Gene-specific DNA methylation in newborns in response to folic acid supplementation during the second and third trimesters of pregnancy: epigenetic analysis from a randomized controlled trial

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Short running head: Maternal folate and DNA methylation in newborns

Abbreviations used: FA, folic acid; FASSTT, Folic Acid Supplementation in the Second and Third Trimesters; GW, gestational week; NTD, neural tube defect; RBC, red blood cell.

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ABSTRACT

Background: Emerging evidence suggests that maternal folate status can impact cognitive development in childhood. Folate-dependent DNA methylation may provide a biological mechanism to link folate status during pregnancy with cognition in the offspring.

Objective: The objective was to investigate the effect of continued folic acid (FA) supplementation beyond the first trimester of pregnancy on DNA methylation in cord blood of epigenetically-controlled genes related to brain development and function.

Design: Using available cord blood samples (n = 86) from the Folic Acid Supplementation in the Second and Third Trimesters (FASSTT) trial in pregnancy, we applied pyrosequencing techniques to analyze cord blood DNA at nine candidate loci known to be regulated by methylation including some previously implicated in observational studies: the widely-dispersed retrotransposon *LINE-1* and eight single-copy loci (*RBM46, PEG3, IGF2, GRB10, BDNF, GRIN3B, OPCML* and *APC2*).

Results: The newborns of mothers who received FA (400 µg/d) during pregnancy, compared to placebo, had significantly lower overall DNA methylation levels at *LINE-1* (57.2 ± 2.1 % vs 56.3 ± 1.7 %; P = 0.024), *IGF2* (51.2 ± 5.1 % vs 48.9 ± 4.4 %; P = 0.021) and *BDNF* (3.1 ± 0.8 % vs 2.7 ± 0.7 %; P = 0.003). The effect of FA treatment on DNA methylation was significant only in female offspring for *IGF2* (P = 0.028) and only in males for *BDNF* (P = 0.012). For *GRB10* and *GRIN3B*, we detected no effect on overall methylation, however, individual CpG sites showed significant DNA methylation changes in response to FA.

Conclusions: Continued supplementation with FA through trimesters 2 and 3 of pregnancy results in significant changes in DNA methylation in cord blood of genes related to brain development. The findings offer a potential biological mechanism linking maternal folate

status with neurodevelopment of the offspring, but this requires further investigation using a genome-wide approach.

The FASSTT trial is registered at: www.isrctn.com/ISRCTN19917787.

Key words: Folic acid, Pregnancy, DNA methylation, Epigenetics

INTRODUCTION

Periconceptional folic acid (FA) supplementation has a proven effect in preventing the first occurrence (1) and recurrence (2) of neural tube defects (NTD). As a result, women planning a pregnancy are recommended to take 400 µg/d FA from preconception until the end of the first trimester (3). Apart from preventing NTD in early pregnancy, emerging evidence shows that maternal folate status may have other roles in offspring health, particularly in relation to cognitive development in childhood (4, 5). Several observational studies have identified a potential role of maternal folate status during pregnancy on the cognitive performance of offspring (6, 7, 8). We previously investigated the children of mothers who had participated in a randomized trial in pregnancy of Folic Acid Supplementation in the Second and Third Trimesters (FASSTT) (9) and, in a preliminary publication, found beneficial effects of FA on cognition in children at age 3 and 6 years (10). Although, the precise biological mechanism explaining the effect of FA during pregnancy on neurodevelopment of the child is unknown, it must involve the essential role of folate in one-carbon metabolism, whereby one-carbon units are transferred and utilized in critical pathways involving amino acid metabolism, biosynthesis of purines and pyrimidines and the methylation of biological substrates including DNA.

Epigenetics refers to heritable changes in gene expression, which occur without altering the underlying DNA sequence, often via histone modification, RNA interference or DNA methylation (11). DNA methylation is the most widely studied epigenetic mechanism for gene regulation and is dependent upon the supply of methyl donors provided by folate and the metabolically-related B vitamins via the formation of S-adenosylmethionine (SAM) within one-carbon metabolism (5). SAM is the universal methyl donor required for the methylation of numerous endogenous substances and the maintenance of DNA methylation (12). Most previous epigenetic studies in humans have used a candidate gene approach to link

