Modulation of DNA methylation states and infant immune system by dietary supplementation with ω-3 PUFA during pregnancy in an intervention study1–5

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ABSTRACT

Background: Early-life exposures to tobacco smoke and some dietary factors have been identified to induce epigenetic changes in genes involved in allergy and asthma development. Omega-3 (n–3) polyunsaturated fatty acid (PUFA) intake during pregnancy could modulate key cytokines and the T helper (Th) cell maturation; however, little is known about the mechanism by which ω-3 PUFA could have a beneficial effect in preventing inflammatory disorders. Objective: We sought to test whether prenatal dietary supplementation with ω-3 PUFA during pregnancy may modulate epigenetic states in the infant immune system. Design: This study was based on a randomized intervention trial conducted in Mexican pregnant women supplemented daily with 400 mg docosahexaenoic acid (DHA) or a placebo from 18 to 22 wk of gestation to parturition. We applied quantitative profiling of DNA methylation states in Th1, Th2, Th17, and regulatory T–relevant genes as well as LINE1 repetitive elements of cord blood mononuclear cells (n = 261). Results: No significant difference in promoter methylation levels was shown between ω-3 PUFA–supplemented and control groups for the genes analyzed; however, ω-3 PUFA supplementation was associated with changes in methylation levels in LINE1 repetitive elements (P = 0.03) in infants of mothers who smoked during pregnancy. Furthermore, an association between the promoter methylation levels of IFNγ and IL13 was modulated by ω-3 PUFA supplementation (P = 0.06).

Conclusions: Our results indicate that maternal supplementation with ω-3 PUFA during pregnancy may modulate global methylation levels and the Th1/Th2 balance in infants. Therefore, the epigenetic mechanisms could provide attractive targets for prenatal modulation and prevention of inflammatory disorders and potentially other related diseases in childhood and adulthood. Am J Clin Nutr doi: 10.3945/ajcn.112.052241.

INTRODUCTION

The deregulation of the immune system and inflammatory responses are important factors in the development of a wide range of human malignancies, although the precise underlying mechanisms remain unknown. Several studies have investigated causes and risk factors of immune-mediated diseases (1, 2); however, no clear molecular links connect the risk factors and the diseases. It is recognized that increases in the incidence of allergic diseases might be a result of a failure of normal immune regulation in early life rather than simply because of the maintenance of the T helper (Th)2 predominance of the immune response present at birth (3).

On encountering foreign antigens displayed by antigen-presenting cells, naïve CD4+ T cells can differentiate into T cell lineages, which are controlled by cytokines produced by innate immune cells. Th1 cells produce interferon-γ and are responsible for cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, and IL-13, which promote B cell activation and class switching, thereby providing protection against infections. The Th17 subset provides a host defense against extracellular bacteria, particularly at mucosal sites. Regulatory T (Treg) cells have a crucial role in maintaining homeostasis of the immune system and preventing the autoimmune reactivity of self-reactive T cells.

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6 Abbreviations used: CAP, capture system for specific serum IgE; CBMC, cord blood mononuclear cell; Th, T helper; Treg, regulatory T.

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Experimental studies reported on the epigenetic regulation of immune development and early immune profiles that contribute to allergic risk. For example, the maintenance of T cell function, including the pattern of Th1, Th2, Th17, and Treg cell differentiation and development, was shown to be epigenetically regulated (4–6). In particular, early life exposures to environmental factors such as dietary intake and exposure to tobacco might induce epigenetic changes in the regulation of inflammatory genes and, thus, lead to altered allergy risk (7, 8). In an animal model, the supplementation of the maternal diet with methyl donors was associated with airway hyperreactivity, eosinophilic inflammation, and IgE production in offspring; these features of allergic airway disease are under epigenetic control by transcriptional regulation through DNA methylation changes (9).

