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Original Article

Urine Microbe-Derived Extracellular Vesicles in Children With Asthma

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ABSTRACT

Purpose: Several studies have found significant associations between asthma and microbiome. However, it is challenging to obtain-sputum and bronchoalveolar lavage samples from pediatric patients. Thus, we used voided urine to show that urine microbe-derived extracellular vesicles (EVs) in asthma are an available source for clinical research.
Methods: Five urine samples were obtained at 2–3-month intervals from each patient with asthma (n = 20), and a single voided urine sample were obtained from each healthy child (n = 20). After isolating EVs, 16S rDNA pyrosequencing was performed. The Chao1 index and principal coordinate analysis (PCoA) were used to assess diversity. To predict microbiota functional capacities, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used based on the Kyoto Encyclopedia of Genes and Genomes pathway database. Eight covariates were included in the EnvFit analysis to identify significant factors in the asthma group.
Results: The asthma group showed lower Chao1 bacterial richness, and PCoA-based clustering differed significantly. Two phyla, and 13 families and genera were enriched or depleted. Functional profiling revealed significant differences between the asthma and control groups. EnvFit analysis of correlation to age, sex, body mass index, infection, season, asthma phenotype, severity, and symptoms was not significant except for infections associated with visit 1 and the season of visit 2.
Conclusions: This study showed that microbe-derived EVs were constantly altered in the urine of children with asthma, consistent with the findings of previous studies indicating microbiome changes in the lung and gut. The urine may reflect the specific pattern of microbiome EVs in children with asthma.

Keywords: Asthma; child; urine; extracellular vesicles; microbiota

INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation. With developments in 16S rRNA sequencing, which have replaced conventional culture methods, the associations between asthma and bacterial communities have been widely examined. Microbiota composition is considered a key mediator of the immune system

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that is associated with the development of asthma. Several studies have investigated airway microbiota in children with asthma. They found that airway hosts had complex microbial communities and suggested that airway dysbiosis and increased bacterial diversity might contribute to asthma.[1-3] Studies have investigated whether airway or gut microbiota affects asthma. Cumulative reports suggest that dysbiosis and low gut microbiota diversity in infancy are associated with asthma.[4-6] The importance of the lung-gut axis is increasingly recognized along with the concept that the microbiome of one organ affects the other.[7,8] Association between lung and gut microbiome with asthma and the potential use of microbiomes as biomarkers have been examined. However, because children under age 5 cannot spit out sputum and it is difficult to obtain bronchoalveolar lavage (BAL) and stool samples, it is almost impossible to study the microbiomes of young children.

Extracellular vesicles (EVs) are potent vehicles mediating intercellular communication in prokaryotes and eukaryotes. EVs are secreted from cells involved in the allergic response, such as bronchial epithelial cells, mast cells, dendritic cells, and T cells.[9] Pathogen-derived EVs have also been shown to affect diseases such as atopic dermatitis-like skin infection, neutrophilic pulmonary inflammation, and cystic fibrosis, indicating that EVs can mediate allergic disease.[10,11] In addition, a previous study showed that bacteria-derived EVs could affect distal host cell sites.[12] A recent study identified dysbiosis in an asthma group using urine bacteria-derived EVs, which may be useful to indicate allergic airway disease in children.[13]

This study uses voided urine to show that urine microbe-derived EVs in asthma are an available source for clinical research. We show the differences in microbe-derived EVs between pediatric patients with asthma and healthy children, and identify factors influencing urine EVs with asthma.

MATERIALS AND METHODS

Participants and sample collection

Patients who were 1–12 years old and visited the outpatient department at Inha University Hospital in 2017–2018 were included in this study. Specifically, we recruited patients with asthma diagnosed by 2 independent pediatric allergists. Under 5 years old, asthma was diagnosed as follows: 1) wheezing or cough without respiratory infection, 2) allergen sensitization as specific immunoglobulin E (IgE) or history of eczema, and 3) symptom relief after use of controller for 2 to 3 months. Over 6 years old, asthma was diagnosed when forced expiratory volume in 1 second > 12% increased from baseline after using a bronchodilator. Inclusion criteria for healthy controls were patients without respiratory diseases, allergic diseases, or genitourinary diseases. Children with chronic diseases or urinary tract infection were excluded from all groups. Overall, the pediatric asthma and control groups contained 20 patients each.

Urine samples were collected from the asthma group 5 times at 2–3-month intervals during outpatient or inpatient visits to the Department of Pediatrics at Inha University Hospital. Controlled status and asthmatic symptoms were assessed at each visit and when the patient had experienced fever or upper respiratory infection symptoms during the prior 3 weeks. Furthermore, 20 single voided urine samples were collected from the control group. Body mass index (BMI) was calculated using the mean height and weight measured at each of 5 visits. BMIs of < 5%, 5%–85%, 85%–95%, and > 95% were classified as underweight, healthy weight, overweight, and obese, respectively. Atopic asthma was defined as a skin prick test

showing a ≥ 3 mm wheal in response to at least 1 common aero allergen (house dust mites, animals, grass, or mold) or ≥ 0.35 kU/L of specific IgE. As described by the Global Initiative for Asthma,[14] asthma symptom control was classified as well-controlled, partly controlled and uncontrolled, and asthma severity was classified as mild, moderate, and severe.

