Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-β levels after fish oil supplementation of pregnant women

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Background: Altered intakes of n-3 and n-6 polyunsaturated fatty acids were suggested to modulate allergic disease, but intervention trials yielded inconclusive results. Because allergies are primed in early infancy and *in utero*, the fetus might be more accessible to nutritional intervention strategies.

Objective: We sought to investigate how supplementation of pregnant women with a fish oil (FO) preparation modulates allergy-related immune parameters in mothers and offspring.

Methods: We performed a multicenter, randomized, double-blind, placebo-controlled trial. Three hundred eleven pregnant women received daily either FO with 0.5 g of docosahexaenoic acid and 0.15 g of eicosapentaenoic acid, 400 μg of methyl-tetrahydrofolic acid, both, or placebo from the 22nd gestational week. T_H1/T_H2-related molecules were quantified in 197 maternal and 195 cord blood samples by using real-time RT-PCR. Data are given as geometric means [95% CIs].

Results: FO supplementation was associated with increased TGF-β mRNA in maternal (0.85 [0.8-0.89]; placebo: 0.68 [0.64-0.72]) and cord blood (0.85 [0.81-0.9]; placebo: 0.75 [0.71-0.79]). IL-1 (0.69 [0.66-0.73]; placebo: 0.83 [0.79-0.88]) and IFN-γ (0.54 [0.51-0.57]; placebo: 0.65 [0.61-0.69]) were decreased in mothers only ($P < .001$). Cord blood mRNA levels of IL-4 (0.54 [0.52-0.57]; placebo: 0.64 [0.61-0.68]), IL-13 (0.61 [0.58-0.65]; placebo: 0.85 [0.80-0.89]), CCR4 (0.70 [0.67-0.73]; placebo: 0.88 [0.84-0.92]; all $P < .001$), and natural killer ($P < .001$) and $CCR3^+CD8^+$ T cells ($P < .04$) were decreased in the FO group.

Conclusion: Supplementation with FO during pregnancy is associated with decreased mRNA levels of T_H2-related molecules in the fetus and decreased maternal inflammatory cytokines. We speculate that both effects are mediated by TGF-β.

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Key words: *Pregnancy, trial, fish oil, folate, cytokine, cord blood, allergy, neonate*

The n-3/n-6 hypothesis suggests that decreased ratios of n-3/n-6 long-chain polyunsaturated fatty acid (LC-PUFA) intakes have contributed to the increase of atopic diseases in industrialized countries.[1] The most common dietary n-6 LC-PUFA, linoleic acid, is a precursor of arachidonic acid, which itself is a substrate for 2-series prostaglandins (PGs) and 4-series leukotrienes. PGE_2 inhibits the production of T_H1 cytokines[2] and promotes synthesis of T_H2 cytokines.[3] Dendritic cells generated in the presence of PGE_2[4] or PGD_2[5] instruct naive T cells to develop into T_H2 cells. In contrast, n-3 eicosapentaenoic acid (EPA) and n-3 docosahexaenoic acid (DHA), which are abundant in fatty sea fish, are metabolized to alternative eicosanoids with low allergenic and inflammatory properties. Because n-3 and n-6 LC-PUFA pathways compete for the same enzymes and eicosanoid receptors, supplementary n-3 LC-PUFAs were suggested to protect against allergies. Although attractive, this concept could not be confirmed in randomized clinical trials.[6,7] Although the majority of these trials were performed in adults or schoolchildren, *in utero* exposure to fatty acids from maternal diet might be more relevant to modulate fetal immunity and the postnatal allergy risk. In a prospective birth cohort, maternal intakes of n-3 LC-PUFAs in the last 4 weeks of pregnancy were negatively associated with the incidence of eczema in the 2-year-old offspring.[8] In a randomized, placebo-controlled, double-blind trial, atopic pregnant women received fish oil (FO) from the 20th week of gestational

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Abbreviations used

DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
FITC: Fluorescein isothiocyanate
FO: Fish oil
FO&MTHF: Fish oil with methyl-tetra-hydrofolic acid
FO±MTHF: Fish oil with or without methyl-tetra-hydrofolic acid
GA: Gestational age
LC-PUFA: Long-chain polyunsaturated fatty acid
MTHF: Methyl-tetra-hydrofolic acid
PE: Phycoerythrin
PG: Prostaglandin

age (GA). Lower levels of cord plasma IL-13[9] and lower IL-10 responses to cat hair extract[10] were found in the FO group, and children were less likely to be sensitized to egg at 1 year of age. A possible prevention of allergic cough by means of long-term FO supplementation in early infancy was shown in an earlier Australian study, in which infants at risk for asthma were provided with n-3 fatty acids together with a house dust mite allergen avoidance intervention at least from the age of 6 months.[11] At 3 years of age, cough was significantly reduced in the atopic, but not in the nonatopic, children. However, at the age of 5 years, respiratory or allergic outcomes no longer differed between the intervention groups[12] and also not after stratification for n-3/n-6 LC-PUFA plasma levels.[13]

Folate is a critical nutrient during pregnancy and required for one-carbon-transfer reactions and DNA and RNA synthesis. Accordingly, it is also important for proper lymphocyte function.[14-16] The methyl-tetrahydrofolate reductase (C677T) TT genotype is associated with reduced folate availability. An association of this polymorphism with an increased prevalence of sensitization to inhalant allergens was observed in a recent single, cross-sectional, population-based study,[17] but further studies are required to substantiate this observation.

