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Increased fecal human beta-defensin-2 expression in preterm infants is associated with allergic disease development in early childhood

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ABSTRACT

Background: This study aimed to investigate whether fecal human beta-defensins (HBD)-2 and eosinophil cationic protein (ECP) expression in preterm infants are associated with allergic disease development by age 2 years.

Methods: Preterm infants' stool samples were collected at the age of 6 and 12 months postnatally. Information regarding medication exposure histories (antibiotics, antipyretics, probiotics) and physician-diagnosed allergic diseases was obtained using age-specific questionnaires and medical records. We compared the 6-month and 12-month fecal HBD-2 and ECP concentrations between the medication exposure and non-exposure group, respectively, and between children who developed allergic diseases and those who did not by 2 years of age. Univariate and multivariable logistic regression analyses were performed to investigate independent variables related to physician-diagnosed allergic diseases by 2 years of age.

Results: Seventy-four preterm infants (gestational age, 31–36 weeks) were included. Fecal HBD-2 levels were significantly increased at 12 months of age among children who developed allergic diseases compared to those who did not (37.18 $\pm$ 11.80 ng/g *vs.* 8.56 $\pm$ 4.33 ng/g, $P = 0.011$). This association was more apparent among allergic children given antibiotics (50.23 $\pm$ 16.15 ng/g *vs.* 9.75 $\pm$ 7.16 ng/g, $P = 0.008$) or antipyretics (46.12 $\pm$ 14.22 ng/g *vs.* 10.82 $\pm$ 6.81 ng/g, $P = 0.018$) during the first year, whereas among allergic children who were previously not exposed to antibiotics or antipyretics, the differences were not significant. Results of the multivariable logistic regression analysis indicated that HBD-2 concentration in 12-month stools was an independent indicator associated with physician-diagnosed allergic diseases by 2 years of age (adjusted odds ratio: 1.03 [95% confidence interval: 1.00–1.05], $P = 0.036$). Our data revealed a lack of association between fecal ECP and allergic diseases.

Conclusions: We found that preterm infants who expressed high fecal HBD-2 at 12 months of age were associated with physician-diagnosed allergic diseases by the age of 2 years. Further studies are needed to determine the role of fecal HBD-2 in the development of allergic diseases.

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INTRODUCTION

The prevalence of pediatric allergic diseases has increased rapidly worldwide in recent decades.[1] In parallel, the rates of prematurity continue to rise; in 2014, the estimated global preterm birth rate was 10.6%.[2] Neonates are born with an immature gastrointestinal tract and immune system.[3] Several perinatal and postnatal factors, such as low birth weight, mode of delivery, feeding type, medication exposure, hygienic measures, as well as the composition of gut microbiota may influence the maturation of the infant immune system and the risk of later atopic diseases.[4,5] Compared to healthy term infants, premature infants are more susceptible to infections and sepsis due to defective immune systems.[6] Nonetheless, data addressing the intestinal innate immune responses in preterm infants are few.[7,8] The association between the immature intestinal inflammatory response of premature infants and an increased risk of childhood allergic diseases is rarely investigated and needs further research.

To elucidate intestinal inflammatory responses in the children born preterm, we analyzed two fecal immunological biomarkers, human beta-defensins (HBD)-2, and eosinophil cationic protein (ECP) in the study. Human defensins exhibit a wide range of antimicrobial activities and immunoregulatory functions, which serve a central role in innate immunity.[9] Of these, HBD-2 and certain beta-defensin subfamilies are capable of inducing chemotaxis in keratinocytes, dendritic cells, macrophages, memory T cells, and mast cells.[10] Recently, a few pediatric studies have reported that alteration of fecal HBD-2 concentrations is associated with necrotizing enterocolitis,[7] inflammatory bowel diseases,[11] and the initiation and development of allergic diseases.[12] Nevertheless, there are conflicting results about whether HBD-2 are predominantly pro- or anti-inflammatory in allergic diseases.[13] On the other hand, fecal ECP is an intestinal inflammatory marker that can be used to monitor intestinal inflammation in infants with food allergies.[14,15] Additionally, elevated ECP in serum and respiratory secretions are of value in predicting atopic eczema,[15] wheezing, and even asthma in infants.[16]

In the present study, we explored the intestinal innate immune response in preterm infants by monitoring fecal immunological biomarkers (fecal HBD-2 and ECP) serially. We investigated if any perinatal and postnatal factors influenced these two fecal biomarker concentrations. Additionally, we assessed whether fecal HBD-2 and ECP expression in preterm infants is associated with subsequent allergic disease development in early childhood.

METHODS

Study subjects

A total of 116 preterm infants at a gestational age (GA) of 31–36 weeks were enrolled from a birth cohort study that aimed to investigate epidemiological and predictive factors for allergic diseases in children.[17,18] All preterm infants were born in the delivery room of one hospital, a perinatal transfer and critical-care center, between March 2012 and August 2016, and were enrolled in the study after obtaining written informed consent from their parents soon after birth. Of the 116 infants, six participants who were small for their gestational age (birth weight $<$10th percentile), 26 participants who either did not follow-up or whose parents did not complete the questionnaires, and 10 participants who did not provide any stool samples were excluded from the analysis (Table 1). Finally, analytical samples of 74 infants were included in the study.

