

PEDIATRIC

## Fatty Acids in Breast Milk Associated with Asthma-Like Symptoms and Atopy in Infancy: A Longitudinal Study

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**Objective.** The relationship between fatty acids (FAs) in breast milk and the risk of childhood allergies is controversial. We prospectively investigated the relationship between FAs in colostrum and breast milk and asthma-like symptoms (AS) and atopy in infancy. **Methods.** Pregnant women were recruited in Columbia and Charleston, South Carolina. Colostrum and mature milk samples were collected. The concentrations of *n*-3 FAs (eicosapentaenoic acid,  $\alpha$ -linolenic acid, docosapentaenoic acid, and docosahexaenoic acid) and *n*-6 FAs (linoleic acid, arachidonic acid, and eicosadienoic acid) were determined by gas chromatography. AS were ascertained at 6 and 12 months of age and atopy (skin prick test) at 12 months. FAs were dichotomized (high vs. median and low). Generalized estimating equations were used to determine the effect of FAs on repeated AS, compensating for intra-individual correlations and adjusting for confounders. Log-linear regression was used to analyze atopy. **Results.** FAs were analyzed in 24 colostrum and 78 breast milk samples. High levels of total *n*-6 (lipid based) FAs in breast milk were associated with an increased risk of AS in infants (risk ratio (RR) = 2.91; 95% confidence interval (CI): 1.37, 6.18), even after controlling for total *n*-3 FAs (RR = 2.07, 95% CI: 1.12, 3.85). High levels of total *n*-3 FAs controlling for *n*-6 FAs decreased the risk of atopy at the age of 12 months. **Conclusions.** High levels of total *n*-6 polyunsaturated fatty acids (PUFAs) in breast milk are associated with an increased risk for AS, whereas high levels of total *n*-3 PUFAs decreased the risk of atopy. These data suggest that the effects of *n*-3 and *n*-6 PUFAs on allergic disorders should be further explored.

**Keywords** human milk, omega-3, omega-6, skin prick testing

### INTRODUCTION

Allergic disorders such as eczema, asthma, and hay fever are among the most chronic and challenging problems in childhood. The 2009 National Health Interview Survey reported that more than 10 million children out of 74 million (14%) under 18 years of age carry a diagnosis of asthma in the United States (1). Despite considerable research, the etiology of childhood asthma and allergies presents an unsolved puzzle. There is a consensus of a protective effect of breastfeeding on lowering the risk or severity of respiratory infections (2, 3). However, for asthma, the question of whether breastfeeding is protective or a risk remains controversial. Some studies reported that exclusive breastfeeding is associated with a reduced risk (4–11), while others showed an increased risk of asthma (12, 13) or no association (14–16). Breast milk contains a variety of immunomodulators including cytokines, chemokines, growth factors, and immunoglobulins (Igs). In addition, human milk contains *n*-3 and *n*-6 polyunsaturated fatty

acids (PUFAs) that are hypothesized to influence the health status of the infant (17–19) and the overall development of the neonatal immune system (20, 21).

Specific breast milk-derived factors that influence the development of allergies and asthma in infants have not yet been clearly identified. The PUFAs in breast milk are known to have pleiotropic effects, including protection against inflammation, platelet aggregation, hypertension, and hyperlipidemia (21). Since the precursors of *n*-3 and *n*-6 fatty acids (FAs), namely  $\alpha$ -linolenic acid (ALA) and linoleic acid (LA), respectively, cannot be synthesized in mammals, diet is important. It has been suggested that either an imbalance in the dietary intake between *n*-6 and *n*-3 FAs is associated with an increased prevalence of atopic diseases, such as asthma in children and adults (22), or *n*-6 FAs are directly involved in allergic diseases, such as atopy and asthma (23). Particularly in infants, since maternal diet will influence the concentration of FAs in breast milk (24), the child's diet, and consequently health, can be affected. There are at least two complementary explanations for why these FAs may affect asthma-like symptoms (AS) in infants. First, PUFAs are incorporated in membrane lipids and modulate the production of lipid mediators and lipid signaling molecules involved in immune regulation and inflammation

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(25). Second, the *n*-3 and *n*-6 FAs are part of the surfactant in the lungs (26), which reduces surface tension and facilitates gas exchange. We sought to explore the relationship between the FAs balance in breast milk and subsequent AS at ages 6 and 12 months and atopy at age 12 months. We hypothesize that high levels of *n*-3 PUFAs in breast milk reduce the risk of AS and atopy in infants, whereas high levels of *n*-6 PUFAs increase the risk.

## METHODS

### Study Design

This work was derived from two ongoing prospective cohort studies, which were approved by the Institutional Review Boards at the University of South Carolina, the Medical University of South Carolina, and the Palmetto Health. All participants signed a written consent form either in English or in Spanish.

### Participants

Expectant mothers were enrolled between April 2008 and January 2010 in Columbia and Charleston, South Carolina, in obstetric clinics and prenatal classes (*n* = 231). Eligibility criteria included (1) aged 18 years or older, (2) no chronic illness (diabetes, thyroid or adrenal disorders, or chronic infections), (3) planned to stay in the area for at least 9 months, and (4) willingness to provide breast milk samples two weeks after giving birth.