For urine samples, 20 mL of midstream clean-catch urine were collected in a sterile urine bottle to prevent contamination.[15] The specimen bottle was labeled with a unique patient identifier, and the time of collection was recorded. Immediately after collection, the specimen was sent to the laboratory in an ice-box. The urine was subdivided into analysis containers and stored at −70°C. This study was approved by the Institutional Review Board of Inha University Hospital (IRB No. 2017-05-008), and informed consent was obtained from all patients and guardians.

EV isolation and DNA extraction

EVs were isolated from human urine samples by centrifugation at 1,000 × *g* for 10 minutes at 4°C. Bacteria and foreign particles were eliminated by filter sterilization of the supernatant through a 0.22 µm filter. To extract DNA from the EV membrane, EVs separated from urine in the previous steps were heated for 40 minutes at 100°C. To eliminate remaining floating particles and waste, the supernatant was centrifuged for 30 minutes at 14,953 × *g* at 4°C and collected. A PowerSoil DNA (MO BIO, Carlsbad, CA, USA) isolation kit was used to remove soluble proteins and to extract EV DNA following the manufacturer's instructions. Then, each sample was quantified using the QIAxpert system (Qiagen, Hilden, Germany).

Bacterial metagenomics analysis

Bacterial genomic DNA was amplified using 16S_V3_F (5-TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGCCTACGGGNGGCWGCAG-3) and 16S_V4_R (5-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3) primers, which are specific for V3–V4 hypervariable regions of the 16S rDNA gene. Libraries were prepared by polymerase chain reaction products according to the MiSeq System guide (Illumina, San Diego, CA, USA) and quantified using QIAxpert (Qiagen). Each amplicon was then quantified, set at an equimolar ratio, pooled, and sequenced using the MiSeq (Illumina) platform according to the manufacturer's recommendations.

16S rRNA amplicon sequencing and analysis

Paired-end reads matching the adapter sequences were trimmed using Cutadapt version 1.1.6.[16] The resulting FASTQ files containing paired-end reads were merged with CASPER and then quality-filtered using Phred (Q) score-based criteria described by Bokulich.[17,18] Any reads shorter than 350 base pairs and longer than 550 base pairs after merging were discarded. To identify chimeric sequences, a reference-based chimera detection step was performed using a VSEARCH against the SILVA gold database.[19,20] Next, sequence reads were clustered into operational taxonomic units using VSEARCH with a *de novo* clustering algorithm based on a threshold of 97% sequence similarity. Representative sequences of the operational taxonomic units were finally classified using the SILVA 128 database with UCLUST (*parallel_assign_taxonomy_uclust.py* script on QIIME version 1.9.1) under default parameters.[21] The Chao index, an estimator of the richness of taxa per individual, was used to measure the diversity of each sample.

Statistical analysis

Mann-Whitney *U* tests to compare age and BMI between the control and asthma groups, χ^2 test to compare sex ratio, and Fisher's exact tests to compare BMI classification were performed

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using SAS 9.4 software (SAS Institute, Cary, NC, USA). Richness was evaluated using the Chao1 index to assess α diversity. Principal coordinate analysis (PCoA) based on Bray-Curtis distance was used to evaluate β diversity. Taxa comprising less than 1% of the average composition of the enriched group were discarded. To predict the functional capacities of the microbiota, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States was used based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.[22] The effect size and significance of each covariate were determined using the 'EnvFit' function in 'vegan' (https://cran.r-project.org/web/packages/vegan/index.html) comparing the difference in centroids of each group relative to the total variation. Ordination was performed using non-metric multidimensional scaling based on Bray-Curtis dissimilarity. The significance value was determined based on 10,000 permutations. In total, 8 covariates with known associations with asthma were included in EnvFit analysis. Specifically, age, sex, asthma phenotype, BMI, infection, season, asthma severity, and asthma symptoms were tested. $P < 0.05$ was considered statistically significant, and all statistical analyses were performed using R version 3.3.2 (R Foundation, Vienna, Austria).

RESULTS

The control group consisted of 20 participants, 13 boys and 7 girls, with a mean age of 10.9 years and sex-based mean ages of 11 and 10.6 years, respectively. The mean BMI was 18.8 kg/m^2; 17 participants had a healthy weight, and 3 participants were obese.

The asthma group consisted of 20 participants, 15 boys and 5 girls, with a mean age of 5 years and sex-based mean ages of 5.4 and 3.8 years, respectively. There was a significant difference in age between the control and asthma groups. The mean BMI of the asthma group was 17.4 kg/m^2; 16 participants had a healthy weight, and 4 participants were obese. For the asthma phenotype, 18 and 2 participants had atopic and non-atopic asthma, respectively. The 20 asthmatic patients visited the outpatient department or were admitted 5 times each, comprising 100 visits. Of these, asthma symptoms were judged to be well-controlled, partly controlled, and uncontrolled on 55, 38, and 7 occasions, respectively. Asthma severity was mild, moderate, and severe on 65, 28, and 7 occasions, respectively. Of 100 total visits, fever or upper respiratory infection symptoms were observed on 28 occasions (**Table 1**).