Both epidemiologic and experimental studies have shown that maternal intake of omega-3 (ω-3; also known as n-3) PUFA, including EPA (20:5n−3) and DHA (22:6n−3), is associated with protection against atopic or allergic outcomes in children (10–12). Omega-3 PUFA intake during pregnancy and early infancy has been shown to modulate key regulatory cytokines and the maturation of Th cells (13). However, data are inconsistent, and little is known about the mechanism by which ω-3 PUFA could have a beneficial effect in preventing immunemediated diseases.

In this study, we tested the hypothesis that prenatal supplementation with ω-3 PUFA modulates epigenetic changes of key genes involved in the development of the immune system in utero, which can affect risk of allergic diseases and asthma in childhood.

SUBJECTS AND METHODS

Study population and design

The study was based on a double-blind, randomized, placebo-controlled intervention trial with ω-3 PUFA supplementation conducted in Mexico (14, 15). Briefly, pregnant women were recruited between July 2005 and May 2007 at the Instituto Mexicano del Seguro Social General Hospital 1 in Cuernavaca, Mexico (14, 15). A screening questionnaire was used to identify women who met the inclusion criteria. Eligible women were 18–35 y old, in gestation weeks 18–22, and residents of Cuernavaca who intended to deliver at Instituto Mexicano del Seguro Social General Hospital 1, remain in the area for the next 2 y, and provided informed consent. Women were randomly assigned to receive either 400 mg algal DHA daily (2 capsules/d) or placebo until delivery. Each tablet provided 200 mg DHA synthesized from an algal source, and placebo capsules contained olive oil and were similar in appearance and taste to the DHA capsules. Study participants and members of the study team remained unaware of the treatment scheme throughout the intervention period of the study. After the study had been explained orally and in writing, everyone in the study population provided written consent to participate in this study. The local ethics committee approved the protocol, and the study was also reviewed and approved by the Ethics Committee of the International Agency for Research on Cancer.

To determine the maternal allergic status, we measured specific IgE to 10 common allergens [milk, cat, egg, dust mite, wheat, Alternaria, grasses (Bermudagrass and timothygrass), and pollen (mountain cedar and ragweed) in blood samples collected during pregnancy by using the capture system for specific serum IgE (CAP) technique (Pharmacia Diagnostic). CAP-specific IgE concentrations were defined as follows: 0, <0.35 IU/mL (negative); 1, 0.35–0.69 IU/mL (very low); 2, 0.70–3.49 IU/mL (moderate); 3, 3.50–17.49 IU/mL (moderate to high); 4, 17.50–49.99 IU/mL (high); and 5, 50–100 IU/mL (very high). Maternal atopy was defined as a CAP class ≥1 for one or more of the allergens tested.

Sample collection

We collected umbilical cord blood samples from infants of supplemented and control mothers including mothers with an allergy (atopic group) and without an allergy (nonatopic group). Umbilical cord blood samples were collected by venipuncture of cord vessels after the cord had been clamped and cut, placed into a tube containing EDTA, and kept at room temperature until delivery to the Instituto Nacional de Salud Pública laboratory for isolation of cord blood mononuclear cells (CBMCs). The isolation procedure was completed in ≤12 h of collection. CBMCs were cryopreserved following a standard protocol. Cord blood was layered on Ficoll-Hypaque (Lymphoprep; Nycomed Pharma). CBMCs were separated by density centrifugation and stored at −80°C for additional analyses. For an additional analysis, we first randomly selected 100 CBMC samples from supplemented mothers and 100 CBMC samples from control subjects. To increase the sample size in the maternal smoking group and further strengthen the association between smoking and DNA methylation, we selected an additional 61 samples (31 samples of which were from the maternal smoking group) for the analysis (which made a total of 261 samples analyzed of which 52 samples were in the smoking group). The distribution of atopic and nonatopic mothers in these subgroups was reasonably balanced.

DNA extraction

The isolation of DNA from CBMCs was performed by using the AllPrep DNA/RNA mini kit (Qiagen) according to the AllPrep DNA/RNA protocol with minor modifications. The quantity and quality of purified DNA were determined with an ND-1000 spectrophotometer (NanoDrop Technologies). DNA was stored at −20°C before use.

