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Immune factors and fatty acid composition in human milk from river/lake, coastal and inland regions of China†

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Abstract

Breast milk fatty acid composition may be affected by the maternal diet during gestation and lactation. The influence of dietary and breast milk fatty acids on breast milk immune factors is poorly defined. We determined the fatty acid composition and immune factor concentrations of breast milk from women residing in river/lake, coastal and inland regions of China, which differ in their consumption of lean fish and oily fish. Breast milk samples were collected on days 3–5 (colostrum), 14 and 28 post-partum (PP) and analysed for soluble CD14 (sCD14), transforming growth factor (TGF)-β1, TGF-β2, secretory IgA (sIgA) and fatty acids. The fatty acid composition of breast milk differed between the regions and with time PP. The concentrations of all four immune factors in breast milk decreased over time, with sCD14, sIgA and TGF-β1 being highest in the colostrum in the river and lake region. Breast milk DHA and arachidonic acid (AA) were positively associated, and γ-linolenic acid and EPA negatively associated, with the concentrations of each of the four immune factors. In conclusion, breast milk fatty acids and immune factors differ between the regions in China characterised by different patterns of fish consumption and change during the course of lactation. A higher breast milk DHA and AA concentration is associated with higher concentrations of immune factors in breast milk, suggesting a role for these fatty acids in promoting gastrointestinal and immune maturation of the infant.

Key words: Breast milk: DHA: Transforming growth factor β: Secretory IgA: Soluble CD14

The WHO[1] promotes exclusive breast-feeding for the first 6 months of life. Along with energy and nutrients, breast milk provides a broad range of immunological factors[2,3] of benefit to an infant during a time of gastrointestinal and immune system maturation. The long-chain PUFA (LC-PUFA) arachidonic acid (AA) and DHA are required for the development of the neonate[4]. Fatty acids in breast milk can be affected by the maternal diet during both gestation[5] and lactation[6]. LC *n*-3 PUFA are associated with reduced production of pro-inflammatory mediators and enhanced production of anti-inflammatory mediators[7]. They may also modulate components of both natural (innate) and acquired (specific or adaptive) immunity[8]. There is evidence that exposure to LC-PUFA can influence aspects of immune function in early life[9] and can have effects on later life[10]. However, the relationship between breast milk fatty acids and immune factors in breast milk is poorly defined.

In China, despite national food-based dietary guidelines[11], there are regional differences in diet due to tradition, local accessibility to food types and socio-economics[12].

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; LA, linoleic acid; LC-PUFA, long-chain PUFA; PP, post-partum; sCD14, soluble CD14; TGF, transforming growth factor.

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† The fatty acid composition of the mature milk studied here has been reported previously in Chinese in Gao Y, Zhang J, Wang C, *et al.* (2011) Fatty acid composition of mature human milk in three regions of China. *Wei Sheng Yan Jiu* **40**, 731–734.

Regional differences in lean fish and oily fish intake provide an excellent opportunity to investigate dietary influences on breast milk composition. In the present study, we investigated differences in breast milk fatty acids and immune factors and the relationships between them. Previous studies in China have demonstrated significant differences in the fatty acid composition of breast milk from coastal, pastoral, rural and urban regions, where the populations differ in their dietary intakes of meat, fish, cereal and dairy products[13–15], and differences in the macro- and micronutrient composition of breast milk between urban and rural areas[16]. However, to our knowledge, there are no studies examining both breast milk fatty acid composition and immune factor concentrations in regions that differ in diet. Here, we report fatty acid composition and immune factor concentrations in breast milk collected at three time points from women living in three regions of China characterised by different patterns of fish consumption (lean *v.* oily and marine *v.* freshwater); women in the coastal region consume lean fish and some oily fish, while those in the river and lake region consume lean fish and little oily fish, and those in the inland region consume little in the way of fish. Furthermore, women in the coastal region consume mainly marine water fish that has a higher content of LC *n*-3 PUFA for an equivalent fat content compared with freshwater fish[17], which is mainly consumed by the women in the river and lake region. In a previous study, differences in the dietary intake of several fatty acids, including AA, EPA and DHA, and of LC-PUFA were seen between pregnant women living in coastal, river/lake and inland regions[18].

Study design and methods used

Study design and subject characteristics

Aquamax China is a cross-sectional observational study of healthy pregnant women from three geographical regions of China associated with different dietary intakes of lean fish and oily fish and therefore of fatty acids. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Ethics Committee of the Institute of Nutrition & Food Safety, Chinese Center for Disease Control and Prevention. All women gave written informed consent. Women with a healthy singleton pregnancy were recruited from hospitals in Jurong county, Jiangusu province (river and lake region, *n* 42), Rizhao county, Shandong province (coastal region, *n* 42) and Xushui county, Hebei province (inland region, *n* 41) between 18 and 22 weeks of gestation. Breast milk samples were collected during the first month post-partum (PP) to represent colostrum (days 3–5 PP), transition milk (day 14 PP) and mature milk (day 28 PP). The milk was collected between 09.00 and 10.00 hours before the baby had been fed (i.e. foremilk) and was manually expressed into sterile containers and immediately stored at −20°C until transfer to the laboratory, where it was then stored at −80°C until analysis.

Assessment of maternal diet

A semi-quantitative FFQ including 213 foods was developed[19,20] and used to estimate energy, macronutrient, fat and fatty acid intakes at 34 weeks of gestation. The FFQ was administered by interview by trained nutritionists or nurses and with the aid of food models, bowls, plates and cups. Nutrient intakes were calculated based on the Chinese Food Composition Tables[21].

Breast milk fatty acid composition

Lipids were extracted from whole milk (100 μl) using chloroform–methanol (2:1, v/v; 5 ml) as published previously[22] with butylated hydroxytoluene (50 mg/l) added as an antioxidant. The mixture was emulsified with 1 M-NaCl (1 ml) and centrifuged at 715 ***g*** for 10 min. The lower organic layer was collected and dried under N_2 at 40°C. Fatty acid methyl esters were prepared by the addition of toluene (400 μl) and 1·5 % H_2SO_4 in methanol (800 μl) followed by incubation for 1 h at 70°C. The mixture was neutralised with 0·1 M-K_2CO_3:0·1 M-$KHCO_3$ (2 ml) and fatty acid methyl esters extracted into hexane (2 ml) and collected by centrifugation at 1120 ***g*** for 10 min; the upper layer was collected and dried under N_2 at 40°C. Hexane (150 μl) was added to redissolve the dried sample, which was then stored at −20°C until it was analysed by GC. Fatty acid methyl esters were resolved on a CP-SIL88 fused silica capillary column (50 m × 250 μm × 0·2 μm; Varian BV) using a HP6890 gas chromatograph (Agilent) equipped with a flame ionisation detector. Each sample (1 μl) was injected onto the column using an automatic injector at a split ratio of 100:1. He gas was used as a carrier. The detector and injector ports were set at 240 and 250°C, respectively. The oven temperature was maintained at 100°C for 4 min and the temperature was programmed to ramp to a maximum of 240°C at 3·5°C/min. Fatty acids of carbon lengths of 14–22 were identified by comparing retention times against authentic individual and mixed fatty acid methyl ester standards (NuChek Prep; Supelco Analytical). Fatty acids as a percentage of total fatty acids were determined from baseline corrected peak areas. Finally, three test samples of breast milk were analysed in duplicate for each new series of milk samples, and the average intra-assay CV was 1·5 % and the inter-assay CV was 4·1 %.