### Clinical Data Collection and Outcomes

Women took part in four telephone or in-person interviews: a core demographic and baseline interview conducted before delivery, and three interviews at 2 weeks, and 6 and 12 months after delivery. At the baseline interview, information was obtained about the women's socio-demographic characteristics, race (African American, Caucasian, or other), maternal age, cigarette use prior to and since the beginning of pregnancy, whether cigarettes were smoked inside the home (categorized into yes vs. no), and education level (less than high school, some college, college graduate, or graduate school). In addition, maternal and paternal history of asthma, wheezing, and whistling in the chest was obtained by asking: "Have you ever had asthma?" and "Have you ever had wheezing or whistling in the chest at any time in the past?" A history of rhinitis was assessed by two questions: "Have you ever had hay fever?" and "Have you ever suffered—in the absence of a cold—from an itchy stuffy or runny nose and/or swollen, itchy eyes?" Eczema history was assessed by the question: "Have you ever had an itchy rash, which was coming and going for at least six months?"

The second interview was conducted 2–4 weeks after delivery to collect data on the specifics of delivery (spontaneous vaginal delivery, after induction vaginal delivery, and cesarean section), infectious exposures during pregnancy, maternal and household smoking during pregnancy period (yes/no), gender of the offspring, maternal use of antibiotics and acetaminophen during pregnancy, and

relationship of the delivery date to pollen seasons in South Carolina. These are fall (September, October, and November) and spring (March, April, and May). The non-pollen seasons are winter (December, January, and February) and summer (June, July, and August).

The third and fourth interviews were based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (27) and ascertained AS at ages 6 and 12 months. The recall period was restricted to either the previous 6 or 12 months. The study outcome of AS was based on a positive answer to any one of the following four questions:

- (1) Has your child had wheezing or whistling in the chest in the last 6 months?
- (2) How many attacks of wheezing has your child had in the last 6 months? (>1 vs. none)?
- (3) Has your child been unwell when breathing in or making noise when doing so in the last 6 months?
- (4) Has your child suffered from a shortness of breath in the last 6 months?

The interview also collected information on duration of breastfeeding (weeks) as well as information on smoking in the house in the last 6 months (yes/no). At 6 and 12 months, the following question was asked for ascertaining different respiratory infections had the child in the last 6 months: "In the last 6 months, was a doctor's diagnosis made in your child of (Yes/No): (1) pneumonia, (2) wheezy bronchitis, (3) infectious bronchitis, or (4) middle ear infection."

Skin prick tests (SPTs) were performed at 12 months with six allergens (cat dander, milk, egg, peanut, mix of *Dermatophagoides farina* and *Dermatophagoides pteronyssinus*, and *Alternaria*) following a standardized technique (28). A positive SPT, a marker of atopy, was defined as a wheal diameter at least 3 mm greater than the negative control. SPTs were performed using the Lincoln Multi-Test II device (Lincoln Diagnostics, Inc., Decatur, Illinois, USA).

### Colostrum and Breast Milk Collection and Preparation

Women were asked to provide a colostrum sample at 2 days after delivery (*n* = 33) and a breast milk sample about 2 weeks after delivery (*n* = 115). Of note, we did not intend to collect colostrum samples from every participant, since such a requirement would likely have reduced the acceptance of the study and thus lowered participation. All women followed detailed colostrum and breast milk collection protocol. Each participant expressed approximately 10 and 15 mL of colostrum and breast milk, respectively, in the morning after putting the baby to breast using an electric breast pump provided by the study. Sample collection was done, on average, 2 days (range: 1–3 days) and 3 weeks (range: 1–8 weeks) after delivery, respectively. All colostrum and breast milk samples were collected in sterile plastic bottles (Medela 80 mL or 2.7 oz). Research staff picked the breast milk sample up at the participants residence. Within 1 hour of collection, all samples were transferred to sterile centrifuge tubes and spun at 2900 rpm for 10 min at 4°C. Fat was removed; centrifugation and fat

removal steps were repeated until all fat was taken out. Finally, the cell pellet was removed. The isolated fat was stored in a  $-80^{\circ}\text{C}$  freezer until preparation for gas chromatography (GC).

#### *Extraction and Measurement of FAs*

Isolated breast milk fats were sent to the Obesity Nutrition Research Center at the University of North Carolina at Chapel Hill for FA analysis. Lipids were extracted using the method of Bligh and Dyer (29). The lower (chloroform) phase was transferred to a clean tube and evaporated to dryness under nitrogen. The residual lipids were saponified and the FAs trans-methylated by sequential 1 mL addition of 4.25% NaOH in  $\text{CHCl}_3\text{:MeOH}$  (2:1, v/v) and 1 N HCl in saline (30). The samples were mixed vigorously; then centrifuged at 1500g for 5 min. The lower phase containing the FA methyl esters was carefully transferred to a clean, dry tube and evaporated to dryness under nitrogen. FA methyl esters were then resuspended in 50 mL undecane and analyzed using capillary GC.

FA methyl esters were analyzed by Fast GC on a PerkinElmer Autosystem XL Gas Chromatograph (PerkinElmer, Inc., Shelton, CT, USA), split injection, with helium as the carrier gas. The methyl esters were separated on a capillary column coated with 70% cyanopropyl polysilphenylene-siloxane (10 m  $\times$  0.1 mm ID-BPX70 0.2 mm; SGE, Austin, TX, USA); injector  $240^{\circ}\text{C}$  and detector  $280^{\circ}\text{C}$ . Data were analyzed with the PerkinElmer Totalchrom Chromatography Software, version 6.2. Heptadecanoic acid (17:0) was added to the samples as an internal standard to correct for recovery and quantitation. Individual FAs were identified by comparing their retention with authentic standards (Nu Chek Prep, Elysian, MN, USA).

