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The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition

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Summary

Background Breast milk contains many immunomodulatory factors (soluble CD14 (sCD14), IgA and cytokines) with the potential to influence infant immune development.

Objective To determine if changes in breast milk ω-3 polyunsaturated fatty acid (n-3 PUFA) composition as a result of maternal dietary fish oil supplementation during pregnancy can modify levels of these immunological parameters in breast milk.

Method In a randomized controlled trial, 83 atopic women received either 4 g fish oil capsules (containing 3.7 g n-3 PUFA) ($n = 40$) or 4 g olive oil capsules ($n = 43$) from 20 weeks gestation until delivery. Breast milk was collected 3 days post-partum and fatty acids were analysed by gas liquid chromatography and IgA, sCD14 and cytokines (IL-5, IL-6, IL-10, TNF-α and IFN-γ) were quantitated by ELISA or time resolved fluorescence (TRF).

Results ω-3 docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) levels were significantly higher ($P < 0.001$) in breast milk from women supplemented with fish oil ($n = 33$, DHA mean 1.15%, SD 0.47% and EPA mean 0.16%, SD 0.07%) than in samples from the control group ($n = 40$, DHA mean 0.50%, SD 0.17% and EPA mean 0.05%, SD 0.02%). Breast milk arachidonic acid (AA; 20:4n-6) levels were significantly lower ($P = 0.045$) in the fish oil group (mean 0.55%, SD 0.12%) compared with the control group (mean 0.61%, SD 0.14%). Breast milk IgA was positively correlated with DHA ($P = 0.046$) and 22:5n-3 ($P = 0.003$), but inversely correlated with linoleic acid (LA; 18:2n-6) ($P = 0.034$). Levels of sCD14 were also positively correlated with 22:5n-3 ($P = 0.009$). Cytokines involved in IgA synthesis (IL-10 and IL-6) were also significantly correlated with both IgA and n-3 PUFA levels, although there were no differences in the levels of breast milk IgA, sCD14 or cytokines between study groups.

Conclusion Supplementation with fish oil during pregnancy significantly alters early post-partum breast milk fatty acid composition. ω-3 PUFA levels were positively associated with IgA and sCD14 levels, suggesting a relationship between fatty acid status and mucosal immune function.

Keywords allergy, breast milk, cytokines, docosahexaenoic acid, fish oil, infants, long chain polyunsaturated fatty acids, pregnancy

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Introduction

In addition to providing essential nutrients, breast milk also contains many factors (including antibodies, cells, cytokines and soluble CD14 (sCD14)) that provide some measure of immunological protection for infants during a period of relative immaturity. As well as protection from infection, immunological factors in breast milk may be important in other aspects of immune development. With increasing rates of allergic disease there has been growing interest in the protective effects of breastfeeding. Although not supported by all studies (discussed in [1, 2]), there remains a substantial body of evidence to suggest that breastfeeding may reduce the risk of developing allergic disease (discussed in [3]). It is not clear if this could be due to direct immunological properties of breast milk, or indirect effects on bacterial colonization or delayed introduction of potential dietary allergens. As such, there is growing interest in the immunological properties of breast milk and how these may be modulated by environmental changes (including maternal diet) and in turn influence infant immune development.

Breast milk contains maternal antibodies to environmental proteins including allergens [4, 5], which may confer some protection from allergic diseases [6–8], possibly by reducing absorption of allergens. Other immune factors may modify intestinal colonization [9], which has also been implicated in the prevention of allergic disease. The soluble form of the bacterial pattern recognition receptor CD14 (sCD14) is present in human milk at levels 20-fold higher than serum [10], suggesting that this may play a role in modulating local innate

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and adaptive immune responses in the newborn. Furthermore, one recent study found a protective relationship between levels of sCD14 in breast milk and the development of subsequent allergic disease [11]. Much less is known about the role of other factors including immune cells and cytokines, which have also been detected in human breast milk [12].

Variations in maternal dietary nutrients influence the composition of breast milk. There is particular interest in the effects of nutrients with known immunomodulatory properties, such as the ω-3 polyunsaturated fatty acids (n-3 PUFA) [5]. These fatty acids modify cellular immune responses *in vitro* and *in vivo* (reviewed in [13, 14]) and have the potential to influence the immunological products secreted in human milk according to their composition. Although there are preliminary reports linking fatty acid and cytokine levels in breast milk [15] the significance is not clear, and other studies have not found this [16]. We have recently reported that dietary supplementation with fish oil (containing 3.7 g n-3 PUFA) during pregnancy resulted in modulation of infant immune response at birth [17, 18]. This paper reports the effects of fish oil supplementation during pregnancy on the breast milk PUFA composition and associated relationships with IgA, sCD14 and cytokine content of breast milk in the same cohort.