Breast milk immune factors

Immune factors were measured in whole milk, as described elsewhere[23]. Intra- and inter-assay CV were determined from four replicates within a single assay and over a total of five separate assays, respectively. The concentrations of soluble CD14 (sCD14), transforming growth factor (TGF)-β1 and TGF-β2 were measured using the Quantikine Human sCD14, TGF-β1 and TGF-β2 immunoassays (R&D Systems Europe). The manufacturer's instructions were followed and whole-milk samples were assayed at dilutions of 10 000 for sCD14, 1·4 for TGF-β1 and 7·0 for TGF-β2. Latent TGF-β in breast milk was activated to the immunoreactive form

as described for cell-culture supernatants with 1·0 M-HCl for 10 min and neutralised with 1·2 M-NaOH/0·5M-HEPES. For sCD14, the limit of detection was 125 pg/ml, the intra-assay CV 4% and the inter-assay 11%. For TGF-β1, the limit of detection was 7·0 pg/ml, the intra-assay CV 5% and the inter-assay CV 15%. For TGF-β2, the limit of detection was 7·0 pg/ml, the intra-assay CV 4% and the inter-assay CV 9%.

The concentration of sIgA was determined using the sIgA ELISA kit reported to have 100% cross-reactivity for human sIgA from breast milk (Demeditec Diagnostics). The manufacturer's instructions were followed but with some adjustment for dilution factor. Whole-milk samples were assayed at a dilution of 15 000, and the limit of detection was 1·2 μg/ml, the intra-assay CV 7% and the inter-assay CV 16%.

All plates were read on a GENios Spectra FLUOR plus (Tecan UK Limited).

Breast milk protein content

The summary of a workshop on immune factors in human milk[24] describes the difficulties in standardising samples when working in the field and recommended measuring immune factors both per unit volume and standardised against another milk component, e.g. K or total protein. Thus, the protein content of breast milk was determined using a standard method based on that of Bradford[25,26]. Human serum albumin (Sigma-Aldrich) was diluted with distilled water to produce a range of protein standards from 0·125 to 2·0 mg/ml. Standards or diluted whole-breast milk samples (10 μl) were mixed with Bradford reagent (500 μl). Immediately, 200 μl aliquots were transferred to wells of a ninety-six-well flat-bottom plate, and absorbance read at 595 nm on a GENios Spectra FLUOR plus. The limit of detection was 0·1 mg/ml, the intra-assay CV was 7·0% and the inter-assay CV was 9·0%.

Statistics

Data were checked for normality with histograms and the Kolmogorov–Smirnov test. Data are expressed as mean and standard deviation or median and 25th-75th percentiles as appropriate. Subject characteristics were analysed with the Kruskal–Wallis test, Fisher's exact test or χ^2 test as appropriate. Breast milk fatty acids were calculated as a percentage of total fatty acids. Breast milk immune factors were calculated as either absolute concentration or concentration per mg protein. All data were transformed into ranks and a rank ANCOVA for the effect of time, region and region × time interaction were determined, adjusting for covariates (parity, maternal age, maternal BMI and self-reported maternal atopy). Where appropriate, *post hoc* tests with Bonferroni correction were determined. Partial correlations of breast milk immune factors with dietary fatty acids were carried out on rank transformed data, controlling for region and repeated measures. Models for predicting breast milk immune factors were developed using multiple regression analysis. The independent variables selected for inclusion in the model were determined from initial significant bivariate correlations with the dependent variable. All multiple regression analyses were applied in conditions that ensured a suitable fit. These conditions were explored using relevant residual analyses and plots. The Statistical Package for Social Sciences version 15 (SPSS, Inc.) was used for all tests, and in all cases, a P value of $<0·05$ was considered as statistically significant ($P<0·05$ adjusted for multiple comparisons).

Results

Subject characteristics

The characteristics of the pregnant women from the three regions are shown in Table 1. Women from the coastal region were significantly older than women from the other two regions, and, in addition, there was a greater proportion of multipara women in the coastal region. Weight and BMI were different between the river/lake and coastal regions, with women from the coastal region weighing more and having a higher BMI. The weights and BMI of women from the inland region were not significantly different from the other two regions. The coastal region had the highest proportion of women self-reporting atopic disease. Overall, the level of education in the inland region was the poorest, with a majority of women having only attended junior high school. The number of women with a university or higher education was significantly higher in the river and lake region. All women were of Han ethnicity.

Maternal diet

Intake of lean fish by women from the river/lake and the coastal regions did not differ, but women from both of these regions had a significantly higher intake of lean fish than those from the inland region (Table 2). Women from the coastal region had a higher intake of oily fish compared with those from the river and lake region (Table 2).

Energy intakes were comparable among pregnant women in the three regions (Table 2). Women from the river and lake region consumed less protein as a percentage of total energy compared with those from the coastal region, and less fat and more carbohydrate as percentage of total energy compared with those from the inland region (Table 2). Women from the river and lake region had the highest intake of total *n*-3 PUFA, but this was mostly contributed by α-linolenic acid (ALA) (Table 2). In contrast, women from the coastal region had the lowest intake of total *n*-3 PUFA, and the highest dietary ratio of total *n*-6:total *n*-3 PUFA (Table 2). However, the intakes of EPA, docosapentaenoic acid (DPA) and DHA were significantly higher for women from the coastal region compared with those in the other two regions (Table 2). Women from the inland region had the highest intake of total *n*-6 PUFA, including linoleic acid (LA) and AA, but the lowest intake of EPA and DPA, while the intake of DHA was comparable with that of the river and lake region (Table 2).

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Table 1. Characteristics of the pregnant women across the three geographical regions of China
(Numbers of subjects and within-group percentages; medians and 25th–75th percentiles)

	River/lake (*n* 42)		Coastal (*n* 42)		Inland (*n* 41)		
	n	%	*n*	%	*n*	%	*P*
Age (years)							<0·001
Median	25·0[a]		27·5[b]		26·0[a]		
25th–75th percentiles	22·8–26·0		25·8–32·0		23·0–28·5		
Height (cm)							0·848
Median	160·0		160·0		160·0		
25th–75th percentiles	158·0–162·7		158·0–162·0		157·5–163·0		
Weight at 19 weeks (kg)							0·012
Median	50·0[a]		57·0[b]		53·0[a,b]		
25th–75th percentiles	46·0–57·5		53·5–61·0		48·0–60·0		
BMI at 19 weeks (kg/m^2)							0·004
Median	19·8[a]		22·2[b]		20·7[a,b]		
25th–75th percentiles	18·1–22·1		20·6–23·4		19·5–23·2		
No. of children including the current pregnancy*							
1	39	92·9	24	57·1	29	70·7	<0·001
2	2	4·8†	17	40·5†	12	29·3	
3	1	2·4	1	2·4	0	0·0	
Education*							
Primary school or below	0	0·0	3	7·1†	0	0·0	0·001
Junior high school	15	35·7	16	38·1	30	73·2†	
Senior high school or college	14	33·3	17	40·5	10	24·4	
Professional training	4	9·5	2	4·8	0	0·0	
University or higher	9	21·4†	4	9·5	1	2·4	
Passive smoking*							
No	33	78·6	27	64·3	29	70·7	0·468
Yes	9	21·4	15	35·7	12	29·3	
Alcohol intake*							
No	40	95·2	42	100	39	95·1	0·468
Yes	2	4·8	0	0·0	2	4·9	
Any atopic disease*‡							
No	39	92·9	26	61·9	35	85·4	0·001
Yes	3	7·1	16	38·1†	6	14·6	

