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 post-natally. 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 ± 11.80 ng/g vs. 8.56 ± 4.33 ng/g, \( P = 0.011 \)). This association was more apparent among allergic children given antibiotics (50.23 ± 16.15 ng/g vs. 9.75 ± 7.16 ng/g, \( P = 0.008 \)) or antipyretics (46.12 ± 14.22 ng/g vs. 10.82 ± 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. In parallel, the rates of prematurity continue to rise; in 2014, the estimated global preterm birth rate was 10.6%. Neonates are born with an immature gastrointestinal tract and immune system. 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. Compared to healthy term infants, premature infants are more susceptible to infections and sepsis due to defective immune systems. Nonetheless, data addressing the intestinal inflammatory responses in preterm infants are few. 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. 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. Recently, a few pediatric studies have reported that alteration of fecal HBD-2 concentrations is associated with necrotizing enterocolitis, inflammatory bowel diseases, and the initiation and development of allergic diseases. Nevertheless, there are conflicting results about whether HBD-2 are predominantly pro- or anti-inflammatory in allergic diseases. On the other hand, fecal ECP is an intestinal inflammatory marker that can be used to monitor intestinal inflammation in infants with food allergies. Additionally, elevated ECP in serum and respiratory secretions are of value in predicting atopic eczema, wheezing, and even asthma in infants.

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. 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

#### Allergic phenotypes by age 2 years, n (%)

| Phenotype                        | Allergic children (N=38) | Non-allergic children (N=36) | Excluded preterm infants (N=42) |
|----------------------------------|--------------------------|-------------------------------|---------------------------------|
| 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

| Factor                                      | Allergic children (N=38) | Non-allergic children (N=36) | Excluded preterm infants (N=42) |
|---------------------------------------------|--------------------------|-------------------------------|---------------------------------|
| 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

| Factor                                      | Allergic children (N=38) | Non-allergic children (N=36) | Excluded preterm infants (N=42) |
|---------------------------------------------|--------------------------|-------------------------------|---------------------------------|
| 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)                       |
| 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)
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.

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.

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.

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. 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

| 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.
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 ± 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 ± 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. 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)    |
| Stool samples            | 74                     | 19.23 ± 4.25          | 60                   | 21.85 ± 6.19        |
|                          |                        |                       |                      | 74                   | 261.85 ± 20.64       |
|                          |                        |                       |                      | 60                   | 257.44 ± 26.68       |
| **Antibiotics exposure history** |                      |                       |                      |                      |
| Any                      | 32                     | 14.58 ± 4.30          | 36                   | 31.76 ± 10.30       |
|                          |                        |                       |                      | 32                   | 260.23 ± 33.62       |
| None                     | 42                     | 21.06 ± 5.43          | 24                   | 8.71 ± 2.65         |
|                          |                        |                       |                      | 42                   | 262.23 ± 27.43       |
| P-value                  | 0.860                  | 0.291                 | 0.950                | 0.195                |
| **Antipyretics exposure history** |                      |                       |                      |                      |
| Any                      | 22                     | 15.00 ± 5.84          | 40                   | 30.05 ± 9.26        |
|                          |                        |                       |                      | 22                   | 246.77 ± 41.14       |
| None                     | 52                     | 22.08 ± 4.03          | 20                   | 7.35 ± 2.88         |
|                          |                        |                       |                      | 52                   | 271.08 ± 23.87       |
| P-value                  | 0.565                  | 0.129                 | 0.590                | 0.841                |
| **Probiotics exposure history** |                      |                       |                      |                      |
| Any                      | 39                     | 19.23 ± 5.55          | 42                   | 22.39 ± 6.94        |
|                          |                        |                       |                      | 39                   | 274.77 ± 27.17       |
| None                     | 35                     | 19.84 ± 6.77          | 18                   | 20.61 ± 13.02       |
|                          |                        |                       |                      | 35                   | 248.50 ± 32.51       |
| 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       |
| Never                    | 35                     | 11.14 ± 3.06          | 27                   | 27.12 ± 12.28       |
|                          |                        |                       |                      | 35                   | 216.97 ± 30.67       |
| 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)
### 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.

|                | 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)     |
| 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                 |
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
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.

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)    | 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          |
| **Antibiotics exposure** history |                       |      |                      |                         |                          |                          |                          |                         |
| Any                  | 13                     | 27.36 ± 10.66 | 7.55 ± 2.22 | 0.018*                  | 19                       | 50.23 ± 16.15           | 17                       | 9.75 ± 7.16          | 0.008* |
| None                 | 25                     | 31.32 ± 12.87 | 11.11 ± 4.28 | 0.563                   | 12                       | 12.00 ± 5.68            | 12                       | 6.92 ± 2.40          | 0.722   |
| **Antipyretics exposure** history |                       |      |                      |                         |                          |                          |                          |                         |
| Any                  | 10                     | 25.22 ± 13.42 | 8.50 ± 2.94 | 0.508                   | 23                       | 46.12 ± 14.22           | 17                       | 10.82 ± 6.81         | 0.018* |
| None                 | 28                     | 34.65 ± 11.39 | 9.63 ± 3.18 | 0.301                   | 8                        | 12.29 ± 7.87            | 12                       | 5.20 ± 1.72          | 0.669   |
| **Probiotics exposure** history |                       |      |                      |                         |                          |                          |                          |                         |
| Any                  | 19                     | 37.31 ± 12.06 | 5.41 ± 1.47 | 0.064                   | 20                       | 40.22 ± 14.38           | 22                       | 8.87 ± 5.07          | 0.013* |
| None                 | 19                     | 28.33 ± 14.25 | 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. *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.
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 confounders of both 12-month fecal HBD-2 concentrations and the risk of allergic disease (P < 0.157). Furthermore,
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 relevant 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.

