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Increased intake of oily fish in pregnancy: effects on neonatal immune responses and on clinical outcomes in infants at 6 mo[1–3]

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ABSTRACT

Background: Long-chain n−3 PUFAs found in oily fish may have a role in lowering the risk of allergic disease.

Objective: The objective was to assess whether an increased intake of oily fish in pregnancy modifies neonatal immune responses and early markers of atopy.

Design: Women (n = 123) were randomly assigned to continue their habitual diet, which was low in oily fish, or to consume 2 portions of salmon per week (providing 3.45 g EPA plus DHA) from 20 wk gestation until delivery. In umbilical cord blood samples (n = 101), we measured n−3 fatty acids, IgE concentrations, and immunologic responses. Infants were clinically evaluated at age 6 mo (n = 86).

Results: Cord blood mononuclear cell (CBMC) production of interleukin (IL)-2, IL-4, IL-5, IL-10, and tumor necrosis factor-α in response to phytohemagglutinin (PHA) and of IL-2 in response to *Dermatophagoides pteronyssinus* allergen 1 (Derp1) was lower in the salmon group (all $P \leq 0.03$). In the subgroup of CBMCs in which an allergic phenotype was confirmed in the mother or father, IL-10 production in response to Toll-like receptor 2, 3, and 4 agonists, ovalbumin, salmon parvalbumin, or Derp1 and prostaglandin E_2 production in response to lipopolysaccharide or PHA was lower in the salmon group (all $P \leq 0.045$). Total IgE at birth and total IgE, incidence and severity of atopic dermatitis, and skin-prick-test positivity at 6 mo of age were not different between the 2 groups.

Conclusion: Oily fish intervention in pregnancy modifies neonatal immune responses but may not affect markers of infant atopy assessed at 6 mo of age. This trial is registered at clinicaltrials.gov as NCT00801502. *Am J Clin Nutr* doi: 10.3945/ajcn.111.022954.

INTRODUCTION

The development of childhood allergic disease is frequently preceded by immunologic differences that are evident in the neonatal period (1). These include immaturity in effector T cell responses (2, 3), differences in the function of regulatory T cells (4), and altered innate immune function (5). Some studies suggest that maternal environmental exposures (such as through diet) can modify neonatal immune function, although the mechanisms are not clear (6). Dietary n−3 LCPUFAs[4], found in oily fish and in fish oils, may represent a means of allergy prevention. Evidence to support this comes from epidemiologic and case-control studies that investigated associations between fish intake in pregnancy, lactation, infancy, and childhood and atopic outcomes in infants and children and from intervention studies with fish-oil supplements in pregnancy, lactation, infancy, and childhood, and atopic outcomes in infants and children (reviewed in 7).

PUFAs of different families are the substrates for the production of eicosanoid mediators, such as the PGs and LTs, which are involved in immunoregulation and in inflammatory processes. Higher concentrations of n−6 PUFAs favor the production of the 2-series PGs (such as PGE_2) and the 4-series LTs (such as LTB_4), both of which are highly inflammatory (8, 9). These mediators are known to promote allergic inflammation and IgE responses (10, 11). n−3 LCPUFAs can decrease the production of these mediators from arachidonic acid and give rise to alternative mediators that are significantly less inflammatory (12). In addition, other n−3 LCPUFA-derived metabolites, including resolvins, act to decrease inflammation (reviewed in reference 13) and have been shown to be effective in murine models of allergic inflammation (14, 15). These differences in lipid-me-

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[4] Abbreviations used: CBMC, cord blood mononuclear cell; Derp1, *Dermatophagoides pteronyssinus* allergen 1; FACS, fluorescence-activated cell sorter; HDM, house dust mite; IFN, interferon; LOD, limit of detection; LT, leukotriene; n−3 LCPUFA, long-chain n−3 PUFA; OVA, ovalbumin; PC, phosphatidylcholine; PE, phycoerythrin; PG, prostaglandin; PGN, peptidoglycan; PHA, phytohemagglutinin; poly I:C, polyinosinic-polycytidylic acid; Sals1, salmon parvalbumin; SCORAD, severity scoring of atopic dermatitis; SiPS, Salmon in Pregnancy Study; Th1, T helper 1 cell; Th2, T helper 2 cell; TLR, Toll-like receptor.

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diator generation and activity provide a biologically plausible mechanism whereby n−3 LCPUFAs might reduce the risk of allergic disease. These fatty acids may also act through other mechanisms involving TLRs (16) and altered T cell signaling and reactivity (17). Studies in human adults have shown that fish-oil supplements reduce T cell proliferation and cytokine production by mitogen-stimulated T cells (18–20) and by lipopolysaccharide-stimulated monocytes (18, 21–23). Studies in infants and children report inconsistent effects of fish oil on cytokine production by stimulated blood immune cells (24–26). A small number of studies have examined the effects of fish-oil intake by pregnant women on immune outcomes in maternal blood (27–29) or umbilical cord blood (27, 30, 31). Krauss-Etschmann et al (27) showed an altered pattern of cytokine messenger RNA expression in whole maternal and umbilical cord blood after pregnant women consumed n−3 LCPUFAs from week 22 of pregnancy. Fish-oil intake by women from week 25 of pregnancy decreased PGE_2, but not cytokine or chemokine, production by lipopolysaccharide-stimulated maternal blood cultures (28) and decreased allergic outcomes in the infants aged 12 mo (29). Finally, fish-oil intake by women from week 20 of pregnancy decreased CBMC cytokine responses to a mitogen and to several allergens, with the most marked effect being a reduction in IL-10 production in response to cat allergen (30). This study showed a decrease in sensitization to hen's egg and in severe atopic dermatitis in infants at age 12 mo whose mothers had consumed fish oil during pregnancy (30).

No studies of the influence of increased oily fish consumption in pregnancy on neonatal immune cell responses or later atopy have been conducted. The SiPS is a randomized controlled trial aimed at identifying whether increased consumption of oily fish (salmon) in pregnancy modifies n−3 LCPUFA status in maternal and umbilical cord plasma (the primary outcome), neonatal immune responses, and early markers of atopy (secondary outcomes).

