DHA supplementation during pregnancy and DNA methylation in cord blood leukocytes

Dear Sir:

Modulation of the level of methylation of CpG nucleotide pairs in the 5′-regulatory regions of genes is an important mechanism in the differentiation of T cells into T helper (Th) 1 or Th2 phenotypes (1). Altered epigenetic regulation of T cell differentiation during fetal development has been implicated as an important mechanism in the etiology of allergic diseases in childhood (2). Therefore, nutritional interventions during pregnancy that may prevent or reverse impaired epigenetic regulation of genes that influence T cell phenotype may be of considerable health benefit.

Relatively few studies have reported the effect of supplementation with n–3 PUFAs present in fish oil, specifically EPA and DHA, on the epigenetic regulation of genes. The addition of EPA to U937 leukemia cells induced demethylation of specific CpG loci in the promoter of the CCAAT/enhancer-binding protein δ (3), whereas treatment of M17 neuroblastoma cells with DHA increased global H3K9 acetylation and decreased levels of transcriptionally repressive histone marks (4). Furthermore, feeding adult rats fish oil induced a reversible increase in the methylation level of specific CpG loci in the Fads2 promoter (5). Increased intake of oily fish during pregnancy has been shown to alter markers of immune function in infants at 6 mo of age (6). Therefore, it is possible that increased intakes of EPA and DHA during pregnancy may induce persistent changes in immune function in the offspring via changes in the epigenetic regulation of T cell phenotypes.

In the August 2013 issue of the Journal, Lee et al (7) reported the findings of a study of the effect of supplementation of the diet of pregnant women with 200 mg/d DHA from midgestation until delivery on the methylation status of CpG loci within the promoter regions of genes involved in immune function in umbilical cord blood mononuclear cells. They showed a nonsignificant trend toward an effect of DHA supplementation on the methylation status of the interferon-γ and IL-13 promoters. They also found significantly higher methylation of long interspersed element (LINE) I sequences in women who took the DHA supplement and who smoked, which became nonsignificant when the data were adjusted for sex, gestational duration, BMI, and batch of laboratory analyses.

Unfortunately, there are elements in the design of the study that suggest that the findings should be interpreted with caution. Different cell types have different patterns of DNA methylation. Thus, in mixed-cell preparations such as cord blood mononuclear cells, variation in the relative numbers of different leukocyte populations may confound the interpretation of differences in DNA methylation (8). It is usual to account for variation in leukocyte numbers in the analysis of DNA methylation in blood, particularly during development when leukocyte numbers change dynamically. However, Lee et al (7) did not test for any potential association between variation in the relative size of leukocyte populations and variation in DNA methylation. Because DNA methylation is an important process in immune function, the incidence of infection should have been recorded and incorporated into the data analysis. There is no statement of statistical power for any of the outcome variables. The addition of 61 selected samples “to strengthen the association between smoking and DNA methylation” (7) to 200 randomly selected samples may have introduced bias into the study. Furthermore, the proportion of atopic mothers was “reasonably balanced” (7), although the numbers were not disclosed, rather than using a case-control design.

The difference in the level of methylation of LINE-1 sequences between women who took the DHA supplement and those who did not was ~1%. Because previous reports have shown the precision of the analysis of LINE-1 sequences by pyrosequencing to vary between 1% and 4% (9), it is possible that variation in methylation of 1% may represent analytic error. This view is supported by the loss of statistical significance where the data were adjusted for potential confounders including sample batch. Furthermore, the detection limit of analysis of DNA methylation by pyrosequencing is ~5% (10). Thus, values <5% should be treated with caution, and the level of precision reported for GATA3 (mean methylation: 0.04–0.28%) is unlikely to be robust. The apparently lower methylation of LINE-1 sequences in women in the control group who smoked appears to be a result of a single outlying sample. To date, there have not been any reports that show that the level of methylation of LINE-1 sequences is an important determinant of T cell differentiation and phenotype. Unfortunately, the study by Lee et al did not include experiments to show whether variations in LINE-1 methylation alter T cell function (7).

Therefore, because of the possible confounding factors discussed above, the lack of experiments to show a causal association between variation in LINE-1 methylation and T cell differentiation or function, and the absence of statistically robust results, the conclusion stated by the authors that “Our results indicate that maternal supplementation with n–3 PUFAs during pregnancy may modulate global methylation levels and the Th1/Th2 balance in infants” (7) appears to be an overinterpretation of the findings.

The author did not declare any conflicts of interest.