In the present study we examined within a European multicenter, randomized, double-blind, placebo-controlled study how supplementation of pregnant women with an FO preparation, 5-methyl-tetra-hydrofolic acid (5-MTHF), both, or placebo from the 22nd week of GA until delivery affects maternal and fetal allergy-related immune parameters.

In particular, T_H2-related CCR4, IL-13, IL-4, CRTH2, T_H1-related CXCR3, IFN-γ, IL-1, and TGF-β were quantified at the mRNA level in cord blood and maternal peripheral blood at delivery. Furthermore, $CCR3^+$, $CCR4^+$, $CCR5^+$, $CD4^+$, or $CD8^+$ T cells, as well as $CD69^+$, $CD19^+$, and $CD16^+$ lymphocyte subsets were quantified in the cord blood of a subcohort.

METHODS

Subjects and study design

Full details of the study design, subjects, data collection, and interventions are described in detail elsewhere.[18] In short, the study was conducted as a multicenter, randomized, double-blind, placebo-controlled clinical trial with a 2-factorial design to assess the effects of increased intakes of FO, 5-MTHF, or both from the 22nd week of GA until delivery on pregnancy and birth outcomes. Study centers were the University Hospital of Granada (Spain), the University of Pecs (Hungary), and the University of Munich (Germany). Apparently healthy women between 18 and 40 years of age with uncomplicated singleton pregnancies who did not use FO supplements and no folate or vitamin B_{12} supplements after the 16th week of GA were considered for inclusion. Written informed consent was obtained from all participating women. The local ethics committees of each participating center approved the study protocol.

Participating women received daily either an FO preparation providing 0.5 g of DHA and 0.15 g of EPA (Pronova Biocare, Lysaker, Norway), 400 μg of 5-MTHF (BASF, Ludwigshafen, Germany), both, or placebo, which were provided as a milk-based supplement (Ordesa Laboratorios, Barcelona, Spain) containing vitamins and minerals in amounts meeting the estimated requirements during the second half of pregnancy for European women (see Table E1 in the Online Repository at www.jacionline.org).[19] Details of the fatty acid composition are given in Table E2 (in the Online Repository at www.jacionline.org). In the present study we investigated mRNA expression levels of T_H1/T_H2–related molecules in maternal peripheral blood at delivery and in cord blood in a random subcohort of the main trial (see Fig E1 in the Online Repository at www.jacionline.org). The subcohort was set up according to the availability of material for mRNA analyses.

Collection of biologic material

For the assessment of plasma phospholipid fatty acids, 10 mL of maternal venous EDTA blood from each subject was collected at study entry and at the 30th week of GA. At delivery, 12 mL of EDTA blood was collected from the mother, together with 12 mL of venous placental cord blood. For mRNA analyses, 2 mL of placental cord blood and 4 mL of peripheral venous blood from the mothers was drawn in 10 mL Trizol LS Reagent (Invitrogen, Life Technologies, Karlsruhe, Germany) at delivery and stored at −80°C until mRNA extraction.

Plasma fatty acid analysis

Initial total lipid extraction was performed according to the method of Kolarovic and Fournier.[20] Briefly, 500 μL of plasma plus 0.5 mL of water was vortexed for 30 seconds with 100 μL of internal standard (0.857 mg/mL PL-17:0 dissolved in chloroform) and mixed with 4 mL of hexane/2-propanol (3:2) with 25 mg/L butylated hydroxytoluene. After centrifugation (10 minutes at 4°C and 1500*g*), the organic layer was transferred into another glass tube. The extraction was repeated 3 times with pure hexane. The combined extracts were dried under a vacuum and dissolved in 200 μL of hexane/methyl-tert-butyl-ether/acetic acid (100:3:0.3 vol/vol/vol). Phospholipids were isolated by means of liquid chromatography with aminopropyl columns (Sep Pak Cartridges; Waters, Milford, Mass), as described by Agren et al.[21] Phospholipid fractions obtained from the columns were evaporated to dryness under a vacuum, and 100 μL of chloroform was added to each tube. Fatty acid methyl esters were formed as described by Lepage et al.[22] A gas chromatograph Model HP-5890 Series II (Hewlett Packard, Palo Alto, Calif) equipped with a flame ionization detector was used for quantification of fatty acid methyl esters by means of gas chromatography. A capillary column (Sp 2330 FS), with 60-m length, 0.32 mm internal diameter, and 20-μm thickness (Supelco, Inc, Bellefonte, Palo Alto, Calif) was used. The injector (split/splitless: 29:1) and the detector were maintained at 250°C and 275°C, respectively, with nitrogen as the carrier gas. DHA and EPA levels were calculated as weight percentages of total detected fatty acids with 14 to 24 C-atoms.