Questionnaires

Participants regularly returned for follow-up clinic visits and were checked by pediatricians for general health and allergic manifestations at

Characteristics	Children born preterm with allergic diseases by age 2 years (Allergic children) (N=38)	Children born preterm without allergic diseases by age 2 years (Non-allergic children) (N=36)	Excluded preterm infants (N=42)
Allergic phenotypes by age 2 years, n (%)			
Atopic dermatitis (AD)	10 (26.3)		
Allergic rhinitis (AR)	8 (21.0)		
AD and AR	13 (34.2)		
AD and wheezing	2 (5.3)		
AR and wheezing	4 (10.5)		
AD, AR and wheezing	1 (2.6)		
Neonatal and environmental factors			
Sex of infants, male (%)	21 (55.2)	20 (55.5)	24 (57.1)
Gestational age (week)	34.6 ± 1.8	34.6 ± 1.6	33.7 ± 3.5
Birth weight (kg)	2.4 ± 0.4	2.3 ± 0.5	2.3 ± 0.7
Cesarean section, n (%)	24 (63.2)	27 (75.0)	25 (59.5)
Breech or other malpresentations	9 (37.5)	16 (59.3)	
Maternal previous cesarean section	8 (33.3)	8 (29.6)	
Uncontrolled maternal pre-eclampsia or hypertension	5 (20.8)	2 (7.4)	
Placental factors or fetal comprise	3 (12.5)	3 (11.1)	
Number of twin births, (pairs of twin)	8 (3)	14 (5)	6 (3)[#]
Intensive care unit admission after birth, n (%)	25 (65.8)	23 (63.9)	27 (64.3)
Exclusive breastfeeding during the first year, month (n)	2.4 ± 4.1 (38)	2.4 ± 4.3 (36)	2.6 ± 4.2 (28)
Maternal factors			
Maternal age during delivery, years	31.5 ± 3.8	31.7 ± 4.1	30.9 ± 5.3
Maternal education level, n (%)			
High school or below	9 (23.7)	14 (38.9)	19 (45.2)

(continued)

Characteristics	Children born preterm with allergic diseases by age 2 years (Allergic children) (N=38)	Children born preterm without allergic diseases by age 2 years (Non-allergic children) (N=36)	Excluded preterm infants (N=42)
College or above	29 (76.3)	22 (61.1)	23 (54.8)
Gestational diabetes, n (%)	4 (10.5)	2 (5.6)	5 (11.9)
Gestational hypertension, n (%)	9 (23.7)	4 (11.1)	3 (7.1)
Parental allergy history, n (%)			
Both parents	10 (26.3)	7 (19.4)	10 (23.8)
Either one	16 (42.1)	12 (33.3)	16 (38.1)
None of parents	12 (31.6)	17 (47.2)	16 (38.1)
Environmental tobacco smoking, n (%)			
Age <1 year	21 (55.2)	17 (47.2)	
Age ≥ 1–2 years	15 (39.5)	16 (44.4)	
Household pets, n (%)			
Age <1 year	10 (26.3)	12 (33.3)	
Age ≥ 1–2 years	7 (18.4)	9 (25.0)	
Medical histories during the first year			
Any antibiotics exposure, n (%)			
By age 6 months	13 (34.2)	19 (52.7)	
By age 12 months	20 (52.6)	20 (55.6)	
Any antipyretics exposure, n (%)			
By age 6 months	10 (26.3)	12 (33.3)	
By age 12 months	29 (76.3)	21 (58.3)	
Any probiotics exposure, n (%)			
By age 6 months	19 (50.0)	20 (55.6)	
By age 12 months	22 (57.8)	27 (75.0)	
Acute bronchiolitis, n (%)	15 (39.5)	13 (36.1)	
Pneumonia, n (%)	6 (15.8)	2 (5.6)	
Acute gastroenteritis, n (%)	7 (18.4)	3 (8.3)	

(continued)

Characteristics	Children born preterm with allergic diseases by age 2 years (Allergic children) (N=38)	Children born preterm without allergic diseases by age 2 years (Non-allergic children) (N=36)	Excluded preterm infants (N=42)
Urinary tract infection, n (%)	4 (10.5)	4 (11.1)	
Sample collections for laboratory tests			
Total serum IgE (kU/l) (n)			
Age 1 year	238.0 ± 459.5 (29)	38.9 ± 59.1 (30)*	
Age 2 years	130.3 ± 132.3 (19)	31.1 ± 30.4 (18)*	
Stool samples, n (%)			
Age 6 months	38 (100)	36 (100)	
Age 1 year	31 (81.6)	29 (80.6)	

Table 1. (Continued) Study population characteristics. *P-value based on chi-square test and one-way analysis of variance. *Indicates significant differences (P-values < 0.05) between allergic and non-allergic children. #Indicates significant differences (P-values < 0.05) between non-allergic children and excluded study subjects.*

postnatal ages 2, 4, 6, 12, 18, and 24 months. The children were diagnosed with atopic dermatitis if they presented in infancy with relapsing itchy skin rashes on the face, extensors, or both, or on the flexors (eg, elbows, wrists, and back of knees), creases in the body, or both, in toddler years.[18] The children were diagnosed with allergic rhinitis if they had a problem with recurrent sneezing or a runny or blocked nose, and with seasonal or day changes apart from colds in the last 12 months.[19] Wheezing was defined as a history of recurrent cough with wheezing, dyspnea, or both, separate from colds, and a history of atopic dermatitis or allergic rhinitis in children in the preceding 12 months.[20] After an evaluation, age-specific questionnaires were administered to parents under the guidance of well-trained research assistants. The questionnaires recorded information regarding demographic data, history of parental allergies, infant feeding practices (eg, breastfeeding and solid foods), and environmental risk factors. Data on medication exposure (eg, antipyretics, antibiotics, probiotics), histories of infectious diseases, and any physician confirmed allergic diseases were collected from the questionnaires and confirmed through medical records from birth to 2 years of age.