maternal status of folate or other one-carbon nutrients with offspring DNA methylation, and reported significant associations at specific loci, including the high copy-number retrotransposon *LINE-1*, the imprinted genes *IGF2* and *PEG3* and the metastable epiallele *RBM46* (13,14). As shown by ourselves (15) and others (16), these imprinted genes and metastable epiallele have the advantage of showing equivalent methylation levels across various tissues and are potentially responsive to early-life nutritional inputs. In addition, a meta-analysis of two epigenome-wide association studies (EWAS) investigating the impact of maternal folate on DNA methylation identified 48 CpGs showing genome-wide significance (after Bonferroni correction) including clusters of sites at *APC2* and *OPCML* (17). Previous studies in the area, however, are observational and thus, by design, cannot provide evidence of a direct link between maternal folate during pregnancy and DNA methylation effects in offspring. Apart from the aforementioned genes identified in previous studies, three other brain related targets known to be regulated by methylation and not previously investigated in relation to folate, could be of potential interest. These are: *GRB10*, an imprinted gene paternally expressed in the brain (18); *GRIN3B*, a transiently imprinted gene regulated by methylation and important for neuronal plasticity during development (19) and *BDNF*, an important neurotrophic factor frequently associated with epigenetic modulation (20).

Therefore, the aim of this study was to investigate the effect of FA supplementation during trimesters 2 and 3 on DNA methylation in cord blood of key epigenetically-controlled genes, many related to brain development and function.

METHODS

Participants and Study Design

Samples for the current investigation were made available from a previous double-blinded randomized controlled trial (RCT) in pregnancy of Folic Acid Supplementation

during the Second and Third Trimesters (FASSTT) conducted in 2005-2006 (**Figure 1**). The methodological details of the FASSTT trial have been described in full elsewhere (9). In summary, healthy pregnant women aged 18-35 y with a singleton pregnancy were recruited at the 14th gestational week from antenatal clinics at the Causeway Hospital, Coleraine, Northern Ireland. Women included in the study had taken FA supplements at the recommended dose (400µg/d) during the first trimester of pregnancy. Women were excluded from the trial if they had not taken FA during the first trimester or had taken FA at a dose >400 µg/d, were taking medications known to interfere with B-vitamin metabolism, had undergone *in vitro* fertilization treatment, or had a previous NTD-affected pregnancy. Although current practice in Northern Ireland (UK) is to recommend FA supplements from pre-conception to the end of the first trimester of pregnancy only, we also excluded from participation any woman who intended to continue taking FA throughout pregnancy. On recruitment, information on micronutrient supplementation was collected, with a particular emphasis on the dose and timing of use of FA supplements.

As previously described, for randomization purposes, FASSTT trial participants at the beginning of the second trimester were stratified into tertiles of homocysteine concentrations (from the blood sample taken at recruitment), and women in each stratum were then randomly assigned to receive either 400 µg FA/d or placebo from the 14th gestational week until the end of pregnancy (9). The randomization process was carried out by a staff member who was not involved in the study, and this approach ensured that both researchers and participants were blinded to the treatment group allocations. Maternal non-fasting blood samples were taken at the 14th (pre-intervention) and 36th (representative of post-intervention) gestational week, with corresponding cord blood samples collected at delivery. The birth weight, birth length, head circumference, mode of delivery and Apgar score for the newborns were collected after delivery. Ethical approval was obtained from the Office for Research Ethics Committees

Northern Ireland (05/Q2008/21), and all participants gave written informed consent at the time of recruitment.

B-vitamin Status Biomarkers

Upon collection, all blood samples were kept at 4℃. They were subsequently processed within 4 h (apart from cord blood samples which were processed within 24 h of collection) and stored at -80°C until required for analysis. Serum and red blood cell (RBC) folate (21) and serum vitamin B-12 (22) were measured by microbiological assay using established methods. Samples were analyzed blind for all assays, and quality control was carried out by repeated analysis of stored batches of pooled samples covering a wide range of values. Intra- and interassay CVs were ≤8.2% for RBC folate and ≤10.4% for serum vitamin B-12. Methylenetetrahydrofolate reductase (*MTHFR*) 677C>T genotype was identified by using polymerase chain reaction amplification followed by HinF1 restriction digestion (23).