We focused on the following *n*-3 FAs: eicosapentaenoic acid (EPA; C20:5), ALA (C18:3), docosapentaenoic acid (DPA; C22:5), and docosahexaenoic acid (DHA; C22:6) and on the following *n*-6 FAs: LA (C18:2), arachidonic acid (AA; C20:4), and eicosadienoic acid (EA; C20:2).

#### *Exposures*

The main exposures were FAs in colostrum and breast milk. Total *n*-6 FAs (LA, AA, and EA), total *n*-3 FAs (EPA, ALA, DPA, and DHA), and their ratio were tested as predictors of AS and positive skin testing (atopy). FA concentrations are expressed as proportion of weight of fat per total weight of milk (wet based; nmol/mg) and as proportion of weight of fat per total fat (lipid based; nmol/mg). For the analysis of AS, to detect non-linear associations, we categorized all FAs into seven groups (septiles). Each group had about 10 observations. Due to their distribution, we contrasted the concentration in the highest septile to all lower levels. Since fewer children participated in SPT ( $n = 46$ ), we dichotomized the individual FAs and total *n*-6 and *n*-3 FAs by using the median as a cutoff for the analysis of atopy.

#### *Data Analyses*

We ran separate analyses for colostrum and mature milk FAs. To investigate the association of colostrum and breast

milk FAs, intra-class and Spearman's correlation coefficients were estimated (31). The statistical analyses were adjusted for the following confounders: maternal characteristics including race, age at pregnancy, smoking during pregnancy, household cigarette use at ages 6 and 12 months (only for AS outcome); history of asthma, rhinitis, and eczema; consumption of antibiotics during pregnancy; and vaginal or urinary infections during pregnancy. Offspring confounders comprised of gender, any respiratory infections at ages 6 and 12 months (only for AS outcome), and season of birth. Indicators of respiratory infections during infancy were a doctor's diagnosis of pneumonia, wheezy bronchitis, infectious bronchitis, and middle ear infection. For the analysis of atopy (positive SPT), we additionally controlled for paternal history of allergies and consumption of acetaminophen during pregnancy.

Log-linear regression was used to test whether predictors were associated with atopy at age 12 months. Generalized mixed models were employed to determine the association between predictors and repeated AS at ages 6 and 12 months. Adjusting for within-participant effects using the regular maximum likelihood method, we started with an unstructured covariance matrix, which requires the least amount of constraints. Other covariance matrices, including compound symmetry and autoregressive, were considered, but they did not significantly improve the fit based on the evaluation of Akaike information criteria and Bayesian Schwarz information criterion (32).

We determined that total *n*-6 FAs, total *n*-3 FAs, and the individual FAs had non-linear relations with AS and positive skin testing. We estimated and presented risk ratios (RRs) and with 95% confidence intervals (CI).

All confounders were simultaneously entered as indicator variables into the generalized mixed and log-linear models. A backward elimination process was used to retain confounders in the final model: covariates which changed the effect of the main association by 10% or more when omitting it from the model were considered as confounders. In our final models, gestational age, maternal education, pet exposure, and mode of delivery were removed.

In an additional analysis, we determined the Spearman's correlation between FAs and immune markers in breast milk whey. If significant correlations were found between FAs and immune markers in whey, the partial correlation between FAs and AS at ages 6 and 12 months (by immune markers in whey) was reported. Path analysis was conducted to corroborate whether certain immune markers in whey were in the causal pathway between FAs in breast milk and AS at ages 6 and 12 months. The same analysis was applied for FAs and positive SPT at age 12 months. All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

## RESULTS

A total of 115 women provided breast milk samples and colostrum from a subsample of 33 women. FAs were measured in 78 (68%) breast milk and 24 (73%) colostrum

samples. Most of the participants were Caucasian (68%) and well educated (39%). A history of maternal eczema, asthma, and rhinitis were reported by 7%, 31%, and 53% of the women, respectively (Table 1). About 5% of the

women reported smoking during pregnancy and 45% had vaginal or urinary infections during pregnancy.

The presence of AS was assessed in 71 infants at 6 months and 51 children at 12 months. The proportion of AS was 15.5% and 19.6% at ages 6 and 12 months, respectively (Table 2). About 23% (6 months) and 55% (12 months) of children had respiratory infections. Approximately 6% of infants at age 6 months and 17% at age 12 months presented both AS and any type of respiratory infections (data not shown). Regarding positive skin testing in children, all parents were invited to participate in an SPT; 40% agreed, resulting in the participation of 46 children. The proportion of positive SPT was 34.7% (16 out of 46). Among those with positive skin testing, 10 mothers and fathers (22.2% each) reported a history of allergy (data not shown). About 83% (38 out of 46) had measurements of FAs. Sixteen percent (6 out of 38) showed a positive SPT for aero allergens and 13% (5 out of 38) for food allergens (Table 3).

