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RESEARCH ARTICLE

The impact of pre-pregnancy folic acid intake on placental DNA methylation in a fortified cohort

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Abstract

Folate plays an important role in the modulation of one-carbon metabolism and DNA methylation through a complex biosynthesis pathway. Folate deficiency during pregnancy has been associated with an increased risk for birth defects. This study investigates the extent to which the availability of folate and S-Adenosylmethionine (SAM) affects placental DNA methylation. We hypothesized that maintaining sufficient levels of folate and SAM is particularly important in individuals carrying the MTHFR C677T polymorphism. Maternal- and cord blood was analyzed to genotype the *MTHFR* rs1801133 SNP. Red blood cell (RBC) folate, vitamin B12, SAM, and S-Adenosylhomocysteine (SAH) were analyzed in cord blood. Epigenome-wide methylation analyses were performed on 90 placenta tissue samples isolated from the fetal side of the placenta; 45 originating from mother-infant dyads homozygous for the MTHFR C677T variant and 45 originating from mother-infant dyads with the homozygous wild type MTHFR677 genotype. Verification of the results was performed using pyrosequencing assays. Genome-wide placental DNA methylation patterns were relatively stable and not significantly affected by levels of one-carbon metabolites. MTHFR genotype was associated with DNA methylation of several loci, including a locus in the *MTHFR* region. RBC folate and particularly the SAM:SAH ratio did affect overall CpG DNA methylation in some CpG regions when the loci were split according to their CpG island relation. This was most evident in participants carrying the MTHFR C677T variant suggesting a stronger influence of the biosynthesis pathway on the overall placental DNA methylation in MTHFR TT individuals than in MTHFR CC individuals.

KEYWORDS
DNA methylation, epigenetics, folate, folic acid, placenta

1 | INTRODUCTION

Women of childbearing age who wish to conceive are advised to ensure adequate folate levels by intake of folic acid supplements prior to conception. Folate deficiency prior to conception has been associated with an increased risk for adverse health outcomes of the pregnancy, including increased risks of miscarriage, prematurity, and congenital malformations, such as heart defects, oral clefts, and, in particular, neural tube defects (NTD), the most common birth defect, in the offspring.[1,2] The importance of maintaining adequate folate levels during development became evident from the gross malformations resulting from folate deficiency observed in folate receptor 1 (Folr1) and reduced folate carrier-1 (Slc19a1) knock out animals[3] as well as by a randomized controlled trial (RCT) in humans.[4] Folate cannot be synthesized by the human body and must derive from the diet, or in the synthetic form of folic acid supplements. Numerous countries, including the United States, South Africa, and Chile, fortify their foods with folic acid to reduce the incidence of NTDs.[5]

Folate, together with S-Adenosylmethionine (SAM), S-Adenosylhomocysteine (SAH), homocysteine, and the vitamins B6 and B12, is an important factor that can modulate one-carbon metabolism and influence the provision of methyl groups for DNA methylation via a complex biosynthesis pathway (Figure S1). Several studies have reported that insufficient folate intake may lead to a disruption of this pathway and to reduced levels of genome-wide DNA methylation in the blood.[6,7] DNA methylation is a major epigenetic factor influencing gene activities.[8] During intrauterine development, it plays an important role in orchestrating fundamental biological processes, such as imprinting,[9] X-chromosome inactivation,[10] and differentiation and pluripotency.[11] Evidence also underlines the importance of DNA methylation and imprinting in the placenta both for placental development and fetal growth.[12–15] The placenta plays an important role during fetal development as a facilitator for the supply of nutrients from the mother to the neonate and protector of the neonate from harmful substances and as a result, aberrant levels of placental DNA methylation may impact fetal development.