[a,b] Values within a row with unlike superscript letters were significantly different ($P<0·05$; Kruskall–Wallis test).
* Groups compared by Fisher's exact test or χ^2 test as appropriate.
† *Post hoc* tests, based on the standardised residual being lower or higher than the critical values −1·96, +1·96, respectively, to be significant at $P<0·05$.
‡ Self-reported eczema, allergic rhinitis, asthma and atopic dermatitis.

Breast milk fatty acid composition

The fatty acid composition of breast milk from the three regions of China is shown in Table 3, where the data are presented as a percentage of total breast milk fatty acids. There was a significant effect of region and/or time and/or a significant region × time interaction for the majority of fatty acids (Table 3).

SFA

There was a significant effect of region ($P<0·001$) and time ($P=0·001$) and a region × time interaction ($P<0·001$) for the proportion of total SFA (Table 3). These were lowest in the colostrum from the river and lake region ($P<0·05$) and highest in the transition and mature milk from the inland region (both $P<0·05$). In the river and lake region and the inland region, the percentage of total SFA increased between the colostrum and transition milk, whereas it decreased in the coastal region (Table 3).

MUFA

There was a significant effect of region and time ($P<0·001$ for both) for the proportion of total MUFA; however, the region × time interaction was not significant ($P=0·121$) (Table 3). MUFA were higher in breast milk from the river and lake region compared with the coastal and inland regions at all time points ($P<0·05$ for all) and higher in the coastal region compared with the inland region in the colostrum and transition milk ($P<0·05$ for both). In the river and lake region, the percentage of oleic acid and of total MUFA decreased with time. However, in the coastal and inland regions, these did not differ significantly over time (Table 3).

PUFA

There was a significant effect of region ($P<0·001$) and time ($P=0·007$) and a region × time interaction ($P<0·001$) for total *n*-6 PUFA (Table 3). The percentage of LA was lowest in the milk from the river and lake region at all three time

Table 2. Daily dietary characteristics of the pregnant women at 34 weeks of pregnancy across the three geographical regions of China

(Mean values and standard deviations; medians and 25th–75th percentiles)

	River/lake		Coastal		Inland		
	Mean	SD	Mean	SD	Mean	SD	*P*
Lean fish (g)*							<0·001
Median	164[b]		157[b]		36[a]		
25th–75th percentiles	96–227		100–233		18–80		
Oily fish (g)*							0·001
Median	0[a]		6[b]		0[a,b]		
25th–75th percentiles	0–0		0–16		0–7		
Energy (MJ)†	11·7	2·6	11·7	2·8	11·4	3·3	0·868
Protein (% TE)†	15·1[b]	3·1	16·9[a]	2·7	15·6[a,b]	3·3	0·035
Fat (% TE)†	34·3[b]	7·0	35·5[a,b]	6·4	38·3[a]	5·4	0·016
Carbohydrate (% TE)†	50·9[a]	7·4	47·8[a,b]	6·6	46·4[b]	5·7	0·015
Total SFA (g)†	23·0	7·0	25·6	6·5	25·5	7·8	0·178
Total MUFA (g)†	40·9	11·7	38·6	8·7	37·2	10·3	0·262
Total PUFA (g)†	28·8[b]	10·1	30·0[b]	9·5	35·9[a]	12·0	0·007
Total *n*-6 PUFA (g)†	24·1[b]	9·3	28·1[b]	8·8	32·5[a]	10·8	0·001
18 : 2*n*-6 (g)†	23·9[b]	9·3	27·9[b]	8·8	32·2[a]	10·7	0·001
20 : 4*n*-6 (mg)*							<0·001
Median	101·1[c]		140·1[b]		170·2[a]		
25th–75th percentiles	85·1–139·9		90·7–159·5		82·3–259·3		
Total *n*-3 PUFA (g)†	4·7[a]	1·5	1·9[c]	1·2	3·4[b]	1·5	<0·001
18 : 3*n*-3 (g)†	4·6[a]	1·5	1·8[c]	1·1	3·4[b]	1·5	<0·001
20 : 5*n*-3 (mg)*							<0·001
Median	27·9[b]		64·6[a]		12·1[c]		
25th–75th percentiles	14·3–40·3		34·8–103·2		5·0–25·0		
22 : 5*n*-3 (mg)*							<0·001
Median	5·1[b]		15·7[a]		3·4[c]		
25th–75th percentiles	3·5–8·6		9·4–25·4		1·6–5·6		
22 : 6*n*-3 (mg)*							<0·001
Median	41·8[b]		93·9[a]		41·1[b]		
25th–75th percentiles	25·4–61·9		56·2–116·8		26·7–63·5		
Total *n*-6:total *n*-3 PUFA†	5·8[c]	4·1	17·8[a]	8·2	12·2[b]	9·1	<0·001

% TE, percentage of total energy.

[a,b,c] Values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Groups compared by Kruskall–Wallis test.

† Groups compared by ANOVA.

points (Table 3). The proportion of LA in the milk from the coastal and inland regions was not significantly different, except for the colostrum, where LA was lower in the coastal region ($P<0·05$) (Table 3). LA increased with time in the river/lake and coastal regions, whereas it did not differ significantly with time in the inland region. There was a significant region × time interaction ($P<0·001$) for the total LC *n*-6 PUFA; in transition milk, they were lower in the river and lake region compared with the coastal region, but the inland region did not differ from either. The total LC *n*-6 PUFA decreased between the colostrum and transition milk from the river and lake region and the inland region and between transition milk and mature milk from the coastal region (Table 3). The proportion of AA did not differ between the regions in the colostrum or mature milk, but was lower in the transition milk from the river and lake region compared with the coastal region; the inland region did not differ from either.

There was a significant effect of region and time and a region × time interaction for total *n*-3 PUFA and total LC *n*-3 PUFA ($P<0·001$ for all). The percentage of ALA was highest in the milk from the river and lake region at all three time points ($P<0·05$ for all), whereas it was comparable in the coastal and inland regions, except for mature milk, where it was higher in the coastal region. EPA, DPA and DHA were also higher in the river and lake region compared with the inland region at all three time points ($P<0·05$ for all). Compared with the coastal region, the river and lake region had higher total LC *n*-3 PUFA in the colostrum ($P<0·05$), a comparable content in transition milk and a lower content in mature milk ($P<0·05$). The total LC *n*-3 PUFA in the river/lake and inland regions decreased with time, which was not seen in the coastal region.