Sample collection and processing

Infants' stool samples were collected at the age of 6 and 12 months postnatally. Infants' stool samples were collected in plastic containers (Greiner Bio-One, product No. 188271, China) within two days before the study subjects returned to the clinics for follow-ups at 6 and 12 months of postnatal age.[17,21] Mothers were instructed to keep the plastic containers in a refrigerator (−18 °C) after stool-sample collection, and to suspend stool-sample collection for 2 weeks, if their child had recent (2 weeks) exposure to medication. After a research assistant collected a stool sample, its weight (g) was determined, an extraction buffer containing protease inhibitor 10 µL (Temecula, California, USA) and 4 mL 1% phosphate buffered saline was added to per gram of each stool sample. Each sample (around 1 g) was mixed and stirred using a vortex mixer for 15 s, and then 1 mL the homogenate was centrifuged for 15 min at 3000 rpm and 4 °C. The supernatant was collected and frozen at −80 °C until use. Furthermore, blood

samples (3–5 mL) were collected from study subjects at 12 and 24 months of postnatal age.

Determining fecal HBD-2 and ECP, and serum total immunoglobulin E (IgE) levels

After we thawed the supernatants from infant stool samples, the 6- and 12- month fecal HBD-2, and ECP concentrations (ng/ml) were determined using the HBD-2 (Immundiagnostik AG, Bensheim, Germany; detection limit 0.1 ng/mL) and Eosinophil cationic protein ELISA kit (MyoBioSource, San Diego, CA; detection limit 0.5 ng/mL). For a few samples, when repeated measurements with the lowest dilution still fell below the detection limit, zero was reported as the result. To calculate HBD-2 and ECP concentrations in the stool of infants (per gram), the measured fecal HBD-2 and ECP units were adjusted and expressed in nanograms per gram of stool (ng/g), respectively. In addition, the serum total IgE was measured by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) at 12 and 24 months of postnatal age.

Definitions used in the study

Children born preterm who developed allergic diseases (allergic children)

According to the questionnaires and medical records, the study participants had been confirmed by any physician to have atopic dermatitis, allergic rhinitis, or one of these conditions combined with wheezing by 2 years of age postnatally.

Children born preterm without allergic diseases (non-allergic children)

According to the questionnaires and medical records, the study participants had not been confirmed by any physician to have allergic diseases as described above at 2 years of age postnatally.

Classification of medication exposure and non-exposure groups

Based on the questionnaires and medical records, study participants who had been exposed to any types of antibiotics, antipyretics, or probiotics between the time from birth to the 6-month (or 12-month) stool sample collection were classified into the "exposure group". Other participants were classified into the "non-exposure group."

Statistical analysis

Demographic data of the children were collected via questionnaires and analyzed. The associations between categorical variables were assessed using chi-square tests. Continuous and normally distributed variables are expressed as mean $\pm$ SD and were analyzed using one-way analysis of variance. Because the concentrations of HBD-2, and ECP were not normally distributed, Mann-Whitney U and Kruskal–Wallis tests were used to compare the concentrations (mean $\pm$ SEM) in 6- and 12-month fecal samples between the following: preterm infants with and without medication exposure (antibiotics, antipyretics, probiotics); with different exclusive breastfeeding duration; a parental history of allergy; lower and higher gestational ages; birth body weight; and with and without allergic diseases by 2 years of age. Histograms were prepared using mean values and SEM.

Univariate analysis was first performed to determine the relationship between HBD-2 levels (6-month and 12-month stools, analyzed independently), clinical variables such as gestational age, twin births, mode of delivery, length of exclusive breastfeeding duration, medication exposure history (antibiotics, antipyretics, and probiotics) by age 6 and 12 months, and physician-diagnosed allergic diseases by 2 years of age. Beta or odds ratios (ORs) with 95% confidence intervals (CIs) are reported. The variables associated with both HBD-2 levels (6-month and 12-month stools) and physician-diagnosed allergic diseases by 2 years of age with values of $P < 0.157$ were considered confounders.[22] Next, fecal HBD-2 levels and confounding factors were entered into the multivariable logistic regression with backward elimination to investigate the independent indicators related to physician-diagnosed allergic diseases by 2 years of age. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 28.0 for Mac (Chicago, IL, USA).

	Fecal HBD-2 at 6 month		Fecal HBD-2 at 12 month		Fecal ECP at 6 month		Fecal ECP at 12 month	
	n	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)
Stool samples	74	19.23 ± 4.25	60	21.85 ± 6.19	74	261.85 ± 20.64	60	257.44 ± 26.68
[a]**Antibiotics exposure history**								
Any	32	14.58 ± 4.30	36	31.76 ± 10.30	32	260.23 ± 33.62	36	227.64 ± 38.20
None	42	21.06 ± 5.43	24	8.71 ± 2.65	42	262.23 ± 27.43	24	329.92 ± 38.16
P-value		0.860		0.291		0.950		0.195
[a]**Antipyretics exposure history**								
Any	22	15.00 ± 5.84	40	30.05 ± 9.26	22	246.77 ± 41.14	40	274.48 ± 37.73
None	52	22.08 ± 4.03	20	7.35 ± 2.88	52	271.08 ± 23.87	20	255.65 ± 40.41
P-value		0.565		0.129		0.590		0.841
[a]**Probiotics exposure history**								
Any	39	19.23 ± 5.55	42	22.39 ± 6.94	39	274.77 ± 27.17	42	201.90 ± 33.53
None	35	19.84 ± 6.77	18	20.61 ± 13.02	35	248.50 ± 32.51	18	317.89 ± 40.33
P-value		0.894		0.597		0.367		0.055
Exclusive breastfeeding duration								
≥ 6 months	10	35.10 ± 20.56	9	24.00 ± 12.70	10	392.50 ± 55.12	9	349.33 ± 115.06
≥ 1–5 months	22	22.09 ± 8.17	17	20.29 ± 8.17	22	278.82 ± 34.06	17	173.53 ± 34.39
Never	35	11.14 ± 3.06	27	27.12 ± 12.28	35	216.97 ± 30.67	27	276.44 ± 35.20
P-value		0.314		0.626		0.007*		0.255
Gestational age (GA)								
GA 35–36 weeks	48	20.48 ± 5.84	42	24.88 ± 8.43	48	265.79 ± 24.74	42	267.66 ± 32.33
GA 31–34 weeks	26	17.60 ± 5.77	18	14.35 ± 5.16	26	256.60 ± 38.98	18	234.17 ± 48.12