Real-time RT-PCR

Total RNA was isolated according to the manufacturer's instructions (Invitrogen, Life Technologies) and reverse transcribed with random hexamer primers (Roche Applied Sciences, Mannheim, Germany) and Supercript II RT (Invitrogen, Life Technologies). Samples in which contamination with genomic DNA was suspected (controlled by cDNA synthesis reactions without reverse transcriptase) were excluded from the analyses. PCR reactions were carried out in 20 μL of final volume with 1 μL of cDNA, 5 pmol/μL primers (see Table E3 in the Online Repository at www.jacionline.org), and 10 μL of SYBR Green IQ Supermix (Bio-Rad, Hercules, Calif) containing Hot-Start Taq Polymerase in an iCycler iQ (Bio-Rad). The amplification steps were repeated 43 times (denaturation: 93°C for 30 seconds; annealing: 55°C for 30

seconds; extension: 72°C for 45 seconds), with fluorescence measurement at 72°C. The relative gene expression was calculated by using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene. Contamination with unspecific byproducts that affect the quantitation of the PCR product was excluded by means of melting curve analyses at the end of each run. The product size was verified by using agarose gel electrophoresis. The specificity of the amplicons was further confirmed by sequencing (Medigenomix, Martinsried, Germany).

Quantitation of mRNA transcripts in low copy numbers was confirmed with hybridization probes (100 nmol/L) tagged at the 5′ end with the fluorescent molecule 6-carboxyfluorescein as a reporter and at the 3′ end with the fluorescent molecule 6-carboxytetramethylrhodamine as a quencher (TibMolBiol, Berlin, Germany). The latter PCRs were carried out in 20 μL of final volume with 1 μL of cDNA, 1 μL of 5 pmol/μL forward and reverse primers, 2 μL of 5 pmol/μL 6-carboxyfluorescein–6-carboxytetramethylrhodamine probe, 10 μL of IQ Supermix (Bio-Rad), and 5 μL of H_2O. Activation of the Taq Polymerase was followed by 43 cycles (denaturation at 93°C for 30 seconds and annealing with fluorescence measurement at 60°C for 1 minute). Both PCR-based methods yielded analogous results ($r = 0.92$, $P < .01$), thus confirming the quantification of the transcripts.

Flow cytometry

Because fresh blood is needed for flow cytometric analyses, 0.5 mL of cord blood was used only from the German participants for 4-color flow cytometry (Calibur, Becton-Dickinson, Heidelberg, Germany) and processed within 24 hours at room temperature. Lymphocytes were gated according to their forward/side-scatter characteristics. The percentages of $CD4^+$ or $CD8^+$ chemokine-receptor double-positive cells were determined within total lymphocytes after defining a cutoff value according to the isotype control by using CD4-allophycocyanine mouse IgG1, CCR4-phycoerythrine (PE) mouse IgG2a, CD69-PE mouse IgG2a, CD16-fluorescein isothiocyanate (FITC) mouse IgG1, CD19-FITC mouse IgG1 (BD PharMingen, Heidelberg, Germany), CD8–phycocyanine 5 mouse IgG1 (Immunotech, Marseille, France), CXCR3-FITC mouse IgG1, CCR3-PE rat IgG2a, CCR4-PE mouse IgG2b, CCR5-PE mouse IgG2b (R&D Systems, Wiesbaden, Germany), mouse IgG1-FITC, rat IgG2a-PE, mouse IgG2b-PE, and mouse IgG2a-PE (Immunotech, Marseille, France).

Statistical analysis

Only corresponding mother-child pairs were included in the analysis. For the description of maternal and cord plasma percentage wt/wt DHA, cytokines, and lymphocyte subpopulations, geometric means and 95% CIs were calculated for the 4 intervention groups. Because the data were not normally distributed, they were transformed by means of natural logarithm to reduce deviation from distributional requirements for 1-way analysis of covariance in an intention-to-treat-analysis.[23] Samples at less than the detection limit were excluded from the analyses (relevant for lymphocyte subpopulations only). Cytokines and the frequencies of lymphocyte subpopulations were adjusted for study center (reference Hungary) and maternal percentage wt/wt DHA at week 20 of gestation. To further adjust for potential confounders, single variables (gravidity, parity, delivery mode, and maternal smoking in the 20th and 30th week of gestation) were included one by one in the model. The significance level was set at an α value of .05, and all P values were corrected for multiple comparisons by using the Tukey-Kramer procedure.

All computations were performed with SAS for Windows, version 9.1.3 (SAS Institute, Cary, NC) by using the procedure "Proc GLM" for Analysis of Variance and Covariance.

RESULTS

Characteristics of mothers and neonates

The population for this study consisted of 197 mothers (90 Spanish, 56 German, and 51 Hungarian) and 195 neonates (90 Spanish, 56 German, and 49 Hungarian) for the mRNA analyses (Fig E1). Twenty-one mothers and 18 neonates were excluded from the analyses because wt/wt percentage DHA was not obtained both at delivery and in cord plasma. Thus 158 mother-child pairs were included in the analysis. After randomization into the 4 intervention groups, the participating women did not differ significantly with respect to parity, height or weight at study entry, smoking habits, or social demographic characteristics (Table I). The neonates showed no significant differences regarding sex, birth weight and length, Apgar score, and parental history of allergy (Table II). The characteristics of the participants of the present study did not differ from those of the main trial. As reported for the main trial,[18] FO intervention, with or without MTHF, was associated with increased percentage wt/wt DHA in cord plasma and maternal plasma in the 30th week of GA and at delivery compared with that seen with placebo or MTHF treatment.