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**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.

| Variable                          | 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*  |
Neonates possess a developing gut immune system. After birth, gut microbiota help to maintain gut epithelial integrity, establish immune tolerance and modify the body’s response to potential allergen. Various factors affecting immune response or microbiota homeostasis, such as exposure to acetaminophen, antibiotics, or both, in early life may precede the development of allergic diseases. To date, there is insufficient evidence regarding early-life probiotic administration as an effective approach in preventing allergic diseases. 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. 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, as well as the strain, timing, and length of administration of probiotics—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. In studies, whether higher HBD-2 expression has adverse or beneficial effects on allergic prevention remains controversial. Some reports concur with our findings, which showed a positive association between HBD-2 concentrations and allergic manifestations, including virus-induced asthma. 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. However, several studies seem to contradict these results. 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 ≥ GA 37 weeks follow a similar trend requires further study.

ECP is an excellent marker of eosinophil activation in various allergic and gastrointestinal diseases. 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 ≥6 months (P = 0.007), which may be relevant to the intestinal inflammatory response against various dietary antigens in the breast milk. 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 ≥ gestational age 36 weeks.

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. 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. 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. 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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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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REFERENCES
1. Pawankar R. Allergic diseases and asthma: a global public health concern and a call to action. World Allergy Organ J. 2014;7:12.
2. Chawanpaiboon S, Vogel JP, Moller AB, et al. Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. Lancet Global Health. 2019;7:e37-e46.
3. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. *Immunity* 2012;37:771-783.

4. Munyaka PM, Khafipour E, Ghia JE. External influence of early childhood establishment of gut microbiota and subsequent health implications. *Front Pediatr*. 2014;2:109.

5. Lodge CJ, Dharmage SC. Breastfeeding and perinatal exposure, and the risk of asthma and allergies. *Curr Opin Allergy Clin Immunol*. 2016;16:231-236.

6. Power Coombs MR, Kronforst K, Levy O. Neonatal host defense against Staphylococcal infections. *Allergy Clin Immunol Int*. 2013;2013:826303.

7. Jenke AC, Zilbauer M, Postberg J, Wirth S. Human β-defensin 2 expression in ELBW infants with severe necrotizing enterocolitis. *Pediatr Res*. 2012;72:513-520.

8. Richter M, Topf HG, Gröschl M, et al. Influence of gestational age, cesarean section, and type of feeding on fecal human beta-defensin 2 and tumor necrosis factor-alpha. *J Pediatr Gastroenterol Nutr*. 2010;51:103-105.

9. Underwood MA, Bevins CL. Defensin-barbed innate immunity: clinical associations in the pediatric population. *Pediatrics*. 2010;125:1237-1247.

10. Yang D, Liu ZH, Tewary P, Chen Q, de la Rosa G, Oppenheim JJ. Defensin participation in innate and adaptive immunity. *Curr Pharmaceut Des*. 2007;13:3131-3139.

11. Kapel N, Benahmed N, Morali A, et al. Fecal beta-defensin-2 in children with inflammatory bowel diseases. *J Pediatr Gastroenterol Nutr*. 2009;48:117-120.

12. Savilahti EM, Kukkonen AK, Haahtela T, Tuure T, Kuitunen M, Savilahti E. Intestinal defensin secretion in infancy is associated with the emergence of sensitization and atopic dermatitis. *Clin Exp Allergy*. 2012;42:405-411.

13. Nyonsaba F, Kiatsurayanon C, Ogawa H. The role of human β-defensins in allergic diseases. *Clin Exp Allergy*. 2016;46:1522-1530.

14. Saarinen KM, Sarnesto A, Sivalahti E. Markers of inflammation in the feces of infants with cow’s milk allergy. *Pediatr Allergy Immunol*. 2002;13:188-194.

15. Majamäki H, Laine S, Miettinen A. Eosinophil protein X and beta-defensin-2 and tumor necrosis factor-alpha. *J Pediatr Gastroenterol Nutr*. 2010;51:103-105.

16. Hua MC, Yao TC, Chen CC, et al. Faecal eosinophil cationic protein and serum immunoglobulin E in relation to infant feeding practices. *Ann Clin Biochem*. 2017;54:246-252.

17. Hua MC, Chen CC, Liao SL, et al. Faecal eosinophil cationic protein and serum immunoglobulin E in relation to infant feeding practices. *Ann Clin Biochem*. 2017;54:246-252.