To date, few studies have investigated the influence of some of the major players in the biosynthesis pathway on DNA methylation patterns in the placenta. This study was designed to investigate the extent to which placental DNA methylation may be affected by the availability of folate and SAM. We hypothesized that maintaining sufficient levels of folate and SAM is particularly important in individuals carrying the MTHFR C677T polymorphism, a polymorphism that can lead to reduced MTHFR activity. This may not only result in higher homocysteine levels in the blood,[16,17] but also an impaired SAM production and decreased folate levels. This hypothesis is further supported by a study by Friso et al., in which the authors observed diminished levels of DNA methylation in individuals carrying the MTHFR C677T polymorphism who had low levels of folate.[18]

To investigate this hypothesis, we explored placental DNA from 90 pregnancies, with 45 originating from mother–child dyads carrying the homozygous wild-type MTFHR genotype and 45 originating from mother–child dyads carrying the MTHFR C677T polymorphism.

Significance Statement

This study was designed to gain insight to which extent the availability of folate and SAM affects placental DNA methylation. Folate is important for the modulation of one-carbon metabolism and DNA methylation and folate deficiency during pregnancy has been associated with an increased risk of adverse health outcomes. The placenta plays an important role during fetal development. Since placental DNA methylation plays an important role in the development of both the placenta and the fetus, investigating the importance of folate in maintaining placenta function and the extent to which DNA methylation is modulated in the placenta can be of major importance in understanding the effect of folic acid intake on the pregnancy and health outcomes.

2 | MATERIALS AND METHODS

2.1 | Study population

Placenta tissue and participant information were collected as a part of the Harvard Epigenetic Birth Cohort (HEBC) and ethics approval was obtained by the Brigham and Women's Hospital Institutional Review Board. Details of the study protocol have been described previously .[19] This study included 90 placental DNA samples isolated from the fetal side of the placenta; 45 originating from mother-infant dyads homozygous for the MTHFR C677T variant (TT participants) and 45 originating from mother-infant dyads with the homozygous wild-type MTHFR677 genotype (CC participants). The TT and CC participants were matched based on maternal age (with averages of 33.04

versus 33.0 years), infant biological sex, birth weight (with averages of 7.66 versus 7.69 lb), birth length (with averages of 19.61 versus 19.64 inches) and gestational age (with averages of 39.02 versus 39.1 weeks). The study was restricted to non-Hispanic Caucasian mothers who had spontaneous conception and who gave birth within 3 weeks of their due date. None of the participants had other conditions, such as HELLP syndrome, hepatitis C, gestational diabetes, or pre-ecclampsia (Table 1).

2.2 | Sample preparation

To collect placental tissue, a 2 cm incision was made in the amnion. Placental tissue was collected from the fetal side of the placenta and the area nearest to the umbilical cord. Placental tissue was immediately snap-frozen and stored in liquid nitrogen until further processing. Maternal blood and cord blood samples were collected into EDTA- tubes and plasma, buffy coat, and RBC aliquots were separated by gradient centrifugation at 4000*g* for 10 min at 4°C. Plasma aliquots were transferred into cryostat tubes and stored in liquid nitrogen until further processing.

Genomic DNA was extracted from fresh frozen placenta tissue and maternal and cord blood buffy coat with the DNA easy blood and tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

2.3 | Measurements of the covariates

Genomic DNA isolated from maternal blood and cord blood was used to genotype the MTHFR rs1801133 SNP using the Pyromark Q24 pyrosequencer.

Red blood cell (RBC) folate from cord blood was measured on the Roche E Modular system (Roche Diagnostics, Indianapolis, IN, USA) and Vitamin B12 was measured using a Roche enzyme immunoassay technique (Roche Diagnostics, Indianapolis, IN, USA) at the Children's Hospital in Boston (MA, USA).

Plasma aliquots of the cord blood samples were used to assess levels of SAM and SAH using HPLC quantification at the Autism Metabolic Genomics Laboratory, Arkansas Children's Hospital Research Institute Little Rock (Arkansas, USA). To determine SAM and SAH, 100 ml of 10% metaphosphoric acid was added to 200 ml plasma to precipitate protein; the solution was mixed well and incubated on ice for 30 min. After centrifugation for 15 min at 18 000*g* at 4°C, supernatants were passed through a 0.2 mm nylon membrane filter and 20 ml was injected into the HPLC system. The separation of metabolites was performed using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (5 mm; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chemsford, MA). A 20 ml aliquot of plasma extract was directly injected into the column using Beckman Autosampler (model 507 E). All plasma metabolites were quantified using a model 5200A