We analyzed α diversity between the control and asthma groups based on the Chao1 value, which represents microbiome richness. Compared to that of the control group, all visits by patients in the asthma group were associated with lower richness (**Fig. 1**). PCoA showed significant differences in clustering between the control and asthma groups (**Fig. 2**). At the phylum level, Verrucomicrobia was significantly more abundant in the control group than in samples from all visits of the asthma group, whereas Cyanobacteria were significantly more abundant in the asthma group than in the control group. At family and genus levels, Pseudomonadaceae and *Pseudomonas* were significantly more abundant in the asthma groups. However, Megamonas, Clostridiaceae, Acinetobacter, Comamonadaceae, Enterobacteriaceae, Pasteurellaceae, Haemophilus, Rhodocyclaceae, Acetobacteraceae, Verrucomicrobiaceae, and Akkermansia were significantly lower in the asthma group (**Supplementary Table S1**). In particular, Acetobacteraceae and *Megamonas* increased by more than 10-fold in the control group, while Cyanobacteria, Pseudomonadaceae, and *Pseudomonas* increased by more than 10-fold in the asthma group (**Table 2**).

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Table 1. Demographics of study subjects

Characteristics	Controls (n = 20)	Asthma (n = 20)	*P*
Age (yr)	10.85 ± 0.81	5.00 ± 2.25	< 0.001*
Boy	11.00 ± 0.81	5.40 ± 2.29	< 0.001*
Girl	10.57 ± 0.79	3.80 ± 1.79	0.004*
Boy:girl	13:7	15:5	0.490†
BMI	18.79 ± 4.57	17.36 ± 3.45	0.110*
Healthy weight:obese	17:3	16:4	1.000‡
Underweight	0	0	
Overweight	0	0	
Asthma phenotype			
Atopic	-	18	-
Non-atopic	-	2	-
Asthma symptom control			
Well	-	55	-
Partly controlled	-	38	-
Uncontrolled	-	7	-
Asthma severity			
Mild	-	65	-
Moderate	-	28	-
Severe	-	7	-
Infection	-	28	-

BMI, body mass index.
P values were calculated compared to controls using *Mann-Whitney *U*, †χ^2, or ‡Fisher's exact tests.