18. Hua MC, Su HM, Kuo ML, et al. Association of maternal allergy with human milk soluble CD14 and fatty acids, and early childhood atopic dermatitis. *Pediatr Allergy Immunol*. 2019;30:204-213.

19. Strachan D, Sibbald B, Weiland S, et al. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr Allergy Immunol*. 1997;8:161-176.

20. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. *Am J Respir Crit Care Med*. 2000;162:1403-1406.

21. Hua MC, Chen CC, Liao SL, et al. Faecal eosinophil cationic protein and serum immunoglobulin E in relation to infant feeding practices. *Ann Clin Biochem*. 2017;54:246-252.

22. Heinze G, Wallisch C, Dunkler D. Variable selection - a review and recommendations for the practicing statistician. *Biom J*. 2018;60:431-449.

23. Cukrowska B, Bierla JB, Zakrzewska M, Klukowski M, Maciorkowska E. The relationship between the infant gut microbiota and allergy. The role of bifidobacterium breve and prebiotic oligosaccharides in the activation of anti-allergic mechanisms in early life. *Nutrients*. 2020;12:946.

24. Baron R, Taye M, van der Vaart IB, et al. The relationship of prenatal antibiotic exposure and infant antibiotic administration with childhood allergies: a systematic review. *BMC Pediatr*. 2020;20:312.

25. Zeng Y, Song B, Gao Y, et al. Cumulative evidence for association of acetaminophen exposure and allergic rhinitis. *Int Arch Allergy Immunol*. 2020;181:422-433.

26. Wickens K, Beasley R, Town I, et al. The effects of early and late paracetamol exposure on asthma and atopy: a birth cohort. *Clin Exp Allergy*. 2011;41:399-406.

27. Bisgaard H, Li N, Bonnelykke K, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol*. 2011;128:646-652.

28. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy*. 2014;44:842-850.

29. Sjögren YM, Jenmalm MC, Böttcher MF, Björkstén B, Sverremark-Ekström E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy*. 2009;39:518-526.

30. D’Elia S, Trambusti I, Verduci E, et al. Probiotics in the prevention and treatment of atopic dermatitis. *Pediatr Allergy Immunol*. 2020;31:43-45.

31. Meirlaen L, Levy EL, Vandenplas Y. Prevention and management with pro-, pre and synbiotics in children with asthma and allergic rhinitis: a narrative review. *Nutrients*. 2021;13:934.

32. Shelley JR, Davidson DJ, Dorin JR. The dichotomous responses driven by β-defensins. *Front Immunol*. 2020;11:1176.

33. Kanda N, Watanabe S. Increased serum human β-defensin-2 levels in atopic dermatitis: relationship to IL-22 and oncostatin M. *Immunobiology*. 2012;217:433-445.

34. Asano S, Ichikawa Y, Kumagai T, Kawashima M, Imokawa G. Microanalysis of an antimicrobial peptide, beta-defensin-2, in the stratum corneum from patients with atopic dermatitis. *Br J Dermatol*. 2008;159:97-104.

35. Duits LA, Nibbering PH, van Strijen E, et al. Rhinovirus increases human beta-defensin-2 and -3 mRNA expression in
36. Kota S, Sabbah A, Chang TH, et al. Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-kappaB-mediated innate antiviral response against human respiratory syncytial virus. J Biol Chem. 2008;283, 22417-2.

37. Hata TR, Kotol P, Boguniewicz M, et al. History of eczema herpeticum is associated with the inability to induce human beta-defensin (HBD)-2, HBD-3 and cathelicidin in the skin of patients with atopic dermatitis. Br J Dermatol. 2010;163:659-661.

38. Ong PY, Ohtake T, Brandt C, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med. 2002;347:1151-1160.

39. Choi IJ, Rhee CS, Lee CH, Kim DY. Effect of allergic rhinitis on the expression of human beta-defensin 2 in tonsils. Ann Allergy Asthma Immunol. 2013;110:178-183.

40. Bogefors J, Kvarnhammar AM, Cardell LO. Up-regulated levels of human beta-defensins in patients with seasonal allergic rhinitis after allergen-specific immunotherapy treatment. Int Forum Allergy Rhinol. 2013;3:99-103.

41. Garcia-Rubio I, Martinez-Cocera C, Zayas L. Eosinophil cationic protein in feces: reference values in healthy and atopic individuals and patients with digestive diseases. Allergy Asthma Proc. 2007;28:468-471.

42. Cave AJ, Atkinson LL. Asthma in preschool children: a review of the diagnostic challenges. J Am Board Fam Med. 2014;27: 538-548.

43. van Meel ER, Jaddoe VWV, Bønnelykke K, de Jongste JC, Duijts L. The role of respiratory tract infections and the microbiome in the development of asthma: a narrative review. Pediatr Pulmonol. 2017;52:1363-1370.

44. Walker WA. The importance of appropriate initial bacterial colonization of the intestine in newborn, child, and adult health. Pediatr Res. 2017;82:387-395.