DNA Methylation Analysis

Table 1 summarizes the candidate genes selected for methylation analysis and their function. For the current analysis, genomic DNA was extracted from cord blood using the QiAMP DNA Blood Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The quality of DNA was evaluated via gel electrophoresis, and then quantified using the Nanodrop 2000 spectrophotometer (Labtech International, Ringmer, UK). The DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Pyrosequencing assays were designed in-house for all genes using PyroMark Assay Design Software 2.0 (Qiagen, Crawley, UK) according to previously published primer sets/regions: Long-interspersed nuclear element-1 (*LINE-1*) (24), RNA binding motif protein-46 (*RBM46*) (14), Paternally-expressed gene 3 (*PEG3*) (25), Insulin-like growth factor-2 (*IGF2*) (26), Growth Factor Receptor Bound Protein 10 (*GRB10*) (27),

Glutamate Ionotropic Receptor NMDA Type Subunit 3B (*GRIN3B*) (15, 19), Opioid Binding Protein/Cell Adhesion Molecule-Like (*OPCML)* and Adenomatosis Polyposis Coli-2 (*APC2*) (17). Brain-derived neurotrophic factor (*BDNF*) was purchased as a commercially available assay (Qiagen, Crawley, UK).

Pyrosequencing analysis was carried out in duplicate and overall methylation was obtained from 5-17 CpG sites for each gene **(Supplemental Table 1)**. Further information on chromosomal position, primer sequences and number of CpG sites analyzed are detailed in **Supplemental Table 1**. Bisulfite converted DNA was amplified using the PyroMark PCR kit (Qiagen, Crawley, UK) with aforementioned primer sets, conditions were: 15 minutes at 95°C, followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 30 seconds at 72°C, with final elongation for 10 minutes at 72°C. Products were verified via gel electrophoresis prior to pyrosequencing analysis, which was performed using the PyroMark Q24 Pyrosequencing platform as per manufacturer's recommendations (Qiagen, Crawley, UK).

Dietary Analysis

Maternal dietary information was collected during the second trimester of pregnancy using a 4-d food diary in combination with a food-frequency questionnaire, a method previously validated for folate and related B-vitamin intakes against biomarker values, as detailed elsewhere (31). Dietary analysis was carried out using the nutritional software package WISP version 3.0 (Tinuviel Software), which had been customized to generate separate values for naturally occurring food folate and FA added to foods; the separate values were then used to calculate dietary folate equivalents, as previously described (31).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS) (Version 22.0; SPSS UK Ltd., Chertsey, UK). The results are expressed as

mean ± SD, except where otherwise stated. For normalization purposes, variables were log transformed before analysis, as appropriate. Differences between treatment groups for participant characteristics were assessed using an independent *t* test for continuous variables or chi-square for categorical variables. Differences in gene-specific DNA methylation between the two treatment groups were assessed by analysis of covariance (ANCOVA) with adjustment for confounders previously reported to influence DNA methylation such as maternal age, smoking during pregnancy, caesarean section, baby's sex and gestational weight. Multiple linear regression analysis was used to examine the maternal and neonatal predictors of gene-specific DNA methylation in cord blood (dependent variable) controlling for common confounders. $P<0.05$ was considered significant.

RESULTS

From the total FASSTT trial sample of 119 participants, 86 cord blood samples were available for the current analysis (9). A comparison of maternal folate status post-intervention between the sub-cohort with (n = 86) versus without (n = 33) available cord blood, showed no significant differences in mean (± SD) RBC folate concentrations (1270 ± 611 nmol/L vs 1279 ± 820 nmol/L; P = 0.942), ensuring that there was no selection bias in the sub-cohort who provided cord blood.

At baseline (14th GW), there were no detectable differences between the treatment groups in general maternal or neonatal characteristics, serum or RBC folate concentrations or dietary folate **(Table 2)**. As a result of treatment with FA during trimesters 2 and 3, maternal serum and RBC folate were significantly increased. Cord serum and RBC folate concentrations were also significantly higher in infants of mothers supplemented with FA compared with those from the placebo mothers. As expected, maternal RBC folate (at the 36th GW) was highly correlated with cord RBC folate (r = 0.619; P = <0.001; data not shown).

DNA methylation levels of the investigated genes in cord blood samples are presented in **Figure 2**. The results showed significantly lower overall DNA methylation levels at *LINE-1*, *IGF2* **(Figure 2)** and *BDNF* in the offspring of mothers who received FA treatment compared to placebo during pregnancy (*BDNF*: Placebo 3.1 ± 0.08 % vs FA 2.7 ± 0.07 %; *P* = 0.003; data not shown), after adjustment for maternal age, smoking during pregnancy, caesarean section, baby's sex and birth weight. The effect of FA treatment on DNA methylation was however significant only in female offspring for *IGF2* and only in males for *BDNF* **(Table 3)**. No other genes showed significant treatment effects for overall DNA methylation levels. When examined separately, individual CpG sites reflected the overall DNA methylation lowering effect of FA found with the complete loci, apart from *GRB10* CpG 3 where FA supplementation resulted in significantly higher DNA methylation (**Table 3**).