We analyzed 78 breast milk samples, and in all samples, we measured LA, AA, docosapentanoic acid (DPA), and DHA; however, only one sample did not have ALA since the values for ALA were low. Regarding EPA, 18 samples had low levels of EPA; 77% of the samples had quantifiable concentrations of EPA. The concentrations of the FAs in colostrum and in breast milk were similar or higher than the previously published data (33). The median lipid-based FA concentrations of the FAs in colostrum and breast milk were similar (Table 4 and 5). Due to its higher fat content, wet-based FA concentrations in colostrum were higher than that in breast milk. Most of the FAs in colostrum and breast milk (lipid based) were associated (correlation range = 0.59–0.86). None of the FAs were correlated with the time of milk collection (data not shown).

#### FA Profiles and Risk of AS at Ages 6 and 12 Months

We tested in the same statistical model whether total *n*-3 and *n*-6 FAs predicted AS in children. There was no linear association between FAs levels in breast milk and

TABLE 1.—Characteristics of the participants (*n* = 78).

| Variables                                                        | Subsample with FAs in breast milk or colostrum<br><i>n</i> (%) |
|------------------------------------------------------------------|----------------------------------------------------------------|
| <i>Maternal race</i>                                             |                                                                |
| African American                                                 | 12 (15.6)                                                      |
| Caucasian or Other <sup>a</sup>                                  | 65 (84.4)                                                      |
| <i>Maternal education</i>                                        |                                                                |
| Less than high school                                            | 5 (6.5)                                                        |
| Some college                                                     | 16 (20.8)                                                      |
| College graduate                                                 | 26 (33.8)                                                      |
| Graduate school                                                  | 30 (38.9)                                                      |
| <i>Mode of delivery</i>                                          |                                                                |
| Spontaneous vaginal delivery                                     | 31 (40.3)                                                      |
| After induction vaginal delivery                                 | 25 (32.5)                                                      |
| Cesarean section                                                 | 21 (27.3)                                                      |
| <i>Maternal smoking during pregnancy</i>                         | 4 (5.2)                                                        |
| <i>Maternal history of allergy</i>                               |                                                                |
| Asthma                                                           | 24 (31.2)                                                      |
| Rhinitis                                                         | 41 (53.3)                                                      |
| Eczema                                                           | 5 (6.5)                                                        |
| <i>Smoked at child age 6 months</i>                              | 3 (5.9)                                                        |
| <i>Smoked at child age 12 months</i>                             | 5 (7.0)                                                        |
| <i>Vaginal or urinary infection during pregnancy<sup>b</sup></i> | 34 (44.2)                                                      |
| Fungal infection                                                 | 16 (20.7)                                                      |
| Group B Streptococcus infection                                  | 19 (24.7)                                                      |
| Genital herpes                                                   | 3 (3.9)                                                        |
| <i>Use of antibiotics during pregnancy</i>                       | 24 (30.8)                                                      |
| <i>Season of child's birth</i>                                   |                                                                |
| Fall                                                             | 16 (20.7)                                                      |
| Spring                                                           | 16 (20.7)                                                      |
| Summer                                                           | 14 (18.2)                                                      |
| Winter                                                           | 31 (40.3)                                                      |
|                                                                  | Mean ( <i>n</i> ; 95% CI 5%, 95%)                              |
| <i>Maternal age (years)</i>                                      | 31.5 (76; 22.6, 40.5)                                          |
| <i>Maternal gestational age (weeks)</i>                          | 39.0 (77; 35.0, 41.0)                                          |

<sup>a</sup>Other race: *n* = 13

<sup>b</sup>Including urinary tract infection, vaginitis or vaginosis, genital herpes, yeast infection, and group B *Streptococcus* infections.

TABLE 2.—Prevalence of AS and respiratory infections at ages 6 and/or 12 months (*N* = 78).

| AS (% (n/N))                                 | 6 months     | 12 months    | 6 or 12 months | 6 and 12 months |
|----------------------------------------------|--------------|--------------|----------------|-----------------|
| Wheezing                                     | 9.9 (7/71)   | 9.8 (5/51)   | 15.3 (11/72)   | 1.4 (1/72)      |
| One or more wheezing attacks                 | 5.8 (4/69)   | 2.0 (1/50)   | 7.1 (5/70)     | 0 (0/70)        |
| Felt unwell and made noise when breathing    | 9.9 (7/71)   | 11.8 (6/51)  | 16.7 (12/72)   | 1.4 (1/71)      |
| Shortness of breath                          | 1.4 (1/71)   | 5.9 (3/51)   | 5.6 (4/72)     | 0 (0/72)        |
| <i>Combined AS</i>                           |              |              |                |                 |
| Any AS                                       | 15.5 (11/71) | 19.6 (10/51) | 26.4 (19/72)   | 2.8 (2/72)      |
| <i>Child respiratory infections</i>          |              |              |                |                 |
| Pneumonia                                    | 0 (0/71)     | 5.9 (3/51)   | 4.2 (3/78)     | 0 (0/78)        |
| Wheezy bronchitis                            | 4.2 (3/71)   | 9.8 (5/51)   | 11.1 (8/78)    | 0 (0/78)        |
| Infectious bronchitis                        | 0 (0/71)     | 3.9 (2/51)   | 2.8 (2/78)     | 0 (0/78)        |
| Middle ear infection                         | 22.5 (16/71) | 52.9 (27/51) | 44.4 (32/78)   | 15.3 (11/78)    |
| <i>Combined child respiratory infections</i> |              |              |                |                 |
| Any respiratory infections                   | 22.5 (16/71) | 54.9 (28/51) | 45.8 (33/78)   | 15.3 (11/78)    |

TABLE 3.—Prevalence of positive SPTs at age 12 months ( $N = 38$ ).

| SPT <sup>a</sup>                                               | % (n)     |
|----------------------------------------------------------------|-----------|
| <i>Food-allergens</i>                                          |           |
| Milk                                                           | 2.6 (1)   |
| Egg                                                            | 10.4 (4)  |
| Peanut                                                         | 5.3 (2)   |
| <i>Aero-allergens</i>                                          |           |
| Cat dander                                                     | 15.8 (6)  |
| Mix of <i>Dermatophagoides farina</i> and <i>Pteronyssinus</i> | 0 (0)     |
| <i>Alternaria</i>                                              | 0 (0)     |
| Positive SPT                                                   | 31.5 (12) |

<sup>a</sup>Wheal diameter at least 3 mm greater than the negative control.