TABLE 1 Subject characteristics

		Total population	Wildtype MTHFHR (C/C) ($n = 45$)	MTHFR C677T (T/T) ($n = 45$)
Folic acid supplements				
Prior to pregnancy	During pregnancy			
No	No	34	15 (33.33%)	19 (42.22%)
No	Yes	12	8 (17.78%)	4 (8.89%)
Yes	No	13	9 (20%)	4 (8.89%)
Yes	Yes	31	13 (28.89%)	18 (40%)
Conception				
Planned		74	35 (77.78%)	39 (86.67%)
Unplanned		16	10 (22.22%)	6 (13.33%)
Sex				
Female		54	27 (60%)	27 (60%)
Male		36	18 (40%)	18 (40%)
Maternal age (years)		33.03 (4.4)	33.02 (4.4)	33.0 (4.48)
Maternal BMI		23.80 (4.3)	24.19 (4.9)	23.42 (3.5)
Gestational age (weeks)		39.06 (1.00)	39.02 (0.99)	39.1 (1.01)
Birth length (inches)		19.61 (0.89)	19.61 (0.89)	19.64 (0.93)
Birth weight (lb)		7.68 (0.91)	7.66 (0.84)	7.69 (1)

Coulochem II and CoulArray electrochemical detection systems (ESA, Inc., Chelmsford, MA) equipped with a dual analytical cell (model 5010), a 4-channel analytical cell (model 6210) and a guard cell (model 5020). The concentrations of plasma metabolites were calculated from peak areas and standard calibration curves using HPLC software. The measurements allow us to perform analyses on the individual SAM and SAH levels, as well as the SAM:SAH ratio, which is frequently used as an indicator of the cellular methylation potential.

Folic acid supplementation was self-reported by the participants via a questionnaire (Yes/No).

2.4 | Epigenome-wide methylation analysis

For comprehensive analyses of epigenome-wide methylation, 1 µg of placental DNA was analyzed using the Illumina Human Methylation 450 Bead Chip at the USC Epigenome Center (Los Angeles, CA, USA). The Illumina Human Methylation 450 Bead Chip covers over 485 000 methylation loci per sample at single-nucleotide resolution, spanning around 99% of the RefSeq genes. Microarray data of 93 samples, including three technical replicates, were processed using the R/Bioconductor package minfi. First, raw signal intensities were extracted from *IDAT files. Second, CpGs with detection *p*-values >.01 or bead counts <3 in more than 10% of the samples were removed. Third, intensities were background corrected, dye-bias normalized, and functional normalization was performed to remove technical variability and batch effects .[20] Lastly, β-values were calculated to form methylated (Meth) and unmethylated (Umeth) signal intensities as suggested by Illumina β = Meth/(Umeth + Meth + 100), and M-values were calculated as logit transformed β-values. Finally, probes that overlap with known SNPs and CpGs on the X- and Y-chromosome were masked, leaving 451 522 CpGs. Before statistical analysis, β-levels of technical duplicates were averaged.

Statistical differences between univariate methylation levels and covariates were assessed using linear modeling and moderated *t*-tests[21] with Benjamini-Hochberg multiple testing correction on M-values as suggested by Du et al.[22] Continuous covariates were divided into tertiles to account for potential outliers.

2.5 | Assay verification

Candidate loci for verification were identified using the microarray analyses. The loci with the largest shift in DNA methylation were chosen for subsequent analyses by pyrosequencing. Pyrosequencing primers were designed using the primer design program 'PSQ assay' (Biotage), using gene sequences that were obtained from the GenBank entry on NCBI. The primers were designed to cover CpG loci that were identified as exhibiting differential methylation on the Infinium HumanMethylation450 BeadChip Assay. Pyrosequencing primers were designed to cover the specific CpG loci of interest plus as many surrounding loci as feasible. For each set of primers, one of the primers included a 5′-biotin label to allow subsequent analysis by pyrosequencing. An overview of primer sequences and their corresponding gene positions can be found in Table S1.