Maternal cytokine expression patterns at delivery

Supplementation with FO was strongly associated with decreased maternal mRNA expression levels of IFN-γ and IL-1 compared with those seen with placebo, MTHF, and the combined supplementation of FO with MTHF (FO&MTHF; Table III). In contrast, TGF-β mRNA was increased compared with placebo or MTHF. Similar to FO intervention, supplementation with FO&MTHF was associated with increased TGF-β levels compared with those seen with placebo and MTHF (trend only, $P = .07$). CCR4 mRNA levels were decreased in the FO intervention groups with or without MTHF (FO±MTHF) and the MTHF intervention groups compared with those seen in the placebo group. Furthermore, CRTH2 mRNA levels were decreased in the FO&MTHF intervention group compared with those seen in the placebo group, whereas FO alone was not associated with altered CRTH2 expression levels.

Cord blood cytokine expression patterns

In cord blood FO supplementation was strongly associated with decreased mRNA levels of the T_H2-associated molecules CCR4, IL-13, and IL-4 compared with placebo or MTHF, whereas levels of T_H1 cytokines remained unaffected (Table IV). Furthermore, FO±MTHF was associated with decreased IL-13 mRNA levels compared with those seen with placebo (but not MTHF). Single MTHF intervention correlated with decreased CXCR3 and IL-1 mRNA levels compared with those seen with FO or placebo. Similar to mothers, TGF-β expression was increased in the FO intervention group, but the combined FO&MTHF intervention was not associated with altered TGF-β mRNA levels. When we stratified for maternal allergic disease, the decrease of IL-13 mRNA levels in the FO group was more pronounced in cord blood samples from nonallergic mothers (see Table E4 in the Online Repository at www.jacionline.org).

Effects of DHA supplementation on lymphocyte subsets in cord blood

Cord blood lymphocytes were quantified by means of flow cytometry in cord blood samples from German participants to assess how the 4 different intervention arms affected lymphocyte

TABLE I. Characteristics of mothers after randomization into intervention groups at study entry

	Placebo (n = 50)	FO (n = 49)	5-MTHF (n = 49)	FO&MTHF (n = 49)	*P* value†
Study center*					.468
Spain	19 (38)	21 (43)	26 (53)	24 (49)	
Germany	19 (38)	16 (33)	9 (18)	12 (24.5)	
Hungary	12 (24)	12 (24)	14 (29)	13 (26.5)	
Weight (kg)	67.3 (64.3-70.4)	69.4 (66.6-72.2)	67.5 (64.4-70.6)	66.7 (63.9-69.4)	.592
Height (cm)	167.6 (163.2-171.9)	164.3 (160.1-168.6)	164.4 (159.9-168.8)	165.7 (162.2-169.2)	.810
Parity					.636
<2	64 (92)	44 (90)	42 (86)	42 (86)	
2	4 (8)	5 (10)	5 (10)	6 (12)	
>2	0 (0)	0 (0)	2 (4)	1 (2)	
Smoking					.976
Yes	4 (8)	5 (10)	5 (10)	5 (10)	
No	46 (92)	44 (90)	44 (90)	44 (90)	
Living					.881
Single	2 (4)	1 (2)	2 (4)	1 (2)	
In partnership	48 (96)	48 (98)	47 (96)	48 (98)	
Job training (father)‡	n = 49	n = 48	n = 48	n = 47	.653
None	20 (41)	19 (40)	23 (48)	13 (28)	
Apprenticeship	8 (19)	10 (21)	11 (23)	14 (30)	
Masters degree	3 (6)	4 (8)	3 (6)	3 (6)	
University degree	18 (37)	14 (29)	11 (23)	17 (36)	
Others	0 (0)	1 (2)	0 (0)	0 (0)	

*Results are expressed as means (95% CIs) for continuous variables and numbers of subjects (percentages) for ordinal data.
†*P* values refer to differences among all intervention groups (1-way ANOVA for continuous data and Pearson χ^2 test for ordinal data).
‡Numbers of subjects are only indicated when they differ from the total number of subjects in each group.

TABLE II. Characteristics of neonates after randomization into intervention groups at study entry

	Placebo (n = 50)	FO (n = 49)	5-MTHF (n = 49)	FO&MTHF (n = 47)	*P* value†
Study center*					.468
Spain	19 (38)	21 (43)	26 (53)	24 (49)	
Germany	19 (38)	16 (33)	9 (18)	12 (24.5)	
Hungary	12 (24)	12 (24)	14 (29)	11 (26.5)	
Female sex	22 (44)	24 (49)	32 (65)	26 (55)	.170
Birth weight (g)‡	n = 50	n = 49	n = 47	n = 47	.924
	3280.7 (3.140-3.422)	3318.1 (3.137-3.499)	3316.4 (3.170-3.463)	3253.6 (3.104-3.403)	
Birth length (cm)	n = 50	n = 48	n = 46	n = 46	
	50.8 (50.0-51.6)	50.6 (49.6-51.6)	50.5 (49.7-51.3)	50.9 (50.1-51.7)	
Apgar score, 5 min	n = 49	n = 49	n = 48	n = 47	.896
	9.8 (9.7-9.9)	9.8 (9.6-9.9)	9.8 (9.7-10.0)	9.6 (9.3-9.9)	
Cord artery pH	n = 44	n = 43	n = 43	n = 38	.929
	7.3 (7.3-7.3)	7.3 (7.3-7.3)	7.3 (7.2-7.3)	7.3 (7.2-7.3)	
Maternal allergy	n = 49	n = 49	n = 49	n = 50	.101
Atopic eczema	4 (8)	4 (8)	1 (2)	5 (10)	
Hay fever	10 (20)	6 (12)	6 (12)	3 (6)	
Asthma	2 (4)	1 (2)	4 (8)	3 (6)	
Others	8 (16)	8 (16)	4 (8)	2 (4)	
Paternal allergy (n)	n = 50	n = 49	n = 49	n = 49	.130
Atopic eczema	0 (0)	1 (2)	0 (0)	2 (4)	
Hay fever	7 (14)	7 (14)	7 (14)	3 (6)	
Asthma	0 (0)	0 (0)	2 (4)	3 (6)	
Others	8 (16)	5 (10)	6 (12)	1 (2)	

