

Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy

C. Thijs^{1,2}, A. Müller³, L. Rist⁴, I. Kummeling¹, B. E. P. Snijders¹, M. Huber⁵, R. van Ree^{6,7}, A. P. Simões-Wüst⁴, P. C. Dagnelie¹ & P. A. van den Brandt¹

¹Maastricht University/CAPHRI School of Public Health and Primary Care, Maastricht, the Netherlands; ²Maastricht University/NUTRIM School of Nutrition, Toxicology and Metabolism, Maastricht, the Netherlands; ³Department of Food Chemistry, University of Hamburg, Hamburg, Germany; ⁴Paracelsus-Hospital Richterswil, Richterswil, Switzerland; ⁵Louis Bolk Institute, Driebergen, the Netherlands; ⁶Sanquin, Department of Immunopathology, Amsterdam; ⁷Academic Medical Center, University of Amsterdam, Department of Experimental Immunology, Amsterdam, the Netherlands

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Correspondence

Dr. Carel Thijs, Department of Epidemiology, Maastricht University, P.O. Box 616, 6200 MD Maastricht, the Netherlands.

Tel.: +31 43 3882389

Fax: +31 43 3884128

E-mail: c.thijs@epid.unimaas.nl

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Abstract

Background: One of the explanations for the increasing prevalence of atopic diseases is a relative low perinatal supply of n-3 fatty acids. However, this does not explain the protective effects of whole-fat dairy products or high levels of transfatty acids in breast milk, observed in some studies. We evaluated the role of perinatal supply of fatty acids in the early development of atopic eczema and allergic sensitisation.

Methods: Fatty acids, including n-3 long-chain polyunsaturated fatty acids (LCPs) as well as ruminant fatty acids (rumenic acid, *cis*-9,*trans*-11-C18:2 conjugated linoleic acid; and vaccenic acid, *trans*-11-C18:1), were determined in breast milk sampled at 1 month postpartum from 310 mother–infant pairs in the KOALA Birth Cohort Study, the Netherlands. Children were followed for atopic outcomes until 2 years of age.

Results: Higher concentrations of n-3 LCPs as well as ruminant fatty acids were associated with lower risk of (1) parent-reported eczema, (2) atopic dermatitis (UK Working Party criteria), and (3) sensitisation at age 1 year (as revealed by specific serum IgE levels to cow's milk, hen's egg and/or peanut). In multivariable logistic regression analysis, the inverse associations between ruminant fatty acid concentrations in breast milk and atopic outcomes were found to be independent from n-3 LCPs.

Conclusions: The results confirm a protective role of preformed n-3 LCPs in the development of atopic disease. Moreover, this is the first study in humans confirming results from animal studies of protective effects of ruminant fatty acids against the development of atopic manifestations.

The increase in atopic diseases (atopic eczema, food and airway allergies, asthma) as seen in the last decades in westernised countries may be explained by the n-6/n-3 balance hypothesis (1): a shift in the balance between dietary polyunsaturated fatty acids (PUFAs) with increased dietary n-6 fatty acids intake [mainly linoleic acid (LA) from vegetable oils] and decreased intake of n-3 fatty acids [alpha-linolenic acid (ALA) from green vegetables and n-3 long-chain polyunsaturated fatty acids (LCPs) from fatty fish]. Consistent with this hypothesis are cross-sectional studies showing that high margarine intake (high in LA) was associated with a higher risk of atopic eczema and airway allergy (2–4) and a study showing that children with asthma had higher dietary n-6/n-3

ratio than controls (5). More recently, two cohort studies have pointed out that the supply of fatty acids is relevant already early in life, by showing that low concentration of n-3 LCPs in breast milk was associated with elevated risk of eczema in the infant (6, 7) and asthma at 4 years of age (7).

However, other observations from dietary studies suggest that also fat from dairy products is relevant: higher intake of full-cream milk and butter was associated with lower risk of atopy and asthma in children and adults (8, 9). Moreover, Wijga et al. (7) showed that a higher concentration of transfatty acids in breast milk was associated with lower risk of atopic symptoms. Vaccenic acid (VA, *trans*-11-C18:1) is one of the most abundant transfatty acids in breast milk (and

also in cow's milk and dairy products). It is the intermediary precursor of rumenic acid (RA, *cis*-9,*trans*-11-C18:2), a conjugated linoleic acid (CLA) produced by bacterial biohydrogenation of fatty acids in the rumen of ruminant livestock like cows. Conjugated linoleic acids have been suggested to have immunomodulating properties (10, 11).

We hypothesize that not only n-3 LCPs but also ruminant fatty acids (VA, RA) are involved in the protection against development of atopy in the infant. In this study, we have analysed the fatty acid composition in breast milk to characterize perinatal fatty acid supply to the child. The objectives were, first, to confirm previous findings on n-3 LCPs and atopic outcomes (eczema and allergic sensitisation) and, second, to investigate the association between ruminant fatty acids in breast milk and these atopic outcomes.

Methods

Study design

This study is part of the KOALA Birth Cohort Study, a prospective birth cohort study in the Netherlands described elsewhere in detail (12). Between 2000 and 2002, healthy pregnant women who participated in a cohort study on development of pregnancy-related pelvic girdle pain ($n = 7020$) were invited to participate with their newborn ($n = 2343$, conventional recruitment group). Additionally, to enrich the cohort with sufficient participants with interesting exposures such as organic food use and prolonged breastfeeding (12), pregnant women with alternative lifestyles were recruited from 2001 to 2002 through organic food shops, anthroposophic doctors and midwives, and Steiner schools ($n = 491$, alternative recruitment group).

