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 ω-3 PUFA group) (5), and thus the proportion of atopic mothers was indeed balanced. We also confirmed that interaction between maternal smoking and ω-3 PUFA on LINE-1 methylation was significant when adjusted for sex, gestational duration, BMI, and batch of laboratory analyses. This result supports ω-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 ω-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.

Non 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 µg/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 µg/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 µmol/L at 36 GWs observed in

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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 µmol/L at 36 GWs observed in
unsupplemented mothers (placebo group), this treatment had no significant impact on fetal homocysteine concentration, with similar homocysteine concentrations in the cord blood of both study groups (the authors’ Table 3). We would comment that maternal homocysteine concentrations reported in the study groups between 14 and 36 GWs could, in part, also be modulated by placental uptake and transport of homocysteine to the fetus (5), a concept not alluded to in the article. Previous studies by Molloy et al (6) have shown that maternal homocysteine concentration had the strongest effect on fetal homocysteine concentration in women with normal pregnancies who routinely took FA supplements, evidenced also by the positive correlation between maternal and cord blood homocysteine concentrations (6). It should be further noted that the authors comment that maternal and fetal vitamin B-12 concentrations had additional effects (6). It is presently unclear whether homocysteine entering the fetal circulation is partly derived from the maternal circulation after transplacental passage (5) and/or from placental methionine metabolism (2). However, homocysteine concentrations in umbilical cord venous plasma are ~1 μmol/L higher than in cord arterial plasma, with these variables being highly correlated to each other (7). Such findings suggest that homocysteine delivered by the placenta into cord venous blood is extracted by the fetus, resulting in fetal uptake of homocysteine, akin to other amino acids (2, 5).

This leads to an interesting question: why does the fetal response to increased maternal plasma homocysteine concentration exhibit divergence between these studies? This is an important aspect to understand mechanistically if the capacity of folate to lower maternal homocysteine concentration is conceived to be of positive benefit with respect to pregnancy outcomes, including those of the neonate, as commented on by the authors. We speculate that one possibility underlying this discordance relates to the impact on folate-dependent homocysteine metabolism, which had become limited by vitamin B-12.

We suggest this because we note that some mothers at 14 GWs had remarkably low serum vitamin B-12 concentrations (<150 pmol/L; the authors’ Table 1) and that advancing gestational age (14–36 GWs) was associated with a significant decline in maternal vitamin B-12 concentration, which could represent a reduction in maternal reserves during pregnancy (8), and a subclinical deficit limiting the amount transported by the placenta (9) to the fetal circulation. Hence, women with low vitamin B-12 status may not be able to provide the optimum amount of this micronutrient required by the fetus (10), with implications for newborn infants including low reserves of vitamin B-12 and possibly a risk of vitamin B-12 deficiency (8). The maternal data concerning vitamin B-12 concentration at 36 GWs were not provided, and although cord vitamin B-12 concentrations were not different between study groups at delivery, the reduction seen in maternal homocysteine concentration with FA treatment was not reflected in the fetal compartment, suggesting limitation by another modulatory factor.

The emergence of vitamin B-12 as an additional factor of importance influencing folate-dependent homocysteine metabolism toward the end of gestation would be in agreement with earlier studies (6). An efficient use of vitamin B-12 both by the placenta and the fetus has been implicated (2, 8), particularly with regard to directing vitamin B-12 to favor methionine synthase activity, providing methionine and tetrahydrofolate to support cell growth. The trial was not extended to the neonatal period to examine the potential impact of maternal FA supplementation intervention during pregnancy on newborn infants. Other studies have reported that over the first few weeks of birth an increase in homocysteine concentration in newborn infants is strongly associated with a reduction in vitamin B-12 concentration, consistent with impaired vitamin B-12 function and not correlated with serum or whole-blood folate (8). Indeed, in newborn infants of healthy, nonvegetarian mothers, impaired vitamin B-12 function appears to be related to their low vitamin B-12 tissue reserves (8).