The total *n*-6:total *n*-3 PUFA ratio and the total *n*-6:total *n*-3 LC-PUFA ratio were significantly different between the three regions (Table 3): both ratios were highest in the inland region and lowest in the river and lake region at all three time points ($P<0·05$ for all) with the exception of mature milk, when the total *n*-6:total *n*-3 LC-PUFA ratio in the river/lake and coastal regions was not significantly different.

The relationships between dietary fatty acids and breast milk fatty acids are shown in Table 4. There were positive correlations between dietary LC *n*-3 PUFA and breast milk LC *n*-3 PUFA; however, there was no correlation between dietary AA and breast milk AA (Table 4). There was a positive correlation between dietary ALA and breast milk ALA and EPA, but no correlation with DPA and DHA. Dietary ALA was negatively

Table 3. Breast milk fatty acid composition (individual fatty acids as a percentage of total fatty acids) at days 3 to 5, 14 and 28 post-partum (PP) across the three geographical regions of China (Medians and 25th–75th percentiles)

		River/lake (*n* 42)		Coastal (*n* 42)		Inland (*n* 41)		*P**		
Fatty acid	Days PP	Median	25th–75th percentile	Median	25th–75th percentile	Median	25th–75th percentile	R	T	R × T
12 : 0	3–5	1·38[a]	0·92–2·20	2·27[b]	1·78–4·26	2·63[b]	1·47–4·66	0·002	<0·001	<0·001
	14	4·79[b]†	4·01–5·44	3·53[a]	1·46–5·04	5·06[b]†	3·56–6·60			
	28	4·26[a,b]†	3·46–4·97	3·65[a]	2·21–5·70	4·84[b]†	3·68–6·34			
14 : 0	3–5	3·32[a]	2·48–4·04	4·30[b]	3·76–5·71	4·14[a,b]	3·02–5·05	0·005	0·089	<0·001
	14	4·11[a,b]†	3·45–5·17	3·76[a]	3·50–5·39	5·05[b]	4·10–6·39			
	28	3·37[a]‡	2·82–4·30	4·90[b]‡	3·77–6·49	4·81[b]	3·74–6·21			
16 : 0	3–5	21·73[a]	20·60–22·69	22·37[a,b]	20·65–24·02	23·08[b]	22·17–24·18	<0·001	<0·001	0·003
	14	19·86[a]†	18·31–21·00	20·41[a]†	19·22–20·95	22·14[b]	20·80–24·00			
	28	19·75[a]†	18·32–20·86	20·14[a]†	18·34–20·34	21·34[b]†	19·80–23·35			
18 : 0	3–5	4·69[a]	4·26–5·85	5·96[b]	5·24–6·66	5·46[b]	4·80–6·28	0·056	<0·001	0·006
	14	5·56†	5·20–6·25	5·85	5·68–6·38	6·10	5·29–6·92			
	28	5·83†	5·27–6·33	6·16	5·14–6·37	5·68	5·26–6·27			
Total medium-chain SFA	3–5	4·57[a]	3·56–6·65	7·34[b]	5·47–9·79	6·49[b]	4·60–9·60	0·009	<0·001	<0·001
	14	8·87[a,b]†	7·74–10·50	7·10[a]	5·23–10·63	10·73[b]†	7·15–13·12			
	28	7·70†	6·20–9·12	8·36	5·99–11·67	9·43†	7·61–12·29			
Total SFA	3–5	31·02[a]	30·38–32·60	35·95[b]	33·43–37·41	35·46[b]	33·31–38·42	<0·001	0·001	<0·001
	14	34·50[a]†	33·29–36·32	32·56[a]†	31·59–36·95	38·41[b]†	35·93–41·44			
	28	32·76[a]†	30·65–36·47	33·39[a]†	32·53–36·44	37·44[b]	33·31–40·33			
16 : 1*n*-7	3–5	1·89	1·73–2·50	1·87	1·59–2·45	1·98	1·62–2·37	0·008	0·664	0·020
	14	2·20[b]	1·87–2·66	1·71[a]	1·59–1·87	2·11[b]	1·77–2·44			
	28	2·20[b]	1·87–2·38	1·61[a]	1·57–1·87	2·11[b]	1·72–2·62			
18 : 1*n*-9	3–5	37·87[c]	36·50–39·04	32·98[b]	29·56–34·11	30·71[a]	29·37–32·72	<0·001	0·022	0·002
	14	35·61[b]†	33·05–38·12	33·10[b]	31·83–34·05	30·13[a]	27·89–32·42			
	28	36·87[c]†	33·13–38·60	32·02[b]	30·56–33·54	30·13[a]	27·76–32·61			
t-18 : 1*n*-9	3–5	3·98[c]	3·77–4·15	3·50[b]	3·23–3·69	3·27[a]	3·03–3·57	<0·001	<0·001	0·192
	14	3·54[c]†	3·27–3·66	2·99[b]†	2·53–3·56	2·96[a]†	2·60–3·24			
	28	3·37[b]†	3·29–3·69	2·80[a,b]†	2·47–3·74	2·87[a]†	2·56–3·14			
Total MUFA	3–5	44·24[c]	42·81–44·83	38·45[b]	35·29–39·99	36·07[a]	34·76–38·10	<0·001	<0·001	0·121
	14	41·11[c]†	38·73–43·36	38·04[b]	37·16–38·47	35·56[a]	33·00–37·81			
	28	42·48[b]†	38·86–44·45	37·06[a]	34·93–38·89	35·43[a]	32·23–38·20			
18 : 2*n*-6 (LA)	3–5	16·02[a]	14·89–16·79	18·35[b]	17·41–21·16	21·63[c]	19·40–24·64	<0·001	<0·001	<0·001
	14	16·99[a]†	15·51–19·10	22·44[b]†	19·43–23·72	20·47[b]	17·62–23·18			
	28	16·68[a]†	15·79–20·10	22·79[b]†	20·91–24·18	21·43[b]	18·03–26·19			
18 : 3*n*-6	3–5	0·00[a]	0·00–0·00	0·04[b]	0·00–0·06	0·07[b]	0·03–0·12	<0·001	<0·001	<0·001
	14	0·13[b]†	0·10–0·17	0·06[a]†	0·00–0·16	0·13[b]†	0·09–0·19			
	28	0·13[b]†	0·09–0·17	0·02[a]‡	0·00–0·08	0·14[b]†	0·10–0·21			
20 : 2*n*-6	3–5	1·13	0·94–1·34	1·17	0·84–1·48	1·16	0·65–1·58	0·014	<0·001	<0·001
	14	0·53[a]†	0·43–0·61	0·75[b]	0·55–1·74	0·58[a]†	0·55–0·71			
	28	0·50[a]†	0·44–0·54	0·60[a,b]†	0·44–1·45	0·58[b]†	0·55–0·65			
20 : 3*n*-6	3–5	0·72	0·57–0·89	0·63	0·55–0·79	0·73	0·56–0·92	0·013	<0·001	<0·001
	14	0·50[a]†	0·43–0·61	0·63[b]	0·55–0·70	0·61[b]†	0·51–0·73			
	28	0·47[a]†	0·36–0·53	0·50[a,b]†‡	0·30–0·68	0·57[b]†	0·49–0·69			
20 : 4*n*-6 (AA)	3–5	0·94	0·82–1·11	0·85	0·76–1·01	0·88	0·82–0·96	0·036	<0·001	0·244
	14	0·85†	0·75–0·95	0·80	0·71–1·00	0·82†	0·73–0·88			
	28	0·76[b]†	0·70–0·92	0·71[a,b]†‡	0·68–0·74	0·70[a]†‡	0·60–0·83			
Total *n*-6 PUFA	3–5	18·78[a]	17·89–19·83	21·61[b]	20·05–24·18	24·38[c]	21·56–27·79	<0·001	0·007	<0·001
	14	19·10[a]	17·57–21·65	25·78[b]†	21·95–26·27	22·60[b]	19·79–25·56			
	28	18·74[a]	17·67–21·94	24·25[b]†	23·74–26·66	23·59[b]	20·76–28·04			
Total LC *n*-6 PUFA	3–5	2·79	2·37–3·34	2·58	2·42–3·18	2·71	2·06–3·45	0·167	<0·001	<0·001