We sampled breast milk in a consecutive subgroup of mothers who entered the cohort between December 2002 and September 2003 and who confirmed the intention to give breastfeeding ($n = 315$). Breast milk was collected 1 month postpartum, together with a short questionnaire at the same day (13).

During pregnancy and the first 2 years postpartum, information on breastfeeding, atopic outcomes, and their determinants was collected by repeated questionnaires (34 weeks of pregnancy, follow-up at 3 and 7 months, and 1 and 2 years postpartum). Home visits were made at 1 and 2 years of age for blood collection, at 2 years combined with a physical examination by a trained research nurse.

Inclusion criteria for this study were (1) availability of complete baseline data from the 34 weeks pregnancy questionnaire and (2) availability of a breast milk sample.

The study was approved by the Medical Ethical Committee of Maastricht University/University Hospital Maastricht, and all parents gave written informed consent.

Collection of breast milk and analysis of fatty acids

Methods of breast milk collection and fatty acid analyses have been described in detail elsewhere (14). In short, mothers were instructed to collect the milk sample in the morning,

before breastfeeding their child from the second breast after finishing the first, and to keep the sample in the refrigerator ($\approx 4^\circ\text{C}$) until it was collected by one of the researchers on the same day. The lipid fraction was stored at -80°C in the Biobank Maastricht until analysis.

After lipid extraction, methyl esters of the following fatty acids were analysed by gas chromatography with flame ionisation detector and Ag+–HPLC (15): C12:0, C14:0, C14:1, C15:0, C16:0, *cis*-7-C16:1, C17:0, C18:0, C20:0, *cis*-9-C20:1, *trans*-6-C18:1, *trans*-7- etc. until *trans*-13-C18:1, *cis*-6-C18:1, *cis*-9-C18:1, *cis*-11-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *trans*-9,*trans*-12-C18:2, *cis*-9,*cis*-12-C18:2, C18:3n-6, C20:3n-6, C20:4n-6, C22:4n-6, C22:5n-6, C18:3n-3, C18:4n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3. Conjugated fatty acid isomers (including CLAs *trans*-9,*trans*-11-C18:2, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2) were separated using Ag+–HPLC – diode-array detection (15). All measurements were taken in duplicate and averaged. Fatty acid concentrations are expressed as proportion of total milk fat (weight percentage, wt%, mg/100 mg).

Definition of atopic outcomes

In the 7, 12, and 24 month postpartum questionnaires, questions on atopic eczema were adapted from the ISAAC questionnaire (16). Parents were asked 'has your child ever had an itchy rash that was coming and going in the past months?' ('past months' referred to the interval between questionnaires). If this question was answered affirmatively, infants were defined as having parent-reported eczema. Cases of only diaper rash, rash around the eyes, and/or scalp scaling were excluded.

At the home visit at 2 years of age, the health nurse took a medical history from the parents and examined the child for signs of atopic eczema using the UK Working Party criteria for children (17): presence of itchy rash (PIR, coded as 0 = absent, 1 = present); history of flexural dermatitis (HFD, 0 = absent, 1 = present); visible flexural dermatitis (VFD, 0 = absent, 1 = present); and onset before age of 2 years (OB2, 0 = absent, 1 = present). The UK Working Party probability score of atopic dermatitis (AD) is then computed as: probability AD = odds (AD)/[odds (AD) + 1], where odds (AD) = $\exp[-4.36 + 1.84 \text{ (HFD)} + 3.46 \text{ (OB2)} + 2.09 \text{ (VFD)} + 1.71 \text{ (PIR)}]$ (17). Atopic dermatitis was considered present if the UK Working Party probability score was higher than 90%.

At 1 year postpartum, a capillary blood spot was collected to determine total IgE and specific IgE against hen's eggs, cow's milk, and peanut (18, 19) with modifications for capillary blood spots (20). At 2 years of age, venous blood was taken to determine total IgE and specific IgE against hen's eggs, cow's milk, peanut, birch pollen, grass pollen, house dust mite, cat and dog, measured as described earlier (21). Allergic sensitisation was considered to be present if specific IgE was > 0.35 IU/ml for one or more of the tested allergens. Specific and total IgE measurements were performed by in-house methods at Sanquin Research, the Netherlands (formerly the National Central Laboratory of the Blood Transfusion Services). Total serum IgE was considered 'high'

if above the 67th percentile (upper tertile), resulting in cut-off values of 10 IU/ml at age 1, and 25 IU/ml at 2 years of age.

Statistical analysis

Differences between groups were evaluated using the student's *t*-test statistic (for continuous variables), and Fisher's exact or the Chi-square test statistics (for categorical variables); all reported *P*-values are two-sided; *P*-values below 0.05 were considered statistically significant.

The association between breast milk fatty acids and the infant's atopic outcomes (eczema, AD, allergic sensitisation, and high total IgE levels) was assessed by multivariable logistic regression analysis. Fatty acids of interest (n-3 LCPs, RA, VA) were categorized into quartiles (based on the conventional group because this group reflects the general population). These quartiles were used in categorical logistic regression analysis, with results expressed as odds ratios (ORs) with their 95% confidence intervals (95% CI). As a test for trend, these fatty acids were entered separately as a continuous variable (after natural log-transformation to reach a normal distribution), using the Wald test.