(continued)

	Fecal HBD-2 at 6 month		Fecal HBD-2 at 12 month		Fecal ECP at 6 month		Fecal ECP at 12 month	
	n	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)
P-value		0.825		0.602		0.538		0.474
Birth weight								
≥ 2500 g	38	21.27 ± 6.75	31	26.03 ± 11.38	38	264.14 ± 27.89	31	241.11 ± 41.58
< 2500 g	36	15.71 ± 5.17	29	18.24 ± 5.50	36	243.84 ± 32.81	29	270.90 ± 38.59
P-value		0.667		0.369		0.482		0.441
Maternal and paternal history of allergy								
Both	17	12.64 ± 5.18	11	15.64 ± 7.80	17	295.41 ± 39.53	11	366.91 ± 79.52
Either one	28	24.64 ± 7.56	26	28.35 ± 12.65	28	215.71 ± 25.24	26	238.81 ± 40.20
None	29	17.76 ± 8.20	23	18.90 ± 7.86	29	287.56 ± 43.78	23	253.86 ± 45.72
P-value		0.646		0.382		0.357		0.317

Table 2. (Continued) Fecal human beta-defensin 2 (HBD-2), and eosinophil cationic protein (ECP) concentrations in terms of medications exposure histories by age 12 months and risk factors for allergy. *The differences between study groups were estimated using Mann-Whitney U and Kruskal–Wallis tests. *P-values < 0.05 were considered statistically significant. [a]Study participants who had been exposed to any types of antibiotics, antipyretics, or probiotics from the time of birth to 6-month (or 12-month) stool sample collection were classified into the "exposure group." Others were classified into the "non-exposure" group*

RESULTS

Clinical characteristics

In total, 74 preterm infants (median: 35 weeks, mean: 34.7 weeks) were included in the study, and 42 (median: 35.3 weeks, mean: 33.7 weeks) were excluded. The participants' demographic characteristics are shown in Table 1. A lower prevalence of twin births was found in children who were excluded from the study than in participants who were non-allergic (ie, absence of allergies by 2 years of age; 14.3% vs. 38.9%, $P = 0.019$). Overall, no significant difference was observed in sex, gestational ages, birth weight, environmental tobacco smoking, household pets, maternal characteristics, the prevalence of parental allergies, the exclusive breastfeeding duration among study groups, as well as the exposure to medications and infectious diseases during the first year (Table 1).

Among included participants, by the age of 2 years, 38 (51.3%) children had physician-diagnosed allergic diseases, and 36 children (48.7%) did not. In total, 74 preterm infants (100%) provided 6-month stool samples, and 60 (81.1%) provided 12-month stool samples. Additionally, the total serum IgE was measured in 59 infants (79.7%) at age 1, and 37 (50.0%) at age 2. There was a significant difference in total serum IgE levels between children who developed allergic diseases and those who did not at 1- and 2 years of age ($P = 0.021$ and 0.008, respectively).

HBD-2 and ECP concentrations in 6-month and 12-month stool samples in terms of medication exposure histories and risk factors for allergy

As shown in Table 2, neither HBD-2 (19.23 ± 4.25 ng/g vs. 21.85 ± 6.19 ng/g, $P = 0.620$) nor ECP concentrations (261.85 ± 20.64 ng/g vs. 257.44 ± 26.68 ng/g, $P = 0.655$) showed a significant difference between 6-month and 12-month stools. When the study groups were divided according to medication-exposure histories, although the differences were not significant, we observed that preterm infants with prior antibiotic