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REFERENCES
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8. Adalsteinsson BT, Gudnason H, Aspelund T, Harris TB, Launer LJ, Eiriksdottir G, Smith AV, Gudnason V. Heterogeneity in white blood cell composition in umbilical cord blood mononuclear cells (CBMCs) may confound the results because the methylation levels obtained represent the average methylation levels in a given cell population analyzed (1). To rule out potential confounding factors that may be derived from heterogeneous cell populations, it would be necessary to collect a larger volume of cord blood at birth and perform analysis of DNA methylation in fresh fluorescence-activated cell sorting (FACS) sorted cell subpopulations. In our study, the original design did not allow us to collect a larger volume of fresh cord blood and perform analysis of FACS-sorted CBMC subpopulations. Therefore, future studies on CBMCs should exploit a recently developed set of analytic tools (2) for inferring changes in the distribution of different white blood cell subpopulations by using DNA methylation patterns (such as the regression calibration algorithm), an approach that circumvents the need for fresh blood cells and extensive flow cytometry sorting (3, 4). Although we were able to address the problem of mixed populations of cells (which could only be addressed by a single-cell methylomics approach) and examine whether ω-3 PUFA may alter epigenetic states indirectly, we were careful in our statements that our study shows that prenatal ω-3 PUFA supplementation may modulate immune response in infants, which correlates with, and not necessarily causes, changes in epigenetic states.

Importantly, we have analyzed mRNA levels as a direct measure of gene transcription and the functional impact of DNA methylation changes on gene activity. We found that mRNA levels of IFNγ were markedly higher (although marginally significant) in the ω-3 PUFA group than in controls, consistent with many previous studies showing an inverse correlation between the expression and DNA methylation levels (H-S Lee and Z Herceg, unpublished data, 2013). Therefore, our results support the notion that ω-3 PUFA supple-


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Reply to GC Burdge

Dear Sir:

We appreciate Burdge’s thoughtful comments, and we thank you for the opportunity to reply to his concerns.

We agree with Burdge’s suggestion that the variability of the epigenome among different cells in tissues can confound the findings. Indeed, the intrinsic variability of the DNA methylome among different cell types in umbilical cord blood mononuclear cells (CBMCs) may confound the results because the methylation levels obtained represent the average methylation levels in a given cell population analyzed (1). To rule out potential confounding factors that may be derived from heterogeneous cell populations, it would be necessary to collect a larger volume of cord blood at birth and perform analysis of DNA methylation in fresh fluorescence-activated cell sorting (FACS) sorted cell subpopulations. In our study, the original design did not allow us to collect a larger volume of fresh cord blood and perform analysis of FACS-sorted CBMC subpopulations. Therefore, future studies on CBMCs should exploit a recently developed set of analytic tools (2) for inferring changes in the distribution of different white blood cell subpopulations by using DNA methylation patterns (such as the regression calibration algorithm), an approach that circumvents the need for fresh blood cells and extensive flow cytometry sorting (3, 4). Although we were not able (similarly to the vast majority of other studies) to resolve the problem of mixed populations of cells (which could only be addressed by a single-cell methylomics approach) and examine whether ω-3 PUFA may alter epigenetic states indirectly, we were careful in our statements that our study shows that prenatal ω-3 PUFA supplementation may modulate immune response in infants, which correlates with, and not necessarily causes, changes in epigenetic states.

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We respectfully disagree with Burdge’s suggestion that the addition of 61 selected samples to 200 randomly selected samples could have introduced bias in the analysis. For clarification, we first randomly selected 100 CBMC samples from supplemented mothers and 100 CBMC samples from controls, and our analysis already showed an association between smoking and ω-3 PUFAs and DNA methylation. However, because the sample size in the maternal smoking group was rather small, we subsequently analyzed an additional 61 samples (to increase sample size, as requested by the reviewers). Importantly, after we significantly increased the sample size in the smoking group, all mothers and offspring were randomized and analyzed blindly with respect to supplementation and smoking status. In addition, all assays were performed at the same time and on the same aliquot of bisulfite-converted DNAs with the use of the same batch of pyrosequencing reagents, thus avoiding a potential batch effect. The quality control for DNA methylation analysis further included a regular assessment of pyrosequencing quality (including peak heights, deviation from the reference sequencing pattern, and unexpected peak heights). Furthermore, although in our analysis we did not routinely include methylation standards, it is to be noted that for a given gene we obtained relatively homogeneous DNA methylation levels across