
Allantoin	5.395(s)	2.16	0.73	0.015	1.85	0.75	0.080	-	0.90	0.542	-	0.90	0.272
Citric acid	2.575-2.515(d)	2.12	0.95	0.845	3.86	0.90	0.296	1.87	0.96	0.963	-	1.00	0.896
Dimethylamine	2.715(s)	1.80	0.80	0.145	-	0.89	0.859	-	1.02	0.673	-	0.99	0.976
3-Aminoisobutyric acid	1.205-1.185(d)	1.67	1.08	0.963	5.21	1.33	0.314	3.18	1.21	0.124	4.32	1.27	0.131
Glycine	3.575-3.565(s)	1.65	1.05	0.688	1.75	0.95	0.888	2.65	0.92	0.435	1.09	1.04	0.488

Succinic acid	2.415-2.405(s)	1.60	0.93	0.661	1.01	0.95	0.815	3.08	0.85	0.963	1.66	0.91	0.849
Lysine	1.765-1.705(m)	1.55	0.95	0.948	2.06	0.93	0.687	1.99	0.93	0.542	-	0.99	0.725
1-Methylnicotinamide	9.285(s)	1.55	0.69	0.007	1.06	0.76	0.090	-	0.92	0.844	-	0.81	0.230
3-Hydroxyisovaleric acid	1.275(s)	1.52	0.92	0.449	1.43	0.92	0.435	1.11	0.93	0.517	-	1.03	0.463
Hypoxanthine	8.215-8.195(s)	1.51	0.85	0.072	1.49	0.83	0.382	-	0.97	0.580	-	0.90	0.186
<i>N,N</i> -Dimethylglycine	2.935-2.925(s)	1.45	1.12	0.963	2.99	0.78	0.481	2.32	0.77	0.119	-	0.92	0.463
Glutamine	2.465-2.425(m)	1.22	1.03	0.801	-	0.99	0.673	1.26	1.02	0.903	1.18	1.04	0.651
Glucose	4.665-4.655(d)	1.18	0.94	0.556	1.53	0.91	0.342	-	0.94	0.815	1.27	0.92	0.438
3-Indoxylsulfate	7.715-7.695(d)	1.09	1.11	0.344	1.40	1.15	0.190	1.93	1.28	0.008	-	1.08	0.427
Isobutyric acid	1.085-1.065(d)	1.01	1.13	0.933	-	1.04	0.271	1.48	1.08	0.024	2.70	1.45	0.349
Alanine	1.495-1.465(d)	-	0.98	0.519	2.88	0.89	0.619	2.49	0.89	0.593	1.09	1.05	0.179
2-Phenylpropionate	1.415(d)	-	0.91	0.647	2.15	0.78	0.032	1.30	0.85	0.247	-	0.97	0.802
Propylene glycol	1.155-1.135(d)	-	1.01	0.647	1.95	0.84	0.948	2.20	0.81	0.757	-	0.94	0.246
Trimethylamine <i>N</i> -oxide	3.275-3.265(s)	-	0.99	0.620	1.66	0.96	0.978	2.88	1.06	0.296	6.46	1.20	0.033
Formic acid	8.465-8.455(s)	-	0.99	0.993	1.63	0.82	0.098	-	1.02	0.632	-	1.09	0.488
<i>N</i> -Acetyltyrosine	6.865-6.875(d)	-	1.05	0.241	1.54	1.16	0.062	-	1.02	0.786	-	0.92	0.802
Betaine	3.905(m)	-	1.04	0.405	1.01	0.95	0.815	1.60	0.94	0.743	-	1.04	0.500
Creatine phosphat	3.955(s)	-	1.03	0.744	-	1.01	0.673	1.68	0.96	0.352	1.02	1.03	0.594
Fucose	1.265-1.245(d)	-	0.99	0.674	-	0.99	0.493	1.14	0.96	0.888	1.55	1.08	0.107
Valine	1.055-1.035(d)	-	1.08	0.634	-	1.09	0.593	1.07	1.11	0.505	1.92	1.22	0.090
Methylmalonic acid	1.235-1.225(d)	-	1.00	0.948	-	1.03	0.687	1.04	1.04	0.529	2.23	1.14	0.193
3-Phenylpropionate	2.495(t)	-	1.07	0.859	-	1.07	0.948	1.03	1.10	0.414	-	1.02	0.526
<i>N</i> -Acetylglucosamine	2.055-2.045(m)	-	0.98	0.556	-	0.99	0.542	-	1.00	0.859	2.04	0.94	0.179

Imidazole	7.335(s)	-	1.17	0.219	-	1.05	0.993	-	1.13	0.714	1.26	1.27	0.309
Glucuronic acid	5.255(d)	-	1.07	0.661	-	1.02	0.918	-	1.01	0.687	1.19	1.17	0.166
<i>N</i> -Methylhydantoin	2.945(s)	-	1.06	0.661	-	1.02	0.859	-	1.00	0.567	1.12	1.16	0.222

*VIP score were obtained from PLS-DA and a VIP score < 1.0 was shown as “–”.

†Fold changes were divided asthmatic patient by healthy control, with/without mite, food and IgE sensitization respectively.

‡All raw *P* values < 0.05, which is in bold, are significant. All FDR-adjusted *P*-values were > 0.5.

VIP, Variable Importance in Projection; s, singlet; m, multiplet; t, triplet; d, doublet; n, nonet.