Multiple linear regression analysis was conducted on the whole cohort (placebo and FA treated groups combined) in order to identify the maternal and neonatal determinants of DNA methylation in cord blood (**Table 4**). Maternal FA treatment was significantly associated with offspring DNA methylation at *LINE-1*, *IGF2* and *BDNF* genes, whereas caesarean section was a determinant of *LINE-1* and *BDNF* methylation. Vitamin B12 concentration in cord (but not maternal) blood was significantly associated with offspring *IGF2* methylation. Neither maternal age nor smoking during pregnancy was significantly related to DNA methylation in the cord blood of any genes examined.

DISCUSSION

This is the first randomized trial of FA supplementation during pregnancy to examine DNA methylation levels in cord blood at a number of important candidate genes, some previously associated with brain development and function. The results showed significantly

lower DNA methylation levels of specific genes, *IGF2*, *BDNF* and *LINE-1*, in cord blood from mothers who received FA supplementation compared with placebo during the second and third trimesters of pregnancy. In addition, sex-specific differences in the response to FA were observed in offspring DNA methylation of *IGF2* and *BDNF*. Not only does the current study present data on relevant genes not previously investigated, but because of the randomized trial design, the findings can clarify the nature of the relationship between maternal folate and offspring DNA methylation as reported in previous observational studies.

The significant effect of folate during pregnancy on gene-specific DNA methylation in cord blood shown here is in broad agreement with the findings of two observational studies (13,17). The first of these was a large cohort study (n = 913) that found lower methylation in cord blood for both *LINE*-1 and *PEG3*, but higher methylation in *IGF2*, in women who reported using FA supplements after the 12th GW of pregnancy (13). Our data showing significantly lower *LINE-1* methylation in response to FA intervention supports this previously reported relationship with maternal folate; however, our results in relation to the effect of FA on *PEG3* (i.e. no methylation change) and *IGF2* (i.e. decrease in methylation) differ from these earlier observations (13). Of perhaps greater relevance, our results are in good agreement with the findings of an epigenome-wide meta-analysis (n = 1988) which found that with increasing maternal folate concentrations (as measured in mid pregnancy; 13th to 18th GW), there were more CpGs with significantly decreased methylation (416 or 94%) than those with increased methylation (27 or 6%) (17). Likewise, we showed that in response to FA intervention during a similar period of pregnancy, more CpG sites have decreases than increases in methylation at the single-copy loci and at *LINE-1*, which indicates a genome-wide methylation decrease, since there are >500,000 copies of this element across the genome (32). Taken together, the current and earlier evidence (17) strongly suggests that the overall effect of maternal folate is to lower, not increase, DNA methylation. The latter report found

250 that the largest number of statistically significant CpG sites were within the *APC2* gene
251 (expressed in fetal and adult brain) and the *OPCML* gene (17). Our results, somewhat
252 unexpectedly however, showed no significant effect of maternal FA supplementation on DNA
253 methylation for either *APC2* or *OPCML* (at any CpG sites investigated), an inconsistency that
254 may relate to differences in the selection of specific CpG sites or to study design differences.
255 Furthermore, time of sampling for maternal folate measurement was not directly comparable,
256 with blood samples collected on either the 13th or 18th GW in the previous study (17) whereas
257 blood samples in the current study represented before and after intervention with FA over 22
258 weeks of pregnancy from the 14th GW.

259 The current and aforementioned studies relate to mid-pregnancy onwards, whereas
260 early pregnancy is considered a sensitive period of plasticity in fetal developmental
261 programming and has thus been of interest for several epigenetic studies of maternal diet and
262 offspring DNA methylation in specific genes (5). One such study, showing that maternal
263 periconceptional FA use (as reported by mothers) was associated with increased methylation
264 of *IGF2* (by 4.5%) in the offspring when measured at 17 months old (33), is at odds with the
265 current results showing a decrease at this locus in response to FA intervention. In addition,
266 one notable previous study conducted in Gambian women reported that the season of
267 conception (which reflects variability in nutrient supply) can influence DNA methylation
268 patterns of the *RBM46* gene in the offspring at 2-8 months (14). In contrast, the current study
269 found no significant effect in offspring *RBM46* methylation in response to FA during
270 trimesters 2 and 3 of pregnancy. The reason for these inconsistencies are unclear, but may
271 relate to the fact that compared with the current RCT which investigated the effect of FA
272 administered from the 14th GW to the end of pregnancy, the latter studies were observational
273 (14, 33) and focused on the periconceptional phase of pregnancy. In addition, the DNA
274 methylation effects observed in these previous studies were examined up to 17 months after