AS. However, controlling for  $n$ -3 concentrations, the highest septile of total  $n$ -6 FAs in breast milk posed an increased risk for AS, both lipid and wet based (Table 6). The RR for the occurrence of AS in infancy was 3.11 (95% CI: 1.35, 7.19) for lipid-based FA concentrations and 2.07 (95% CI: 1.12, 3.85) for wet-based FA concentrations. For AS, no statistically significant effect was found for higher levels of  $n$ -3 FAs (Table 6). The adjusted analyses of the repeated measurements of AS indicated that none of the individual FAs (except for the high levels of AA) in colostrum and breast milk influenced AS (data not shown). After adjusting for total  $n$ -3 FAs, high levels of AA (lipid based) increased the risk of AS (RR = 2.48,  $p = .03$ ). No association was found between the ratio of total  $n$ -6 to  $n$ -3 FAs and AS (data not shown).

Regarding the associations between FAs and immune markers in breast milk, we found that  $n$ -6 FAs LA (wet and lipid based), EA (wet based), and total  $n$ -6 FAs (wet and lipid based) were moderately correlated with interleukin-5 (IL-5) in whey (Table 7). In addition, the wet-based  $n$ -3 FAs, DPA and DHA, were associated with IL-5 in whey. Given that the IL-5 marker was associated with LA, EA, DPA, and DHA, the partial correlations of these FAs and AS at ages 6 and 12 months were determined. None of the partial correlations were significant. To corroborate these results, we performed a path analysis. IL-5 cytokine did not act as an intervening variable between the FAs in human milk and AS at ages 6 and 12 months (data not shown).

TABLE 5.—Median and range of high and low levels of total  $n$ -3 and total  $n$ -6 FAs.

| Fatty acids <sup>a</sup><br>(nmol/mg) | FAs in breast milk<br>(lipid based)<br>Median (range) | FAs in breast milk<br>(wet based)<br>Median (range) |
|---------------------------------------|-------------------------------------------------------|-----------------------------------------------------|
| Total $n$ -6                          |                                                       |                                                     |
| High levels                           | 9377.83 (8320.37, 19500.48)                           | 213.86 (169.88, 1500.30)                            |
| Low levels                            | 5334.06 (736.62, 8252.39)                             | 77.75 (4.74, 164.78)                                |
| Total $n$ -3                          |                                                       |                                                     |
| High levels                           | 425.78 (351.81, 688.64)                               | 9.32 (8.05, 26.40)                                  |
| Low levels                            | 203.48 (56.62, 338.80)                                | 3.14 (0.31, 7.94)                                   |

<sup>a</sup>Fatty acids were categorized into septiles and the lower levels (1 to 6) were used as reference (highest septile vs. all lower levels).

### FA Profiles and Risk of Positive Skin Testing at Age 12 Months

There was no evidence that colostrum FA profiles were associated with positive skin testing at age 12 months (data not shown). In mature milk, total  $n$ -3 FAs were protective (RR = 0.15, 95% CI: 0.02, 1.05) for the lipid-based FA concentration and for wet-based FA concentrations (RR = 0.14, 95% CI: 0.05, 0.39) against the development of positive skin testing even after controlling the total  $n$ -6 FAs (Table 8). Regarding individual FAs in mature milk, all the three  $n$ -3 FAs (EPA, lipid based, RR = 0.07,  $p < .0001$ ; DHA, lipid based, RR = 0.09,  $p = .0006$  and wet based, RR = 0.02,  $p < .0001$ ; and DPA, lipid based, RR = 0.25,  $p = .01$ ) had a protective effect against the development of positive skin testing after controlling for confounders and total  $n$ -6 FAs (data not shown). Additionally, we tested whether FAs were associated with specific aero- and food-allergens separately; however, no association was found (data not shown). In an aside analysis, we tested whether the ratio of total  $n$ -6 to total  $n$ -3 and individual ratios of  $n$ -6 to  $n$ -3 were associated with atopy. We did not identify an association between atopy and the two ratios.