*Results are expressed as means (95% CIs) for continuous variables and numbers of subjects (percentages) for ordinal data.
†*P* values refer to differences among all intervention groups (1-way ANOVA for continuous data and Pearson χ^2 test for ordinal data).
‡Numbers of subjects are only indicated when they differ from the total number of subjects in each group.

subpopulations (n = 30). Because the number of samples in the MTHF group was too low for statistical evaluation, only the FO±MTHF and placebo groups were evaluated (see Table E5 in the Online Repository at www.jacionline.org). The number of natural killer (NK) cells ($CD16^+$) was significantly decreased in the FO intervention group compared with that seen in the placebo group ($P < .001$). Furthermore, $CCR3^+CD8^+$ T cells were decreased in the FO group ($P = .04$).

TABLE III. Estimated geometric mean levels of maternal cytokines at delivery by treatment group

	Placebo	FO	5-MTHF	FO&MTHF	*P* value
CCR4	0.84* (0.80-0.88)	0.78 (0.74-0.81)	0.76† (0.73-0.79)	0.76† (0.72-0.79)	.01†
IL-13	0.68 (0.64-0.73)	0.69 (0.65-0.73)	0.65 (0.62-0.69)	0.62 (0.58-0.65)	
IL-4	0.67 (0.64-0.71)	0.67 (0.64-0.71)	0.65 (0.62-0.68)	0.62 (0.59-0.65)	
CRTH2	0.83 (0.79-0.86)	0.80 (0.77-0.84)	0.79 (0.76-0.81)	0.76† (0.73-0.79)	.02†
CXCR3	0.75 (0.72-0.79)	0.76 (0.73-0.79)	0.75 (0.73-0.78)	0.78 (0.75-0.82)	
IFN-γ	0.65 (0.61-0.69)	0.54† (0.51-0.57)	0.63† (0.60-0.66)	0.63‡ (0.60-0.66)	<.00†‡
IL-1	0.83 (0.79-0.88)	0.69† (0.66-0.73)	0.79‡ (0.75-0.82)	0.77‡ (0.74-0.81)	<.00†‡; .02‡
TGF-β	0.68 (0.64-0.72)	0.85† (0.80-0.89)	0.75‡ (0.71-0.78)	0.82† (0.78-0.86)	<.00†; .01‡

*Data are expressed as geometric means (95% CIs). One-way analysis of covariance adjusted for center (reference: Hungary) with maternal percentage wt/wt DHA at week 20 of gestation as a covariate was performed. All *P* values are corrected for multiple comparisons by using the Tukey-Kramer procedure.
†Versus placebo.
‡Versus FO.

TABLE IV. Estimated geometric mean levels of fetal cytokines by treatment group

	Placebo	FO	5-MTHF	FO&MTHF	*P* value
CCR4	0.88* (0.84-0.92)	0.70†§ (0.67-0.73)	0.87 (0.83-0.91)	0.75†§ (0.71-0.78)	<.00†§
IL-13	0.85 (0.80-0.89)	0.61†§ (0.58-0.65)	0.76† (0.72-0.80)	0.73†‡ (0.69-0.77)	<.00†§‡; 0.03†
IL-4	0.64 (0.61-0.68)	0.54†§ (0.52-0.57)	0.65 (0.62-0.68)	0.53†§ (0.50-0.55)	<.00†§
CRTH2	0.80 (0.77-0.83)	0.78 (0.76-0.81)	0.78 (0.75-0.81)	0.76 (0.74-0.79)	
CXCR3	0.81 (0.78-0.84)	0.79 (0.76-0.81)	0.73†‡ (0.71-0.76)	0.77 (0.74-0.79)	<.00†; 0.04‡
IFN-γ	0.60 (0.58-0.63)	0.59 (0.56-0.61)	0.60 (0.57-0.63)	0.59 (0.56-0.62)	
IL-1	0.85 (0.81-0.89)	0.81 (0.78-0.85)	0.75† (0.72-0.79)	0.80 (0.76-0.83)	<.00†
TGF-β	0.75 (0.71-0.79)	0.85† (0.81-0.90)	0.75 (0.72-0.79)	0.76‡ (0.73-0.80)	<.00†; 0.02‡

*Data are expressed as geometric means (95% CIs). One-way analysis of covariance adjusted for center (reference Hungary) with maternal percentage wt/wt DHA at week 20 of gestation as a covariate was performed. All *P* values are corrected for multiple comparisons by using the Tukey-Kramer procedure.
†Versus placebo.
‡Versus FO.
§Versus MTHF.