Potential confounders that were assessed and adjusted in the multivariable regression analyses were as follows: recruitment group (conventional *vs* alternative) and all other characteristics shown in Table 1. The model was chosen that had a regression coefficient for the main determinant closest to the model with all potential confounders (within a margin of 5%) and a narrower confidence interval, according to Kleinbaum's method of confounding and precision assessment (22). Differences in results between conventional and alternative recruitment groups were assessed by test for interaction in the multivariate regression models. No statistically significant interaction was found. Odds ratios mentioned in the text are always adjusted for confounders.

Results

Baseline data were complete for 312 (99%) of the 315 participating mother-infant pairs (Table 1). Fatty acids were determined in breast milk of 312 mothers and were complete for 310 of them. Several factors differed at baseline between the conventional and alternative groups (reflecting the intended enrichment, e.g. prolonged breastfeeding >6 months); all factors shown in Table 1 were evaluated for confounding in the logistic regression analyses.

Data on parent-reported eczema until 2 years of age were complete in 304 (97%) of the 312 children, while clinical examination for AD was completed in 207 (66%) children at the home visit at 2 years of age, and blood sampling was completed in 223 (71%) and 204 (65%) at 1 and 2 years of age, respectively.

Fatty acids in breast milk

Figure 1 shows the distribution of the LCPs and transfatty acids in breast milk. The three main n-3 LCPs eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosa-

hexaenoic acid (DHA) were highly correlated (Pearson's correlation coefficient (*r*) > 0.84, *P* < 0.001) and were therefore summed up into a new variable ('main n-3 LCPs'). The concentration of VA was moderately correlated with RA (*r* = 0.67, *P* < 0.0005). The concentration of main n-3 LCPs was weakly correlated with RA (*r* = 0.18, *P* = 0.002) but not with VA (*r* = 0.08, *P* = 0.16).

Compared with the conventional recruitment group, the alternative recruitment group had somewhat higher mean concentrations and higher standard deviations of main n-3 LCPs [mean (SD) 0.78 (0.38) *vs* 0.71 (0.27) wt%, *P* = 0.04], RA [0.29 (0.10) *vs* 0.25 (0.07) wt%, *P* = 0.00001], and VA [0.54 (0.20) *vs* 0.48 (0.20) wt%, *P* = 0.01].

Thirty-seven per cent of the mothers had a personal history of atopy. We found no statistically significant differences between atopic and nonatopic mothers in any breast milk fatty acid or ratio (total n-6/n-3, n-6/n-3 LCPs).

Infant atopic outcomes

The cumulative incidence of parent-reported eczema until 2 years of age was 31% (95/304). At the home visit at 2 years of age, 15% (31/207) of the children were found to have AD according to the UK Working Party criteria. Allergic sensitisation was present in 13% (28/220) of the children at 1 year of age, and 25% (51/204) at 2 years of age.

Parent-reported eczema discriminated fairly for sensitisation at 2 years of age: 42% (25/60) of children with eczema were sensitised compared with 18% (26/142) of children without eczema (difference *P* = 0.001). By contrast, AD discriminated less: 39% (12/31) of children with AD were sensitised at 2 years of age compared with 23% (39/173) of children without AD, *P* = 0.07. For sensitisation at 1 year of age, the reverse was true: it did not differ much between children with and without parent-reported eczema [16% (11/67) *vs* 11% (16/151), *P* = 0.27], but differed more than threefold between children with *vs* without AD: 31% (8/26) *vs* 9% (14/150) were sensitised at 1 year of age (*P* = 0.006).

Breast milk fatty acids and infant atopic outcomes

The risk of parent-reported eczema was lowest in the highest quartiles of main n-3 LCPs (Table 2). The trend of decreasing risk of eczema with increasing level of main n-3 LCPs was statistically significant (*P*-value for trend 0.040, adjusted for potential confounders). A further trend towards a lower risk of eczema was present with increasing concentrations of RA (*P* for trend 0.020) and VA (*P* for trend 0.024). The trend was less obvious for the quartiles of RA with OR in the two middle quartiles showing a greater fluctuation, but it should be noted that RA values in these quartiles showed a narrow range so that the statistically significant trend is mainly determined by the upper and lower ends of the RA distribution. The results for AD confirmed those of parent-reported eczema (Table 2).