birth, a period during which factors other than maternal folate during pregnancy may have influenced the results. The totality of evidence suggests that there are different windows of susceptibility to maternal changes in the folate-dependent one-carbon pathway, and therefore periods beyond periconception may have important roles in influencing epigenetic changes in the offspring.

Although significant, the offspring DNA methylation changes in response to maternal FA treatment found here are small. The magnitude of change we showed is however in good agreement with our previous studies showing that small changes affected by drug treatment can cause transcriptional alterations including at imprinted genes (15, 19). Additionally, the small changes that we observed may lead to an altered balance at imprinted loci globally (34). Like the current study, previous studies have also reported sex-specific differences in DNA methylation in offspring in response to nutrition. During the Dutch Hunger Winter, when there was a reduced supply of essential nutrients including folate, *IGF2R* methylation was found to be higher by 2.6% in males, whereas DNA methylation of *LEP*, *IL10* and *APOC1* was lower by 1.5-2.9%, compared with female offspring (35). Furthermore, periconceptional micronutrient supplementation of Gambian women was found to lower offspring methylation in males only for *GTL2*-DMR_2 (by 6.5%) and in females only for *IGF2R*-DMR (by 8.6%) (36). Likewise, the current results showed sex-specific effects of FA treatment for certain genes, with the reduction in methylation found to be significant in female (for *IGF2*) or in male (for *BDNF*) offspring only. The findings in the current study of sex-differences in DNA methylation in *IGF2* and *BDNF* in response to FA in pregnancy may be related to the fact that they are considered estrogen-responsive genes (37, 38), but the mechanisms underlying these sex-specific effects shown here and elsewhere remain to be elucidated.

Apart from maternal FA treatment, vitamin B12 status and caesarean section delivery were found to be significant predictors of gene-specific DNA methylation in the offspring

when regression analysis was conducted on the whole cohort (placebo and FA treated groups combined). After adjustment for covariates, our results showed that increasing cord blood vitamin B12 concentration was associated with decreasing *IGF2* methylation. The finding that vitamin B12 may also influence DNA methylation in a similar way to folate is not surprising as it acts synergistically with folate within the one-carbon metabolic cycle and both vitamins are required for the generation of SAM (12). Therefore, although the current study focused on the effects of intervention with FA during pregnancy, our regression results suggest a mechanism whereby vitamin B12 status during pregnancy may also have a role in influencing DNA methylation in the offspring. In relation to caesarean section, the current results are in line with previous evidence that DNA methylation is higher in infants delivered by caesarean section than by vaginal delivery (39), an effect that may be owing to maladaptive perinatal stress associated with this type of delivery.

The main strength of this study is that it is a randomized trial and therefore has the ability to investigate causal links between maternal FA intervention and DNA methylation of the offspring. However, this study was not without limitations. The candidate gene approach means that whilst specific genes of potential interest were identified, other genes and CpG sites not investigated may have been affected by FA supplementation during pregnancy. In addition, as per the design of the FASSTT trial, whereby participants were included only if they had taken FA during the first trimester (9), all women received FA periconceptionally and therefore no conclusions can be made as regards FA responsive epigenetic effects at this early stage of pregnancy. Finally, since neural tissue could not be obtained, we cannot exclude the possibility that the DNA methylation changes we observed in blood are not reflected in the brain, although methylation at imprints (16) and many of the other loci investigated (12-16, 24-27) are known to be similar across different tissues.

In conclusion, the current study presents the first evidence from an RCT that continued FA supplementation after the first trimester of pregnancy affects offspring DNA methylation of specific genes, including those related to offspring brain. DNA methylation may thus offer a potential biological mechanism linking maternal folate status with offspring neurodevelopment. This area of research is still in its infancy and much remains unknown as to how an individual's DNA methylation profile is established during early development, the contributing factors and the long-term health effects. Future studies using an EWAS approach will be necessary to more fully explore the epigenetic mechanisms explaining the impact of maternal FA supplementation on offspring cognitive health.