DISCUSSION

The present study demonstrates that FO supplementation of pregnant women is associated with decreased mRNA levels of IL-4, IL-13, and CCR4 in cord blood and with decreased frequencies of cord blood NK cells and $CCR3^+CD8^+$ T cells. In contrast, mRNA levels of the regulatory cytokine TGF-β are increased in maternal peripheral blood at delivery, as well as in cord blood. Furthermore, mRNA levels of IL-1 and IFN-γ were decreased in FO-supplemented mothers, whereas the T_H2-associated chemokine receptor CCR4 is decreased in FO&MTHF-supplemented women.

Our finding of reduced cord blood T_H2-related molecules confirms and extends the results of Dunstan et al,[9] who found decreased IL-13 plasma levels in offspring from atopic mothers supplemented with 1.2 g of DHA. Our study further shows that the decrease of T_H2-related molecules is not restricted to neonates at risk of atopy. Whether mRNA levels were altered by changes in cell proportions or by modified cytokine production is unknown because T_H1 and T_H2 cells are difficult to quantify in unstimulated blood. The decreased frequency of cord blood $CCR3^+CD8^+$ cells points to a downmodulation of fetal T_H2 responses at the cellular level. Nevertheless, FO might act both at the cellular and transcriptional levels. The reduction of NK cells after FO intervention is in line with the results of previous studies.[24]

FO supplementation seems to exert different effects on the fetal immune system compared with the mature immune system of an adult. Thus FO supplementation was primarily associated with reduced IL-1 and IFN-γ mRNA levels in mothers. Suppression of inflammatory cytokines is a well-known feature of n-3 LC-PUFAs, especially of DHA and EPA (reviewed by Calder[25]). Because pregnancy is associated with a T_H2 shift of the maternal immune response, downmodulation of T_H1 immune responses might be beneficial. On the other hand, an overly strong T_H1 suppression could impair immune responses toward pathogens. However, in our main trial analysis, supplemented mothers did not experience more infections compared with nonsupplemented mothers,[18] nor was this reported from other intervention studies in pregnant women.[26] In contrast to mothers, mainly T_H2-associated mRNA transcripts were downmodulated in cord blood samples from the FO group.

TGF-β mRNA levels were increased in FO-supplemented mothers, as well as their offspring. TGF-β is produced by a variety of cell types, including regulatory T cells. Regulatory T cells suppress responses toward self-antigens and NK cell activity, which is extremely important for the maintenance of the fetal allograft. Correspondingly, maternal TGF-β plasma levels increase during pregnancy, with the highest levels in late gestation.[27] The different effects of maternal FO supplementation on fetal and maternal immune parameters might be explained by a common regulation through TGF-β, which suppresses the respective predominant immune response in mother and fetus. Furthermore, TGF-β has been shown to be involved in tolerance toward allergens,[28] and reduced cord blood TGF-β production was found in offspring from atopic mothers.[29] The increase of cord plasma DHA in the FO group is within the range of the difference observed in plasma from atopic versus nonatopic individuals.[30,31] Whether the increase of TGF-β levels in offspring of FO-supplemented mothers ultimately helps to protect against allergic

disease remains to be determined because the children of our study are too young at present to assess allergic outcomes.

The association of MTHF supplementation with decreased maternal CCR4 and fetal CXCR3 and IL-1 mRNA levels indicates a potential relation of MTHF with cytokine mRNA transcription. Although folate influences lymphocyte effector functions,[14-16] explanations for an underlying mechanism remain speculative. Because folate can reverse homocysteine–induced trophoblast apoptosis,[32] it might affect placental cytokine synthesis and hence indirectly affect fetal cytokine production. However, this does not explain why maternal CCR4 mRNA levels were decreased. Alternatively, a potential synergy between the MTHF and DHA pathways has been suggested.[33-37] Although this model could explain why FO&MTHF was superior to FO to suppress maternal CCR4 mRNA levels, it is not clear why fetal CXCR3 and IL-1 levels were decreased by MTHF alone but not in combination with FO. Thus other mechanisms must exist to explain these findings. The decrease of maternal IL-1 and IFN-γ levels in the FO group was not replicated in the FO&MTHF group. Similarly, fetal IL-1 levels were decreased in the MTHF group but not in the combined FO&MTHF group. Although no interaction of MTHF with FO was found in our main trial analysis,[18] we cannot exclude that our study was underpowered to detect a significant interaction between both supplements. Why maternal and fetal IL-1 levels were decreased in the FO and the MTHF groups, respectively, is difficult to explain. Aside from differences that are related to the developing fetal immune system as opposed to the mature maternal immune system, pregnancy itself is likely to affect the maternal immune system. Furthermore, it can be speculated that different hormone status, growth factors, or baseline fatty acid profiles in mothers compared with those in their offspring contributed to the observations.

In conclusion, the present European multicenter study shows that long-term FO supplementation during pregnancy has differential effects on maternal and fetal immune parameters. The most prominent findings were a decrease of maternal inflammatory/T_H1 cytokines and a decrease of fetal T_H2-related cytokines. We speculate that these different effects are mediated through TGF-β. Furthermore, 5-MTHF was independently associated with altered fetal CXCR3 and IL-1 mRNA levels. The underlying mechanisms are unknown at present, and this observation needs to be confirmed in further randomized trials. The follow-up of this study will reveal whether the altered balance between fetal TGF-β and T_H2-related mRNA expression levels translates into a decreased incidence of allergic diseases in offspring of FO-supplemented mothers.

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Clinical implications: FO supplementation of pregnant women might be an option for primary allergy prevention.