There were also statistically significant trends towards a lower risk of allergic sensitisation at 1 year of age with increasing concentrations of main n-3 LCPs, RA, and VA, but not for allergic sensitisation at 2 years of age (Table 3). The

Table 1 Characteristics of participants with breast milk sample, KOALA Birth Cohort Study ($N = 312$)

Characteristics		Recruitment group		
		Conventional ($n = 146$)	Alternative ($n = 166$)	
Age of mother at delivery	Mean (SD), years	32.4 (3.3)	34.0 (4.2)	***
Highest education of mother	Lower-mid (lower or middle vocational)	61 (41.8)	40 (24.1)	**
	Mid-higher (higher vocational)	65 (44.5)	79 (47.6)	
	Higher (university)	20 (13.7)	47 (28.3)	
Gender of child	Male, no. (%)	71 (48.6)	82 (49.4)	n.s.
No. of siblings and their atopic history	No siblings, no. (%)	70 (47.9)	59 (35.5)	n.s.
	1 sib, non-atopic, no. (%)	45 (30.8)	53 (31.9)	
	1 sib, atopic	13 (8.9)	19 (11.4)	
	2 or more sibs, none atopic	9 (6.2)	17 (10.2)	
	2 or more sibs, at least one atopic	9 (6.2)	18 (10.8)	
Parental atopic history†	None, no. (%)	54 (37.0)	65 (39.2)	n.s.
	Father atopic	37 (25.3)	40 (24.1)	
	Mother atopic	33 (22.6)	31 (18.7)	
	Both atopic	22 (15.1)	30 (18.1)	
Maternal smoking during pregnancy and/or lactation and/or smoking in presence of the infant	Yes, no. (%)	16 (11.0)	14 (8.4)	n.s.
Place of birth	Birth at home, no. (%)	71 (48.6)	92 (55.4)	n.s.
Timing of breast milk collection, no. of days postpartum	Mean (SD), days	32.5 (9.1)	31.8 (8.7)	n.s.
Season of breast milk collection	December 2002–February 2003, no. (%)	66 (45.2)	48 (28.9)	***
	March–May 2003	62 (42.5)	61 (36.7)	
	June–September 2003	18 (12.3)	57 (34.3)	
Duration (and exclusivity of) breastfeeding	<6 months breastfeeding, no. (%)	72 (49.3)	35 (21.1)	***
	6 + months (at most 4 exclusive)	29 (19.9)	43 (25.9)	
	6 + months (at least 4 exclusive)	45 (30.8)	88 (53.0)	
n-3 or fish fatty acids supplements used by mother‡	Yes, no. (%)	4 (2.7)	11 (6.6)	n.s.
Probiotic supplement used by mother (at least weekly)‡	Yes, no. (%)	3 (2.1)	9 (5.4)	n.s.
Probiotic dairy products used by mother (at least weekly)‡	None, no. (%)	89 (61.0)	72 (43.4)	***
	Biogarde or other milk products with live bacteria	26 (17.8)	67 (40.4)	
	Specific probiotic milk products (e.g. Yakult)	31 (21.2)	27 (16.3)	
Antibiotic used by mother during lactation§	Yes, no. (%)	25 (17.1)	19 (11.4)	n.s.
Antibiotic used by infant§	Yes, no. (%)	24 (16.4)	20 (12.0)	n.s.
Regular vaccination schedule§	Yes, no. (%)	136 (93.2)	88 (53.0)	***
Dampness of the home	No, no. (%)	113 (77.4)	122 (73.5)	n.s.
	Only in none-living rooms	24 (16.4)	27 (16.3)	
	Yes with mould on walls	9 (6.2)	17 (10.2)	
Pet animal (dog, cat, furry pet) in the home§	Yes, no. (%)	73 (50.0)	68 (41.0)	n.s.

†Parental atopy: self-reported seasonal rhinoconjunctivitis (hay fever) or doctor's diagnosed atopic eczema, aeroallergen allergy, or asthma.

‡In last 6 weeks of pregnancy or first 3 months postpartum.

§In the child's first 6 months of life (regular vaccination schedule according to the national childhood vaccination programme: DPPT and Hib).

n.s., not significant: $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$ for the difference between conventional and alternative recruitment groups.

presence of high total IgE (highest tertile, data not shown) was only associated with low VA at 1 year of age (P for trend = 0.028), but not at 2 years of age (P for trend = 0.30).

Atopic outcomes were not associated with breast milk concentrations of linoleic (C18:2n-6, LA) and alpha-linolenic acid (C18:3n-3, ALA) nor with individual n-6 LCPs

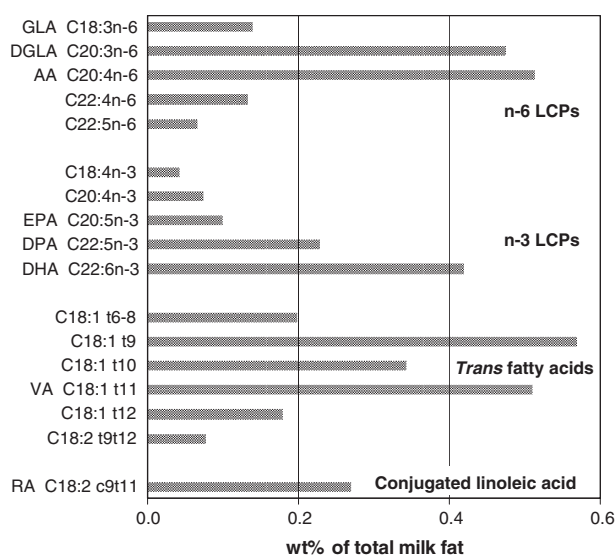


Figure 1 Distribution of LCPs in breast milk (KOALA Birth Cohort Study, the Netherlands). The figure shows mean concentrations of fatty acid in breast milk 1 month postpartum ($n = 310$ mothers), expressed as weight-percentage (wt%, mg/100 mg) of total breast milk fat. Only fatty acids with concentrations >0.05 wt% are shown. LCPs, long-chain polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; C18:1 t6-8 denotes a mixture of *trans*-6, *trans*-7 and *trans*-8 isomers; C18:1 t9 etc.: *trans*-9-C18:1; VA, vaccenic acid; RA, ruminic acid, C18:2 c9t11: *cis*-9,*trans*-11-C18:2.