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Conflict of Interest Statement: Aoife Caffrey, Rachelle Irwin, Helene McNulty, JJ Strain, Diane Lees-Murdock, Mary Ward, Colum P Walsh and Kristina Pentieva have no conflicts of interest to declare.

Authors' Contributions were as follows KP, HM and CPW planned and designed the research. AC and RI conducted the epigenetic laboratory work and AC analyzed the data. CPW and DLM interpreted the methylation data. BM conducted the original FASSTT trial under the supervision of HM, KP, MW and JJS. AC and RI wrote the initial draft of the manuscript and all authors provided important revisions. KP and HM had primary responsibility for the final content. All authors read and approved the final manuscript.

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TABLE 1

Candidate genes for methylation analysis and their function

Gene	Gene Description	Function	Reference
LINE-1	Long interspersed nuclear element-1	Highly repeated retrotransposon thus surrogate marker for global DNA methylation.	Beck *et al*. 2011 (28)
RBM46	RNA Binding Motif Protein 46	Metastable epiallele variably expressed due to epigenetic modifications established during early development.	Dominguez-Salas *et al*. 2014 (14)
PEG3	Paternally Expressed Gene 3	Maternally imprinted gene implicated in placental development p53-mediated apoptosis.	He & Kim. 2014 (29)
IGF2	Insulin Like Growth Factor 2	Maternally imprinted gene required for development and growth.	Chao & D'Amore. 2008 (30)
GRB10	Growth Factor Receptor Bound Protein 10	Growth factor receptor-binding protein that both interacts with insulin-like growth-factor receptors in embryo and mediates social behavior in adult.	Garfield *et al.* 2011 (18)
BDNF	Brain-Derived Neurotrophic Factor	Neurotrophic factor, promotes neuron growth, maturation and survival, shows frequent epigenetic alteration.	Roth & Sweatt. 2011 (20)
GRIN3B	Glutamate Ionotropic Receptor NMDA Type Subunit 3B	cAMP signaling pathway, NMDA receptor found primarily in motor neurons.	Irwin *et al*. 2014 (19)
OPCML	Opioid Binding Protein/ Cell Adhesion Molecule-Like	Associated with neurocognitive conditions.	Joubert *et al*. 2016 (17)
APC2	Adenomatosis Polyposis Coli 2	Regulation of *Wnt* signaling pathway.	Joubert *et al.* 2016 (17)

TABLE 2
General characteristics of mother and offspring participants from the FASSTT Trial[1]

	Placebo (n = 45)	Folic Acid (n = 41)	P value[1]
Maternal characteristics[2]			
Age (y)	28.9 ± 3.5	29.4 ± 3.9	0.513
BMI (kg/m^2)	25.2 ± 3.9	24.9 ± 4.6	0.768
Smoker *n* (%)	8 (18)	6 (15)	0.693
Gestation at baseline (wk)	13.7 ± 2.2	14.1 ± 2.4	0.432
Duration of FA use at baseline (wk)	14.4 ± 10.1	11.9 ± 6.8	0.175
Parity (n)	1.0 ± 1.1	1.0 ± 1.0	0.915
Caesarean section *n* (%)	11 (24)	10 (24)	0.995
MTHFR 677TT genotype *n* (%)	5 (11)	2 (5)	0.291
Dietary Intakes			
Energy (MJ/d)	8.170 ± 1.717	7.732 ± 1.595	0.280
Dietary Folate Equivalents (µg/d)	364 ± 172	387 ± 152	0.582
Vitamin B12 (µg/d)	4.1 ± 1.9	3.9 ± 3.9	0.791
B-vitamin Biomarkers			
Preintervention (14 GW)			
Serum folate (nmol/L)	48.8 ± 19.8	45.8 ± 19.5	0.469
RBC folate (nmol/L)	1185 ± 765	1181 ± 649	0.978
Serum B12 (pmol/L)	224 ± 79	217 ± 79	0.601
Postintervention (36 GW)[3]			
Serum folate (nmol/L)	23.6 ± 17.9	46.5 ± 24.8	<0.001
RBC folate (nmol/L)	991 ± 404	1556 ± 658	<0.001
Serum B12 (pmol/L)	168 ± 51	157 ± 60	0.229
Neonatal characteristics			
Gestational age (wk)	40.1 ± 1.3	40.0 ± 1.1	0.540
Sex, Male *n* (%)	22 (49)	22 (54)	0.659
Birth weight (g)	3610 ± 475	3557 ± 464	0.601
Birth length (cm)	51.5 ± 2.6	51.1 ± 2.2	0.499
Head circumference (cm)	34.9 ± 1.2	34.8 ± 1.4	0.907
Apgar score at 1 min	8.4 ± 1.1	8.6 ± 0.6	0.269
Apgar score at 5 min	8.9 ± 0.4	9.0 ± 0.3	0.220
Breastfed *n* (%)	15 (33)	14 (34)	0.240
MTHFR 677TT genotype *n* (%)	6 (13)	4 (10)	0.605
Cord Blood B-vitamin Biomarkers			
Serum folate (nmol/L)	68.3 ± 24.8	91.7 ± 36.7	0.004
RBC folate (nmol/L)	1518 ± 597	1877 ± 701	0.024
Serum B12 (pmol/L)	276 ± 155	251 ± 107	0.776