Methods

Participants

In a double-blind, randomized controlled study, 98 pregnant atopic women in Western Australia were recruited because their babies were considered to be at high risk of allergic disease. All women had a history of doctor diagnosed allergic rhinitis (AR) and/or asthma and one or more positive skin prick test to common allergens (house dust mite, grass pollens, moulds, cat, dog and cockroach extracts; Hollister-Stier Laboratories, Spokane, WA, USA). Women were ineligible for the study if they smoked, had other medical problems, complicated pregnancies, seafood allergy or their normal dietary intake exceeded two meals of fish per week. The study was approved by the Ethics Committees at St John of God Hospital and Princess Margaret Hospital, and all women gave informed consent.

Study design and intervention

The groups were block randomized according to parity (no previous term childbirth vs. one or more), pre-pregnancy body mass index (BMI), age and maternal allergy (AR or asthma). Women in the fish oil group ($n = 52$) received four (1 g) fish oil capsules per day (Ocean Nutrition, Halifax, Nova Scotia, Canada), comprising a total of 3.7 g of n-3 PUFA with 56.0% as docosahexaenoic acid (DHA) and 27.7% as eicosapentaenoic acid (EPA) (confirmed by gas liquid chromatography). The control group ($n = 46$) received four (1 g) capsules of olive oil per day (containing 66.6% n-9 oleic acid, and less than 1% n-3 PUFA) (Pan Laboratories, Moorebank, NSW, Australia). This amount was chosen as approximately equivalent to one fatty-fish meal per day [19]. Compliance was monitored by measuring the incorporation of DHA and EPA into the cell membranes of erythrocytes.

Collection and analysis of breast milk samples

Breast milk was collected 3 days post-partum into sterile containers either by manual expression or using a breast pump, frozen immediately and stored at −80 °C until analysis. Milk was thawed (warmed to 37 °C) and fatty acid analysis performed on whole milk samples. The aqueous milk phase was removed from below the lipid phase (with a needle and syringe) following centrifugation of the samples for 10 min at 7000 ***g*** and used for cytokine and IgA analysis.

Fatty acid composition

Lipids were extracted with chloroform–methanol (2 : 1) using butylatedhydroxyanisole as an antioxidant, as published previously [20]. Fatty acid methyl esters from milk lipid extracts were prepared by acid transmethylation using 1% H_2SO_4 in methanol. Fatty acid methyl esters were separated using a gas chromatograph (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan) equipped with a 50 m capillary column (0.32 mm internal diameter) coated with BPX-70 (0.25 μm film thickness; SGE Pty Ltd, Ringwood, Australia) using a method modified from [21]. Each sample (3 μL) was injected on to the column using an automatic injector (Shimadzu AOC-14, Shimadzu Corporation) at a split ratio of 30 : 1. The injector temperature was set at 250 °C and the detector (flame ionization) temperature at 300 °C. The initial oven temperature was 140 °C and was programmed to rise to 230 °C at 5 °C/min. Helium was used as the carrier gas at a velocity of 4 mL/min. Fatty acids were identified based on retention time to authentic lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA).

Quantitation of cytokines and immunoglobulin A levels in breast milk

Total breast milk IgA levels were detected by Nephelometry (Beckman Coulterin, Immage, Fullerton, CA, USA), measuring the intensity of scattered light particles suspended in a cuvette using a 670 nm laser as the light source. The light scatter signal measured was proportional to the IgA immunoprecipitation complexed to anti-IgA antibodies (Beckman Coulterin 446460). The lowest level of detection for total IgA was 11.1 μg/mL.

Cytokine and soluble CD14 analysis

Levels of sCD14, IL-13, TNF-α and IL-5 were measured by ELISA as previously described [22]. IL-10, IL-6 and IFN-γ were quantified using time resolved fluorometry (TRF) (DELPHIA, Perkin-Elmer, Life Sciences, Boston, MA, USA) as previously described [18]. Briefly the ELISA method was followed using paired antibodies (Pharmingen, BD, North Ryde, NSW, Australia) and the biotinylated antibody was detected using Europium-labelled streptavidin. Fluorescence dissociated by the addition of low pH enhancement buffer was quantified using a fluorometer (WALLAC VICTOR^2, Perkin-Elmer, Life Sciences). The detection limit was 3 pg/mL for IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and 6 pg/mL for TNF-α. For the Quantikine Human sCD14 Immunoassay (R&D Systems, Minneapolis, MN, USA) the lower detection limit by ELISA was 250 pg/mL.