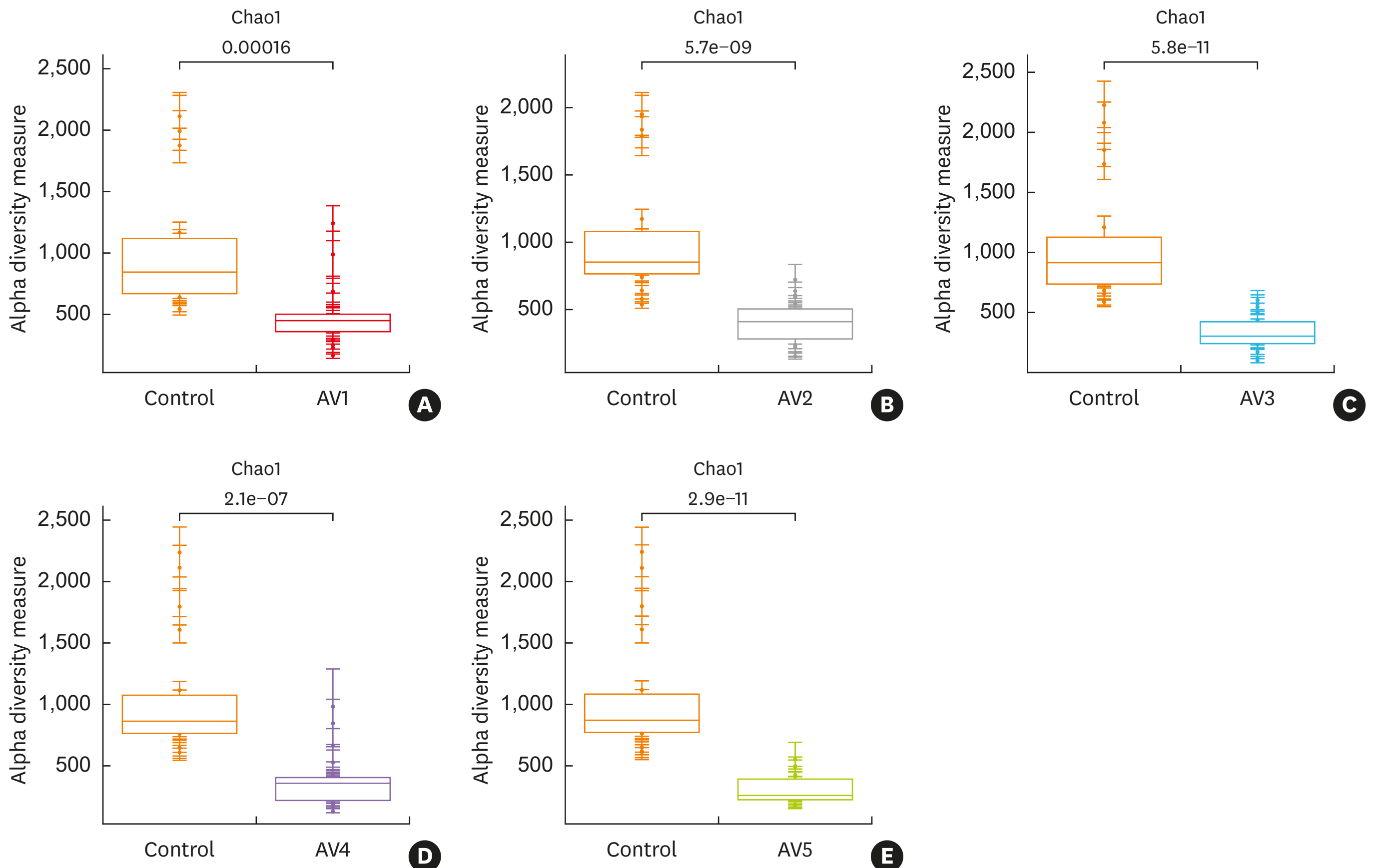

Fig. 1. The α diversity based on Chao1 richness index for asthma vs. control groups. Bacterial richness in controls was significantly higher than in asthma groups. (A) Control vs.AV1, (B) control vs.AV2, (C) control vs. AV3, (D) control vs. AV4, (E) control vs. AV5.
AV1, asthma visit 1; AV2, asthma visit 2; AV3, asthma visit 3; AV4, asthma visit 4; AV5, asthma visit 5.

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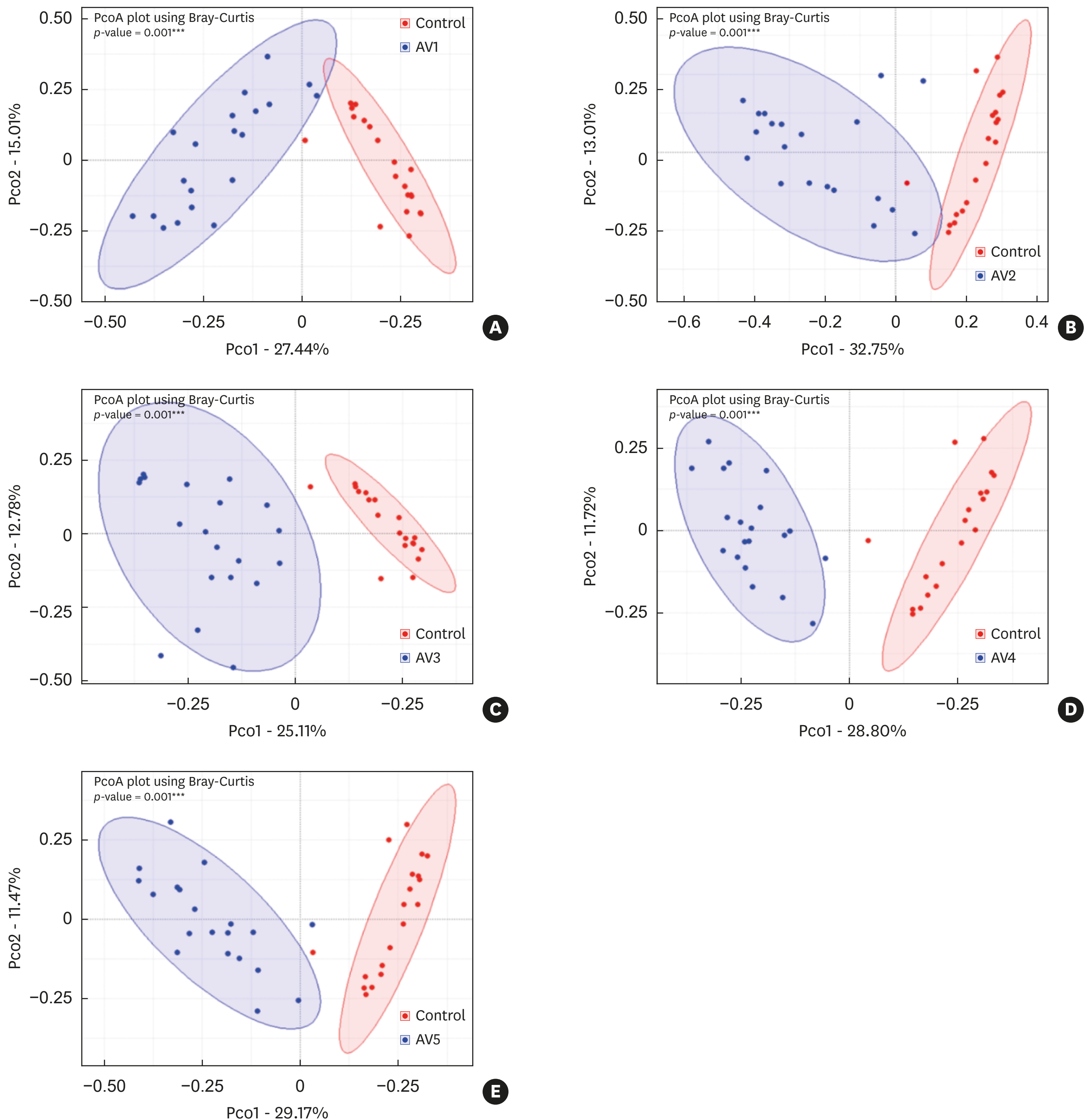