Genomic DNA was newly isolated from the stored placenta tissue from the same set of participants. Genomic DNA was subsequently treated with bisulfite salt using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendation. DNA was amplified in 20 µl PCR reactions, containing 10 µl of Hot StarTaq Master Mix Kit (Qiagen, Valencia, CA, USA), 150 ng of each primer, and ~20 ng modified DNA. PCR was performed with one 15 min cycle of 95°C, 40 cycles with 30 s of 95°C, 30 s of primer-specific annealing temperature, and 30 s of 72°C followed by one 5 min cycle of 72°C.

Pyrosequencing was performed on a PyroMark Q24 MD pyrosequencer (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Assay validation was carried out on samples of known methylation status, which was created from the whole genome amplified DNA, representing hypomethylation, and DNA treated with CpG methyltransferase M.SssI, representing hypermethylation (New England Biolabs, Ipswich, MA, USA). Pyrosequencing analyses were performed in duplicate. If the duplicates of the individual samples showed a difference of <5% methylation, the average methylation of the two measurements was used for further analyses. When the difference was >5% a third measurement was performed and the average methylation of the three measurements was used for further analyses.

Each PCR plate contained the individual DNA samples as well as three dH_2O samples as non-template controls and three samples with known methylation status as positive controls. The quality of the pyrosequencing assays was analyzed using the internal control of the pyrosequencer and the pyrograms and the reproducibility of the pyrosequencing runs was high for all the assays included in our analyses.

2.6 | Statistical analyses

To investigate potential associations between the one-carbon metabolism and overall placental DNA methylation, CpG loci were first grouped according to their CpG island relation described by Sandoval et al.,[23] resulting in

four subgroups, i.e., CpG islands, shores, shelves, and open sea and their average methylation level was derived. CpG-islands are defined by a genomic region 200 bp < x < 500 bp with a CG content of >50%. The regions up and downstream next to CpG-islands are defined as shores (0–2 kb) and shelves (2–4 kb) and CpGs outside these regions are defined as the open sea. Second, student's *t*-tests were performed between averaged methylation levels for the categorical covariate (MTHFR genotype) and Spearman correlation coefficients were calculated for the continuous covariates (RBC folate, SAM, SAH, SAM:SAH, and vitamin B12). All analyses were conducted using R version 3.2.2 software and visualized using the ggplot2 package.

3 | RESULTS

3.1 | Measurements of the covariates

None of the covariates measurements, i.e. levels of RBC folate, vitamin B12, SAM, SAH, SAM:SAH ratio, and folate intake, was significantly different between participants from the two genotypic subgroups, although a trend toward significance was observed for vitamin B12 ($p = .08$). Levels of vitamin B12 were higher in the TT group, with median levels of 832.76 pg/ml. In contrast, median levels of 711.21 pg/ml were assessed in the CC group (Figure 1).

3.2 | Correlations between overall placental DNA methylation and our covariates

No association between average placental DNA methylation and MTHFR genotype was observed (*t*-test, $p > .05$) for any of the four CpG island relations (Figure 2A).

In contrast, we observed positive correlations between DNA methylation and RBC folate levels, which were significant ($p < .05$) in the CpG island and the CpG shelf regions for all samples. The correlation between RBC folate and DNA methylation was strongest in the mother–child dyads carrying the TT genotype compared to those carrying the CC genotype for all CpG regions, but in particular for the open sea, shore, and shelves regions ($p < .05$; Figure 2B).

Similar results were found for SAM and SAH levels, in which the strongest correlations were observed in the TT participants. While SAM levels were mostly positively correlated to DNA methylation, in particular in the open sea and shore region for the TT individuals (Figure 2C), SAH levels were negatively correlated with DNA methylation (Figure 2D). Consequently, we observed significant positive correlations between the SAM:SAH ratio and DNA methylation in the open sea and shore region (Figure 2E).

Lastly, the vitamin B12 levels were positively correlated for the TT genotype and negatively correlated with the CC genotype albeit not significantly (Figure 2F).