SUBJECTS AND METHODS

Subjects

The study design, the subjects and their characteristics, aspects of the subjects' diet, and subjects' compliance were described in detail elsewhere (32). In brief, a total of 123 pregnant women in the area of Princess Anne Hospital (Southampton, United Kingdom) were enrolled in the study (**Figure 1**). Inclusion criteria were as follows: age 18–40 y; <19 wk gestation; healthy uncomplicated singleton pregnancy; infant at risk of atopy (one or more first-degree relatives of the infant affected by atopy, asthma or allergy by self-report); consumption of <2 portions oily fish per month, excluding tinned tuna; and no use of fish-oil supplements currently or in the previous 3 mo. The exclusion criteria were as follows: age <18 or >40 y; >19 wk gestation; no first-degree relatives of the infant affected by atopy, asthma, or allergy; consumption of >2 portions oily fish per month, excluding tinned tuna; use of fish-oil supplements within the previous 3 mo; participation in another research study; known diabetes; presence of any autoimmune disease; learning disability; terminal illness; and mental health problems. All procedures were approved by the Southampton and South West

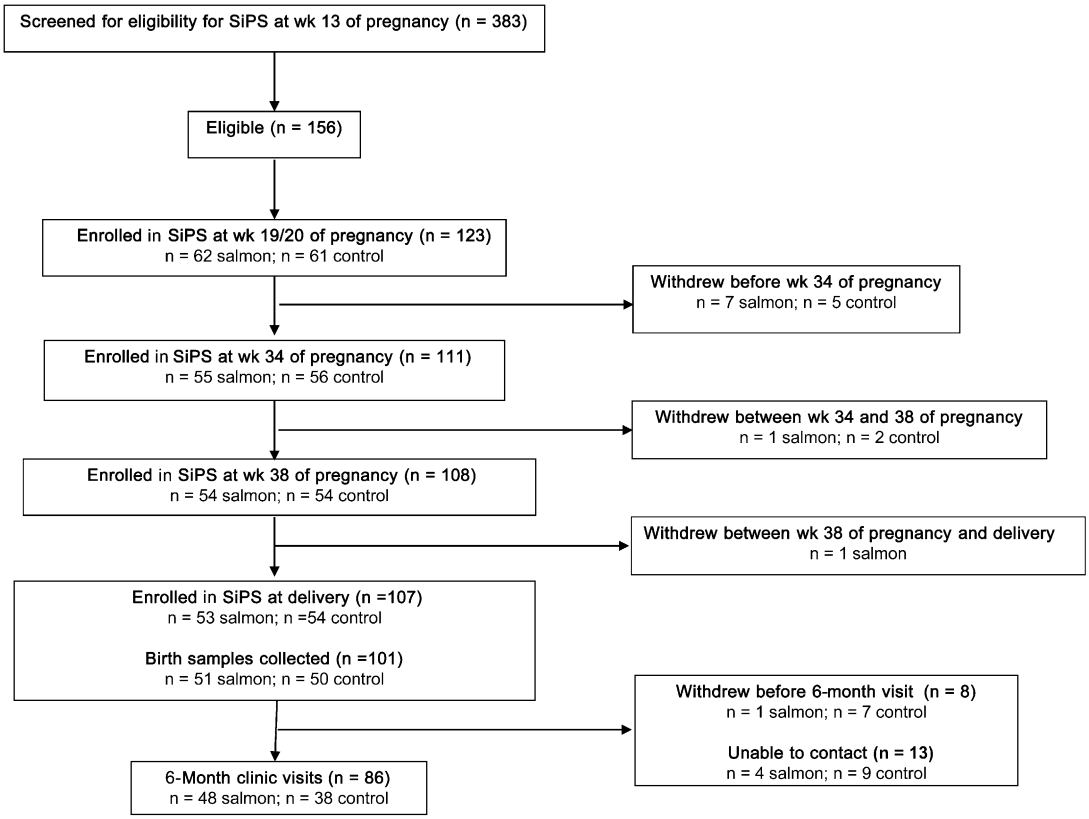

FIGURE 1. CONSORT (Consolidated Standard of Reporting Trials) diagram: progress of participants through the trial. SiPS, Salmon in Pregnancy Study.

Hampshire Research Ethics Committee (07/Q1704/43). The study was conducted according to the principles of the Declaration of Helsinki, and all women gave written informed consent.

Study design

The women were allocated to 1 of 2 groups according to a previously generated random number table. Women in the control group ($n = 61$) were asked to continue their habitual diet, and women in the salmon group ($n = 62$) were asked to incorporate 2 portions of farmed salmon (150 g/portion) into their diet per week from study entry (week 20) until they gave birth. Farmed salmon for use in the SiPS were raised with the use of dietary ingredients selected to contain low concentrations of contaminants. Each 150-g salmon portion contained (on average) 30.5 g protein, 16.4 g fat, 3.56 g total n−3 PUFAs (0.57 g EPA, 0.35 g docosapentaenoic acid, and 1.16 g DHA), 4.1 mg α-tocopherol, 1.6 mg γ-tocopherol, 6 μg vitamin A, 14 μg vitamin D_3, and 43 μg Se. Thus, 2 portions of salmon per week would typically provide 3.45 g EPA + DHA, 28 μg vitamin D_3, and 86 μg Se. The contaminants provided <12.5% of the FAO/WHO provisional tolerable weekly intake for dioxin and dioxin-like polychlorinated biphenyls, <11.5% for arsenic, <0.00000008% for cadmium, 0.0000025% for mercury, and <0.00000002% for lead. Researchers responsible for assessing outcome measures (both laboratory and clinical) remained blinded to the groups.

Fifteen subjects were not able to complete the study for various reasons (delivery before appointment, clinic visits cancelled because of feeling tired, too busy, or an unspecified injury), which left a total of 54 subjects in the control group and 53 subjects in the salmon group at the time of delivery of the infant.