Fig. 2. PCoA based on Bray-Curtis distance of asthma vs. control groups. Clustering of asthma groups was significantly different from that of control group. (A) Control vs. AV1, (B) control vs. AV2, (C) control vs. AV3, (D) control vs. AV4, (E) control vs. AV5.
PCoA, principal coordinates analysis; AV1, asthma visit 1; AV2, asthma visit 2; AV3, asthma visit 3; AV4, asthma visit 4; AV5, asthma visit 5.

We used 16S data with KEGG pathway abundances to assess whether the functional potential of microbe-derived EVs differed between the asthma and control groups; this analysis revealed significant differences between asthma and control groups. Classification of human disease, organismal functions, and unclassified were excluded. One pathway for the first KEGG Orthology (KO) categories and 10 pathways for the second KO categories

Table 2. Families and genera significantly more than 10-fold enriched or depleted in asthma groups compared to control group

Phylum/family and genus	Control	AV1	*P*	Control	AV2	*P*	Control	AV3	*P*	Control	AV4	*P*	Control	AV5	*P*
Cyanobacteria	0.0087 ± 0.0255	0.1425 ± 0.1347	< 0.01*	0.0087 ± 0.0255	0.1684 ± 0.1483	< 0.01*	0.0087 ± 0.0255	0.1300 ± 0.1339	< 0.01*	0.0087 ± 0.0255	0.0999 ± 0.0711	< 0.01*	0.0087 ± 0.0255	0.1283 ± 0.1320	< 0.01*
Pseudomonadaceae	0.0070 ± 0.0046	0.0786 ± 0.0738	< 0.01*	0.0070 ± 0.0046	0.1174 ± 0.0803	< 0.01*	0.0070 ± 0.0046	0.0847 ± 0.0719	< 0.01*	0.0070 ± 0.0046	0.0851 ± 0.0557	< 0.01*	0.0070 ± 0.0046	0.0918 ± 0.0839	< 0.01*
Pseudomonas	0.0069 ± 0.0045	0.0778 ± 0.0730	< 0.01*	0.0069 ± 0.0045	0.1141 ± 0.0783	< 0.01*	0.0069 ± 0.0045	0.0838 ± 0.0708	< 0.01*	0.0069 ± 0.0045	0.0804 ± 0.0528	< 0.01*	0.0069 ± 0.0045	0.0892 ± 0.0843	< 0.01*
Acetobacteraceae	0.0109 ± 0.0464	0.0000 ± 0.0000	< 0.01*	0.0109 ± 0.0464	0.0000 ± 0.0001	< 0.01*	0.0109 ± 0.0464	0.0005 ± 0.0016	0.01*	0.0109 ± 0.0464	0.0003 ± 0.0011	< 0.01*	0.0109 ± 0.0464	0.0004 ± 0.0020	< 0.01*
Megamonas	0.0211 ± 0.0121	0.0007 ± 0.0026	< 0.01*	0.0211 ± 0.0121	0.0001 ± 0.0004	< 0.01*	0.0211 ± 0.0121	0.0003 ± 0.0011	< 0.01*	0.0211 ± 0.0121	0.0026 ± 0.0117	< 0.01*	0.0211 ± 0.0121	0.0010 ± 0.0044	< 0.01*

Values are presented as mean ± standard deviation. *P* values were determined by comparing asthma group to controls using Mann-Whitney *U* test.
AV1, asthma visit 1; AV2, asthma visit 2; AV3, asthma visit 3; AV4, asthma visit 4; AV5, asthma visit 5.
*Significant.

were confirmed to show a significant difference in common. In the first KO categories, "environmental information" processing was significantly more abundant in the control groups (**Fig. 3**). In the second KO categories, "energy metabolism", "folding, sorting and degradation," "cell growth and death," "metabolism of cofactors and vitamins," "enzyme families," and "biosynthesis of other secondary metabolism pathways" were more abundant in the asthma group compared to controls (**Table 3**). Next, the third KO categories were identified, and 17 pathways were significantly different. In particular, "terpenoid backbone biosynthesis," "porphyrin and chlorophyll metabolism", "peptidases", "oxidative phosphorylation", "arginine and proline metabolism," "RNA degradation," "cell cycle-Caulobacter," "nicotinate, and nicotinamide metabolism," "glycolysis/gluconeogenesis," "protein export," and "purine metabolism pathways" were abundant in all asthma visit groups (**Table 4**).