Bisulfite conversion and pyrosequencing

A DNA methylation analysis was performed by pyrosequencing after DNA extraction from CBMCs and bisulfite conversion as previously described (16). In our selection of relevant genes that are potential targets of DNA methylation associated with dietary supplementation and the immune response, we were guided by 2 criteria as follows: genes that may be associated with the immune system or sequences that may be used as a surrogate for global methylation. We established pyrosequencing assays for quantitative measurement of DNA methylation levels in the promoter region of the following target genes in ω-3 PUFA and control groups (Table 1; see Supplemental Figure 1 under “Supplemental data” in the online issue): IFNγ, TNF-α, IL13, GATA3, STAT3, IL10, FOXP3 (17), and LINE1 repetitive elements (18). Target CpG sites (regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide) were evaluated by converting the
resulting pyrograms to numerical values for peak heights. The percentage of methylation was calculated as described previously (19). Primer sequences for target genes are shown in Table 1.

### Statistical analysis

Baseline characteristics of the study population were compared between ω-3 PUFA and control groups by using the *t* test for continuous variables or the chi-square test for categorical ones. Main covariates included maternal age (y), height (cm), weight (kg), BMI (kg/m²), educational level (0–6, 7–12, or 13–16 y), socioeconomic level (low, medium, or high), maternal smoking during pregnancy, paternal smoking status, sex, birth weight (g), and gestational duration (wk). Correlations between promoter methylation levels of genes encoding Th1 (*IFNγ* and *Th2* (*IL13*) cytokines were assessed by using Pearson’s correlation analysis to determine whether the correlation was changed by ω-3 PUFA supplementation. Multivariable linear regression was used to estimate the associations between DNA methylation and ω-3 PUFA supplementation. The final multivariate model was obtained considering a significance level of *P < 0.05* (sex, gestational duration, and BMI) and biological plausibility. All models were conducted in all children and after stratification by maternal smoking. Interactions between ω-3 PUFA supplementation and maternal smoking and BMI were tested by including an interaction term in the multivariable linear regression when *P ≤ 0.15*, as suggested by Hosmer and Lemeshow (20) (see Supplemental Table 1 under “Supplemental data” in the online issue). Interaction terms were considered significant at *P ≤ 0.20* as proposed by Selvin (21) for studies with a limited sample size. All analyses were conducted with SAS statistical software (version 9.2; SAS Institute).

### RESULTS

#### Characteristics of study population

Baseline characteristics of the study population by ω-3 PUFA intake are shown in Table 2. There were no significant differences in maternal atopic status, paternal smoking status, socioeconomic level, or gestational duration between the ω-3 PUFA–supplemented group and control subjects. However, the mean BMI before pregnancy of the ω-3 PUFA group was lower than that of control subjects (and this difference was significant; 25.9 ± 30; *P = 0.01*), and 13.5% of the ω-3 PUFA–supplemented group and 20% of control subjects were obese (BMI ≥ 30; *P = 0.97*).

#### Modulation of global DNA methylation levels by ω-3 PUFA supplementation during pregnancy

To examine whether prenatal supplementation with ω-3 PUFA may modulate DNA methylation states in the immune system of infants, we first examined global methylation levels in CBMCs by analyzing DNA methylation in *LINE1* sequences, which are repeated retrotransposons commonly used as surrogates for the global 5-methylcytosine content (22). As shown in Figure 1, the overall global DNA methylation was similar between ω-3 PUFA–supplemented subjects and control subjects in the
maternal nonsmoking group (71.60% compared with 71.60%; P = 0.99). In addition, we showed that CBMCs from infants of mothers who smoked during pregnancy did not exhibit significantly different global methylation levels compared with those of nonsmoking mothers (P = 0.67). However, in the maternal smoking group, the mean global DNA methylation level was significantly higher in the ω-3 PUFA subgroup than in the nonsupplemented subgroup (P = 0.03) (Figure 1, A and B). This finding was strengthened because the interaction test between maternal smoking during pregnancy and ω-3 PUFA supplementation was significant (P = 0.11) (see Supplemental Table 1 under “Supplemental data” in the online issue). These results suggested that maternal smoking during pregnancy may influence global methylation levels in repetitive DNA elements in infants, and ω-3 PUFA supplementation may increase global methylation levels, consistent with the notion that retrotransposon mobility may be counteracted, and epigenome of infant cells stabilized, through dietary intervention.