The women and their partners were skin prick tested by using a standardized technique (33) to common allergen extracts (HDM, cat, dog, grass mix, tree mix, molds; ALK Abello) and to histamine as a positive control and to glycerine as a negative control. A wheal diameter of ≥3 mm indicated a positive result to the skin-prick test. In total, 86 women ($n = 48$ in the salmon group; $n = 38$ in the control group) and 76 men ($n = 44$ in the salmon group; $n = 32$ in the control group) agreed to be skin-prick tested. No significant differences in sensitization (ie, any positive result to the skin-prick test) were observed between the sexes in either of the groups (women: $n = 31$ sensitized in the salmon group and $n = 21$ sensitized in the control group; men: $n = 31$ sensitized in the salmon group and $n = 20$ sensitized in the control group; $P = 0.380$ and 0.466, respectively). The characteristics of the study subjects are shown in **Table 1**.

Serum, plasma, and mononuclear cell preparation

Blood was collected from the umbilical cord at birth ($n = 51$ in the salmon group; $n = 50$ in the control group). Serum and plasma (from heparin-treated blood) were prepared. CBMCs were isolated from heparin-treated blood by density-gradient centrifugation on Histopaque (Sigma-Aldrich) for 29 subjects in the control group and 32 subjects in the salmon group; the characteristics of the subjects with and without isolated CBMCs were not significantly different (data not shown). Purified CBMCs were cryopreserved by using standard techniques (1). Previous experiments established that the responses of cryopreserved mononuclear cells are not distinguishable from those of mononuclear cells that had not been cryopreserved (34, 35).

Fatty acid analysis

Fatty acid analysis of umbilical plasma PC was carried out by gas chromatography with flame ionization detection as previously described (32).

CBMC culture

To assess functional responses to TLR ligation, the pattern and magnitude of cytokine production after activation with specific microbial ligands for TLR2 (*Staphylococcus aureus* PGN; InvivoGen), TLR3 (poly I:C; InvivoGen), and TLR4 (ultrapure *Escherichia coli* K12 lipopolysaccaride; InvivoGen) were assessed. CBMCs (2×10^6/mL) were cultured in duplicate in 96-well round-bottomed plates in RPMI medium plus 10% (vol:vol) autologous plasma, either alone or with optimized doses of PGN, poly I:C, or lipopolysaccharide at 37°C with 5% CO_2.

TABLE 1
Characteristics of the study population[1]

	Control group	Salmon group
Mother's age (y)	28.4 ± 0.6 (61)[2]	29.5 ± 0.5 (62)
Mother's height (cm)	165.6 ± 0.9 (61)	165.4 ± 0.8 (62)
Mother's weight in early pregnancy (kg)	71.3 ± 2.0 (61)	67.5 ± 1.6 (62)
First pregnancy (n)	23 (61)	27 (62)
Duration of gestation (d)	277 ± 2 (54)	282 ± 1 (53)
Mode of delivery (*n*)		
Normal vaginal delivery	35	35
Elective cesarean delivery	2	3
Emergency cesarean delivery	7	5
Instrumental	10	10
Infant birth weight (g)	3425 ± 82 (54)	3449 ± 72 (53)
Infant head circumference at birth (cm)	34.7 ± 0.2 (54)	34.5 ± 0.2 (53)
Apgar score at 1 min	8.5 ± 0.2 (54)	8.5 ± 0.2 (53)
Apgar score at 5 min	9.1 ± 0.1 (54)	9.1 ± 0.1 (53)

[1] There were no significant differences between the groups.
[2] Mean ± SEM; *n* in parentheses (all such values).

After 24 h, the supernatant fluid was collected and stored at −20°C for cytokine analysis.

To assess lymphocyte responses to allergen and mitogen stimulation, 2×10^6 CBMCs/mL were cultured in duplicate in 96-well round-bottomed plates in RPMI plus 10% autologous plasma for 48 h with or without (control) LoTox natural Derp1 (10 μg/mL; Indoor Biotechnologies), low endotoxin OVA (200 μg/mL; Profos AG), Sals1 (15 μg/mL), or the mitogen PHA 16 (7.5 μg/mL; Sigma-Aldrich) at 37°C with 5% CO_2. After 48 h the supernatant fluid were collected and stored at −20°C for cytokine analysis.

Cytometric bead array immunoassays for cytokines

Supernatant fluid from the cultures described above was analyzed for cytokines with flow cytometry–based multiplex assays. Human Th1/Th2 6-plex kit II (for IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α) and human inflammation 6-plex kit (for IL-1β, IL-6, IL-8, IL-10, TNF-α, and IL-12p70) were purchased from BD Bioscience. All reagents were provided with the CBA kit, and all reagents were prepared according to the manufacturer's protocol booklet provided. The assays were performed as described by the kit manufacturers, and data were collected on a FACSCalibur flow cytometer. LOD concentrations for each cytokine were as follows: IL-1β (7.2 pg/mL), IL-2 (2.6 pg/mL), IL-4 (2.6 pg/mL), IL-5 (2.4 pg/mL), IL-6 (2.5 pg/mL), IL-8 (3.6 pg/mL), IL-10 (2.8 pg/mL), IL-12(p70) (1.9 pg/mL), TNF-α (2.8 pg/mL), and IFN-γ (7.1 pg/mL). Values below the LOD were set at half the LOD for all cytokines.

Prostaglandin E_2 production by mononuclear cells

Supernatant fluid from cell cultures stimulated with TLR4 ligand (lipopolysaccharide) and mitogen (PHA) were analyzed for PGE_2 concentrations by using an ELISA and following the manufacturer's instructions (R&D Systems). The lower LOD was 30.9 pg/mL.

Flow cytometric analysis of leukocyte phenotypes

Whole-blood samples were stained by using optimized amounts of fluorochrome-conjugated antibodies: anti-CD3 fluorescein isothiocyanate (UCHT-1), anti-CD4 PE (RPA-T4), anti-CD8 (LT8), anti-CD14 (TUK4), anti-CD16 (3G8), anti-CD19 (LT19), anti-TLR2 (CD282) (TLR2.3), anti-CD127 (R34.34), and anti-CD25 PE-Cy5 (CD25-3G10). All of the antibodies were purchased from AbD Serotec, except anti-CD127, which was purchased from Beckman Coulter. Appropriate isotype controls were always included. Contaminating erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After staining, cells were fixed by using BD Cell Fix (BD Pharmingen) and were analyzed within 24 h on a FACSCalibur (BD Pharmingen) by using CellQuestPro software.