TABLE 4.—Concentrations and correlations between FAs in colostrum and breast milk.

| FAs                               | Concentration of FAs (lipid based) (median) |             |      |            | Spearman correlation | $P$ -value | Concentration of FAs (wet based) (median) |             |        |            | Spearman correlation | $P$ -value |
|-----------------------------------|---------------------------------------------|-------------|------|------------|----------------------|------------|-------------------------------------------|-------------|--------|------------|----------------------|------------|
|                                   | Colostrum                                   | Breast milk | ICC  | $P$ -value |                      |            | Colostrum                                 | Breast milk | ICC    | $P$ -value |                      |            |
| <i><math>n</math>-6 (nmol/mg)</i> |                                             |             |      |            |                      |            |                                           |             |        |            |                      |            |
| LA                                | 4442.0                                      | 5615.1      | 0.73 | <.0001     | 0.60                 | .001       | 120.70                                    | 87.87       | −0.001 | .99        | −0.12                | .55        |
| AA                                | 143.33                                      | 169.68      | 0.68 | .0003      | 0.71                 | <.0001     | 3.41                                      | 2.65        | 0.16   | .49        | 0.05                 | .79        |
| EA                                | 109.13                                      | 127.88      | 0.64 | .002       | 0.59                 | .002       | 2.47                                      | 1.95        | 0.23   | .39        | 0.05                 | .81        |
| <i><math>n</math>-3 (nmol/mg)</i> |                                             |             |      |            |                      |            |                                           |             |        |            |                      |            |
| EPA acid                          | 34.23                                       | 45.51       | 0.80 | <.0001     | 0.80                 | <.0001     | 0.62                                      | 0.66        | 0.34   | .20        | 0.32                 | .18        |
| ALA                               | 73.11                                       | 101.45      | 0.82 | <.0001     | 0.85                 | <.0001     | 1.78                                      | 1.41        | 0.30   | .14        | 0.15                 | .46        |
| Docosapentaenoic acid             | 23.84                                       | 33.81       | 0.79 | <.0001     | 0.76                 | <.0001     | 0.57                                      | 0.46        | 0.09   | .68        | 0.06                 | .77        |
| DHA acid                          | 57.82                                       | 79.51       | 0.87 | <.0001     | 0.86                 | <.0001     | 1.41                                      | 1.11        | 0.49   | .99        | 0.06                 | .76        |

TABLE 6.—Adjusted effects of FAs in breast milk on AS at ages 6 or 12 months: a repeated measurement analysis.

| FAs <sup>a</sup> (nmol/mg)             | FAs in breast milk (lipid based) |         | FAs in breast milk (wet based) |         |
|----------------------------------------|----------------------------------|---------|--------------------------------|---------|
|                                        | RR <sup>b</sup> (95% CI)         | P-value | RR <sup>b</sup> (95% CI)       | P-value |
| Total <i>n</i> -6                      |                                  |         |                                |         |
| High levels                            | 2.91 (1.37, 6.18)                | .005    | 1.89 (0.92, 3.86)              | .08     |
| Low levels                             | Reference                        |         | Reference                      |         |
| Total <i>n</i> -3                      |                                  |         |                                |         |
| High levels                            | 0.94 (0.30, 2.94)                | .92     | 1.17 (0.34, 4.03)              | .80     |
| Low levels                             | Reference                        |         | Reference                      |         |
| Model with <i>n</i> -6 and <i>n</i> -3 |                                  |         |                                |         |
| Total <i>n</i> -6 (high)               | 3.11 (1.35, 7.19)                | .007    | 2.07 (1.12, 3.85)              | .01     |
| Total <i>n</i> -3 (high)               | 1.35 (0.41, 4.41)                | .61     | 0.78 (0.26, 2.30)              | .65     |

<sup>a</sup>Gestational age, maternal education, pet exposure, and mode of delivery were removed from the models because they were not confounding.

<sup>b</sup>FAs were categorized into septiles and the lower levels (1–6) were used as reference (highest septile vs. all lower levels). All FAs were adjusted for child's sex, maternal age during pregnancy, maternal race, smoking during pregnancy, vaginal or urinary infection during pregnancy, maternal history of asthma, consumption of antibiotics during pregnancy, season of child's birth, any respiratory infection during infancy, and household cigarette use at ages 6 and 12 months.

TABLE 7.—Correlations between wet- and lipid-based FAs in breast milk and IL-5 and AS at ages 6 and 12 months and partial correlation (IL-5) of FAs in breast milk whey and AS.

| FAs (nmol/mg)                     | Spearman correlation with IL-5 |                                 | Spearman correlation             |  | Partial correlation with AS correcting for IL-5 |             |
|-----------------------------------|--------------------------------|---------------------------------|----------------------------------|--|-------------------------------------------------|-------------|
|                                   | <i>r</i> (P-value)             | AS at 6 m<br><i>r</i> (P-value) | AS at 12 m<br><i>r</i> (P-value) |  | AS <i>r</i> (p-value)                           |             |
|                                   |                                |                                 |                                  |  | 6 m                                             | 12 m        |
| <i>n</i> -6                       |                                |                                 |                                  |  |                                                 |             |
| LA (wet based)                    | 0.30 (.007)                    | −0.06 (.59)                     | 0.12 (.37)                       |  | −0.16 (.20)                                     | 0.12 (.38)  |
| LA (lipid based)                  | 0.27 (.01)                     | −0.06 (.59)                     | 0.12 (.37)                       |  | −0.16 (.20)                                     | 0.12 (.38)  |
| EA (wet based)                    | 0.27 (.01)                     | −0.04 (.68)                     | 0.10 (.45)                       |  | −0.13 (.26)                                     | 0.10 (.47)  |
| <i>n</i> -3                       |                                |                                 |                                  |  |                                                 |             |
| Docosapentaenoic acid (wet based) | 0.25 (.02)                     | 0.02 (.83)                      | −0.06 (.64)                      |  | −0.05 (.66)                                     | −0.07 (.58) |
| DHA acid (wet based)              | 0.28 (.01)                     | 0.05 (.65)                      | < 0.01 (.99)                     |  | −0.02 (.85)                                     | −0.01 (.94) |
| Total <i>n</i> -6 (wet based)     | 0.30 (.009)                    | −0.06 (.59)                     | 0.12 (.37)                       |  | −0.16 (.20)                                     | 0.12 (.38)  |
| Total <i>n</i> -6 (lipid based)   | 0.27 (.01)                     | 0.001 (.98)                     | 0.18 (.20)                       |  | −0.08 (.48)                                     | 0.18 (.21)  |