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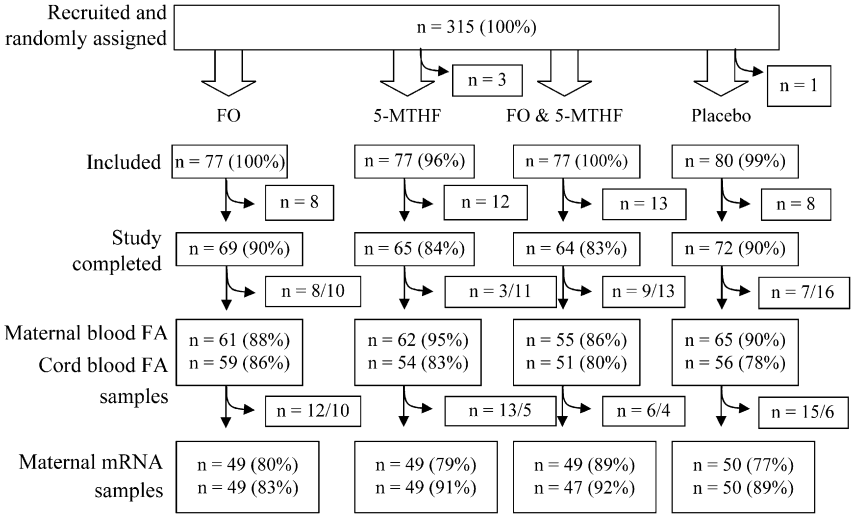

FIG E1. Numbers of enrolled subjects and blood samples for fatty acid and mRNA analyses. Four subjects did not fulfill the inclusion criteria. Dropouts were due to noncompliance (n = 2), relocation (n = 1), bad taste of the supplement (n = 9), and lost contact (n = 2). In 17 cases the reasons for dropout are unknown. Forty-six maternal and 25 cord blood samples were not included in the mRNA analyses because not enough material was available or contamination with genomic DNA could not be excluded.

TABLE E1. Composition of supplements according to manufacturers analyses per sachet (15 g)

	Placebo	FO	5-MTHF	FO&MTHF
DHA (mg)	0	500	0	500
EPA (mg)	0	150	0	150
5-MTHF (μg)	0	0	400	400
Energy (kcal)	70	71	70	71
Protein (g)	2.9	2.5	2.9	2.5
Fat (g)	2.9	3.1	2.9	3.1
Carbohydrate (g)	8.0	8.2	8.0	8.2

Identical for all supplementation groups—minerals: 300 mg of calcium, 240 mg of phosphorus, 93 mg of magnesium, 3 mg of zinc, and 66 μg of iodine; vitamins: 330 μg of vitamin A, 1.5 μg of vitamin D, 3 mg of vitamin E, 0.36 mg of vitamin B_1, 1.5 mg of vitamin B_2, 4.5 mg of vitamin B_3, 1.9 mg of vitamin B_6, 3.5 μg of vitamin B_{12}, and 270 mg of vitamin C.

TABLE E2. Fatty acid composition of the FO supplements used in this study

Fatty acid	Placebo	FO
C4:0	0.92*	0.67
C6:0	1.41	0.87
C8:0	1.16	0.65
C10:0	3.07	1.66
C12:0	3.94	2.16
C14:0	12.88	7.33
C15:0	1.32	0.85
C16:0	36.45	22.03
C16:1 n-7	2.13	1.64
C17:0	0.85	0.88
C18:0	10.37	8.82
C18:1 n-9+n-7	22.48	20.76
C18:2 n-6	1.74	2.02
C20:0	0.21	0.36
C18:3 n-3	0.53	0.69
C20:1 n-9	BD	1.01
C20:4 n-6	0.14	1.16
C22:1 n-9	BD	0.17
C20:5 n-3	BD	5.58
C22:4 n-6	BD	1.02
C22:5 n-3	0.11	1.66
C22:6 n-3	BD	17.84

The data are mean values of 2 batches used in the study. The supplements were provided as a cow's milk–based powder. Thus the fatty acid profile corresponds to cow's milk fatty acids. Placebo contained around 90% and the experimental product contained around 65% of this ingredient. FO was added instead of milk fat, leading to more PUFAs and less saturated fatty acids in the intervention product compared with placebo. Linoleic acid and linoleneic acid were similar in both supplements.
BD, below detection limit.
*Percentage by weight of total fatty acids.

TABLE E3. Primer pairs

mRNA target	Oligonucleotides	Product size (bp)
CCR4	F: TggTTCTggTCCTgTTCAAA	175
	R: TATCTATCAATgCTCATgAgCATgA	
	H: gCTAggTCTgTgCAAgATgATTTCC	
IL-13	F: CCTCCCTCTACAgCCCTCA	160
	R: gAATCCgCTCAgCATCCTCT	
	H: CCAgAAggCTCCgCTCTgCAA	
IL-4	F: AgAAgACTCTgTgCACCgAgTTgA	259
	R: CTCTCATgATCgTCTTTAgCCTTT	
	H: AAgCAgCTgATCCgATTCCTgAAACg	
CRTH2	F: CCAgTTTCAgggCTAACgAC	214
	R: TCAACTTCTAACgACCgAAg	
CXCR3	F: ggTCCTTgAggTgAgTgACC	113
	R: CgAAgTTCAggCTgAAgTCC	
IFN-γ	F: gCATCCAAAAgAgTgTggAg	192
	R: ATgCTCTTCgACCTCgAAAC	
	H: ACgCAAAgCAATACATgAACTCA	
IL-1	F: CAgggACAggATATggAgCAA	232
	R: ATgTACCAgTTggggAACTg	
TGF-β	F: CATCAACgggTTCACTACC	130
	R: CTCCgTggAgCTgAAgCA	
	H: CCACCATTCATggCATgAACCgg	
GAPDH	F: gAAggTgAAggTCggAgTCR: gAAg ATggTgATgggATTTC	188