Measurements of total IgE concentrations

Total serum IgE was measured in blood samples collected at delivery (cord blood) and at 6 mo of age. High-sensitivity IgE was measured with a Phadia ImmunoCAP 250 (Immunology Department, Southampton University Hospitals Trust, Southampton, United Kingdom); the lower LOD was 0.01 IU/mL.

Clinical outcomes at age 6 mo

Parents recorded various infant symptoms on prospective diary cards from birth to age 6 mo. Parents who noted "noisy breathing" of any kind were asked to give a detailed description of this to the research nurse (NDD), which was used to determine whether the symptoms were likely to be "wheeze," "stridor," or the result of secretions in the upper airway.

Infants were clinically evaluated at 6 mo of age ($n = 48$ in the salmon group; $n = 38$ in the control group), which included a detailed history and examination by the same research nurse (NDD). A diagnosis of atopic dermatitis was made in infants with typical skin lesions (36), and its severity was determined by using the SCORAD index as previously described (37).

Skin-prick testing was performed with common allergen extracts (HDM, cat, dog, tree mix, grass mix, hen's egg, salmon, and raw cow milk) applied to the volar surface of the forearm. Separate sterile lancets were used for each allergen tested. Histamine was used as a positive control, and glycerine was used as a negative control. All allergens other than salmon (purchased from Merck), including the positive and negative controls, were purchased from ALK Abello. A wheal diameter of ≥2 mm was considered positive.

Sample size and statistical analysis

SiPS was powered according to an anticipated increase in maternal plasma PC EPA content (primary outcome) and an anticipated reduction in sensitization to egg in the infants at 6 mo of age (a secondary outcome). It was calculated that a sample size of 50 women per group would have 93% power to detect a 50% higher plasma PC EPA content in the salmon group than in the control group and 70% power to detect 50% lower egg sensitization in the infants at 6 mo in the salmon group than in the control group.

Cytokine and serum IgE data were not normally distributed and could not be normalized by using logarithmic transformation. Therefore, these data were analyzed as continuous data, reported as medians and interquartile ranges, and as dichotomous data (detected or not detected). Differences in continuous data between groups were assessed by Mann-Whitney U test, whereas differences in dichotomous data between groups were determined by Pearson's chi-square test. Fatty acid and blood leukocyte data were normally distributed; values were expressed as mean percentages and SDs, and differences between groups were determined by Student's t test. Differences in clinical outcomes (categorical data) between the 2 groups at 6 mo were assessed by Pearson's chi-square test, except for the SCORAD index, which used Student's t test. Because of the recognized limitations associated with parental self-reported allergic history (38), a subgroup analysis was also performed, which used a strict definition of parental allergic disease (ie, one or both parents having a positive response to skin-prick testing in addition to one or more first-degree relatives of the infant being affected by atopy, asthma, or allergy by self-report). Results for the subgroup ($n = 46$ in the salmon group; $n = 29$ in the control group) are indicated accordingly and were analyzed in the same way as for the group as a whole. Statistical analysis was performed by using SPSS software (version 17.0 for Windows XP; SPSS Inc). A P value <0.05 was considered statistically significant for all analyses.

RESULTS

Effects of dietary salmon during pregnancy on cord blood n−3 LCPUFAs

The content of both EPA and DHA (as % of total fatty acids) was higher in cord plasma PC in the salmon group than in the control group [EPA: 0.3 ± 0.1 in the control group and 0.6 ± 0.3 in the salmon group ($P < 0.001$); DHA: 6.4 ± 1.3 in the control group and 7.4 ± 1.4 in the salmon group ($P = 0.001$)]. Conversely, the content of the n−6 PUFA arachidonic acid was lower ($P < 0.001$) in cord plasma PC in the salmon group (16.6 ± 1.8) than in the control group (18.3 ± 2.4).

Effects of dietary salmon during pregnancy on cord blood leukocyte phenotypes

CBMC subsets are shown elsewhere (*see* Supplemental Table 1 under "Supplemental data" in the online issue). There were no significant differences between the 2 groups in the percentages of T helper cells ($CD3^+CD4^+$), T cytotoxic cells ($CD3^+CD8^+$), natural killer cells ($CD3^-CD16^+$), B cells ($CD3^-CD19^+$), regulatory T cells ($CD4^+CD25^+CD127^{lo/-}$), and monocytes ($CD14^+TLR2^+$).

Effects of dietary salmon during pregnancy on TLR2-, TLR3-, and TLR4-mediated CBMC inflammatory cytokine responses

Dietary salmon intervention during pregnancy was not associated with any specific effects on CBMC cytokine responses to TLR2, TLR3, or TLR4 stimulation (**Table 2**). No differences in the frequency or magnitude of cytokine responses were found between the groups.

Effects of dietary salmon during pregnancy on allergen-specific and polyclonal CBMC cytokine responses

CBMC IL-2 responses to Derp1 were significantly lower ($P = 0.01$) in the salmon group than in the control group (**Table 3**). This difference was maintained when a subgroup analysis, using data only from those with confirmed parental allergy, was carried out ($P = 0.02$). No other differences in the magnitude of IL-2, IL-4, IL-5, IFN-γ, or TNF-α production in response to antigen-specific stimuli (OVA, Sals1, or Derp1) between CBMCs were observed between the 2 groups (Table 3). The IL-2 ($P = 0.01$), IL-4 ($P = 0.02$), IL-5 ($P = 0.03$) and TNF-α ($P = 0.01$) responses to a polyclonal (PHA) stimuli were all significantly lower in the salmon group than in the control group (Table 3). These relations were no longer statistically significant when a subgroup analysis, using data only from those with confirmed parental allergy, was carried out.