TABLE 8.—Adjusted RRs of FAs in breast milk on positive skin testing at age 12 months.

| FAs <sup>a</sup> (nmol/mg)             | FAs in breast milk (lipid based) |         | FAs in breast milk (wet based) |         |
|----------------------------------------|----------------------------------|---------|--------------------------------|---------|
|                                        | RR <sup>b</sup> (95% CI)         | P-value | RR <sup>b</sup> (95% CI)       | P-value |
| Total <i>n</i> -6                      |                                  |         |                                |         |
| High levels                            | 0.76 (0.26, 2.20)                | .72     | 0.30 (0.10, 0.92)              | .03     |
| Low levels                             | Reference                        |         | Reference                      |         |
| Total <i>n</i> -3                      |                                  |         |                                |         |
| High levels                            | 0.15 (0.02, 1.05)                | .05     | 0.14 (0.05, 0.39)              | .0002   |
| Low levels                             | Reference                        |         | Reference                      |         |
| Model with <i>n</i> -6 and <i>n</i> -3 |                                  |         |                                |         |
| Total <i>n</i> -6 (high)               | 1.25 (0.60, 2.61)                | .53     | 0.64 (0.19, 2.16)              | .47     |
| Total <i>n</i> -3 (high)               | 0.14 (0.02, 1.06)                | .05     | 0.18 (0.05, 0.64)              | .008    |

<sup>a</sup>Gestational age, maternal education, pet exposure, and mode of delivery were removed from the models because they were not confounding.

<sup>b</sup>FAs were dichotomized (by using the median as a cutoff) by using the low values as reference. All FAs were adjusted for child's sex, maternal age during pregnancy, maternal race, smoking during pregnancy, vaginal or urinary infection during pregnancy, maternal history of asthma, eczema, rhinitis, paternal history of allergies, consumption of antibiotics and acetaminophen during pregnancy, and season of child's birth.

## DISCUSSION

Results show that high levels of total *n*-6 FAs in breast milk are associated with a significantly increased risk for AS (RR = 2.91, 95% CI: 1.37, 6.18). Even after controlling for total *n*-3 FAs, high levels of total *n*-6 FAs in

breast milk remained associated with AS. Finally, infants exposed to high levels of total *n*-3 FAs were at lower risk of atopy by skin testing at 12 months even after adjusting for total *n*-6 FAs in the explanatory model. Taken together, these findings suggest that breast milk FAs

composition plays a role in the subsequent development of AS and atopy.

The strengths of this study include its prospective design, the independent collection and analysis of clinical data and breast milk FAs. With regard to selection bias, participation in studies during pregnancy and infancy depends on volunteering and a high level of dedication to study requirements. We received breast milk from 115 participants and colostrum samples from 33 participants. Due to limited funding, we could analyze only 78 breast milk samples and 24 colostrum samples. Regarding maternal race, education, history of asthma, and smoking, there were no differences in maternal characteristics when comparing the samples with and without measurements of FAs (data not shown). In addition, we did not detect any association between the early presence of AS symptoms in offspring and the probability of providing breast milk/colostrum samples. Moreover, we used statistical techniques to control for potential confounders. Of note, we did not control for breastfeeding duration since the FAs are intervening variables between breastfeeding and AS (i.e., only those infants who are breastfed will receive FAs in breast milk). For the model, it is not appropriate to split a chain of responses (breastfeeding and FAs) up into their elements and assess them individually since the elements of one chain are dependent on one another. We did note large differences between concentrations of wet- and lipid-based FAs in colostrum and in breast milk, which has given the high level of variations in fat content in breast milk (34). However, similar results, independent of wet- or lipid-based FA concentrations, were found showing the associations between FAs, AS, and positive skin testing, which strengthens our confidence in the conclusions. The concentrations of the FAs in colostrum and in breast milk were similar or higher than those of a previously published study (33).

A limitation of the analysis of AS in infancy is that there is no consensus in the literature as to what these symptoms in infancy represent (35, 36). Respiratory viruses are one of the factors that may be related to AS early in life (36), but not allergies. Nonetheless, early symptoms of asthma may develop into allergies (36). Therefore, AS in infancy may be a sign of a non-specific response to an external allergen. There is a need to better understand AS in infancy. It is not satisfactory to claim that there is no asthma in infancy, when infants have symptoms such as wheezing and shortness of breath. We believe that additional information should be gathered in future studies on what makes infants wheeze and have shortness of breath. We speculate that the level of surfactant in the lungs may be affected by FAs. For instance, a recent study showed that FAs in exhaled breath condensates (EBCs) are constituents of the surfactant in the lungs of children (26). Human pulmonary surfactant, which minimizes the surface tension in the alveoli and provides a barrier against disease and respiratory distress, is composed of an array of ~80% phospholipids, 8% neutral lipids I (cholesterol and free FAs), and 12% proteins (37). Hence, to shed more light on the role of FAs in AS in infancy, investigations on

EBC may provide critical information that would lead to new treatment options for wheezing and shortness of breath in infancy.