Against this background, the reduction in maternal vitamin B-12 concentrations by 36 GWs mentioned in the FA supplementation trial has particular relevance, because it is consistent with the notion that fetal vitamin B-12 requirements may well have exceeded the capacity for placental vitamin B-12 transport to meet fetal demand. This commentary supports the view that vitamin B-12 transported by the placenta has relative importance, particularly with regard to the fetal methylation of homocysteine to methionine, as well as the influence on the prevalence of impaired vitamin B-12 status in the neonatal period. This should motivate vitamin B-12 intervention studies in pregnancy and in newborn infants.

None of the authors declared a conflict of interest.

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Reply to SW D’Souza et al

Dear Sir:

D’Souza et al share some interesting thoughts regarding our article on the effects of folic acid supplementation in the second and third trimesters of pregnancy (1). They point out, quite rightly, that whereas folic acid supplementation influenced maternal homocysteine by preventing the increase in homocysteine usually observed in the third trimester (2), we did not observe a difference in cord blood homocysteine concentrations between the placebo and folic acid treatment groups. D’Souza et al suggest that low vitamin B-12 status might explain the anomaly. Serum total vitamin B-12 is known to decrease by ∼30% over the course of pregnancy (3), and in this study between gestational weeks 14 and 36 we observed (although did not report) a decline in maternal vitamin B-12 concentrations of 22% (from 211 ± 82 pmol/L to 165 ± 59 pmol/L; P = 0.001) in the placebo group and 25% (from 235 ± 94 pmol/L to 176 ± 75 pmol/L; P = 0.001) in the folic acid–supplemented group, with no significant treatment effect (P = 0.59). We interpret this as indicating that vitamin B-12 affected the relation between maternal and cord blood homocysteine in both groups, although this was not an outcome of primary interest to the study.

Although it was not included in the article, we used multiple regression analysis to examine each of the measured variables in our study as determinants of the fetal (cord blood) homocysteine. Neither folate nor vitamin B-12 were significant predictors of cord blood homocysteine in either the placebo or the folic acid treatment group (P = 0.98 and 0.09 for cord blood folate and vitamin B-12, respectively, in the placebo group; and P = 0.26 and 0.45, respectively, in the treatment group). The only significant predictor of fetal homocysteine was the maternal homocysteine concentration, but the overall relation in the multiple regression analysis was much weaker in the folic acid treatment group (adjusted R² = 13.7% compared with 54.7% in the placebo group).

D’Souza et al suggest that the cord blood homocysteine concentration may be limited by another modulatory factor, and we agree. As in our previous observational study (4), in the current randomized controlled trial we found that maternal homocysteine was significantly associated with fetal homocysteine, but this relation was relatively weaker in the folic acid treatment group. However, the data do not indicate that the limiting factor in our study is vitamin B-12. Although we would not deny the importance of vitamin B-12 in pregnancy and in early neonatal life, there are many gaps in our understanding of vitamin B-12 status at these critical times. Several studies have shown that, whereas the total serum vitamin B-12 decreases dramatically during pregnancy, the fraction of vitamin B-12 that is available for uptake into tissue (ie, holotranscobalamin) does not appear to decline (3, 5), indicating that the interpretation of biomarkers of vitamin B-12 during pregnancy is complex, a point made by others in earlier studies (6, 7). This appears to be the case in our study, in which low maternal concentrations of vitamin B-12 at gestational week 36 do not predict functional vitamin B-12 deficiency in the fetus at birth, at least where the functional biomarker homocysteine is concerned.

We would also comment that although D’Souza et al consider the importance of transplacental transfer of homocysteine and placental methionine metabolism, they have overlooked other pregnancy-related factors that affect maternal and fetal homocysteine (8). One relevant micronutrient not considered by D’Souza et al, and not measured in our study, is choline, for which there is a very high fetal requirement. We previously found maternal homocysteine concentration to be strongly associated with cord blood choline, consistent with a high maternal de novo synthesis of choline through hepatic phosphatidylethanolamine methyltransferase (9). Clearly, all of these factors need to be considered, and although the call for intervention studies with vitamin B-12 in pregnancy and newborns is well made, we need first to know how to interpret the relevant biomarkers of vitamin B-12 status in pregnancy.

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