(C18:3n-6; C20:3n-6; C20:4n-6; C22:4n-6; and C22:5n-6). Also, the ratios between LA and ALA and between total n-6 and n-3 PUFAs were not associated with atopic outcomes.

Independence of the associations of n-3 LCPs and ruminant fatty acids with atopic outcomes

To test whether the associations between ruminant fatty acids and atopic outcomes were independent of n-3 LCPs, we compared models with the ruminant fatty acids (sum of RA and VA) and n-3 LCPs (sum of EPA, DPA and DHA) separately and together in the logistic regression analysis. Table 4 shows the results expressed as OR for an increment of 2 standard deviations in the fatty acid concentrations. For parent-reported eczema, the inverse associations with main n-3 LCPs (MODEL A.1) and ruminant fatty acids (MODEL A.2) are slightly attenuated but remain statistically significant for ruminant fatty acids ($P = 0.022$) in the model with both groups of fatty acids included (MODEL A.3). Specifically, the OR for ruminant fatty acids was 0.49 (95% CI 0.28–0.85) (MODEL A.2) and after adjustment for n-3 LCPs remained 0.52 (0.29–0.91) (MODEL A.3), showing that the association between ruminant fatty acids with eczema was almost completely independent from n-3 LCPs. For AD, the results were largely similar, but did not reach statistical significance in the model with both n-3 LCPs and ruminant fatty acids (MODEL B.3; note that the number of subjects is smaller

than for parent-reported eczema so that the statistical power is lower).

For allergic sensitisation at 1 year of age (not shown in the tables), the OR for two standard deviations increment of ruminant fatty acids was 0.34 (0.14–0.79, $P = 0.013$) without adjustment for n-3 LCPs, changing to 0.41 (0.17–0.96, $P = 0.040$) after adjustment, which indicates partial independence. For the association between n-3 LCPs and allergic sensitisation, the OR was 0.35 (0.13–0.90, $P = 0.029$) without ruminant fatty acids in the model, changing to 0.42 (0.16–1.08, $P = 0.073$) after adjustment for ruminant fatty acids.

For total IgE at 1 year of age (upper tertile), the OR for two standard deviations increment of ruminant fatty acids was 0.54 (0.30–0.98, $P = 0.042$) and after adjustment of n-3 LCPs, this changed only slightly to 0.52 (0.29–0.96, $P = 0.035$), which indicates almost complete independence. Total IgE at 1 year of age was not associated with n-3 LCPs [OR 1.07 (0.65–1.78, $P = 0.79$) before adjustment for ruminant fatty acids, and 1.17 (0.70–1.97, $P = 0.55$) after adjustment].

Discussion

In the present study, we found that higher concentrations of main n-3 LCPs (sum of EPA, DPA, and DHA) in human breast milk were associated with a lower risk of parent-reported eczema and AD at 2 years of age (according to the UK working party criteria), and a lower risk of allergic sensitisation at 1 year of age (but not at 2 years of age). Most interestingly, our data reveal for the first time a consistent association between high breast milk concentrations of ruminant fatty acids (VA and RA) and lower risk of parent-reported eczema, AD, allergic sensitisation, and total IgE at 1 year, which was largely independent from the levels of n-3 LCPs.

Two similar studies, one from Australia and one from the Netherlands, have also reported an association between low n-3 LCP concentrations in breast milk and atopic outcomes. In the first study, n-3 LCPs were associated with a lower risk of nonatopic eczema at 6 months of age, but not at 5 years of age (6). In the second study, low n-3 LCP concentrations were associated with asthma at 4 years of age and persistence of eczema or asthma until 4 years of age, but only in children with mothers with allergy (7). Both studies showed no association with allergic sensitisation as measured by skin prick testing (6) or specific IgE (7), suggesting that the effect is mediated by an IgE-independent (nonatopic) mechanism. However, we did find an association between low n-3 LCP concentration and allergic sensitisation (specific IgE, at least at age 1 year), suggesting that IgE-mediated sensitisation is involved in the relation between n-3 LCPs and early development of atopic symptoms.

The human body is able to synthesize n-3 LCPs from dietary ALA (derived mainly from green vegetables but also from dairy products), but the contribution of this synthesis to n-3 LCP availability is limited (23). Therefore, n-3 LCP status and levels in breast milk are mainly determined by intake of preformed n-3 LCPs, and fatty fish is the main dietary source. In several recent studies, high maternal fish intake during

Table 2 Association between fatty acid concentrations in breast milk and parent-reported eczema and atopic dermatitis in the child