[1]Differences between groups were assessed using an independent *t* test (continuous variables) or chi-square test (categorical variables). Values expressed as means ± SD except where otherwise stated. P<0.05 was considered significant.

[2]Maternal characteristics assessed at the 14th gestational week (pre-intervention) unless where otherwise stated.
[3]Postintervention refers to 36th gestational week.
Abbreviations: FASSTT, Folic Acid Supplementation in the Second and Third Trimesters; GW, gestational week RBC, red blood cell.

TABLE 3
CpG site-specific DNA methylation (*LINE-1*, *IGF2*, *BDNF*, *GRB10* and *GRIN3B*) in cord blood by maternal treatment group[1]

	Genomic location	Placebo (n = 45)	Folic Acid (n = 41)	P value[1]
Maternal RBC folate status (36 GW; nmol/L)		991 ± 404	1556 ± 658	<0.001
Cord RBC folate status (nmol/L)		1518 ± 597	1877 ± 701	0.024
Cord DNA methylation (%)				
LINE-1[2]	Promoter			
CpG 1		83.5 ± 4.7	83.6 ± 3.9	0.679
CpG 2		62.8 ± 3.9	59.9 ± 4.2	0.002
CpG 3		37.1 ± 2.4	36.4 ± 3.5	0.301
CpG 4		20.4 ± 3.0	18.9 ± 3.0	0.045
CpG 5		57.9 ± 4.4	57.3 ± 4.3	0.489
CpG 6		81.6 ± 2.7	81.7 ± 3.1	0.933
Overall (all CpG sites)		57.2 ± 2.1	56.3 ± 1.7	0.024
Males		57.0 ± 2.3	56.5 ± 1.8	0.067
Females		57.4 ± 2.0	56.1 ± 1.7	0.038
IGF2	DMR 2 (somatic)[3]			
CpG 1		43.4 ± 3.7	40.0 ± 5.2	0.001
CpG 2		47.1 ± 6.5	43.7 ± 6.5	0.017
CpG 3		54.4 ± 5.9	52.7 ± 5.7	0.102
CpG 4		50.0 ± 5.8	48.5 ± 5.7	0.190
CpG 5		68.0 ± 9.2	65.0 ± 6.2	0.071
CpG 6		42.8 ± 6.3	40.6 ± 4.2	0.050
CpG 7		52.5 ± 5.8	52.0 ± 6.5	0.428
Overall (all CpG sites)		51.2 ± 5.1	48.9 ± 4.4	0.021
Males		50.2 ± 4.6	49.3 ± 3.4	0.201
Females		52.1 ± 5.5	48.5 ± 5.3	0.028
BDNF	Exon 1/Promoter			
CpG 1		2.1 ± 0.8	1.6 ± 0.6	0.001
CpG 2		6.1 ± 1.5	5.8 ± 2.1	0.229
CpG 3		2.1 ± 0.7	1.6 ± 0.7	<0.001
CpG 4		3.1 ± 1.1	2.9 ± 1.1	0.301
CpG 5		1.8 ± 0.8	1.4 ± 0.5	0.003
Overall (all CpG sites)		3.1 ± 0.8	2.7 ± 0.7	0.003
Males		3.2 ± 0.8	2.7 ± 0.7	0.012
Females		2.9 ± 0.7	2.6 ± 0.7	0.212