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Table 3. *Continued*

Fatty acid	Days PP	River/lake (*n* 42)		Coastal (*n* 42)		Inland (*n* 41)		*P**		
		Median	25th–75th percentile	Median	25th–75th percentile	Median	25th–75th percentile	R	T	R × T
	14	1·87[a]†	1·74–2·02	2·43[b]	1·89–3·52	1·99[a,b]†	1·84–2·29			
	28	1·80†	1·57–1·90	1·91†‡	1·45–2·84	1·88†	1·70–2·07			
18 : 3*n*-3 (ALA)	3–5	1·39[b]	1·10–1·71	0·83[a]	0·52–1·03	0·80[a]	0·53–0·99	<0·001	<0·001	0·136
	14	1·71[b]†	1·33–1·95	0·96[a]	0·80–1·18	0·90[a]	0·61–1·15			
	28	1·84[c]†	1·36–2·19	1·25[b]†‡	1·08–1·32	0·92[a]†	0·66–1·44			
20 : 3*n*-3	3–5	0·19[b]	0·17 –0·23	0·09[a]	0·06–0·14	0·08[a]	0·06–0·12	<0·001	<0·001	<0·001
	14	0·08[b]†	0·07–0·10	0·10[b]	0·04–0·14	0·05[a]†	0·04–0·07			
	28	0·09[b]†	0·07–0·11	0·08[b]	0·00–0·15	0·05[a]†	0·04–0·07			
20 : 5*n*-3 (EPA)	3–5	0·05[b]	0·00–0·09	0·03[b]	0·00–0·06	0·00[a]	0·00–0·03	<0·001	<0·001	0·002
	14	0·07[b]†	0·05–0·09	0·00[a]	0·00–0·07	0·03[a]†	0·00–0·04			
	28	0·06[b]†	0·05–0·08	0·04[b]	0·00–0·26	0·03[a]†	0·00–0·04			
22 : 5*n*-3 (DPA)	3–5	0·43[b]	0·31–0·53	0·27[a]	0·21–0·33	0·23[a]	0·17–0·30	<0·001	<0·001	<0·001
	14	0·22[b]†	0·19–0·27	0·25[b]	0·17–0·41	0·16[a]†	0·13–0·21			
	28	0·22[b]†	0·19–0·27	0·29[c]	0·23–0·37	0·16[a]†	0·12–0·20			
22 : 6*n*-3 (DHA)	3–5	0·66[b]	0·51–0·85	0·61[b]	0·44–0·76	0·41[a]	0·33–0·54	<0·001	<0·001	0·008
	14	0·53[b]†	0·44–0·65	0·53[b]	0·44–0·62	0·35[a]†	0·29–0·41			
	28	0·50[b]†	0·40–0·60	0·55[b]	0·43–1·38	0·27[a]†	0·23–0·35			
Total *n*-3 PUFA	3–5	2·63[c]	2·51–3·16	1·68[b]	1·54–2·40	1·59[a]	1·22–2·12	<0·001	<0·001	<0·001
	14	2·64[c]	2·32–2·88	2·09[b]	1·64–2·35	1·50[a]	1·26–1·79			
	28	2·67[b]	2·27–3·22	2·43[b]†‡	2·13–3·15	1·54[a]	1·23–2·06			
Total LC *n*-3 PUFA	3–5	1·26[c]	1·10–1·62	0·98[b]	0·83–1·26	0·75[a]	0·61–0·96	<0·001	<0·001	<0·001
	14	0·91[b]†	0·78–1·09	0·90[b]	0·81–1·25	0·60[a]†	0·50–0·68			
	28	0·85[b]†	0·75–1·04	1·18[c]	0·83–1·97	0·49[a]†	0·43–0·65			
Total *n*-6 : *n*-3 PUFA	3–5	6·87[a]	5·75–7·77	11·82[b]	10·58–13·74	14·99[c]	12·67–17·67	<0·001	<0·001	<0·001
	14	7·86[a]	5·64–9·43	12·27[b]	10·57–15·87	15·24[c]	13·16–17·08			
	28	7·57[a]	5·69–9·08	10·38[b]	8·46–11·05	15·39[c]	12·54–18·09			
n-6:*n*-3 LC-PUFA	3–5	2·12[a]	1·90–2·38	2·66[b]	2·35–3·24	3·56[c]	2·97–4·44	<0·001	0·102	0·033
	14	1·99[a]	1·73–2·46	2·51[b]	2·30–3·35	3·44[c]	3·17–4·18			
	28	2·08[a]	1·57–2·44	2·39[a]	0·74–3·25	3·60[b]	3·06–4·21			

R, region; T, time; LA, linoleic acid; AA, arachidonic acid; LC, long chain; ALA, α-linolenic acid; DPA, docosapentaenoic acid.
[a,b,c] Median values within a row with unlike superscript letters were significantly different ($P<0·05$).
* *P* values for two-factor rank ANCOVA adjusting for maternal age, BMI, parity and atopy.
† Median values were significantly different from the day 3–5 sample (colostrum) ($P<0·05$).
‡ Median values were significantly different from the day 14 sample (transition milk) ($P<0·05$).

Table 4. Relationship between dietary fatty acid intakes (g/d)† and breast milk fatty acids (% of total fatty acids)‡

	Dietary fatty acid					
Breast milk fatty acid	LA	AA	ALA	EPA	DPA	DHA
18 : 2*n*-6 (LA)	0·164**	0·064	−0·257***	0·043	0·088	0·128*
20 : 4*n*-6 (AA)	−0·024	−0·069	0·038	0·015	0·028	−0·043
18 : 3*n*-3 (ALA)	−0·209***	−0·247***	0·341***	−0·041	−0·009	−0·170**
20 : 5*n*-3 (EPA)	−0·186***	−0·207***	0·201***	0·065	0·051	−0·037
22 : 5*n*-3 (DPA)	−0·105*	−0·094	−0·018	0·304***	0·297***	0·217***
22 : 6*n*-3 (DHA)	−0·192***	−0·243***	−0·098	0·296***	0·316***	0·141**

LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid.
* $P<0·05$,** $P<0·01$, *** $P<0·001$.
† Dietary intakes of fatty acids are from FFQ administered at 34 weeks of pregnancy.
‡ Values are partial correlation coefficients of rank-transformed data, for the whole population, controlling for repeated measures.

correlated with milk LA and, conversely, dietary LA was positively correlated with milk LA and negatively correlated with milk LC *n*-3 PUFA and ALA (Table 4).