Effect of dietary salmon during pregnancy on CBMC regulatory cytokine production

On the basis of reports that the antiinflammatory effects of n−3 LCPUFAs might be mediated through IL-10–related regulatory mechanisms (30), we examined the production of this cytokine. At birth, IL-10 responses to TLR4 ligation were

TABLE 2
Effect of maternal salmon intervention during pregnancy on umbilical cord blood mononuclear cell cytokine responses to TLR ligands 2, 3, and 4[1]

	Whole group					Subgroup[2]				
	Control (n = 31)		Salmon (n = 25)			Control (n = 17)		Salmon (n = 20)		
Cytokine and TLR	Median	25th, 75th percentile	Median	25th, 75th percentile	P[3]	Median	25th, 75th percentile	Median	25th, 75th percentile	P[3]
IL-6 (pg/mL)										
2	17,665	14,526, 18,610	16,971	15,458, 18,131	0.55	17,896	15,023, 18,736	16,786	13,815, 18,130	0.15
3	11,289	3984, 16,440	14,140	3405, 16,104	0.84	12,705	1637, 17,434	13,647	3400, 15,692	0.38
4	17,872	14,850, 18,558	17,543	14,883, 17,895	0.22	18,131	14,140, 18,616	17,424	13,413, 17,893	0.17
IL-8 (pg/mL)										
2	32,189	22,223, 38,219	35,300	27,567, 39,686	0.42	29,975	21,125, 37,945	34,437	22,427, 38,295	0.46
3	26,584	19,504, 33,064	27,450	14,215, 34,234	0.79	27,444	18,643, 32,685	28,386	18,607, 34,679	0.66
4	35,714	27,867, 39,558	35,584	30,902, 38,646	0.90	35,575	26,521, 39,348	34,057	28,568, 38,018	0.82
IL-1β (pg/mL)										
2	3498	909, 7144	2858	677, 5617	0.52	3668	1364, 10,806	2695	531, 5649	0.24
3	500	97, 1141	475	104, 1241	0.70	661	99, 1146	447	101, 1150	0.74
4	6214	3492, 9258	5371	3430, 6949	0.66	7227	2725, 9537	5085	2805, 6755	0.45
TNF-α (pg/mL)										
2	591	255, 1850	486	185, 931	0.48	751	338, 2114	578	186, 1020	0.10
3	64.2	21.0, 138	38.5	9.32, 87.2	0.34	96.1	28.5, 146.1	34.8	8.8, 68.5	0.06
4	247	124, 448	190	126, 500	0.33	334	151, 905	189	99, 501	0.17
IL-12p70 (pg/mL)										
2	0.0	0.0, 1.25	0.0	0.0, 1.82	0.22	0.0	0.0, 1.27	0.0	0.0, 1.89	0.36
3	1.45	0.0, 4.08	1.33	0.0, 4.44	0.95	2.15	0.0, 4.67	0.62	0.0, 5.22	0.50
4	0.0	0.0, 1.39	0.0	0.0, 1.95	0.19	0.0	0.0, 1.68	0.0	0.0, 1.76	0.89

[1] Toll-like receptor.
[2] Subgroup refers to either a mother or father confirmed as being allergic through both self-report and a positive skin-prick-test result.
[3] Determined by Mann-Whitney U test; there were no significant differences between the groups.

TABLE 3
Effect of maternal salmon intervention during pregnancy on umbilical cord blood mononuclear cell cytokine responses to allergen-specific and polyclonal stimuli[1]

	Whole group					Subgroup[2]				
	Control (n = 32)		Salmon (n = 30)			Control (n = 18)		Salmon (n = 25)		
Cytokine and stimulant	Median	25th, 75th percentile	Median	25th, 75th percentile	P[3]	Median	25th, 75th percentile	Median	25th, 75th percentile	P[3]
IL-2 (pg/mL)										
Derp1	1.30	1.30, 2.43	1.30	1.30, 1.30	0.01[4]	1.30	1.30, 4.31	1.30	1.30, 1.30	0.02[4]
OVA	1.30	1.30, 1.30	1.30	1.30, 1.30	0.49	1.30	1.30. 3.57	1.30	1.30, 1.30	0.17
Sals1	1.30	1.30, 1.30	1.30	1.30, 1.30	0.14	1.30	1.30, 1.30	1.30	1.30, 1.30	0.19
PHA	196.4	86.8, 509.9	69.5	28.7, 185.3	0.01[4]	160.4	29.2, 382.3	72.81	28.7, 223.8	0.28
IL-4 (pg/mL)										
Derp1	1.30	1.30, 1.30	1.30	1.30, 1.30	0.36	1.30	1.30, 1.30	1.30	1.30, 1.30	0.26
OVA	1.30	1.30, 1.30	1.30	1.30, 1.30	0.36	1.30	1.30, 1.30	1.30	1.30, 1.30	0.26
Sals1	1.30	1.30, 1.30	1.30	1.30, 1.30	0.36	1.30	1.30, 1.30	1.30	1.30, 1.30	0.26
PHA	6.89	2.41, 14.1	1.30	1.30, 7.22	0.02[4]	4.58	1.30, 8.95	2.98	1.30, 7.72	0.44
IL-5 (pg/mL)										
Derp1	1.20	1.20, 1.20	1.20	1.20, 1.20	0.47	1.20	1.20, 2.56	1.20	1.20, 1.20	0.50
OVA	1.20	1.20, 1.20	1.20	1.20, 1.20	0.80	1.20	1.20, 1.20	1.20	1.20, 1.20	0.45
Sals1	1.20	1.20, 3.48	1.20	1.20, 3.50	0.90	1.20	1.20, 3.03	1.20	1.20, 3.50	0.97
PHA	12.3	3.93, 34.1	4.89	1.20, 20.5	0.03[4]	7.07	1.84, 28.22	5.77	1.20, 20.9	0.49
IL-10 (pg/mL)										
Derp1	41.8	12.1, 63.6	17.2	5.36, 60.9	0.19	57.64	24.4, 100.9	17.23	4.4, 54.3	0.02[4]
OVA	46.4	14.8, 84.2	16.7	7.89, 75.6	0.09	76.86	42.7, 106.1	15.73	5.4, 50.1	<0.01[4]
Sals1	62.2	30.2, 87.3	26.8	13.8, 77.4	0.07	83.48	58.1, 111.4	26.84	13.3, 65.2	<0.01[4]
PHA	106.9	56.2, 166.3	43.3	18.4, 132.1	0.02[4]	103.7	63.9, 213.8	43.3	18.37, 110.0	0.04[4]
IFN-γ (pg/mL)										
Derp1	3.55	3.55, 14.7	3.55	3.55, 3.55	0.11	3.55	3.55, 10.6	3.55	3.55, 3.55	0.21
OVA	3.55	3.55, 12.6	3.55	3.55, 9.50	0.63	3.55	3.55, 11.2	3.55	3.55, 11.1	0.65
Sals1	3.55	3.55, 26.9	3.55	3.55, 9.03	0.45	3.55	3.55, 21.4	3.55	3.55, 13.9	0.65
PHA	121.2	34.4, 286.4	86.6	24.5, 178.2	0.22	164.6	16.52, 410.8	87.06	22.43, 178.1	0.36
TNF-α (pg/mL)										
Derp1	39.4	11.4, 79.1	21.3	8.64, 44.8	0.15	42.25	11.4, 106.7	19.95	6.4, 44.8	0.11
OVA	35.8	18.8, 92.1	22.5	9.50, 54.3	0.19	37.36	15.7, 122.8	18.90	6.67, 52.8	0.12
Sals1	54.6	26.4, 97.3	33.1	19.7, 53.4	0.07	60.84	23.3, 200.8	33.07	19.7, 53.3	0.07
PHA	224.9	62.4, 429.5	67.0	26.6, 129.4	0.01[4]	191.8	37.4, 467.5	67.0	26.6, 129.4	0.13