We found that high levels of total *n*-6 FAs in breast milk posed an increased risk for AS, but total *n*-3 FAs did not. However, for atopy, high levels of total *n*-3 FAs protected the child from developing atopy. It has been considered that *n*-3 PUFAs work against inflammation (38). For instance, previous studies have found that low intake of oily fish (rich in *n*-3 PUFAs) during pregnancy increases the risk of asthma, wheezing, and atopy (39–41). It has also been considered that high intake of *n*-3 PUFAs reduces the production of eicosanoid mediators, such as prostaglandins and leukotrienes (23), thus influencing the polarization of the naive Th cell toward a non-atopic Th1 immune response (22, 42). However, high consumption of *n*-6 PUFAs may enhance the synthesis of Th2-like cytokines and IgE (23).

The association between total *n*-6 FAs in breast milk and AS is in agreement with one previous study (43). Two studies on breast milk FAs found no association with asthma or wheezing episodes (6, 33). The latter study by Lowe et al. reported that *n*-6 FAs in breast milk were not associated with atopic diseases such as asthma in children at ages 6 and 7 years (33). FAs (total *n*-3, total *n*-6, *n*-6 to *n*-3 ratio, and individual FAs) in colostrum and in breast milk were analyzed as continuous variables. Similarly, in a recent study by Giwercman et al., breast milk FAs (total *n*-6 FAs, total *n*-3 FAs, or trans-FAs) were not found to be risk factors for wheezing episodes during the first 2 years of life (6). The Dutch Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort study found that low *n*-3 PUFAs (dichotomous variable) concentrations were associated with childhood asthma in children (aged 4 years) of mothers with allergy, but not with positive skin testing (43). We investigated AS at ages 6 and 12 months and SPT at 12 months of age. Ascertainment of outcomes at different ages and additional exposures experienced during childhood may explain why associations with FAs in breast milk may differ. Lowe et al. (33) and Giwercman et al. (6) reported asthma at age 6 years and during the first 2 years, respectively, which also may explain the differences between our results and theirs. In addition, the discrepancies found between our study and the previous investigations may also be due to the definition of FAs. Defining FA as the absolute concentration a total FA content in our study and as a weight percent of total FAs in the studies by Lowe et al. and Giwercman et al. have resulted in conflicts between findings. In addition, the use of a continuous versus dichotomized FAs variable may explain conflicting findings. We did not find any effect when we kept FAs as a continuous variable, but we and the Dutch study dichotomized the concentration since the relation between breast milk FAs and AS was not linear. Finally, divergent results may also be due to the various ways AS were assessed.

Our results on atopy (positive SPT) are in agreement with one of three other studies, which demonstrated a protective effect of *n*-3 FAs (43–45). The KOALA

(Translation from Dutch: Child, Parent and health: Lifestyle and Genetic constitution) study reported that high concentration of *n*-3 long-chain PUFAs (categorized into quartiles) was associated with a lower risk of sensitization at age 1 year (44). The authors defined a positive sensitization as an IgE of >0.35 IU/mL for one or more of the tested allergens (eggs, cow's milk, peanut, birch pollen, grass pollen, house dust mite, cat, and dog). The PIAMA study found no association between *n*-3 FAs (dichotomous variables) and allergic sensitization in children of 4 years of age (43). Both the KOALA and PIAMA studies used radioallergosorbent tests to define atopy; however, the discrepancy found in their results on atopy may be due to the age of the child (age 1 vs. age 4). Oddy et al. found no association of FAs (continuous variable) with atopy in 6-month-old infants (45). SPT was conducted for *Alternaria*, house dust mite, cat, ryegrass, *Aspergillus*, milk, and egg. Unlike the previous studies, we reported atopy at age 12 months rather than at the 4 years and 6 months reported by Wijga et al. (the PIAMA study) (43) and Oddy et al. (45), respectively. In addition, the discrepancy found between our study and the previous reports may be due to the definition of FAs. We define FAs as the absolute concentration of FAs, while Oddy et al. and Wijga et al. (the PIAMA study) defined FAs as a percentage of total FA content. Like the PIAMA study, we dichotomized FAs; however, we found an association with atopy. The difference found in their results on atopy may be due to the age of the child when atopy was measured (age 1 vs. age 4).

In the preceding analyses, we found that immune markers such as higher levels of IL-5 pose a risk for AS at ages 6 and 12 months. Since, with the exception of AA and total *n*-6 FAs, no statistically significant associations between individual FAs and AS were found, we explored whether the FAs have an indirect effect on AS by altering immune factors in whey. We did not find that IL-5 in breast milk whey acts as a mediator.

#### CONCLUSIONS/KEY FINDINGS

We conclude that high levels of total *n*-6 FAs in breast milk are associated with AS in infancy even after controlling for total *n*-3 FAs. This finding is in agreement with one of three prior studies. In addition, the risk of atopy seems to be lower among infants who were exposed to high levels of *n*-3 FAs in breast milk even after adjusting for total *n*-6 FAs. Since there are a number of conflicting reports, further studies are needed to test associations between total breast milk *n*-6 and *n*-3 FAs and AS and atopy.

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#### DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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