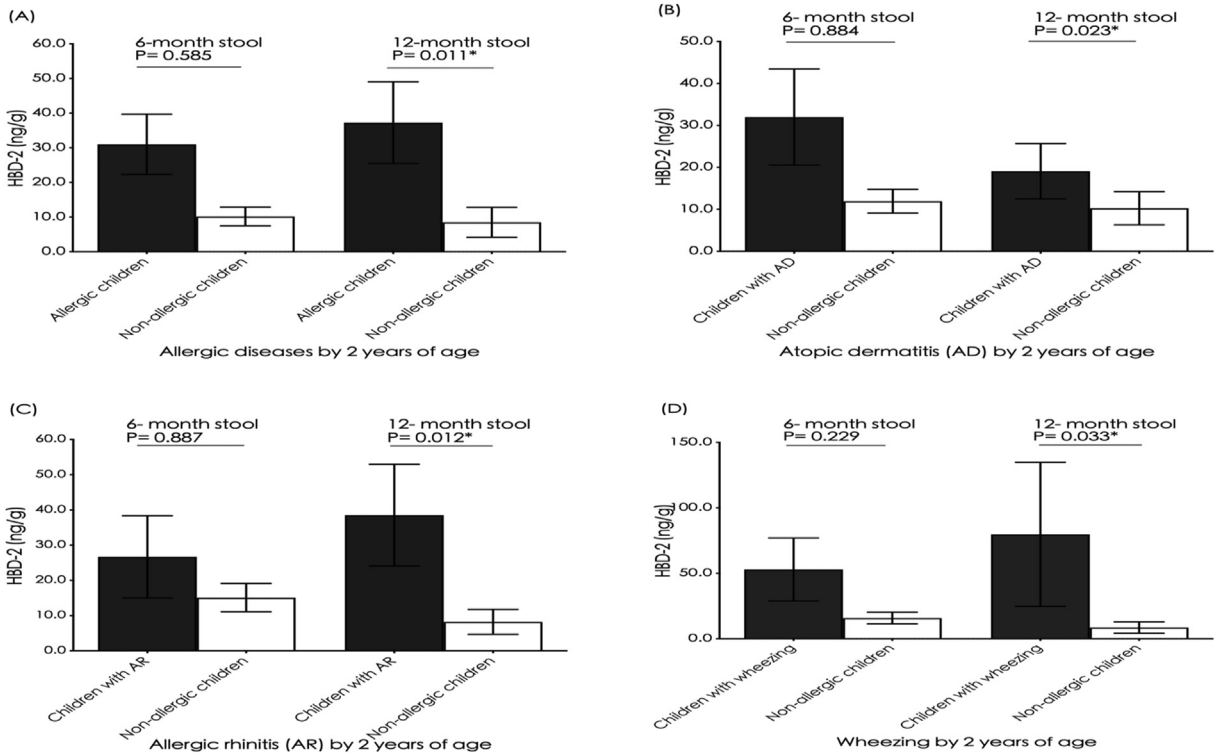

Fig. 1 Comparison of Human Beta-Defensin 2 (HBD-2) concentrations (ng/g) in the 6- and 12-month stool samples in terms of any physician-diagnosed (A) allergic diseases, (B) atopic dermatitis, (C) allergic rhinitis, and (D) wheezing by 2 years of age, respectively. Bar charts are shown as mean along with SEM. The differences between study groups were estimated using the Mann-Whitney *U* test. **P*-values < 0.05 were considered statistically significant

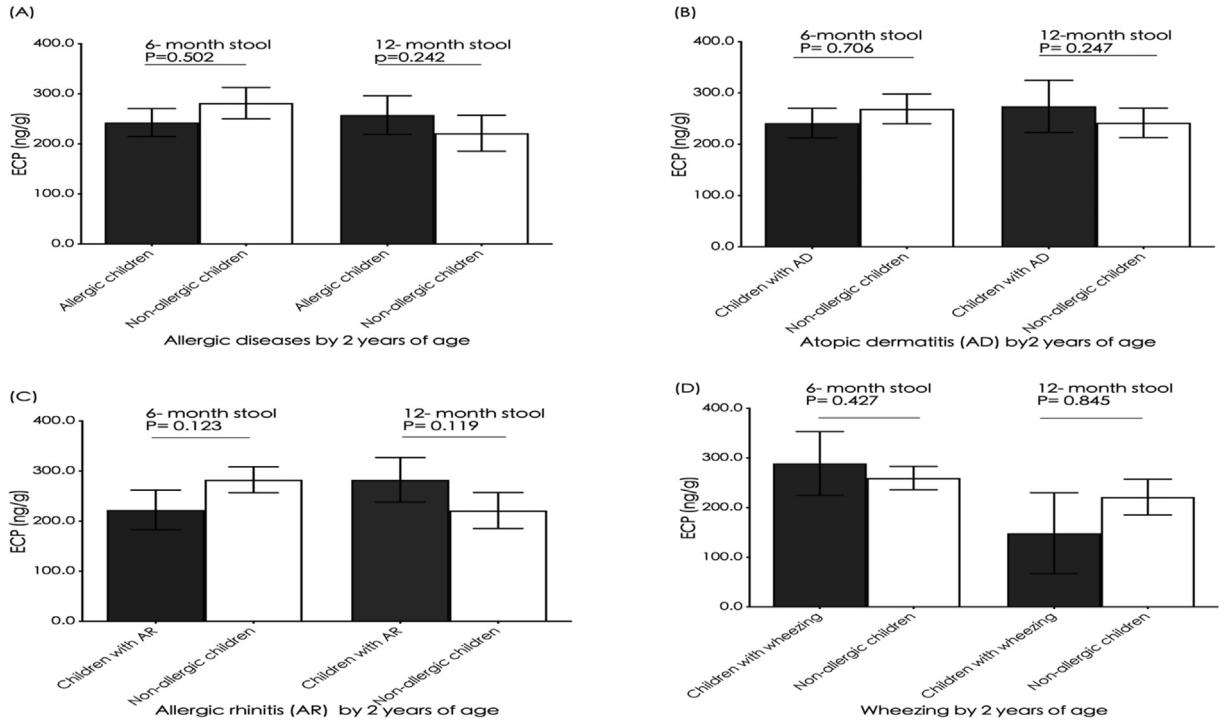

Fig. 2 Comparison of Human Eosinophil cationic protein (ECP) concentrations (ng/g) in the 6- and 12-month stool samples in terms of any physician-diagnosed (A) allergic diseases, (B) atopic dermatitis, (C) allergic rhinitis, and (D) wheezing by 2 years of age, respectively. Bar charts are shown as mean along with SEM. The differences between study groups were estimated using the Mann-Whitney *U* test. **P*-values < 0.05 were considered statistically significant

(31.76 ± 10.30 ng/g vs. 14.58 ± 4.30 ng/g, $P = 0.427$) or antipyretic exposure (30.05 ± 9.26 ng/g vs. 15.00 ± 5.84 ng/g, $P = 0.459$) displayed higher HBD-2 levels at 12 months than at 6 months of age. In addition, HBD-2 concentrations in 12-month stools were higher in the antibiotic (31.76 ± 10.30 ng/g vs. 8.71 ± 2.65 ng/g, $P = 0.291$) and antipyretic exposure groups (30.05 ± 9.26 ng/g vs. 7.35 ± 2.88 ng/g, $P = 0.129$) than in the non-exposure controls (Table 2).