[1] Derp1, *Dermatophagoides pteronyssinus* allergen 1; IFN, interferon; OVA, ovalbumin; PHA, phytohemagglutinin; Sals1, salmon parvalbumin.
[2] Subgroup refers to either a mother or father confirmed as being allergic through both self-report and a positive skin-prick-test result.
[3] Determined by Mann-Whitney U test.
[4] Significantly different from the control group, $P < 0.05$.

significantly lower (P = 0.04) in the salmon group than in the control group (**Figure 2**A). No differences in IL-10 production were observed when cultures were stimulated via TLR2 or TLR3 or with antigens (OVA, Sals1, or Derp1). On the basis of data for only those with confirmed parental allergic disease, production of IL-10 was significantly lower (on the basis of CBMCs) in the salmon group when stimulated via a TLR2-specific ligand, PGN (P = 0.004); a TLR3-specific ligand, poly (I:C) (P = 0.045); and a TLR4-sepecific ligand, lipopolysaccharide (P = 0.002) (Figure 2A). Furthermore, CBMC IL-10 responses to Derp1 (P = 0.022), OVA (P = 0.004), Sals1 (P = 0.002), and mitogen (PHA) (P = 0.044) were all significantly lower in the salmon group than in the control group (Figure 2B).

Effect of dietary salmon during pregnancy on CBMC PGE_2 production

No differences in PGE_2 production after CBMC stimulation with TLR4 ligand (lipopolysaccharide) or mitogen (PHA) were found between the 2 groups (**Figure 3**). On the basis of data for only those with confirmed parental allergic disease, PGE_2 production from lipopolysaccharide- or PHA-stimulated CBMCs was significantly lower in the salmon group than in the control group (P = 0.003 and 0.010, respectively; Figure 3).

Effects of dietary salmon during pregnancy on neonatal and infant serum IgE

The serum total IgE (IU/mL) concentration [median (interquartile range)] was higher in infants aged 6 mo than at birth [0.16 (0.08, 0.51) at birth compared with 5.34 (5.00, 8.89) at 6 mo in the control group; 0.15 (0.65, 0.44) at birth compared with 5.98 (5.00, 8.97) at 6 mo in the salmon group]. No significant difference in IgE concentrations were found between groups at either time point (P = 0.332 and 0.866, respectively).

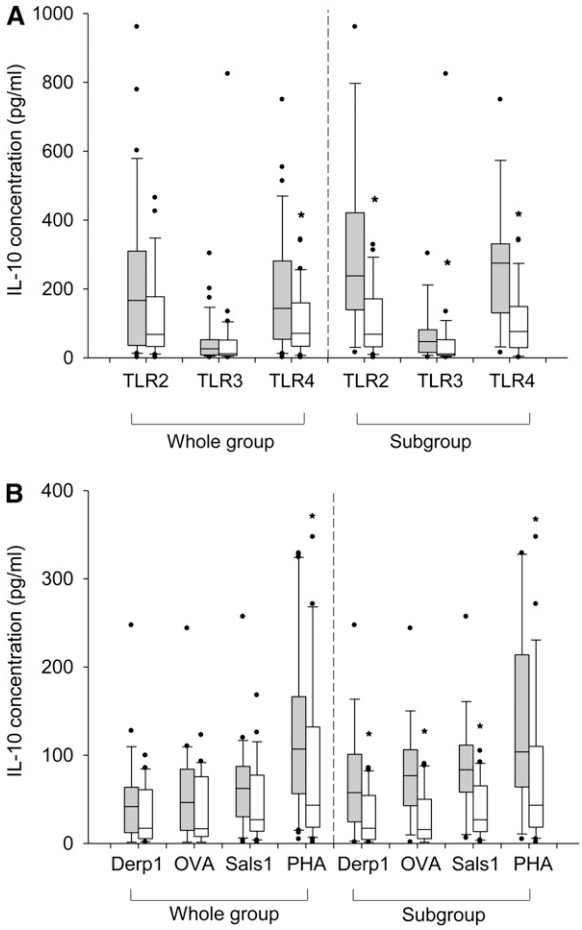

FIGURE 2. Effect of maternal salmon intervention during pregnancy on umbilical cord blood mononuclear cell IL-10 responses to TLR agonists (A) and allergens and a T cell mitogen (PHA) (B). Data are shown as medians and interquartile ranges for the whole-group analysis [control group ($n = 32$ samples; gray bars); salmon group ($n = 27$–30 samples; white bars)] and for the subgroup analysis [control group ($n = 15$–18 samples; gray bars); salmon group ($n = 16$–24 samples; white bars)]. *Significantly different from the control group, $P < 0.05$ (Mann-Whitney U test). Derp1, *Dermatophagoides pteronyssinus* allergen 1; OVA, ovalbumin; PHA, phytohemagglutinin; Sals1, salmon parvalbumin; TLR, Toll-like receptor.