Primers were designed with Primer Premier 5 primer design software (PREMIER Biosoft International, Palo Alto, Calif) and supplied by TibMolBiol (Berlin, Germany).
F, Forward; *R*, reverse; *H*, hydrolysis probe; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

TABLE E4. Estimated geometric mean levels of fetal cytokines by treatment group stratified for maternal allergy

	Placebo	FO	5-MTHF	FO&MTHF
CCR4	0.71* (0.64-0.78)	0.72 (0.67-0.77)	0.83 (0.76-0.90)	0.86 (0.80-0.93)
CCR4	0.75 (0.71-0.80)	0.69 (0.65-0.73)	0.88 (0.83-0.93)	0.89 (0.83-0.95)
IL-13	0.66 (0.59-0.73)	0.65 (0.60-0.70)	0.77 (0.70-0.84)	0.79 (0.73-0.86)
IL-13	0.75 (0.71-0.80)	0.59 (0.55-0.63)	0.76 (0.72-0.81)	0.88 (0.82-0.94)
IL-4	0.55 (0.50-0.60)	0.55 (0.52-0.59)	0.61 (0.57-0.67)	0.61 (0.57-0.66)
IL-4	0.53 (0.50-0.56)	0.53 (0.50-0.57)	0.66 (0.62-0.70)	0.65 (0.61-0.70)
CRTH2	0.78 (0.72-0.83)	0.81 (0.77-0.85)	0.74 (0.69-0.78)	0.78 (0.74-0.83)
CRTH2	0.76 (0.73-0.80)	0.77 (0.73-0.80)	0.80 (0.76-0.83)	0.80 (0.76-0.84)
CXCR3	0.75 (0.69-0.81)	0.81 (0.76-0.86)	0.76 (0.71-0.81)	0.82 (0.76-0.87)
CXCR3	0.77 (0.74-0.80)	0.77 (0.74-0.81)	0.73 (0.70-0.75)	0.80 (0.77-0.84)
IFN-γ	0.54 (0.50-0.59)	0.61 (0.57-0.64)	0.55 (0.52-0.59)	0.61 (0.57-0.65)
IFN-γ	0.60 (0.57-0.64)	0.58 (0.54-0.61)	0.61 (0.58-0.65)	0.60 (0.57-0.64)
IL-1	0.82 (0.76-0.89)	0.79 (0.75-0.84)	0.73 (0.68-0.78)	0.83 (0.78-0.88)
IL-1	0.80 (0.76-0.84)	0.82 (0.77-0.87)	0.76 (0.72-0.81)	0.86 (0.81-0.92)
TGF-β	0.77 (0.69-0.87)	0.86 (0.79-0.93)	0.69 (0.63-0.76)	0.72 (0.66-0.78)
TGF-β	0.76 (7.72-0.81)	0.84 (0.79-0.90)	0.78 (0.74-0.83)	0.76 (0.71-0.82)

*Data are expressed as geometric means (95% CIs) and are stratified for presence or absence *(italics)* of maternal allergies.

TABLE E5. Percentages of lymphocyte subpopulations within total lymphocytes in the intervention groups

	Placebo	FO	FO&MTHF
CD4	38.0* (32.4-44.6)	42.7 (35.3-51.6)	47.0 (35.6-62.0)
CD8	20.9 (16.5-26.4)	18.9 (14.3-25.1)	28.4 (18.8-42.8)
CD19	9.4 (5.6-15.8)	4.9 (2.6-9.0)	11.4 (4.7-28.1)
CD16	14.6 (12.3-17.2)	9.0† (7.5-11.0)	13.7 (10.3-18.2)
CD69	13.5 (5.8-31.3)	9.9 (4.0-24.8)	9.1 (2.3-36.0)
CCR3CD4	2.2 (0.5-9.6)	0.9 (0.2-4.4)	0.6 (0.08-4.8)
CCR3CD8	1.5 (0.5-4.5)	0.2‡ (0.1-0.6)	1.2 (0.2-8.2)
CCR4CD4	7.0 (5.1-9.6)	4.4 (3.0-6.4)	6.7 (3.9-11.6)
CCR4CD8	0.4 (0.2-1.0)	0.2 (0.1-0.5)	0.2 (0.0-1.1)
CCR5CD4	0.1 (0.1-0.2)	0.2 (0.1-0.4)	0.4 (0.1-1.1)
CCR5CD8	0.1 (0.0-0.3)	0.1 (0.1-0.2)	0.2 (0.0-1.4)

*Data are expressed as geometric means (95% CIs). Numbers of subjects in the 5-MTHF group were too low for meaningful analysis. One-way analysis of variance adjusted for center (reference Hungary) with maternal percentage wt/wt DHA at week 20 of gestation as a covariate was performed. All *P* values are corrected for multiple comparisons by using the Tukey-Kramer procedure.

†$P < .00$ versus placebo.

‡$P < .04$ versus placebo.