At age 6 months, preterm infants who were exclusively breastfed had significantly higher ECP concentrations compared with those who were never exclusively breastfed (392.50 ± 55.12 ng/g vs. 216.97 ± 30.67 ng/g, $P = 0.006$) (Table 2). Otherwise, we observed that none of the HBD-2 and ECP concentrations in the 6-month or 12-month stool samples showed statistical differences, regardless of whether the preterm born children have higher or lower gestational ages, birth weight, parental allergies, or probiotics exposure history.

HBD-2 and ECP concentrations in 6-month and 12-month stool samples in terms of physician-confirmed allergic diseases by age 2 years

On investigating HBD-2 concentrations in 6-month and 12-month stool samples, we detected higher expression of HBD-2 in children who developed allergic diseases. The concentrations were significantly increased at 12-months-of-age compared with those that did not develop allergies by 2 years of age (37.18 ± 11.80 ng/g vs. 8.56 ± 4.33 ng/g, $P = 0.011$; Fig. 1A). When study subjects were divided according to their allergic phenotypes (Fig. 1B–D), a similar pattern was evident in children who developed atopic dermatitis, allergic rhinitis, or wheezing. They expressed significantly higher fecal HBD-2 levels at 12 months of age than non-allergic children ($P = 0.023$, 0.012, and 0.033, respectively; Fig. 1B–D). Furthermore, between children who developed allergy and those that did not, none of the ECP in the stools of 6- or 12-month old were significantly different ($P > 0.05$; Fig. 2A), regardless of their allergic phenotypes (Fig. 2B–D).

	Fecal HBD-2 at 6 month					Fecal HBD-2 at 12 month				
	Allergic children		Non-allergic children		*P*-value	Allergic children		Non-allergic children		*P*-value
	n	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)		*n*	Mean ± SEM (ng/g)	*n*	Mean ± SEM (ng/g)	
	38	31.03 ± 8.71	36	10.91 ± 2.80	0.585	31	37.18 ± 11.80	29	8.56 ± 4.33	0.011*
[a] **Antibiotics exposure history**										
Any	13	27.36 ± 10.66	19	7.55 ± 2.22	0.018[a]	19	50.23 ± 16.15	17	9.75 ± 7.16	0.008*
None	25	31.32 ± 12.87	17	11.11 ± 4.28	0.563	12	12.00 ± 5.68	12	6.92 ± 2.40	0.722
a **Antipyretics exposure history**										
Any	10	25.22 ± 13.42	12	8.50 ± 2.94	0.508	23	46.12 ± 14.22	17	10.82 ± 6.81	0.018*
None	28	34.65 ± 11.39	24	9.63 ± 3.18	0.301	8	12.29 ± 7.87	12	5.20 ± 1.72	0.669
a **Probiotics exposure history**										
Any	19	37.31 ± 12.06	20	5.41 ± 1.47	0.064	20	40.22 ± 14.38	22	8.87 ± 5.07	0.013*
None	19	28.33 ± 14.25	16	14.29 ± 4.89	0.899	11	33.00 ± 23.15	7	5.60 ± 2.75	0.679

Table 3. Fecal human beta-defensin 2 (HBD-2) concentrations in terms of medications exposure histories and physician-diagnosed allergic diseases by age 2 years. *The differences between study groups were estimated using Mann-Whitney U test. *P-values < 0.05 were considered statistically significant. [a]Study participants who had been exposed to any types of antibiotics, antipyretics, or probiotics from the time of birth to 6-month (or 12-month) stool sample collection were classified into the "exposure group." Others were classified into the "non-exposure" group*

	Fecal HBD-2 at 6 month (ng/g)		Fecal HBD-2 at 12 month (ng/g)	
	Beta (95% CI)	*P*-value	*Beta* (95% CI)	*P*-value
Physician-diagnosed allergic diseases by 2 years of age	20.12 (1.60–38.64)	0.034*	28.73 (3.19–54.27)	0.028*
Gestational age (week)	0.291 (−4.79–5.37)	0.909	4.72 (−4.37–13.81)	0.303
Twin births	−13.99 (−32.3–4.28)	0.131#	−18.91 (-44.82–7.01)	0.150#
Mode of delivery	3.55 (−14.64–21.73)	0.699	−10.78 (−37.37–15.80)	0.420
Parental history of allergy	0.60 (−15.11–16.30)	0.940	6.66 (−19.71–33.03)	0.615
Exclusive breastfeeding duration (month)	3.81 (−0.06–7.69)	0.054#	0.40 (−2.85–3.61)	0.803
Medication exposure history				
Antibiotics	−5.98 (−22.51–10.55)	0.473	23.41 (-1.10–47.92)	0.061#
Antipyretics	−6.02 (−24.61–12.57)	0.521	23.05 (-2.32–48.42)	0.074#
Probiotics	−5.10 (−17.80–16.79)	0.954	1.78 (−25.31–28.91)	0.896

Table 4. Univariate analysis of physician-diagnosed allergic diseases by the age of 2 years and clinical variables in relation to fecal human beta-defensin 2 (HBD-2) concentrations.
*Abbreviations: CI, confidence interval; HBD, human beta-defensin 2. *P-values < 0.05: statistically significant. #P-values < 0.157: a correlation to be considered*

HBD-2 concentrations in 6-month and 12-month stool samples in terms of medication exposure histories and physician-confirmed allergic diseases by 2 years of age