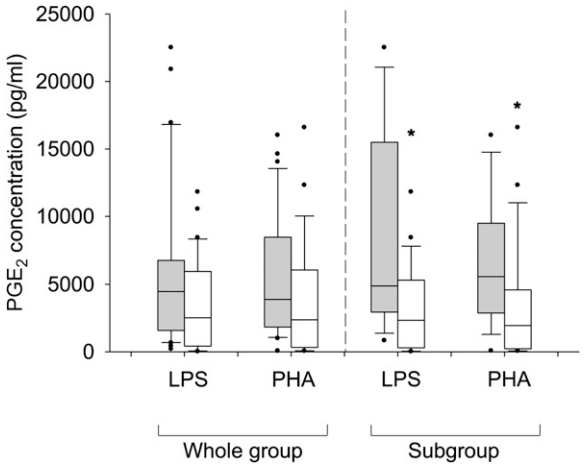

FIGURE 3. Effect of maternal salmon intervention during pregnancy on umbilical cord blood mononuclear cell PGE_2 responses to the Toll-like receptor 4 agonist LPS and a T cell mitogen (PHA). Data are shown as median and interquartile ranges for the whole-group analysis [control group ($n = 31$ samples; gray bars); salmon group ($n = 28$–30 samples; white bars)] and for the subgroup analysis [control group ($n = 18$ samples; gray bars); salmon group ($n = 24$–25 samples; white bars)]. *Significantly different from the control group, $P < 0.05$ (Mann-Whitney U test). LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin.

Clinical outcomes at 6 mo of age

A total of 86 infants attended the clinic visit at 6 mo of age: $n = 48$ in the salmon group and 38 in the control group. The clinical characteristics are shown in **Table 4**. No significant differences in the incidence of atopic dermatitis, the severity of atopic dermatitis in those infants who exhibited atopic dermatitis (SCORAD index), wheeze, bronchiolitis, chest infections, itchy/dry skin, or sensitization rates were observed between the salmon and control groups at 6 mo of age.

DISCUSSION

Allergic disease and sensitization essentially reflect a failure of the host to establish effective immunologic tolerance to a nonpathogenic inhalant or dietary antigen (39). Substantial scientific data on the innate and adaptive immune responses that contribute to the development of tolerance are available, with growing appreciation that innate and adaptive immunity do not function independently of each other (40). Dietary modifications are among the many complex environmental changes implicated in the allergy epidemic (41). The recognized effects of many nutrients on immune function (including immune tolerance) make dietary change one of the potential factors underlying the rise in immune disease (6, 41). To our knowledge, this was the first study to examine the effects of increased intake of salmon (providing n−3 LCPUFAs) during pregnancy on the development of neonatal immune responses and early markers of atopy. The study is based on earlier findings with fish-oil supplements in nonpregnant adults (18–23), infants and children (24–26), and pregnant women (27–31) that indicate altered immune responses. Of particular relevance, fish-oil supplementation in pregnant women altered the pattern of cytokine messenger RNA expression in whole umbilical cord blood (27), decreased allergic outcomes in the infants at 12 mo of age (29, 30), and decreased CBMC cytokine responses to a mitogen and to several allergens (30), especially to IL-10.

The current study found that increased intakes of salmon during pregnancy, which effectively increased the n−3 LCPUFA content of cord plasma, affected neonatal cytokine production; data from all subjects showed that the production of IL-2, IL-4, IL-5, and TNF-α in response to the polyclonal mitogen PHA was lower in the salmon group. However, we found no evidence that increased intake of salmon during pregnancy had any significant effect on TLR2-, TLR3-, or TLR4-mediated proinflammatory neonatal immune responses, although production of the regulatory and antiinflammatory cytokine IL-10 was lower in the salmon group after lipopolysaccharide stimulation of CBMCs. To assess adaptive memory responses, we stimulated CBMCs with both inhalant (Derp1) and food (OVA and Sals1) allergens. Overall, we found limited cytokine responsiveness to these stimuli, possibility attributable to the low precursor frequency of these effector cells in cord blood. Even so, IL-2 production in response to Derp1 was lower in the salmon group. Using the polyclonal stimulus (PHA), we found differences in the capacity

TABLE 4
Effect of maternal salmon intervention during pregnancy on clinical characteristics of the infants at age 6 mo[1]

Characteristic	Control	Salmon	P^2
	n/N (%)	*n/N* (%)	
Atopic dermatitis	7/38 (18.4)	12/48 (25.0)	0.46
SCORAD index[3]	10.0 ± 8.9	7.4 ± 3.5	0.37
Wheeze	7/37 (18.9)	11/46 (23.9)	0.58
Pneumonia/bronchiolitis	1/37 (2.7)	1/46 (2.1)	0.88
Chest infection	3/37 (8.1)	1/46 (2.1)	0.21
Itchy skin	8/37 (21.6)	10/45 (22.2)	0.95
Dry skin	12/37 (32.4)	14/45 (31.1)	0.90
Sensitized	5/38 (13.2)	6/48 (12.5)	0.93
Derp1	0 (0.0)	0 (0.0)	—
Cat (Feld1)	1 (2.6)	1 (2.1)	0.87
Dog	0 (0.0)	2 (4.2)	0.20
6 grass mix	0 (0.0)	0 (0.0)	—
3 tree mix	0 (0.0)	0 (0.0)	—
Salmon (Sals1)	0 (0.0)	0 (0.0)	—
Hen's egg	4 (10.5)	3 (6.3)	0.47
Raw cow milk	2 (5.3)	2 (4.2)	0.81

[1] Derp1, *Dermatophagoides pteronyssinus* allergen 1; Feld1, *Felis domesticus* allergen; Sals1, salmon parvalbumin; SCORAD, severity scoring of atopic dermatitis.
[2] Determined by Pearson's chi-square test, except for the SCORAD index (Student's *t* test).
[3] Values are means ± SDs.

of neonatal cells to produce the regulatory cytokine IL-10, the production of which was lower in the salmon group. The clear effect of increased salmon intake on IL-10 production by CBMCs is consistent with the earlier observations of Dunstan et al (30) after fish-oil consumption by pregnant women.