Next, significant factors associated with urine EVs were identified. For statistical analysis, covariates were analyzed by stratifying samples into discrete time points (visits 1, 2, 3, 4, and 5). There were no significant differences except for between infections associated with visit 1 and the season of visit 2 (**Fig. 4**).

DISCUSSION

This study showed that urine microbe-derived EVs differ in diversity, specific bacterial taxa, and functional profiling between patients with asthma and healthy controls. In addition, several factors that were predicted to affect airway or gut microbiomes did not affect urine EVs. Studies so far have shown that EVs are an important factor associated with asthma.[23] In BAL samples, the composition of lipid mediators driven by EVs differed between patients with asthma and controls.[24] Nasal exosomes influence innate immune cells, which may be important for defense against allergens.[25] A recent study used a new non-invasive method of exhaled breath condensate (EBC) to show a difference in EBC miRNA profiles between patients with asthma and healthy controls.[26] The study of miRNA using EBC is fertile ground for clinical biomarker discovery. However, these methods are difficult to apply to children. There was an effort to use urine EVs as a source of biomarkers in neurologic diseases based on evidence that EVs can cross the blood-brain barrier and distribute to distal organs.[27] However, the specific source and relative contribution of microbe-derived EVs passing through the body to different EV pools of biofluids remain unknown.

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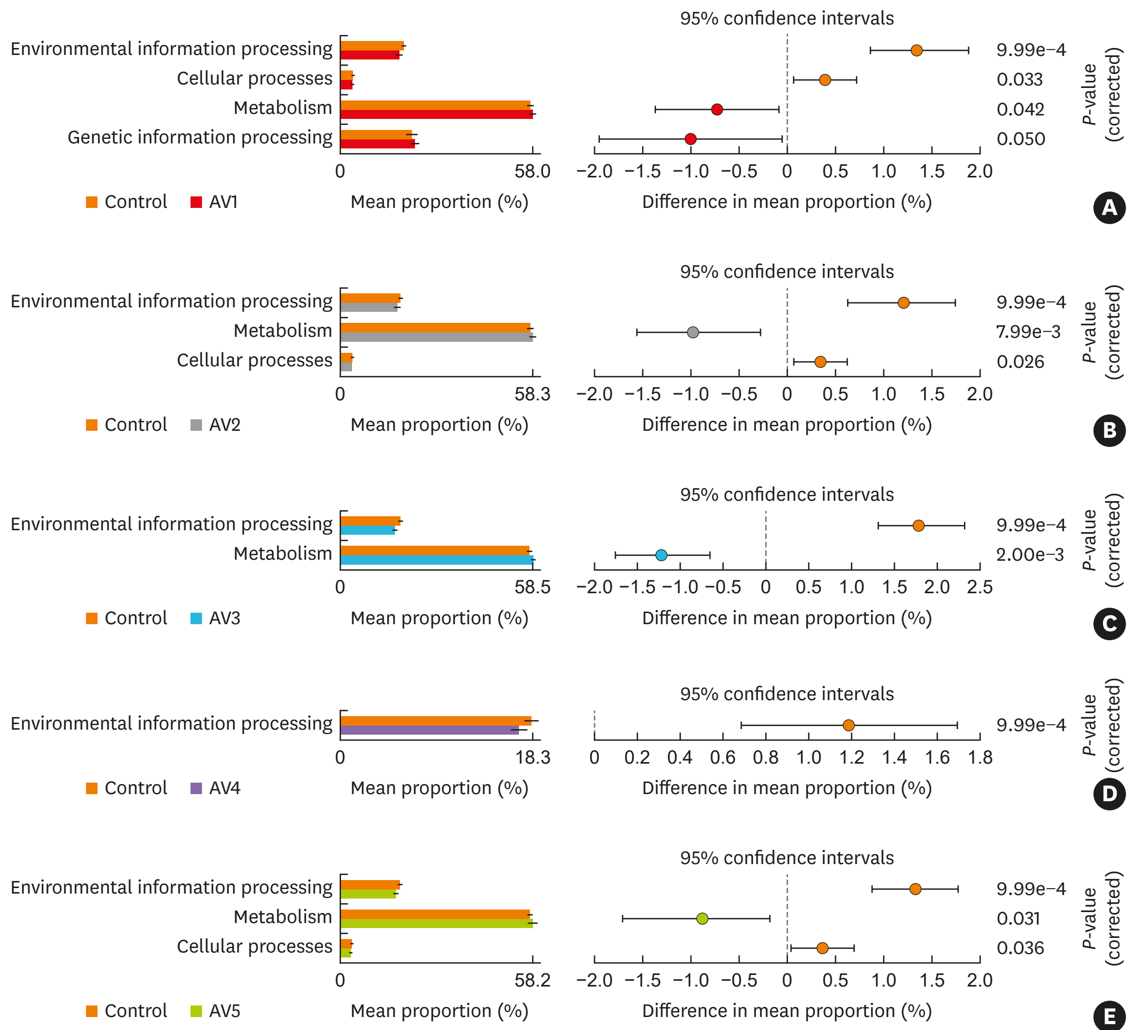

Fig. 3. Mean relative abundance of predictive functions at level 1 for asthma vs. control groups. (A) Control vs.AV1, (B) control vs. AV2, (C) control vs. AV3, (D) control vs. AV4, (E) control vs.AV5.
AV1, asthma visit 1; AV2, asthma visit 2; AV3, asthma visit 3; AV4, asthma visit 4; AV5, asthma visit 5.