DNA methylation states in specific genes are not modulated by ω-3 PUFA supplementation during pregnancy

To examine the impact of ω-3 PUFA supplementation on DNA methylation in immune-response genes, we performed a quantitative measurement of DNA methylation levels in IFNγ, TNF-α, IL13, GATA3, STAT3, IL10, and FOXP3 genes. The mean promoter methylation level was slightly lower (although the difference was not significant; P = 0.11) in the ω-3 PUFA group than in control subjects for IFNγ, and the difference was also not evident after adjusting for potential confounding factors (P = 0.15) (Table 3). No difference was observed in DNA methylation levels of the other genes studied (TNF-α, IL13, STAT3, IL10, and FOXP3) between ω-3 PUFA and control groups (Table 3), which suggested that ω-3 PUFA supplementation may not affect methylation levels in specific genes. The interaction between maternal smoking during pregnancy and ω-3 PUFA supplementation was significant only for LINE1 (P = 0.11) (see Supplemental Table 1 under “Supplemental data” in the online issue).

Omega-3 PUFA supplementation during pregnancy may influence Th1/Th2 balance in infants

Omega-3 PUFA has been shown to normalize the Th1/Th2 balance (23). In addition, the DNA methylation of Th cell cytokine genes is associated with gene expression and allergic phenotypes, which suggests that DNA methylation could affect the balance of Th cells. Therefore, we further investigated whether ω-3 PUFA can influence the ratio of methylation levels of Th1- and Th2-specific genes. Our pairwise comparison between Th1, Th2, T17, and Treg genes revealed a correlation between DNA methylation levels of IFNγ and IL13 (r = 0.23, P = 0.02) in the ω-3 PUFA–supplementation group; when the methylation level of IL13 increased, the IFNγ methylation level also increased (Figure 2A). In the control group, DNA methylation levels of IFNγ and IL13 were not correlated (r = −0.10, P = 0.30). The difference in correlation coefficients between groups was not significant (P = 0.43). No significant correlations were identified between other gene pairs tested (data not shown).

We further assessed the correlation between DNA methylation levels of IFNγ and IL13 in the maternal smoking group. We showed that the correlation between DNA methylation levels of IFNγ and IL13 was significantly changed by ω-3 PUFA supplementation in the maternal smoking group (Figure 2B; r = 0.43, P = 0.05). In the control group, the correlation was r = −0.14 (P = 0.54). The difference in correlation coefficients between groups was P = 0.06, which suggested that ω-3 PUFA can alter the ratio of methylation levels of some, although not all, Th1- and Th2-specific genes.