Data analysis

Only mothers of healthy infants (born $\geqslant$36 weeks gestation) were included in the data analysis (to avoid the confounding effects of pre-maturity). Fatty acids were expressed as mean percentage and standard deviation of the total fatty acids measured. Differences between the groups were determined by independent *t*-test.

Cytokine data were analysed as continuous data, described by the median and interquartile range, and as dichotomous data (detected or non-detected). Differences between the groups were determined by Mann–Whitney test for non-parametric data. Differences between the groups for dichotomous data were determined by χ^2. The two groups were also combined ($n = 73$ with breast milk samples) to determine associations between individual fatty acid proportions and cytokine and IgA levels using Spearman's correlation. Factors with potential confounder effects were tested by Spearman's correlation.

All statistical analyses were performed using SPSS software (Version 10 for Macintosh). A *P*-value <0.05 was considered statistically significant for all analyses.

Results

There was no significant difference in age, pre-pregnancy BMI, allergic status (asthma or AR) or parity between the women completing the study in the fish oil ($n = 40$) and control groups ($n = 43$). Breast milk samples were collected from 73 women (33 in the fish oil group and 40 in the olive oil group) intending to breastfeed their infants.

Breast milk concentrations of DHA (22:6n-3), 22:5n-3 and EPA (20:5n-3) were significantly higher ($P<0.001$) in fish oil supplemented mothers compared with controls (Fig. 1). arachidonic acid (AA) was significantly lower ($P = 0.045$) in the fish oil group.

The DHA proportion in breast milk was positively correlated with other n-3 PUFA; 22:5n-3 ($r = 0.932$, $P<0.001$) and EPA ($r = 0.849$, $P<0.001$), but not to any n-6 PUFA. EPA was negatively correlated to n-6 PUFA; 20:3n-6.

There was no significant difference in the detection (Table 1) or level of free cytokines or IgA between the two groups (Table 2). However when the two groups were combined there were significant correlations between IgA and individual proportions of fatty acids in breast milk (Table 3). IgA was positively associated with DHA (22:6n-3) ($r = 0.235$, $P = 0.046$) and 22:5n-3 ($r = 0.344$, $P = 0.003$) and inversely associated with linoleic acid (LA) (18:2n-6) ($r = -0.249$, $P = 0.034$). Breast milk levels of sCD14 were positively correlated with 22:5n-3 ($r = 0.306$, $P = 0.009$). Breast milk IgA and cytokine content was not affected by maternal age, parity or allergic status (data not shown).

IgA levels in the breast milk samples were positively correlated with levels of IL-10 ($r = 0.459$, $P<0.001$), IL-6 ($r = 0.288$, $P = 0.013$), IL-13 ($r = 0.375$, $P = 0.001$) and sCD14 ($r = 0.335$, $P = 0.004$), in the samples.

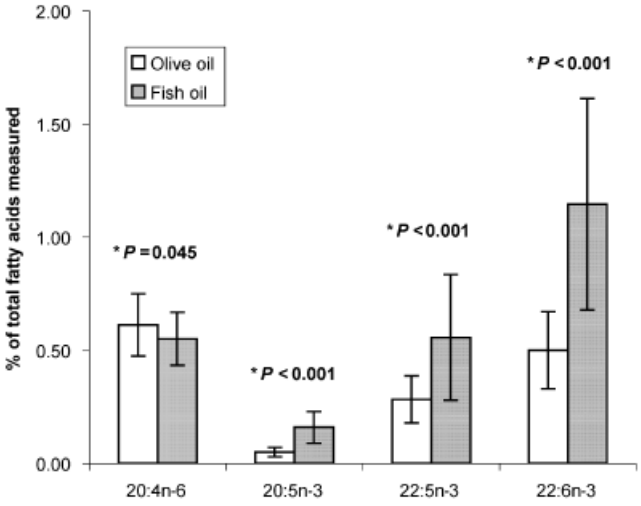

Fig. 1. Fatty acids present in breast milk collected 3 days post-partum from women supplemented with fish oil ($n = 33$, shaded boxes) compared with the control group ($n = 40$, clear boxes). Levels are shown as means and SD. A *P*-value of <0.05 was considered statistically significant.