Because of the recognized limitations associated with parental self-reported allergic history (38), a subgroup analysis was also performed that used a strict definition of parental allergic disease (ie, both positive sensitization and self-report). The use of only data for those with confirmed parental allergic disease showed that increased intake of salmon during pregnancy had significant effects on both regulatory cytokine (IL-10) production in response to a range of stimuli (innate and adaptive) and on eicosanoid (PGE_2) production from lipopolysaccharide- and PHA-stimulated neonatal cord cells.

It is now recognized that an increased risk of allergic disease might be the result of a failure of normal immune regulation in early life, rather than to a simple "Th2-skewing" of immune responses (42). IL-10, now recognized as a major product of regulatory T cells (among others), is believed to play a key role in downregulating inappropriate Th1-and Th2-type responses (43). Here, we showed that dietary salmon intervention during pregnancy resulted in attenuation of the production of IL-10 in response to a range of stimulants, including TLR ligands and allergens. The effects on IL-10 production occurred in the absence of changes in the nature of the immune cell subsets present, including regulatory T cells. This suggests that salmon decreases the activity of IL-10–producing cells rather than their abundance. The potent and broad-spectrum tolerogenic functions of IL-10 have been established in various models of infection, inflammation, and cancer (44), and modulation of IL-10 might therefore contribute to the immunomodulatory effects of PUFAs. Whether this might increase or decrease the risk of atopy and allergic disease is not clear; however, in the current study these were not affected in infants at age 6 mo and the immune differences identified at birth could have a longer-term effect on disease. Dunstan et al (30) showed that fish-oil supplementation in pregnant women reduced IL-10 production by CBMCs in response to allergens (HDM, OVA, and cat) compared with that in the olive oil control group. The current findings agree with and extend those of earlier observations. In the study by Dunstan et al, sensitization to a range of food allergens tended to be lower in infants aged 1 y in the fish-oil group, and the incidence of severe atopic dermatitis was significantly reduced (30).

It is not clear how components of salmon, including n−3 LCPUFAs, might affect IL-10 production. One possibility is that n−3 LCPUFAs modify the types and/or amounts of lipid mediators produced. Through action on dendritic cells, T cell proliferation and Ig class switching in B cells, some eicosanoids, such as PGE_2, are believed to play a role in promoting sensitization to allergens (12). PGE_2 is also a potent inducer of IL-10 (45). In the current study, PGE_2 production from lipopolysaccharide- and PHA-stimulated CBMCs was lower in the salmon group than in the control group. This is consistent with the reported effects of fish-oil supplements (12). The lower PGE_2 production most likely resulted from the increased provision of EPA and DHA from the mother to the fetus and the subsequent decreased incorporation of arachidonic acid into CB immune cells, which resulted in the decreased availability of arachidonic acid for eicosanoid synthesis. The lower PGE_2 production could, in turn, result in less induction of IL-10 production. Thus, the observations from the current study of lower PGE_2 production and lower IL-10 production in the salmon group may be linked. The precise cellular source of IL-10 is not clear, and further experiments will be necessary to determine this.

The current study had several limitations. First, the sample size was modest and there was some loss of subjects from the study (Figure 1). Second, the number of infants sensitized to allergens at age 6 mo was fewer than predicted, which limited our ability to identify any effect on this clinical outcome. Third, the age at which the infants were clinically evaluated (6 mo) for signs of atopy and allergic disease was rather early; further follow-up will be necessary to examine both immune and clinical effects in the longer term. Fourth, allergy was not confirmed in all parents by skin-prick testing. Finally, the method used to identify the regulatory T cell ($CD4^+CD25^+CD127^{lo/-}$) population was not optimal, and this assessment would have been more robust if we had performed intracellular nuclear staining for the transcription factor FOXP3.

The findings from the current study have been interpreted in the context of an increased intake of n−3 LCPUFAs and have been discussed in comparison with studies that used fish-oil supplements. However, it is important to note that the salmon also supplied significant amounts of other nutrients, particularly vitamin D and selenium. Because these nutrients are also immunomodulatory (46), it is possible that some of the observed effects were due to these nutrients or to the combination of these nutrients with n−3 LCPUFAs.

In conclusion, we showed that consumption of oily fish in pregnancy modified some CBMC responses, but that this did not translate into differences in early atopic sensitization or in the incidence or severity of atopic dermatitis in the infants at age 6 mo. Follow-up of the infants is needed to further assess the effects

of the oily fish (salmon) intervention during pregnancy on postnatal allergen-specific immune responses and expression of allergic disease beyond 6 mo.

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The authors' responsibilities were as follows—EAM, KMG, and PCC: responsible for designing the study; PCC: overall responsibility for all aspects of the study; MV, L-SK, and NDD: recruited and screened volunteers, carried out the intervention, and collected the blood samples and anthropometric, questionnaire, and compliance data; NDD: administered the skin-prick tests and conducted the clinical evaluations of the infant under supervision of PCC and ME-L; MV, L-SK, and PSN: carried out the laboratory analysis, supervised by EAM and PCC; PSN: conducted the statistical analysis of the data; APW: contributed to the discussion and interpretation of the data; and PSN: drafted the manuscript. All authors contributed to and approved the final version of the manuscript. None of the authors had any conflicts of interest. The authors alone were responsible for the content and writing of the manuscript.

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