To evaluate whether taking medication modified the expression of fecal HBD-2, we compared 6- and 12-month fecal HBD levels between infants with and without medication exposure (antibiotics, antipyretics, probiotics) according to their allergic outcome by 2 years of age. As shown in Table 3, among study subjects previously exposed to antibiotics, children who developed allergic diseases had significantly higher HBD-2 concentrations at 6 months of age (27.36 ± 10.66 ng/g vs. 7.55 ± 2.22 ng/g, $P = 0.018$) and 12 months of age (50.23 ± 16.15 ng/g vs. 9.75 ± 7.16 ng/g, $P = 0.008$), compared to those in non-allergic children. Similarly, among antipyretics and probiotics exposure groups, the 12-month fecal HBD-2 concentrations were significantly higher in those children who developed allergic diseases than in those who did not (46.12 ± 14.22 ng/g vs. 10.82 ± 6.81 ng/g, $P = 0.018$ and 40.22 ± 14.38 ng/g vs. 8.87 ± 5.07 ng/g, $P = 0.013$, respectively). Nonetheless, among study groups of those previously not exposed to antibiotics, antipyretics, or probiotics, although the 6-month and 12-month HBD-2 concentrations are more likely to be higher in the allergic children, the differences were not significant ($P > 0.05$).

Identification of independent variables relate to physician-confirmed allergic diseases by 2 years of age

As shown in Tables 4 and 5, univariate analysis was first used to determine the relationships between HBD-2 levels (6-month and 12-month stools, separately), clinical variables by age 6 and 12 months, and physician-diagnosed allergic diseases by 2 years of age. Our data revealed that increased HBD-2 in the 6- and 12-month stool samples are positively associated with physician-diagnosed allergic diseases by the age of 2 years (OR [95% CI] = 1.02 [0.99–1.04] and 1.02 [0.99–1.05], respectively) (Table 5). Although not significant, a trend suggests that children who were twin births and exposed to antipyretics during the first year may be cofounders of both 12-month fecal HBD-2 concentrations and the risk of allergic disease ($P<0.157$).[22] Furthermore,

	Physician-diagnosed allergic diseases by age 2 years	
	OR (95% CI)	*P*-value
Gestational age (week)	0.99 (0.75–1.30)	0.933
Twin births (yes)	0.37 (0.13–1.06)	0.064[#]
Mode of delivery (cesarean section)	0.57 (0.21–1.56)	0.274
Parental history of allergy (yes)	1.62 (0.62–4.29)	0.328
Fecal HBD-2 (ng/g)		
By age 6 months	1.02 (0.99–1.04)	0.061[#]
By age 12 months	1.02 (0.99–1.05)	0.072[#]
Exclusive breastfeeding duration (month)		
By age 6 months	1.02 (0.83–1.25)	0.837
By age 12 months	1.00 (0.90–1.12)	0.937
Any antibiotics exposure		
By age 6 months	0.71 (0.28–1.81)	0.476
By age 12 months	1.23 (0.49–3.09)	0.665
Any antipyretics exposure		
By age 6 months	0.88 (0.44–1.78)	0.802
By age 12 months	2.58 (0.95–6.98)	0.062[#]
Any probiotics exposure		
By age 6 months	0.86 (0.52–1.43)	0.640
By age 12 months	0.34 (0.12–1.03)	0.056[#]

Table 5. Univariate analysis of fecal human beta-defensin 2 (HBD-2) concentrations and clinical variables in relation to physician-diagnosed allergic diseases by the age of 2 years. *Abbreviations: OR, odds ratio; CI, confidence interval; HBD, human beta-defensin 2. .* [#]*P-values < 0.157: a correlation to be considered*

we observed that antibiotic exposure during the first year after birth may increase HBD-2 levels in 12-month stools (*Beta* [95% CI] = 23.41 [−1.10 to 47.92]) (Table 4), and probiotic exposure during the first year after birth may reduce the risk of allergic diseases (OR = 0.34 [0.12–1.03]) (Table 5).

To identify the independent variable relate to physician-diagnosed allergic diseases by 2 years of age, the HBD-2 levels in 12-month stools, twin births, and antipyretic exposure during the first year were entered in the multivariable logistic regression model. In results, fecal HBD-2 concentrations at age 12 months was significantly associated with allergic diseases by 2 years of age (adjusted OR [95% CI] = 1.03 [1.00–1.05], $P = 0.036$).

DISCUSSION

To the best of our knowledge, this is the first study to explore the fecal HBD-2 and ECP changes in preterm infants with gestational age 31–36 weeks, and examined the relationship between these 2 fecal biomarkers and allergic disease development in early childhood. We observed that preterm infants who expressed high fecal HBD-2 concentrations at age 12 months were associated with physician-diagnosed allergic diseases by the age 2 years. This association was more apparent among allergic children who were exposed to antibiotics or antipyretics during the first year compared to those children without allergies. Moreover, results of the multivariate logistic regression analysis indicated that HBD-2 concentrations in the 12-month stools was an independent variable positively correlated with allergic diseases by 2 years of age. Based on these results, we suggest that increased fecal HBD-2 expression at age 12 months may be relevant to the development of allergic disease in early childhood.