Table 1. Detection of IgA, sCD14 and cytokines in breast milk in women supplemented with fish oil ($n = 33$) compared with the control group ($n = 40$)

Parameters in breast milk	Control group, $n = 40$	Fish oil, $n = 33$	*P*-value
IgA	40 (100.0%)	43 (100%)	
sCD14	40 (100%)	33 (100%)	
IL-5	0 (0%)	1 (3.0%)	0.452
TNF-α	5 (12.5%)	4 (12.1%)	1
IL-13	24 (60.0%)	20 (60.6%)	0.958
IFN-γ	2 (5.0%)	1 (3.0%)	1
IL-10	12 (30.0%)	16 (48.5%)	0.106
IL-6	31 (77.5%)	30 (90.9%)	0.124

The number (and proportion) of samples with detectable levels are shown. *P*-values were determined by χ^2 and $P<0.05$ was considered statistically significant.

Table 2. Levels of IgA, sCD14 and cytokines in breast milk collected 3 days post-partum from women supplemented with fish oil ($n = 33$) during pregnancy compared with the control group ($n = 40$)

	Control group		Fish oil		
Cytokine	Median	IQR	Median	IQR	*P*-value
IgA (mg/mL)	1.0	0.7–1.4	1.2	0.8–1.6	0.409
sCD14 (μg/mL)	16.9	15.6–19.1	18.2	14.7–20.2	0.270
IL-13 (pg/mL)	6.7	0–14.4	6.3	0–15.3	0.973
IL-10 (pg/mL)	0	0–3.4	0	0–4.2	0.196
IL-6 (pg/mL)	38.7	15.6–81.7	45.7	24.6–89.7	0.323

Median levels and interquartile ranges (IQR) are shown. Groups were compared using the Mann–Whitney non-parametric test. A *P*-value of <0.05 was considered statistically significant.

Discussion

The early events in the gastrointestinal tract are believed to be central in the development of immune tolerance mechanisms. There has been much speculation that breast milk may have important immunomodulatory properties that facilitate appropriate immune development in the fetus. While there have been some associations between breast milk n-3 PUFA, immunological parameters and allergy [5, 23, 24], this remains

Table 3. The relationship (correlation) between immunological parameters (IgA, sCD14 and cytokines) and fatty acids in breast milk

Immunological parameters	BM fatty acids				
	18:2n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
IgA	−0.249*	0.190	0.072	0.344**	0.235*
sCD14	−0.025	0.197	0.023	0.306**	0.182
IL-13	0.027	0.023	0.087	0.083	−0.002
IL-10	−0.003	0.100	0.154	0.261*	0.194
IL-6	0.030	0.221	−0.029	0.336**	0.190

The correlation coefficients are shown for the whole study population ($n = 73$), and were calculated using the Spearman correlation test.
Significance levels are annotated where *$P < 0.05$ and **$P < 0.01$.

controversial and the nature of these relationships are unclear. To our knowledge this is the first study to examine the relationships between PUFA levels and immunological parameters such as sCD14, IgA and a wide range of cytokines in breast milk of women who received either n-3 supplements or placebo in pregnancy.

We confirmed that fish oil supplementation was associated with significantly higher levels of DHA and EPA and significantly lower levels of AA in breast milk compared with the control group, presumably as a direct result of improving maternal n-3 PUFA status. This is consistent with the findings of Helland et al. [25] who administered cod liver oil (2.6 g n-3 PUFA per day) from 20 weeks gestation until 3 months postpartum.

Despite the altered PUFA status in the breast milk of fish oil supplemented women there were no differences in immunological parameters in breast milk between the study groups. However there were significant relationships between breast milk PUFA levels and both IgA and sCD14 levels in breast milk. In both cases higher n-3 PUFA levels were associated with higher levels of these potentially protective factors.

Although the relationship between breast milk IgA levels and the development of allergic disease is unclear, several studies have suggested that both total IgA and cow's milk specific IgA in breast milk levels are associated with reduced risk of developing cow's milk allergy in children [6–8, 26]. These mucosal antibodies may directly bind allergen (and reduce absorption) or have other immunomodulatory effects on local immune networks. Our findings suggest that increasing the n-3 PUFA (and decreasing n-6 PUFA) content of breast milk is one way of increasing breast milk IgA levels. It remains to be seen if these modest changes in maternal IgA in breast milk have any clinical effects on the infants. We also observed that breast milk IL-10 and IL-6 (but not TNF-α) levels were significantly correlated with IgA levels (and n-3 PUFA). These cytokines are involved in IgA synthesis, and these associations are consistent with those previously observed by Böttcher et al. [27]. There has been speculation that these maternally derived cytokines could also promote local mucosal IgA production in infants [27], but this remains to be determined.