Breast milk protein

There was a significant effect of region ($P=0·009$) and time ($P<0·001$) and a region × time interaction ($P=0·001$) for breast milk total protein (Table 5). The protein concentration was highest in the colostrum from the river and lake region, and it was comparable across all three regions in the transition milk and lowest in the mature milk from the coastal region (Table 5). There was no significant effect of parity, maternal age, BMI or maternal self-reported atopy on breast milk total protein (data not shown).

Breast milk immune factors

There was a 100 % detection rate for each of the four immune factors measured. The concentrations of the breast milk immune factors (sCD14, sIgA, TGF-β1 and TGF-β2) for each region are shown in Table 5. Unadjusted analysis (Kruskall–Wallis) identified significant differences between the regions for all immune factors at at least one time point (data not shown). A more detailed analysis using rank-transformed data in an ANCOVA (rank ANCOVA), which considered maternal age, BMI, parity and self-reported atopy as covariates, identified a significant effect of time and a significant region × time interaction for each of the immune factors and also a significant region effect for TGF-β1 ($P=0·043$; Table 5). The concentrations of sCD14, sIgA and TGF-β1 were higher in the colostrum from the river and lake region compared with the coastal and inland regions (mean 1·5-, 3·0- and 1·4-fold higher, respectively; $P=0·05$ for all) and did not differ between the coastal and inland regions. Colostrum TGF-β2 was also higher in the river and lake region compared with the coastal region (1·3-fold) and the inland region (1·7-fold); however, in each case, this was not statistically significant. In transition milk, sCD14 and sIgA were not significantly different across the regions, but TGF-β1 in the coastal region was 2·2-fold higher than that in the river and lake region ($P=0·05$) and 1·5 fold higher than that in the inland region (NS). TGF-β2 concentration in the transition milk from the coastal region was 1·9-fold higher than that from the river and lake region ($P=0·05$) and 1·4-fold higher than that from the inland region (NS). In mature milk, only TGF-β1 differed significantly between the regions, again being higher in the coastal region compared with the river/lake (1·6-fold, $P=0·05$) and the inland regions (1·3-fold, NS); the river and lake region and the inland region were not significantly different from each other. For all regions, each of the immune factors decreased with time (Table 5). When the data were adjusted for total protein content (concentration per mg protein), a significant region × time interaction remained for all immune factors ($P<0·01$ for all; data not shown), with the exception of sCD14, where there was a trend towards significance ($P=0·077$; data not shown). There was also a significant effect of time for each of the immune factors ($P<0·01$ for all; data not shown), an effect of region for TGF-β1 ($P=0·001$; data not shown) and a trend towards an effect of region for TGF-β2 ($P=0·078$; data not shown).

There were positive partial correlations (controlling for region and repeated measures) among all the immune factors: TGF-β1 with TGF-β2 (r 0·681), sIgA (r 0·541) and sCD14 (r 0·536); TGF-β2 with sIgA (r 0·443) and sCD14 (r 0·450); sIgA with sCD14 (r 0·564) ($P<0·001$ for all).

There were no significant effects of maternal age, BMI and parity on breast milk immune factors. There was a significant maternal atopy × time interaction for TGF-β1 and TGF-β2; both were higher in the transition milk from atopic women compared with non-atopic women ($P<0·05$ for both; data not shown). However, when region was used as a covariate in an ANCOVA, the effect of maternal atopy on TGF-β1 was no longer significant.

The influence of individual variables in multiple linear regression analyses of the breast milk immune factors is shown in Table 6. In both models, maternal age, BMI, self-reported atopy and parity were included but their effects are not shown in the table; maternal self-reported atopy was not a significant predictor for any of the immune factors measured. Maternal age showed a trend towards being a weak positive predictor of TGF-β1 ($\beta=0·100$, $P<0·075$) and TGF-β2 ($\beta=0·123$, $P<0·05$). Parity had a trend towards being a weak negative predictor for sCD14 ($\beta=0·090$, $P<0·075$) and TGF-β2 ($\beta=0·114$, $P<0·060$), while BMI was a significant but weak positive predictor of sIgA ($\beta=0·080$, $P<0·05$) and

Table 5. Breast milk protein and immune factor concentrations at days 3 to 5, 14 and 28 post-partum (PP) across the three geographical regions of China
(Medians and 25th–75th percentiles)

		River/lake (*n* 42)		Coastal (*n* 42)		Inland (*n* 41)		*P**		
Immune factor	Days PP	Median	25th–75th percentile	Median	25th–75th percentile	Median	25th–75th percentile	R	T	R × T
Protein (mg/ml)	3–5	19·0[a]	16·3–19·8	14·5[b]	13·5–15·7	15·0[b]	12·7–18·9	0·009	<0·001	0·001
	14	12·4†	11·6–13·2	11·9†	9·2–13·2	11·8†	10·9–13·1			
	28	11·6[a]†‡	10·2–12·9	9·2[b]†‡	8·0–11·3	11·8[a]†	10·7–13·0			
sCD14 (μg/ml)	3–5	19·7[b]	14·2–25·3	13·6[a]	10·5–18·6	13·4[a]	10·3–17·6	0·340	<0·001	<0·001
	14	9·0†	6·9–10·7	8·6†	7·0–11·1	9·6†	7·1–12·8			
	28	7·5†	5·9–10·0	7·0†‡	5·8–8·1	7·9†	6·3–10·9			
sIgA (μg/ml)	3–5	3940[b]	2275–6035	1236[a]	739–2117	1384[a]	617–2677	0·357	<0·001	<0·001
	14	546†	438–753	596†	471–1348	620†	446–741			
	28	438†	370–667	511†‡	430–584	488†	384–736			
TGF-β1 (pg/ml)	3–5	1151[b]	837–2156	903[a]	583–1274	846[a]	539–1339	0·043	<0·001	<0·001
	14	303[a]†	243–432	680[b]	498–1112	451[b]†	389–635			
	28	336[a]†	249–469	538[b]†	450–772	404[a,b]†	338–482			
TGF-β2 (pg/ml)	3–5	6370	3331–12 735	4901	2530–7266	3734	2350–8745	0·371	<0·001	<0·001
	14	1763[a]†	1127–3241	3364[b]	2815–4617	2457[a,b]†	1801–3908			
	28	1816†	1133–3625	2346	1920–8503	2224†	1465–2873			

R, region; T, time; sCD14, soluble CD14; sIgA, secretory IgA; TGF-β, transforming growth factor-β.
[a,b,c]Median values within a row with unlike superscript letters were significantly different ($P<0·05$).
* *P* values for two-factor rank ANCOVA adjusting for maternal age, BMI, parity and atopy.
† Median values were significantly different from the day 3–5 sample (colostrum) ($P<0·05$).
‡ Median values were significantly different from the day 14 sample (transition milk) ($P<0·05$).