These results suggest that RBC folate, as well as SAM, SAH, and the SAM:SAH ratio, are associated with overall DNA methylation in several CpG regions, most notably in participants carrying the MTHFR C677T variant.

3.3 | Associations between loci-specific placental DNA methylation and one-carbon metabolism by MTHFR genotype

To investigate the effect of one-carbon metabolism on placental DNA methylation, DNA methylation was regressed on the MTHFR genotype and levels of RBC folate, SAM, SAH, SAM:SAH ratio, and vitamin B12.

The MTHFR genotype was significantly associated with placental DNA methylation at three CpG loci after

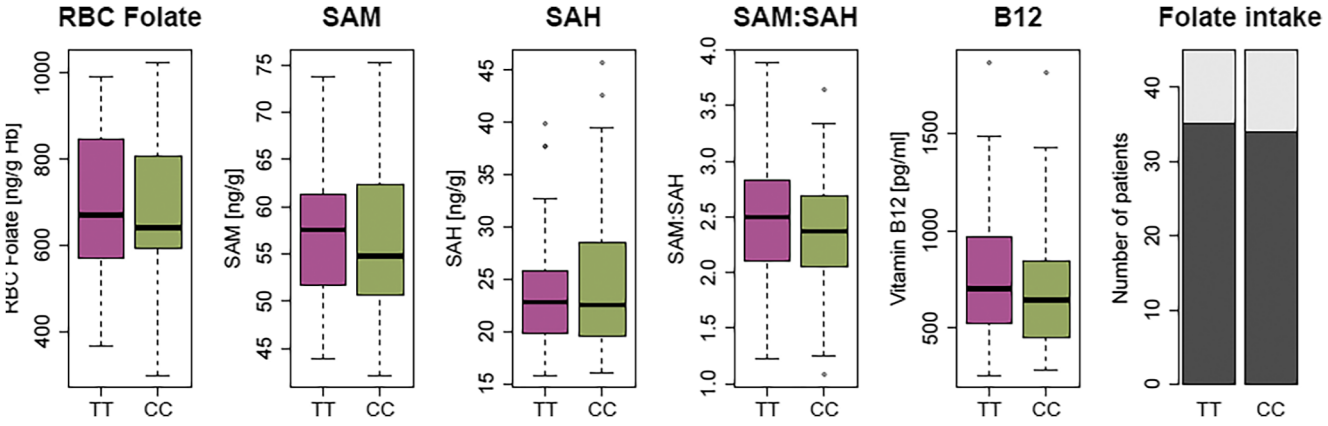

FIGURE 1 No differences were observed in the covariates included between subject groups. Measurements of the covariates in cord blood plasma do not significantly differ between our MTHFR subject groups, although a trend is observed for vitamin B12 ($p = .08$).