GRB10	DMR (gametic)[3]			
CpG 1		82.2 ± 3.1	80.6 ± 3.8	0.041
CpG 2		84.9 ± 6.8	82.8 ± 5.9	0.198
CpG 3		59.9 ± 4.8	61.7 ± 2.9	0.022
CpG 4		59.8 ± 3.7	59.8 ± 3.7	0.973
CpG 5		77.0 ± 3.6	76.9 ± 3.8	0.929
CpG 6		61.8 ± 3.9	62.2 ± 2.8	0.586
CpG 7		88.0 ± 9.2	87.3 ± 7.5	0.781
CpG 8		59.2 ± 3.7	60.0 ± 3.6	0.400
Overall (all CpG sites)		71.6 ± 3.4	71.5 ± 3.0	0.903
	Males	70.9 ± 3.9	71.4 ± 3.0	0.442
	Females	72.2 ± 2.9	71.5 ± 3.1	0.278
GRIN3B	DMR (gametic)[3]			
CpG 1		97.4 ± 1.3	96.7 ± 1.6	0.023
CpG 2		81.0 ± 5.4	82.5 ± 5.9	0.247
CpG 3		98.3 ± 2.0	97.4 ± 2.5	0.101
CpG 4		58.0 ± 13.0	60.7 ± 16.9	0.424
CpG 5		93.0 ± 8.1	86.2 ± 18.3	0.030
Overall (all CpG sites)		85.5 ± 3.9	84.7 ± 6.6	0.471
	Males	84.7 ± 3.8	85.3 ± 6.5	0.806
	Females	86.4 ± 3.8	83.9 ± 6.8	0.179

Data are expressed as mean ± SD. All genes were investigated; those showing significant difference between treatment groups are shown.

[1]Differences between groups were analyzed by ANCOVA adjusting for covariates: maternal age, smoking, caesarean section, baby's sex and gestational weight. $P<0.05$ was considered significant.

[2]Highly-repeated DNA retrotransposon, chromosomal location unavailable. Assay designed from Florea *et al.* (2013).

[3]Gametic DMR, inherits methylation from gamete; somatic DMR, methylation acquired during somatic development. Gametic DMR often occur at imprint control regions that regulate more than one gene, while somatic DMR are usually associated with regulation of the cognate gene only.

Abbreviations: GW, gestational week; RBC, red blood cell; CpG, cytosine-phosphate-guanine; DMR, differentially methylated region.

TABLE 4
Maternal and newborn determinants of DNA methylation in cord blood (*n* 86)[1]

	Cord DNA Methylation (%)					
	LINE-1[2]		*IGF2*		*BDNF*	
	β	*P* value	β	*P* value	β	*P* value
Maternal Characteristics						
Folic Acid Treatment	-0.247	0.029	-0.226	0.020	-0.301	0.006
Maternal Age	0.114	0.322	0.170	0.137	0.111	0.317
Smoking in pregnancy	0.141	0.213	-0.080	0.472	-0.136	0.219
C-section birth	0.230	0.045	-0.006	0.955	0.296	0.008
Vitamin B12 (36 GW)[3]	-0.099	0.492	-0.151	0.185	-0.002	0.990
Neonatal Characteristics						
Sex (M)	0.067	0.572	-0.034	0.764	0.111	0.329
Birth weight	-0.197	0.104	-0.095	0.399	-0.094	0.419
Cord Vitamin B12	0.038	0.790	-0.236	0.030	0.012	0.932

[1]Multiple linear regression analysis performed with gene DNA methylation as dependent variable. $P<0.05$ was considered significant.
[2]Regression for cord DNA methylation was performed for each gene with adjustment for significant covariates, as appropriate. All genes were investigated; those showing significant relationships (for maternal or neonatal characteristic) are shown.
[3]36th GW refers to post-intervention.
Abbreviations: GW, gestational week; RBC, red blood cell.

FIGURE LEGENDS

FIGURE 1. Flowchart showing study design of participants in the FASSTT trial and cord blood collection.

[1]Reasons for exclusion: withdrawal from study, pregnancy complications, prescribed folic acid, fetal death or transferred to a different hospital. For full details, see original report by McNulty et al. 2013 (9).

Abbreviations: FASSTT, Folic Acid Supplementation in the Second and Third Trimesters.

FIGURE 2. Overall DNA methylation (%) at candidate loci in cord blood by maternal treatment group.

Data are expressed as median ± IQR. Differences were analyzed by ANCOVA adjusting for maternal age, smoking, caesarean section, baby's sex and gestational weight. DNA methylation results for BDNF not shown in the figure (Placebo: 3.1 ± 0.08 %; Folic Acid: 2.7 ± 0.07 %; $P = 0.003$). $P<0.05$ considered significant.