Fatty acid quartiles (wt%)*	Parent-reported eczema until age 2 years			Atopic dermatitis at age 2 years (UK working party)		
	Cases, no. (%) (n = 95)	Total, no. (n = 304)	OR (95% CI)†	Cases, no. (%) (n = 31)	Total, no. (n = 207)	OR (95% CI)†
Main n-3 LCPs						
Lowest 0.30–0.56	26 (36.1)	72	1.00 (reference)	10 (23.3)	43	1.00 (reference)
0.56–0.65	21 (30.9)	68	0.80 (0.37–1.71)	7 (15.9)	44	0.66 (0.19–2.32)
0.65–0.78	24 (32.4)	74	0.89 (0.43–1.88)	8 (14.8)	54	0.80 (0.24–2.71)
Highest 0.78–2.55	24 (26.7)	90	0.62 (0.30–1.29)	6 (9.1)	66	0.24 (0.07–0.91)
Total	95	304	<i>P</i> -value for trend 0.040	31	207	<i>P</i> -value for trend 0.024
Rumenic acid						
Lowest 0.01–0.21	28 (40.0)	70	1.00 (reference)	10 (22.7)	44	1.00 (reference)
0.21–0.24	13 (22.0)	59	0.32 (0.13–0.77)	7 (16.7)	42	0.83 (0.27–2.57)
0.24–0.28	19 (38.0)	50	1.11 (0.49–2.54)	5 (13.9)	36	0.46 (0.13–1.59)
Highest 0.28–0.56	35 (28.0)	125	0.48 (0.24–0.96)	9 (10.6)	85	0.34 (0.12–0.97)
Total	95	304	<i>P</i> -value for trend 0.020	31	207	<i>P</i> -value for trend 0.040
Vaccenic acid						
Lowest 0.08–0.34	27 (43.5)	62	1.00 (reference)	10 (26.3)	38	1.00 (reference)
0.34–0.44	17 (29.3)	58	0.52 (0.23–1.19)	6 (14.3)	42	0.41 (0.10–1.58)
0.44–0.57	24 (27.3)	88	0.48 (0.22–1.02)	5 (7.9)	63	0.23 (0.06–0.95)
Highest 0.57–1.66	27 (28.1)	96	0.47 (0.22–0.97)	10 (15.6)	64	0.51 (0.14–1.85)
Total	95	304	<i>P</i> -value for trend 0.024	31	207	<i>P</i> -value for trend 0.031

LCPs, long-chain polyunsaturated fatty acids.

*Main n-3 LCPs: sum of EPA (C20:5n-3, eicosapentaenoic acid), DPA (C22:5n-3, docosapentaenoic acid) and DHA (C22:6n-3, docosahexaenoic acid); breast milk concentrations of main n-3 LCPs, rumenic, and vaccenic were categorized according to quartiles in the conventional recruitment group (with 36 or 37 mothers in each category, total 146); fatty acid concentration are expressed as weight-percentage (wt%, mg/100 mg) of total breast milk fat.

†Adjusted odds ratios (OR) with 95% confidence intervals (CI) based on categorical logistic regression analysis; *P*-value for trend based on logistic regression with the fatty acids as continuous variable (after natural log-transformation). Co-variables adjusted in all models were: recruitment group, maternal age, maternal education, infant's gender, number of older siblings and their atopic history, parental atopic history, maternal smoking during pregnancy and/or smoking in presence of the infant, place of birth, season of breast milk collection, duration and exclusivity of breastfeeding, maternal n-3 fatty acids supplement use, maternal probiotic supplement use, maternal probiotic dairy use, maternal antibiotic use during lactation, infant's antibiotic use, vaccination schedule, dampness of the home, pet animals in the home. Bold text signifies, *P* < 0.05.

pregnancy was associated with lower risk of eczema, atopic wheeze, asthma, and/or skin prick positivity for food allergens in the child (24–28). In addition, a Danish prospective cohort study showed that high fish intake during the first year of life was associated with lower risk of eczema, allergic rhinitis, and asthma, as well as allergic sensitisation at 4 years of age (29), indicating that n-3 LCP intake by the infant after weaning is also relevant. This may explain why the association between low n-3 LCPs and allergic sensitisation in our study was present at age 1 but not any more at 2 years of age, when most children are no longer dependent on maternal fatty acid supply during pregnancy and lactation, and their n-3 LCP status becomes mainly determined by their own food intake.

Rumenic acid and VA in breast milk originate mainly from dietary ruminant fat, although RA can be produced from VA by the mammary gland to a small extent (contributing to up to 10% of the breast milk level) (30). The main source of ruminant fat in the diet is dairy fat, and to a smaller extent ruminant meat, at least in the Netherlands where mean daily intake of RA and VA by adults was estimated to be about 200 and 700–800 mg, respectively (31).

Rumenic acid is the most abundant isomeric form of CLA in breast milk, with concentrations of the same magnitude as n-3 LCPs (Fig. 1), whereas other isomeric forms of CLAs are only present in trace amounts in breast milk. Mechanisms through which CLAs modulate immune function include influences on the production of cytokines, eicosanoids (prostaglandins, leukotrienes) and nitric oxide, inhibition of eosinophilic cationic protein, decreased availability of arachidonic acid or inhibition of cyclooxygenase, mRNA expression of phospholipase A2, NFκB DNA-binding activity, and binding to PPAR-γ (10, 11). In rats, RA supplementation reduced the production of prostaglandin E2 and leukotriene B4 (pro-inflammatory mediators produced from arachidonic acid), and other pro-inflammatory cytokines (32). In mouse models of allergic airway disease, combined dietary RA and VA supplementation reduced airway inflammation and allergen-specific IgE response (33), and RA supplementation reduced IgE production and allergen-induced airway hyper responsiveness (34). Our study is the first to confirm these observations in children, by showing a protective effect of these fatty acids on the development of atopic manifestations.