### TABLE 2
Baseline characteristics of the study population by ω-3 PUFA intake

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Control subjects (n = 130)</th>
<th>ω-3 PUFA group (n = 131)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal atopy [n (%)]</td>
<td>72 (55.8)</td>
<td>70 (53.4)</td>
<td>0.71</td>
</tr>
<tr>
<td>Total IgE concentration in cord blood (IU/mL)</td>
<td>0.22 (0.14, 0.29)</td>
<td>0.32 (0.12, 0.53)</td>
<td>0.33</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>155.6 (154.6, 156.5)</td>
<td>155.3 (154.3, 156.3)</td>
<td>0.68</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.4 (63.2, 67.5)</td>
<td>62.3 (60.5, 64.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0 (26.2, 27.8)</td>
<td>25.9 (25.1, 26.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>Educational level [n (%)]</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–6 y</td>
<td>14 (10.8)</td>
<td>9 (6.9)</td>
<td></td>
</tr>
<tr>
<td>7–12 y</td>
<td>57 (43.8)</td>
<td>58 (44.3)</td>
<td></td>
</tr>
<tr>
<td>13–16 y</td>
<td>59 (45.4)</td>
<td>64 (48.9)</td>
<td></td>
</tr>
<tr>
<td>Socioeconomic level [n (%)]</td>
<td></td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Low</td>
<td>46 (35.4)</td>
<td>49 (37.4)</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>41 (31.5)</td>
<td>47 (35.9)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>43 (33.1)</td>
<td>35 (26.7)</td>
<td></td>
</tr>
<tr>
<td>Maternal smoking status during pregnancy [n (%)]</td>
<td>26 (22.2)</td>
<td>26 (21.7)</td>
<td>0.99</td>
</tr>
<tr>
<td>Paternal smoking status [n (%)]</td>
<td>50 (42.7)</td>
<td>56 (46.7)</td>
<td>0.60</td>
</tr>
<tr>
<td>Sex, M [n (%)]</td>
<td>64 (49.2)</td>
<td>69 (53.1)</td>
<td>0.62</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3264.7 (3184.7, 3344.7)</td>
<td>3263.7 (3191.0, 3336.5)</td>
<td>0.98</td>
</tr>
<tr>
<td>Gestational duration (wk)</td>
<td>39.24 (38.95, 39.53)</td>
<td>39.37 (39.04, 39.71)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*P* test was used for continuous variables, and the chi-square test was used for categorical variables.

1 Mean; 95% CI in parentheses (all such values).

2 Smoking data were missing for 24 participants (13 control subjects and 11 subjects in the ω-3 PUFA group).
DISCUSSION

We have established pyrosequencing assays for the quantitative and sensitive analysis of global DNA methylation and methylation levels in the promoter region of immune-response genes (IFNγ, TNF-α, IL13, GATA3, STAT3, IL10, and FOXP3) and combined these assays with a randomized trial in pregnant women who were supplemented daily with ω-3 PUFA. We showed that ω-3 PUFA supplementation may influence the global 5-methylcytosine content (LINE1 repetitive sequences) without influencing methylation levels in specific immune response genes in infants of mothers who smoked during pregnancy. This study suggested that prenatal ω-3 PUFA supplementation can modulate global DNA methylation levels, although it is unclear whether it can modulate genes involved in the immune response in infants.
Therefore, our results on the decrease of maturation of immune competence in the developing fetus (27) in the fetal circulation appear to be a good indicator of the evidence exists that low IFN for group than in control subjects, whereas the opposite was true methylation level was significantly higher in the v environment factors on prenatal T cell maturation (27, 36, 37).

A protective role against allergy, supporting a direct influence of environmental factors on prenatal T cell maturation (27, 36, 37). Several studies have reported that prenatal exposures such as to tobacco smoke and maternal diet can cause alterations in DNA methylation, which subsequently might alter a child's risk of developing atopic diseases (9, 24–28). However, data are lacking for a better understanding of the mechanisms of early life immune programming. Prenatal exposure to tobacco smoke has been associated with risk of diseases including asthma, allergy, obesity, type 2 diabetes, and cancer (29–32). Of potential mechanisms, DNA adduct formation, gene mutations, chromosome aberrations, and DNA strand breaks have been evoked (33). DNA methyltransferases have been shown to bind DNA at sites of DNA damage, which results in altered methylation patterns on these regions, suggesting an epigenetic mechanism for the generation of aberrant DNA methylation by exposures to chemicals such as tobacco smoke (34). Prenatal exposure to smoking has been reported to be associated with increased genomic DNA methylation in adulthood (35). We showed that ω-3 PUFA supplementation altered mean methylation levels of LINE1 sequences in the maternal smoking group. For LINE1, the mean promoter methylation level was significantly higher in the ω-3 PUFA group than in control subjects, whereas the opposite was true for IFNγ, although this effect was not significant. Strong evidence exists that low IFNγ promoter methylation levels play a protective role against allergy, supporting a direct influence of environmental factors on prenatal T cell maturation (27, 36, 37). High concentrations of cytokines, such as interferon-γ and IL-4, in the fetal circulation appear to be a good indicator of the maturation of immune competence in the developing fetus (27). Therefore, our results on the decrease of IFNγ promoter methylation related to ω-3 PUFA supplementation suggested a possible protective effect against allergic responses in infants prenatally exposed to maternal smoking.