TGF-β1 ($\beta = 0·114$, $P<0·01$). In general, the change from colostrum to transition milk predicted a decrease in the concentration of each of the immune factors and the change from transition to mature milk predicted a further decrease in sCD14 and sIgA (Table 6). Breast milk γ-linolenic acid was a negative predictor and breast milk AA a positive predictor of all immune factors, except for TGF-β2, where AA was not a significant predictor (Table 6). Due to concerns of multi-collinearity, breast milk *n*-3 PUFA were analysed in a separate model and DHA was a positive predictor of all immune factors and EPA a negative predictor for almost all, with a trend towards being a negative predictor for TGF-β2 (Table 6). When AA and DHA were replaced by dihomo-γ-linolenic acid and DPA in the two separate models, respectively, again to avoid multi-collinearity, both were positive predictors of each of the immune factors measured ($P<0·001$ for all, data not shown) except for TGF-β2, where milk dihomo-γ-linolenic acid was not a significant predictor.

There were no significant partial correlations (controlling for region and time) between dietary stearic acid, total SFA, oleic acid and total MUFA during pregnancy with any of the breast milk immune factors (data not shown). The partial correlations between milk immune factors and dietary intakes of *n*-6 and *n*-3 fatty acids during pregnancy are shown in Table 7. Dietary intakes of EPA, DPA and DHA were positively correlated with TGF-β1 and TGF-β2, and there was a negative correlation of dietary ALA intake with TGF-β1 (Table 7). Dietary intakes of LA and AA did not correlate with any of the immune factors (Table 7).

Discussion

Women in the three geographical regions studied here have different patterns of consumption of several foods, including freshwater/marine and lean/oily fish. Women in the coastal region consume lean and some oily fish, while those in the river and lake region consume lean fish and those in the inland region consume little in the way of fish. Consequently, pregnant women in these three regions have different intakes of EPA, DHA and total LC *n*-3 PUFA, as reported previously for pregnant Chinese women living in such regions[18]. Previous studies have reported differences in breast milk fatty acids in Chinese women living in urban, rural, pastoral, coastal and inland regions, which are largely attributable to diet[13–15]. The present study confirms significant differences in the fatty acid composition of breast milk from regions of China differing in their consumption of aquatic foods. However, for the first time, it demonstrates differences in the concentrations of breast milk immune factors between these regions and, further, that consumption of fatty acids by the women during pregnancy and the fatty acid composition of breast milk are important predictors of breast milk immune factor concentrations. Breast milk immune factors play a key role in the defence of the newborn infant[2] and in their early gastrointestinal and immune maturation[3]. Thus, these observations suggest an important link between maternal diet, breast milk fatty acids, breast milk immune factors and infant development.

Table 6. Influence of individual variables in multiple linear regression models for the breast milk immune factors*
(β Coefficients and *P* values)

	Breast milk immune factor							
	sCD14		sIgA		TGF-β1		TGF-β2	
Individual variable	β†	*P*	β	*P*	β	*P*	β	*P*
Time PP; colostrum to transition milk	−0·325	<0·001	−0·333	<0·001	−0·211	<0·001	−0·157	0·009
Time PP; transition to mature milk	−0·129	<0·001	−0·173	<0·001	−0·080	0·081	−0·053	0·335
Breast milk 18 : 3*n*-6	−0·238	<0·001	−0·334	<0·001	−0·478	<0·001	−0·356	<0·001
Breast milk 20 : 4*n*-6	0·254	<0·001	0·116	<0·001	0·109	0·010	0·064	0·211
Adjusted R^2	0·461	<0·001	0·484	<0·001	0·479	<0·001	0·247	<0·001
Time PP; colostrum to transition milk	−0·429	<0·001	−0·462	<0·001	−0·336	<0·001	−0·266	<0·001
Time PP; transition to mature milk	−0·161	<0·001	−0·161	<0·001	−0·046	0·340	−0·023	0·670
Breast milk 20 : 5*n*-3	−0·119	0·022	−0·137	0·008	−0·377	<0·001	−0·108	0·065
Breast milk 22 : 6*n*-3	0·203	<0·001	0·161	0·002	0·309	<0·001	0·324	<0·001
Adjusted R^2	0·383	<0·001	0·402	<0·001	0·392	<0·001	0·218	<0·001

sCD14, soluble CD14; sIgA, secretory IgA; TGF-β, transforming growth factor-β; PP, post-partum.
* The characteristics of the mother (age, BMI, self-reported atopy and parity) were included in each of the models but the results are not shown.
† β Standardised regression coefficient.

Previous reports have shown that breast milk lipid content varies significantly between mothers[27] and increases between the start and the end of a feed (i.e. between foremilk and hindmilk)[27,28], and that the difference in breast milk lipid content between the end of one feed and the start of another is inversely related to the duration of time between the feeds[27]. However, despite reported differences in breast milk lipid content between foremilk and hindmilk, there was no significant difference in the breast milk fatty acid composition between foremilk and hindmilk[29]. In the present study, breast milk samples were collected before the baby had been fed (i.e. foremilk) and they were analysed for breast milk fatty acid composition and not breast milk lipid content. The consumption of an oily fish meal during lactation has a transient acute effect on breast milk LC *n*-3 PUFA composition, and the time lapse between eating the oily fish meal and the sampling of breast milk influences the size of the effect observed[30]. In the present study, breast milk samples were collected at the same time each day between 09.00 and 10.00 hours. It has previously been reported that breast milk protein does not vary significantly between foremilk and hindmilk but does vary significantly between mothers[31].

In the present study, the proportion of LC *n*-3 PUFA in the colostrum was highest in the river and lake > coastal > inland region and the proportion of milk DHA was of the order river and lake = coastal > inland region, even though women in the coastal region had the highest intake of preformed dietary LC *n*-3 PUFA. However, women in the river and lake region had the highest intake of total *n*-3 PUFA, mainly due to ALA, compared with the coastal and inland regions. Fatty acids in breast milk can be derived from the diet[6], from adipose tissue stores[5] or from endogenous synthesis[32]. Although conversion of ALA to DHA in humans is limited, it has been shown to be enhanced in women taking the contraceptive pill containing 17α-ethynyloestradiol[32]. Thus, it is possible that the higher circulating oestrogen levels during pregnancy enhance the conversion of ALA to DHA and so increase the content of DHA in adipose tissue. Indeed, studies in rodents have shown that consuming a diet rich in ALA from conception results in DHA accretion rates that are similar to those achievable with dietary DHA[33,34] and that fetal DHA deficiency can be largely reversed with maternal ALA consumption[35]. However, a study of pregnant women supplemented with ALA and LA did not report a significant increase in maternal and neonatal DHA status, only in EPA and DPA[36]. This may have been due to the fact that women were only supplemented during the second and third trimesters with 2·8 g ALA/d, whereas women from the river and lake region in the present study had a high habitual intake of ALA (mean 4·6 g/d). Interestingly, total LC *n*-3 PUFA in milk from the river/lake and inland regions decreased with time, whereas women from the coastal region had the

Table 7. Relationships between the breast milk immune factors and dietary fatty acid intakes† (g/d) during pregnancy‡

	Dietary fatty acid					
Immune factor	LA	ALA	AA	EPA	DPA	DHA
sCD14	0·041	0·027	−0·098	0·013	0·066	−0·067
sIgA	0·014	0·055	0·023	0·021	0·042	−0·006
TGF-β1	0·010	−0·195***	0·008	0·265***	0·315***	0·222***
TGF-β2	0·002	−0·086	−0·045	0·142**	0·207***	0·119*

LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; sCD14, soluble CD14; sIgA, secretory IgA; TGF-β, transforming growth factor-β.
* $P<0·05$,** $P<0·01$, *** $P<0·001$.
† Dietary intakes of fatty acids are from FFQ administered at 34 weeks of pregnancy.
‡ Values are partial correlation coefficients of rank-transformed data, controlling for region and time.

highest dietary intake of preformed LC *n*-3 PUFA, and milk proportions of LC *n*-3 PUFA did not change significantly over time, so that in mature milk, the levels in the coastal region were the highest (coastal > river and lake > inland).