sCD14 is another immunologically active constituent of breast milk with the potential to modify immune events at infants' mucosal surfaces [10]. Although the processes involved in normal oral tolerance are still poorly understood, a number of bystander factors may modify responses to allergens including enteric flora. Immune stimulation through bacterial pattern recognition receptors such as sCD14 may provide key signals for immune maturation, as evidenced by the failure of oral tolerance in germ free animals [28]. sCD14 in breast milk may stimulate intestinal cells to the release of immunomodulatory cytokines [29] and is a potentially important immune modulator in homeostasis and in the defense of the neonatal intestine. Accordingly, one recent report noted a protective relationship between sCD14 levels in breast milk and the subsequent development of allergic disease [11]. In the present study, we observed a significant positive relationship between n-3 PUFA composition and sCD14 levels in breast milk. This suggests a further avenue of immunomodulation for n-3 PUFA.

Maternal cytokines in breast milk may also influence the immunological milieu in the infant gut, and it has been postulated that these mediators may contribute to the possible allergy-preventive effect of breastfeeding [9]. Although the significance is unclear, there have also been some reports that milk from allergic mothers is different from that of non-allergic mothers with regard to both cytokine levels [27] and PUFA composition [5, 23, 24]. Böttcher et al. [27] reported higher IL-4 levels in breast milk of allergic than non-allergic mothers, but this did not appear to influence the risk of infant allergic disease or sensitization [26] and has not been confirmed by other groups [15] who found that allergic mothers had lower concentration of TGF-β_2 in breast milk, but no difference in IL-4 or other cytokines. Other studies have found differences in PUFA levels in breast milk from allergic women [5, 23], or PUFA metabolism [30], leading to speculation that allergic disease may be associated with altered lipid metabolism. This has been of particular interest given the known immunological properties of n-3 PUFA [14]. However, others have found no differences in breast milk fatty acid composition of allergic women compared with those without allergies [31]. To our knowledge, only one other group has assessed the effects of maternal supplementation with PUFA-rich fish oil (during lactation) on breast milk cytokines. They showed no relationship between PUFA levels and pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) [32] and TGF-β1 and TGF-β2 levels [16] but did not examine other parameters. We have previously reported that cord plasma T-helper type 2 (Th2) IL-13 levels were significantly lower in babies of allergic mothers supplemented with fish oil than those in a control group [17]. In the present study, which also only included allergic women, we observed that n-3 PUFA levels were correlated with levels of some cytokines (IL-6 and IL-10), but not with Th2 cytokines detected in milk. Although 60% of the breast milk samples contained detectable amounts of IL-13, there was no significant difference in the levels between the groups or any correlations between IL-13 and PUFA levels.

The immunomodulatory effects of PUFA on peripheral blood mononuclear cells have been extensively reviewed [13, 14] and include effects on signal transduction, gene expression and eicosanoid (including prostaglandin and leukotriene production). However specific effects on primary cells in human milk (macrophages and epithelial cells) [33] and mammary tissue [34], which are the main source of cytokines in breast milk have not been determined. Lactating mammary glands are part of the integrated mucosal immune system and sIgA in

breast milk results from antigenic stimulation of the gut and airways [35]. It has recently been shown that LC-PUFA may enhance the beneficial effects of probiotic *Lactobacillus species* by promoting their adhesion to gut mucosal cells [36]. Once adhered, *Lactobacilli* stimulate the mucosal immune response towards the production of Th1 cytokines, IgA (from B cells) [37] and oral tolerance, which all prevent the development of atopy. This is supported by a recent study in which ingestion of *Lactobacillus GG* decreased the incidence of atopy in infants at high risk of developing atopy [38]. Further studies are needed to confirm the beneficial interaction between LC-PUFA and probiotics in the prevention of allergic disease.

We have already demonstrated that maternal n-3 PUFA status in pregnancy can influence neonatal immune function. The findings of this study contribute to a growing body of evidence that maternal dietary n-3 PUFA may also contribute to variations in the immunological properties of breast milk. The associations between maternal n-3 PUFA status and a number of immunological parameters in breast milk (sCD14, IgA and some cytokines) suggest a further avenue of influence of maternal diet on infant immune development in the postnatal period. Further studies are required to determine if these perinatal associations are related to the subsequent development of infant allergic disease.

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