Neonates possess a developing gut immune system.[3] After birth, gut microbiota help to maintain gut epithelial integrity, establish immune tolerance and modify the body's response to potential allergen.[23] Various factors affecting immune response or microbiota homeostasis, such as exposure to acetaminophen, antibiotics, or both, in early life,[24–26] may precede the development of allergic diseases.[27–29] To date, there is insufficient evidence regarding early-life probiotic administration as an effective approach in preventing allergic diseases.[30,31] In the present study, we found that 12-month fecal HBD-2 expression seemed to be affected by antibiotics and antipyretics, whereas we did not observe fecal HBD changes among infants with prior probiotic exposure (Table 4). On the other hand, although a trend suggests probiotics exposure during the first year may reduce the risk of allergic diseases in our participants (OR = 0.34 [0.12–1.03]) (Table 5), the prevalence of medication exposure history (antibiotic, antipyretic, and probiotic) showed no difference at the age of 12 months between children that developed allergic diseases and those who did not (Table 1). We proposed that the types, dosage, and frequency of antibiotics or antipyretics,[24,25] as well as the strain, timing, and length of administration of probiotics[30,31]—rather than exposure prevalence—may be more important factors influencing intestinal HBD-2 production and allergic disease development.

Accumulating evidence indicates that HBDs have antimicrobial and immunoregulatory properties, and may behave both like pro-as well as anti-inflammatory peptides.[9,13,32] In studies, whether higher HBD-2 expression has adverse or beneficial effects on allergic prevention remains controversial.[9,13] Some reports concur with our findings, which showed a positive association between HBD-2 concentrations and allergic manifestations,[33,34] including virus-induced asthma.[35,36] Similarly, in a randomized trial investigating the effect of synbiotics among infants with high genetic risk for allergies, Savilahti EM et al observed that high fecal HBD-2 concentrations at age 6 months were associated with an increased risk for sensitization by the age of 5 years.[12] However, several studies seem to contradict these results.[37–40] A possible explanation may be related to the differences in samples collected (eg, skin, serum, airway epithelium), differences in allergic phenotypes, the methods used for diagnosis, and the duration or severity of disease conditions. To date, no study has assessed the association between fecal HBD-2 and subsequent allergic disease development in prematurely born children. Our findings suggest there is a positive association between 12-month fecal HBD concentrations and allergic risk in preterm infants, whether infants born $\geq$ GA 37 weeks follow a similar trend requires further study.

ECP is an excellent marker of eosinophil activation in various allergic and gastrointestinal diseases.[41] However, there was no evidence showing that 6-month and 12-month fecal ECP concentrations correlated to medication exposure histories (eg, antibiotics, antipyretics, probiotics), as well as the development of physician-diagnosed allergic diseases by age 2 years. We only found a significantly increased fecal ECP concentration at age 6 months from preterm infants who were exclusively breastfed $\geq$6 months ($P = 0.007$), which may be relevant to the intestinal inflammatory response against various dietary antigens in the breast milk.[21] Consistently, our previous study showed that 6-month and 12-month fecal ECP concentrations were not associated with serum IgE levels at age 1 year, and it might not be a useful indicator of atopy in infants born $\geq$ gestational age 36 weeks.[21]

Several imitations of the study must be considered. A major limitation is that some misclassification regarding medication exposure and allergic histories in the children cannot be excluded; particularly, early childhood wheezing and asthma are variable expressions, and standard definitions for the type and severity of symptoms for preschool children are lacking.[42] This is the case despite the use of the following in an attempt to reduce misclassifications: regular follow-ups by our pediatricians, physician-confirmed asthma specifications, allergic rhinitis or atopic dermatitis included in the questionnaires, in conjunction with reviewing the medical records in our hospital (0–2 years). Next, despite the prospective design of the study, causality could not be established from the cross-sectional analysis of the fecal concentrations and the relationships with the outcome of allergic diseases at 2 years of age. Whether increased fecal

HBD-2 acts as pro-inflammatory or anti-inflammatory in the development of allergic diseases needs further study.[13] Another limitation is that all preterm infants were enrolled from a single hospital, 51.3% children had physician-diagnosed allergic diseases by age 2 years and may therefore not reflect all preterm populations. The study sample size was relatively small when we restricted our analyses to preterm infants with gestational age 31–36 weeks. Additionally, 30.5% of our preterm infants either lost to follow-up, did not complete questionnaires, or did not provide any stool sample for analysis, were excluded from the study, implying selection bias cannot be ruled out. Finally, we did not evaluate the types of antibiotics or antipyretics, strains of probiotics, frequency effects, or intervals of medication administration with respect to the timing of stool collection, nor did we evaluate the number or severity of infectious episodes, and their relationship with intestinal inflammatory markers. Each factor may have influenced the maturation process of the infant immune system and later atopy.[24–26,43,44] Therefore, our data should be interpreted with caution.

In conclusion, we found that preterm infants (GA 31–36 weeks) who had increased fecal HBD-2 expression at age 12 months were associated with physician-diagnosed allergic diseases by 2 years of age. Additional well-designed, prospective, and larger sample size studies of children born preterm are needed to determine the role of fecal HBD-2 in the emergence of allergic diseases later in childhood.

Abbreviations
HBD, human beta-defensins; ECP, eosinophil cationic protein; IgE, immunoglobulin E.

Ethics statements
This study was approved by the Research Ethics Committee of Chang Gung Memory Hospital (201901820A3, 201601904A3, 103-6519A3, 100-0225B) and complied with the declaration of Helsinki. Written informed parental consent was obtained.

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This manuscript has not been published and is not under consideration for publication elsewhere.

Consent for publication
All authors consented to the publication of this work.

Authors' contributions
All authors were involved in the study design, participates recruitment, and written consent. Hua MC, and Chen CC involved in the laboratory work, statistical analysis and interpretation of its results. Liao SL was responsible for the prematurity follow-up clinic visits. Hua MC wrote the first draft of the manuscript, and Huang JL edited it. All authors reviewed the manuscript and approved the final version of the manuscript.

Data availability statement
The datasets generated for this study are available from the corresponding author upon reasonable request.

Declaration of competing interest
There are no conflicts of interest to disclose.

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