Milk production is stimulated by prolactin, which suppresses oestrogen activity, which in turn could down-regulate the previously enhanced conversion of ALA to DHA. Thus, it is plausible that the endogenous conversion of ALA to LC *n*-3 PUFA is enhanced under the influence of oestrogen during pregnancy, but not maintained during lactation, when oestrogen is suppressed. Indeed, lactating women who were supplemented for 4 weeks with flax seed oil (providing 10·7 g ALA/d) did not experience an increase in milk DHA[37]. The ratio of *n*-6:*n*-3 PUFA in the diet may also provide a plausible explanation for the differences in the proportions of LC *n*-3 PUFA in breast milk among the three regions. Although women in the coastal region had the highest intake of preformed LC *n*-3 PUFA, the dietary *n*-6:*n*-3 PUFA ratio was 15:1 compared with 5:1 in the river and lake region and 10:1 in the inland region. Diets high in LA are associated with a reduced incorporation of preformed LC *n*-3 PUFA into tissue phospholipids in non-human primates[38], and decreasing the LA content of infant formula is as effective as increasing the ALA content with regard to increasing EPA and DHA in infant erythrocytes[39]. In the present study, there were weak, but significant, negative correlations between dietary LA and both breast milk ALA and LC *n*-3 PUFA, and positive correlations between dietary ALA and breast milk ALA and EPA.

The concentrations of each of the immune factors decreased within the first month of lactation, as previously reported by others[40]; this coincides with an increase in the volume of breast milk consumed by the infant over the same period of time[41]. Between days 3 and 28 PP, milk intake by the infant approximately doubles[32]. In the present study, the concentrations of the milk immune factors typically decreased by 50–60 % between these times (although sIgA decreased by 90 % in the river and lake region), suggesting that, in general, the actual intake of the immune factors (except sIgA in the river and lake region) would not be altered. There were also significant regional differences in immune factors, which were influenced by the stage of lactation. Colostrum from the river and lake region, which had the highest proportion of LC *n*-3 PUFA, had the highest concentrations of sCD14, sIgA and TGF-β1; the concentration of TGF-β2 was also higher, but this was not statistically significant. There was a positive association of milk TGF-β1 with milk DPA, DHA, dihomo-γ-linolenic acid and AA, which agrees with the findings of Ferrucci *et al.*[42], where there was a positive correlation of TGF-β with DHA and AA in human plasma. However, in a previous intervention study with oily fish[23] and in a high-dose fish oil intervention study[43], increasing dietary LC *n*-3 PUFA during the latter half of pregnancy did not significantly affect the concentrations of TGF-β1 or TGF-β2 in breast milk, and there was no association of milk DHA with milk TGF-β. This suggests that factors other than dietary fatty acid intake and/or breast milk fatty acid composition play a role in the regional differences in breast milk immune factors.

Breast milk sCD14 was higher in the colostrum from women from the river and lake region. There was a positive association between sCD14 and breast milk AA and a negative association with milk γ-linolenic acid, as previously reported by others in maternal serum and breast milk, respectively[44]. Dunstan *et al.*[45] were the first to report a positive correlation between sCD14 and milk DPA (but not DHA) in the colostrum after a high-dose fish oil intervention. In the present study, there was a positive association between sCD14 and DPA during the first month of lactation, and sCD14 was positively associated with milk DHA and negatively with milk EPA. The lack of a correlation between sCD14 and milk DHA and EPA content in the study of Dunstan *et al.*[45] may possibly be explained by the fact that they measured sCD14 only in the colostrum, whereas we report sCD14 concentrations at three time points throughout the first month of lactation. Also, the DHA content of the colostrum in the Dunstan *et al.* study[45] was much higher at >1·0 % of fatty acids, which coincided with a concomitant decrease in AA.

Breast milk sIgA was positively associated with AA and negatively with EPA. Some studies have reported that the AA-derived mediator, PGE_2, promotes IgA synthesis[46,47] and since LC *n*-3 PUFA may reduce PGE_2, this could explain the negative association with EPA. However, there was also a positive association of sIgA with DPA and DHA in both the present study and that of Dunstan *et al.*[45], and this may be linked to the positive association between DPA, DHA and TGF-β, the latter of which is involved in the switching of B cells to produce sIgA[48].

TGF-β is one of the most abundant cytokines in human milk[49]. It has pleiotropic effects, including promoting oral tolerance[50], cell proliferation and differentiation[51,52] and protection against allergy-related outcomes in infancy and childhood[53]. There have been contrasting reports on the effect of maternal atopy on concentrations of TGF-β in breast milk[23,53]. In the present study, TGF-β1 and TGF-β2 were higher in the transition milk of atopic women compared with non-atopic women. However, the majority of atopic women lived in the coastal region and when this was considered in the analysis, the effect of atopy was no longer significant.

sCD14 provides the neonate with protection against pathogenic invasion, since the CD14-independent pathway in response to endotoxin is lacking in neonates[54]. Several studies have associated higher concentrations of sCD14 in breast milk with a protective effect towards infant and childhood atopy[55–57], although this is not unequivocal[58]. Also, newborns do not produce their own protective levels of sIgA until 30 d PP, but breast-fed neonates benefit from the supply and passive protection afforded by sIgA within their mothers' milk[59]. Reductions in these immune factors could potentially alter the maturation of the neonatal immune system, the risk of allergy[53,60] and the overall health[61] of the infant.

The present cross-sectional study took advantage of dietary contrasts in three geographical regions, and while it is unable to deduce causality, it provides valuable insight into the associations between breast milk immune factors and dietary

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and breast milk fatty acids. It is likely that factors other than dietary and breast milk fatty acids play a role in regional differences in breast milk composition; many women in China still follow traditional cultural practices during the first month PP[62], with restrictions on diet, hygiene and physical activity, but available details regarding these practices are limited.

In conclusion, breast milk fatty acids and immune factors differ between the regions in China characterised by different patterns of lean fish and oily fish consumption and the change during the course of lactation. A higher breast milk DHA and AA concentration is associated with higher concentrations of immune factors in breast milk, suggesting a role for these fatty acids in promoting gastrointestinal and immune maturation of the infant.

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