Table 3. Predictive functions of level 2 with significantly different functions between asthma visit and control groups

Functions	Control vs. all visits for asthma	
	Group	P^*
Lipid metabolism	Control	< 0.01
Carbohydrate metabolism	Control	< 0.01
Membrane transport	Control	< 0.01
Transcription	Control	< 0.05
Energy metabolism	Asthma	< 0.01
Folding, sorting and degradation	Asthma	< 0.01
Cell growth and death	Asthma	< 0.01
Metabolism of cofactors and vitamins	Asthma	< 0.01
Enzyme families	Asthma	< 0.01
Biosynthesis of other secondary metabolites	Asthma	< 0.01

Each pathway represents abundant group. *P* values were determined by comparing asthma group to controls using Mann-Whitney *U* test.
*Significant.

Table 4. Predictive functions of level 3 with significantly different functions between asthma and control groups, with each pathway representing an abundant group

Functions	Control vs. all visits for asthma	
	Group	P^*
ABC transporters	Control	< 0.01
Butanoate metabolism	Control	< 0.01
Transcription factors	Control	< 0.01
Glycerophospholipid metabolism	Control	< 0.01
Glyoxylate and dicarboxylate metabolism	Control	< 0.01
Propanoate metabolism	Control	< 0.01
Terpenoid backbone biosynthesis	Asthma	< 0.01
Porphyrin and chlorophyll metabolism	Asthma	< 0.01
Peptidases	Asthma	< 0.01
Oxidative phosphorylation	Asthma	< 0.01
Arginine and proline metabolism	Asthma	< 0.01
RNA degradation	Asthma	< 0.01
Cell cycle-caulobacter	Asthma	< 0.01
Nicotinate and nicotinamide metabolism	Asthma	< 0.01
Glycolysis/gluconeogenesis	Asthma	< 0.01
Protein export	Asthma	< 0.01
Purine metabolism	Asthma	< 0.01

Each pathway represents abundant group. *P* values were determined by comparing asthma group to controls using Mann-Whitney *U* test.
ABC, ATP-binding cassette.
*Significant.

Many previous studies have assessed the relationship among asthma and the diversity of airway and gut microbiomes. Samples associated with airways, such as bronchial epithelial brushing and sputum, reveal an increased bacterial diversity in asthmatic patients.[1,28] In feces, a lower diversity of the total microbiota in infancy was associated with asthma. However, the correlation was not observed after 1 year, and no association between asthma and bacterial diversity was found in adults.[4] Our study shows reduced α diversity and significantly different clustering of urine EVs based on PCoA in asthmatic children compared to controls. These results suggest that asthma, a disease that has occurred in the airways, affects the pool of urine microbe-derived EVs.

Many studies have found special bacterial groups associated with asthma. For example, one study showed that Comamonadaceae, Sphingomonadaceae, Oxalobacteraceae,