We also showed that the mean LINE1 methylation level was significantly lower in infants prenatally exposed to maternal smoking than in unexposed ones, which suggested that prenatal exposure to smoking results in a significant loss of global methylation, consistent with several previous studies (38). This result was consistent with the “developmental origins hypothesis,” which proposes that susceptibility to diseases in childhood and adulthood is strongly influenced through adaptive responses to in utero and early life conditions (39).

Lower global methylation levels (as revealed by LINE1 methylation) in the maternal smoking group could be counteracted by ω-3 PUFA supplementation. Because of their high copy numbers and correlation with the total genomic 5-methylcytosine content, the LINE1 methylation level is often used as a surrogate for the global methylation level (40). The methylation of LINE1 repetitive elements is thought to play a role in maintaining genomic stability. Thus, our results suggested that prenatal ω-3 PUFA supplementation maintains genomic stability and protects
infant DNA from damage induced by smoking and potentially other in utero exposures.

In this study, we showed that DNA methylation levels of genes encoding Th1 and Th2 cytokines (IFN-γ and IL13, respectively) were changed by ω-3 PUFA supplementation. One interpretation of these findings is that DNA methylation plays an important role in the Th1/Th2 balance mediated by ω-3 PUFA. However, the possibility that observed methylation changes reflected a ω-3 PUFA supplementation-induced shift in the proportions of Th1 and Th2 cells could not be ruled out. Therefore, the mechanistic understanding of the potential involvement of ω-3 PUFA supplementation in DNA methylation regulation and the Th1/Th2 balance requires additional investigation. Although no correlation was observed between methylation levels of IL13 and IFN-γ in the control group, promoter methylation levels of IFN-γ and IL13 were positively correlated in the ω-3 PUFA group. A Th2-skewed immune response is known to underlie the development of allergic asthma. Th1-dominant diseases are chronic inflammatory diseases such as multiple sclerosis, diabetes, and rheumatoid arthritis. In addition, the process of Th1 and Th2 differentiation from naïve CD4+ T cells is accompanied by genetic and epigenetic modifications of Th1 and Th2 cytokine genes (41). IFN-γ is a potent suppressor of Th2-driven allergic immune response and is affected by environmental factors during prenatal T cell maturation (42). Previous studies have reported that IFN-γ and IL13 levels in infants could be modulated by maternal ω-3 PUFA (43, 44). Our finding that the correlation between DNA methylation levels of IFN-γ and IL13 was changed by ω-3 PUFA supplementation, particularly in infants prenatally exposed to tobacco smoke, suggested that ω-3 PUFA can be important for balancing the immune response by altering promoter methylation of Th cell cytokines. A limitation of our study was its small sample size, which decreased our power to find significant associations, in particular when we conducted analyses stratified by maternal smoking during pregnancy. Therefore, our results need to be confirmed in larger studies.

In conclusion, prenatal ω-3 PUFA supplementation appears to modulate global methylation levels and the Th1/Th2 balance in infants. In addition, maternal smoking may be a key factor for epigenetic modulation in inflammatory genes. These epigenetic mechanisms, if confirmed, could provide attractive targets for prenatal modulation and the prevention of inflammatory disorders and other related human diseases.

The authors’ responsibilities were as follows—IR, ZH, PDS, and UR: designed the study; H-SL and HH-V: carried out experiments; H-SL, AB-V, and CB: performed analyses; ZH and IR: wrote the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

REFERENCES