Table 3 Association between fatty acid concentrations in breast milk and allergic sensitization of the child

Fatty acid quartiles (wt%)*	Allergic sensitisation at age 1 year			Allergic sensitisation at age 2 years		
	Cases, no. (%) (n = 28)	Total, no. (n = 220)	OR (95% CI)†	Cases, no. (%) (n = 51)	Total, no. (n = 204)	OR (95% CI)†
Main n-3 LCPs						
Lowest 0.30–0.56	8 (16.3)	49	1.00 (reference)	10 (24.4)	41	1.00 (reference)
0.56–0.65	6 (13.3)	45	0.80 (0.21–3.08)	13 (29.5)	44	1.29 (0.45–3.68)
0.65–0.78	9 (15.5)	58	0.68 (0.21–2.20)	15 (28.3)	53	1.32 (0.47–3.72)
Highest 0.78–2.55	5 (7.4)	68	0.17 (0.04–0.77)	13 (19.7)	66	0.89 (0.32–2.50)
Total	28	220	<i>P</i> -value for trend 0.029	51	204	<i>P</i> -value for trend 0.92
Rumenic acid						
Lowest 0.01–0.21	9 (20.5)	44	1.00 (reference)	11 (25.6)	43	1.00 (reference)
0.21–0.24	6 (14.0)	43	0.74 (0.19–2.96)	12 (28.6)	42	0.99 (0.34–2.93)
0.24–0.28	2 (5.4)	37	0.16 (0.02–1.04)	10 (28.6)	35	1.57 (0.49–5.06)
Highest 0.28–0.56	11 (11.5)	96	0.39 (0.12–1.33)	18 (21.4)	84	0.85 (0.31–2.33)
Total	28	220	<i>P</i> -value for trend 0.028	51	204	<i>P</i> -value for trend 0.81
Vaccenic acid						
Lowest 0.08–0.34	9 (20.9)	43	1.00 (reference)	15 (39.5)	38	1.00 (reference)
0.34–0.44	4 (9.8)	41	0.27 (0.06–1.18)	9 (21.4)	42	0.45 (0.15–1.35)
0.44–0.57	6 (9.4)	64	0.47 (0.13–1.72)	12 (19.7)	61	0.38 (0.14–1.07)
Highest 0.57–1.66	9 (12.5)	72	0.34 (0.10–1.17)	15 (23.8)	63	0.48 (0.18–1.30)
Total	28	220	<i>P</i> -value for trend 0.020	51	204	<i>P</i> -value for trend 0.17

LCPs, long-chain polyunsaturated fatty acids.

*Main n-3 LCPs: sum of EPA (C20:5n-3, eicosapentaenoic acid), DPA (C22:5n-3, docosapentaenoic acid) and DHA (C22:6n-3, docosahexaenoic acid); breast milk concentrations of main n-3 LCPs, rumenic, and vaccenic were categorized according to quartiles in the conventional recruitment group (with 36 or 37 mothers in each category, total 146); fatty acid concentration are expressed as weight-percentage (wt%, mg/100 mg) of total breast milk fat.

†Adjusted odds ratios (OR) with 95% confidence intervals (CI) based on categorical logistic regression analysis; *P*-value for trend based on logistic regression with the fatty acids as continuous variable (after natural log-transformation). Co-variables adjusted in all models were: recruitment group, maternal age, maternal education, infant's gender, number of older siblings and their atopic history, parental atopic history, maternal smoking during pregnancy and/or smoking in presence of the infant, place of birth, season of breast milk collection, duration and exclusivity of breastfeeding, maternal n-3 fatty acids supplement use, maternal probiotic supplement use, maternal probiotic dairy use, maternal antibiotic use during lactation, infant's antibiotic use, vaccination schedule, dampness of the home, pet animals in the home. Bold text signifies, *P* < 0.05.

Dietary studies have revealed an association between high dairy fat intake and lower risk of atopy and asthma (8, 9), and these observations can be explained by the content of RA, and its precursor VA, in dairy fat. In the KOALA cohort at large (over 2500 children), we found that the risk of eczema until 2 years of age was lower if the child consumed dairy from organic origin (produced with organic agriculture and processing methods) compared with dairy from conventional origin and that the eczema risk in the child was also lower when its mother had consumed dairy from organic origin during pregnancy (21). Higher intake of RA and VA from organic dairy may explain these observations, because breast milk concentrations of RA and VA were higher in women using organic dairy products compared with conventional dairy products (14). Because consumption of farm milk has been associated with lower risk of atopy in many studies, and milk from traditional or organic farming methods contains relatively high levels of n-3 fatty acids, RA, and VA (35), this also offers a possible explanation for the effect of farm milk, in addition (or as an alternative) to the alleged microbial burden of unpasteurised farm milk (36).

A strength of the present study is the enrichment with the alternative recruitment group, which enhanced the variation in n-3 LCPs, RA, and VA (as witnessed by higher mean levels and higher standard deviations), favouring the statistical power for our hypothesis, as anticipated in the design phase of our study (12). This is in line with the higher use of organic dairy products in our alternative group and the higher concentrations of RA and VA in these products compared with conventional dairy products (21).

