


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’s may alter epigenetic states directly, we were careful in our statements that our study shows that prenatal ω-3 PUFA supplementation can 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studies showing an inverse correlation between the expression and DNA methylation levels (H-S Lee and Z Herczeg, unpublished data, 2013). Therefore, our results support the notion that ω-3 PUFA supplementation could modulate IFNγ expression through promoter methylation changes, which may ultimately affect the inflammatory response. These results also argue that gene expression changes indeed accompanied the changes in DNA methylation, which is unlikely to happen if the observed changes represented analytic error in estimating DNA methylation.

Burdge also draws attention to potential infections that may cause spurious effects on the immune system and DNA methylation. In this study we analyzed methylation levels in DNA extracted from CBMCs. Although the mothers were not checked for the presence of infection at delivery, all mothers and offspring were healthy at the time of birth without any clinical signs of infection. It is important to mention that all pregnancies were low risk (the inclusion criterion for the study was pregnancy without risk) and that the mean birth weight of the offspring was in the normal range, attesting to the lack of major fetal suffering. Although we agree with the remark that the presence of infections may, in principle, affect the immune system and alter blood cell composition, it is unlikely that such an event would affect specifically one group of subjects and that our results are an indirect consequence of changes in the immune system of newborns.

We agree that the statistical power of our study is limited by the sample size, although we included additional samples (n = 261). This is particularly true when conducting stratified analyses. Therefore, our analyses have focused on only the major strata of maternal characteristics. Despite these limitations, we could detect significant changes in DNA methylation and hence believe that our study is sufficiently powered to detect moderate to large changes in DNA methylation, although we might have missed minor changes. We agree that further studies with a larger sample size are needed to confirm our results and evaluate methylation changes in a larger array of genes.

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 PUFA’s 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 maternal smoking group, the overall trend with lower methylation levels associated with smoking remained unchanged. Therefore, our findings of the association between smoking and ω-3 PUFA’s and DNA methylation are unlikely to be influenced by the analysis of additional samples.

We also disagree with Burdge’s assertion that the precision of the analysis of long interspersed element (LINE) 1 sequences by pyrosequencing may not be sufficiently robust to reliably detect small differences between different groups. To avoid potential biases, in our study for each pyrosequencing assay the samples 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...
different sample categories, despite the fact that average methylation levels were strikingly different among the assays, ranging from \(-0\%\) for *GATA3* and *STAT3* to as high as \(70\%\) and \(100\%\) for LINE1 and FOXP3, respectively (our Table 3) (5). These observations strongly rule out the existence of incomplete bisulfite converted DNA, which would otherwise result in increased methylation levels in all genomic regions with no genes exhibiting methylation levels as low as \(0\%\) methylation. We are thus confident that our methylation assays were quantitatively sensitive and that the impact of different biases could be ruled out. With regard to the impact of the outliers, as one can see from our Figure 1A, even after including additional 61 samples in the analysis the scatter for the nonsmokers is markedly greater than for smokers. Whereas the reason for this observation is unknown (one can speculate that it is the result of smoking status), a single outlying sample cannot result in significantly lower methylation of LINE-1 sequences of the entire group, especially considering the number of samples in each group. Furthermore, the numbers of atopic mothers in the experimental groups were provided in our Table 1 (\(n = 72\) in the control group compared with \(n = 70\) in the \(\omega-3\) PUFA group) (5), and thus the proportion of atopic mothers was indeed balanced. We also confirmed that interaction between maternal smoking and \(\omega-3\) PUFA on LINE-1 methylation was significant when adjusted for sex, gestational duration, BMI, and batch of laboratory analyses. This result supports \(\omega-3\) PUFA involvement in LINE-1 methylation changes depending on smoking status.

We appreciated Burdge’s point with regard to the need to take our study a step further in mechanistic terms. Indeed, one limitation of our study is the lack of experiments to show a causal link between changes in LINE-1 methylation and T cell differentiation/function. Because we had a limited amount of frozen CBMCs, there was a technical challenge in performing functional tests and thus we were not able to carry out mechanistic experiments with the use of CBMC samples. Because \(\omega-3\) PUFA-supplemented and control groups were well balanced for all main covariates (including maternal age, height, weight, BMI, educational level, socioeconomic level, maternal smoking during pregnancy, paternal smoking status, sex, birth weight, and gestational duration), these are unlikely to be confounding factors. Therefore, the main findings of our study are unlikely to be influenced by confounding factors and technical biases.

None of the authors declared a conflict of interest.

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Fetal vitamin B-12: a modulator of folate-dependent homocysteine remethylation?

Dear Sir:

We recently read with interest an article by McNulty et al (1) reporting outcomes of a double-blinded randomized controlled trial of folic acid (FA) supplementation (400 \(\mu\)g/d), taken from the start of the second trimester over 14–36 gestational weeks (GWs), in women who also used FA supplements in the first trimester (400 \(\mu\)g/d) and whose dietary intakes compared favorably with current UK reference values, with no difference in total dietary folate intake between groups. In the trial, FA intervention increased maternal folate status, preventing the gestational decline observed at 36 GWs in the placebo group, and this was associated with a significant increase in cord blood folate concentration at birth, which is entirely consistent with the concept that fetal folate concentration reflects trends in maternal folate concentration, underscored by active transport of folate across the placenta (2). Perhaps not unexpectedly, FA intervention had no significant impact on infant size at birth, according well with studies that have also shown a relative lack of dependency with maternal folate status (3) or improvement in birth weight in randomized controlled trials of FA supplementation (4).

What is intriguing about the observations in this article is that, whereas a change in maternal folate status after the intervention of FA supplementation prevented the gestational increase in maternal homocysteine concentration of \(-1\) \(\mu\)mol/L at 36 GWs observed in

REFERENCES


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