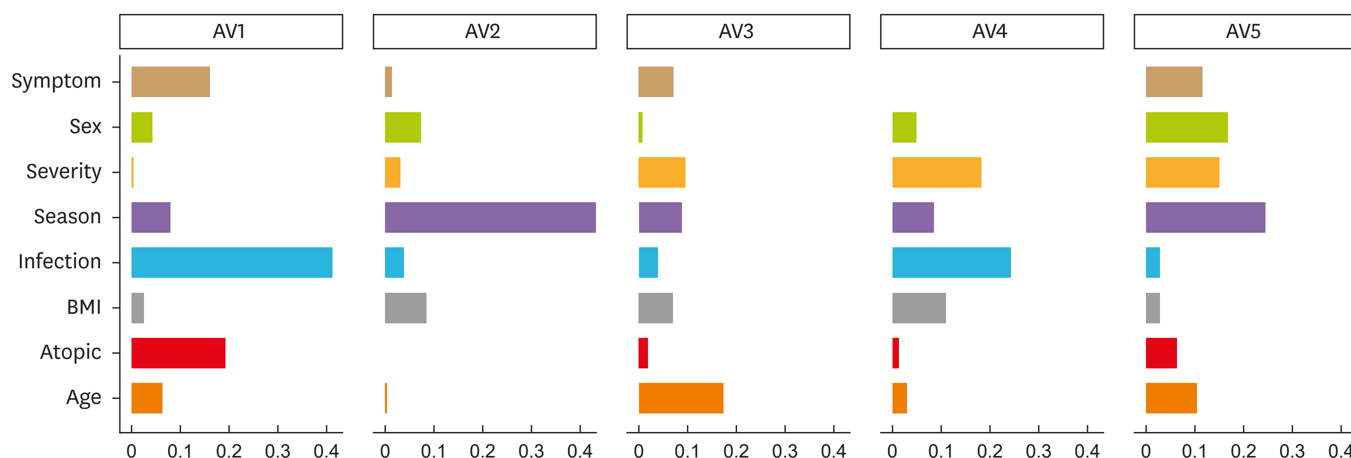

Fig. 4. Significance and explained variance of 8 microbiome covariates modeled by EnvFit. Horizontal bars show amount of variance (r2) explained by each covariate in the model as determined by EnvFit.
AV1, asthma visit 1; AV2, asthma visit 2; AV3, asthma visit 3; AV4, asthma visit 4; AV5, asthma visit 5.

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Nitrosomonadaceae, and Pseudomonadaceae were associated with the degree of bronchial hyperresponsiveness using samples of bronchial epithelial brushings.[1] In addition, reduced bifidobacteria were related to long-term asthma.[29] In this study, several bacterial EV groups were associated with asthma. In particular, Cyanobacteria, Pseudomonadaceae, and *Pseudomonas* were associated with asthma, while Acetobacteraceae and Megamonas were frequently observed in subjects without asthma. Previous studies of the airway and gut microbiomes demonstrated that urine is affected by EVs produced by the whole body. EVs produced by airway and gut microbiomes alone would not have had a statistical impact. Because specific bacterial EV groups differed between patients with asthma and controls, we suggest that asthma impacts the specific bacterial EV groups of urine.

Several predictive functional profiles distinguished between patients with asthma and healthy controls. Some pathways were related to asthma in recent studies of samples obtained from the airway, gut, and serum. In a previous study, acetate, propionate, and butyrate were major short-chain fatty acids[30] that bind G-protein-coupled receptor 43 to affect inflammatory responses in the gut.[31] In the lung, ATP-binding cassette (ABC) transporters are modulated to increase the level of ornithine metabolism (polyamines and proline).[32,33] Plasma levels of nicotinamide were significantly higher in patients with asthma than in controls.[34] In animal studies, allergen-induced early asthmatic response in rat lungs was associated with glycolysis,[35] and purine metabolism was affected in the blood of ovalbumin-induced asthmatic mice.[36] In this study, “propanoate metabolism,” “butanoate metabolism,” and “ABC transporter pathways” were significantly more abundant in the control group. In contrast, “arginine and proline metabolism,” “nicotinate and nicotinamide metabolism,” “glycolysis/gluconeogenesis,” and “purine metabolism pathways” were more abundant in the asthma groups. We expect that urine microbe derived-EVs are a useful tool for identifying patients with asthma because the bacterial diversity, specific bacterial taxa, and functional profiling differ between patients with asthma and control patients.

Previous studies have shown that factors, such as age, sex, asthma phenotype, BMI, infection, season, asthma severity, and asthma symptoms, are related to the microbiome. When the gut microbial composition was immature at 1 year old, the asthma risk rises at age 5 years.[37] The infant gut microbiota showed a difference between boys and girls when there was maternal asthma during pregnancy.[38] A recent report demonstrated that bacterial richness, diversity, and composition differ between neutrophilic and non-neutrophilic asthma.[39] Moreover, obesity changes the bacterial community of the gut and may play a role in obesity-related asthma.[40] Another study that evaluated changes in the nasopharyngeal microbiome according to the seasons showed that specific nasopharyngeal bacterial groups were different between summer and fall.[41] In addition, a report found that specific microbiota is altered in Asthma Control Questionnaire scores and the use of inhaled corticosteroids.[2] Almost all covariates in this study, such as age, sex, BMI, infection, season, asthma phenotype, severity, and symptoms, did not show any significant difference in urine EVs from asthmatic children. This may be because these covariates do not affect urine EVs, but further studies of more patients are needed.

This is the first study to analyze urine EVs. The strength of this study is that urine samples were collected 5 times each from 20 children each, and that the impact of changes in 8 covariates was evaluated. However, this study also involves some limitations. First, it included a relatively small number of participants. Among 100 visits, groups with uncontrolled asthma symptoms or with severe asthma accounted for only 7 visits.

Secondly, the age range of asthma and control groups did not completely match. Age was not a significant covariate in our study, but previous studies reported that the urinary microbiome differs between children and adults.[42] Infant gut microbial communities remain remarkably dynamic until approximately 3 years of age.[43] Thirdly, we could not investigate some factors that may affect the microbiome, including food habits, visitation of other clinics, and medication prescribed at other clinics. Further studies are needed to determine changes in urine EV compositions with age.

In conclusion, this study showed that urine microbe-derived EVs of children with asthma differ as shown in previous studies, indicating that changes in lung and gut microbiomes were associated with allergic airway diseases. We suggest that urine microbe-derived EVs can be a source for clinical research.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary Table S1

Families and genera significantly enriched or depleted in the asthma groups compared to in the control group

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