Limitations of the present study are the still limited range of n-3 fatty acid concentrations in breast milk, which is because of the generally low fish consumption in the Netherlands, the single sampling of breast milk, as well as the reliance on parent-reported information for part of the atopic outcomes (but not for the laboratory-based outcomes). Parent report of eczema probably leads to over diagnosis, or at least reporting of mild symptoms that may not be related to atopy. However, it appeared that parent-reported eczema discriminated fairly well for sensitisation at 2 years of age, and even better than AD, whereas AD at 2 years of age discriminated better for sensitisation at 1 year of age.

Table 4 Multivariable analysis of fatty acids in breast milk and parent reported eczema and atopic dermatitis in the child

	Parent reported eczema until age 2 (95 cases, <i>n</i> = 304)		Atopic dermatitis at age 2 years (31 cases, <i>n</i> = 207)	
	Fatty acids separately	Fatty acids together	Fatty acids separately	Fatty acids together
	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value
Main n-3 LCPs*	MODEL A.1 0.60 (0.37–0.98) <i>P</i> = 0.040	MODEL A.3 0.65 (0.40–1.05) <i>P</i> = 0.080	MODEL B.1 0.33 (0.13–0.87) <i>P</i> = 0.024	MODEL B.3 0.39 (0.15–1.06) <i>P</i> = 0.065
Ruminant fatty acids†	MODEL A.2 0.49 (0.28–0.85) <i>P</i> = 0.011	0.52 (0.29–0.91) <i>P</i> = 0.022	MODEL B.2 0.39 (0.15–1.01) <i>P</i> = 0.053	0.49 (0.18–1.36) <i>P</i> = 0.17

LCPs, long-chain polyunsaturated fatty acids.

*Main n-3 LCPs: sum of EPA (C20:5n-3, eicosapentaenoic acid), DPA (C22:5n-3, docosapentaenoic acid) and DHA (C22:6n-3, docosahexaenoic acid).

†Ruminant fatty acids: sum of rumenic acid and vaccenic acid.

MODELS: main n-3 LCPs and ruminant fatty acids were included separately (MODEL A.1 and A.2; B.1 and B.2) and together (MODEL A.3 and B.3) in the logistic regression model as continuous variables (after natural log-transformation of the concentration, wt%). Adjusted for potential confounding factors (recruitment group, maternal age, maternal education, infant's gender, number of older siblings and their atopic history, parental atopic history, maternal smoking during pregnancy and/or smoking in presence of the infant, place of birth, season of breast milk collection, duration and exclusivity of breastfeeding, maternal n-3 fatty acids supplement use, maternal probiotic supplement use, maternal probiotic dairy use, maternal antibiotic use during lactation, infant's antibiotic use, vaccination schedule, dampness of the home, pet animals in the home). Adjusted odds ratios (OR) with 95% confidence intervals (CI) are computed from the logistic regression models, using a contrast of two standard deviations of the fatty acid concentrations in the conventional group (on the natural log scale). Bold text signifies, *P* < 0.05.

Some misclassification may have occurred in both outcome measures and may have led to bias towards the null, i.e. underestimation of the strength of the true associations with fatty acids. We find it reassuring that the results for parent-reported eczema were largely confirmed with the stricter UK working party criteria for AD and allergic sensitisation at 1 year of age.

The follow-up rate was high for parental questionnaires, but lower for measurements made at the home visit (AD, sensitisation, total IgE). Differential follow-up is a concern in cohort studies with low follow-up rates, especially when the participant is not blinded for the exposure; however, in this study, the exposure was fatty acid composition of breast milk, and the levels were not known to the participating mothers. Therefore, bias by differential follow-up is not likely. Again, the consistency of these outcomes with parent-reported outcomes is reassuring.

Another limitation is inherent in the multivariable method to assess independence of the associations of n-3 LCPs and ruminant fatty acids. It assumes perfect estimation of their levels, and measurement error and biological variation lead to imperfect adjustments so that association seem more independent than they actually are.

Only randomised trials can provide conclusive evidence of causal and independent effects of dietary fatty acids in the aetiology of atopic diseases. We suggest that future supplementation trials should consider background levels of dietary intake of fish as well as ruminant fat and evaluate combinations of fish (oil) and ruminant (dairy) fats. This is also important for observational studies of the protective effect of

breastfeeding on atopy and asthma, because these studies have a long history of inconsistent results (37). Differences in fatty acid status between mothers may modify the protective effect of breastfeeding, and this may explain inconsistencies between studies in different populations with different background intake of fish, ruminant fats, and trans fatty acids from other sources.

We conclude that development of atopic eczema and allergic sensitisation is associated with low n-3 LCP and ruminant fatty acids supply to the infant, during lactation, and possibly also during pregnancy. Ruminant fatty acids deserve further investigations for their role in early human immune development and are potential candidates to explain the protective effects of dairy fat as well as organic dairy (and possibly also farm milk) on the development of atopic conditions in early life.

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All authors have declared that they have no conflicts of interest.

Contributions of the authors

Conception and design (CT, LR, MH, PD, PAVdB), acquisition of data (CT, IK, BS), analysis and interpretation of data (CT, AM, BS, RvR, APS-W), drafting of the manuscript

(CT, AM, APS-W), critical revision (all), statistical analysis (CT, AM, BS, IK), obtaining funding (CT, LR, MH, PD, PAVdB), administrative, technical and material support (IK, BS, AM, RvR, APS-W), and supervision (LR, PD, PAVdB).

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