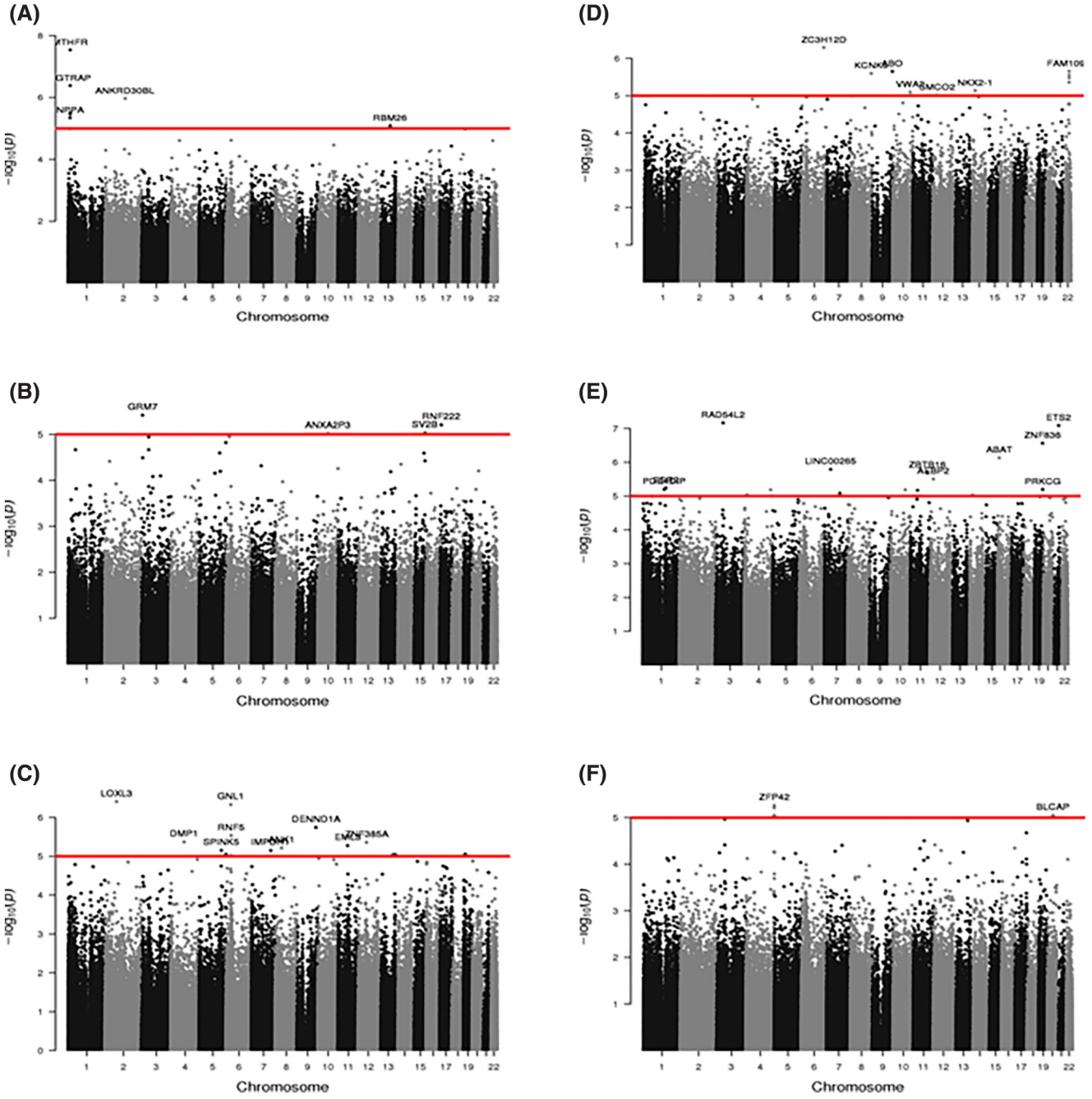

FIGURE 2 Differential DNA methylation of specific loci associated with the different variates. Manhattan plots show differential DNA methylation of specific loci associated with the different variates. (A) Genotype is significantly associated with three loci located in the genes *MTHFR, GTRAP*, and *ANKRD30BL*. (B) None of the loci were significantly associated with RBC folate levels. (C) Sam was associated with 2 loci located in the genes *LOXL3* and *GNL1*. (D) SAH was significantly associated with a locus located in the gene *ZC3H12D*. (E) SAM:SAH was significantly associated with loci located in the genes *RAD54L2*, *ETS2*, *ZNF836*, and *ABAT*. (F) None of the loci were significantly associated with vitamin B12 levels.

correction for multiple testing. These loci were located in the genes *MTHFR, GTRAP*, and *ANKRD30BL* gene ($p \leq 1.08$ E-06). Several other loci were close to reaching statistical significance, including 2 additional loci located in the *MTHFR* gene (Figure 3A). The significant locus located in the *MTHFR* gene (cg21864959) was selected for verification by pyrosequencing. The pyrosequencing results supported the robustness of the observed association and significantly higher levels of DNA methylation were observed in the TT participants than in the CC participants ($p = .04$), with median DNA methylation levels of 16.5% and 13.8%, respectively (Figure S2).

Several loci on the microarray were associated with RBC folate and placental DNA methylation. However,

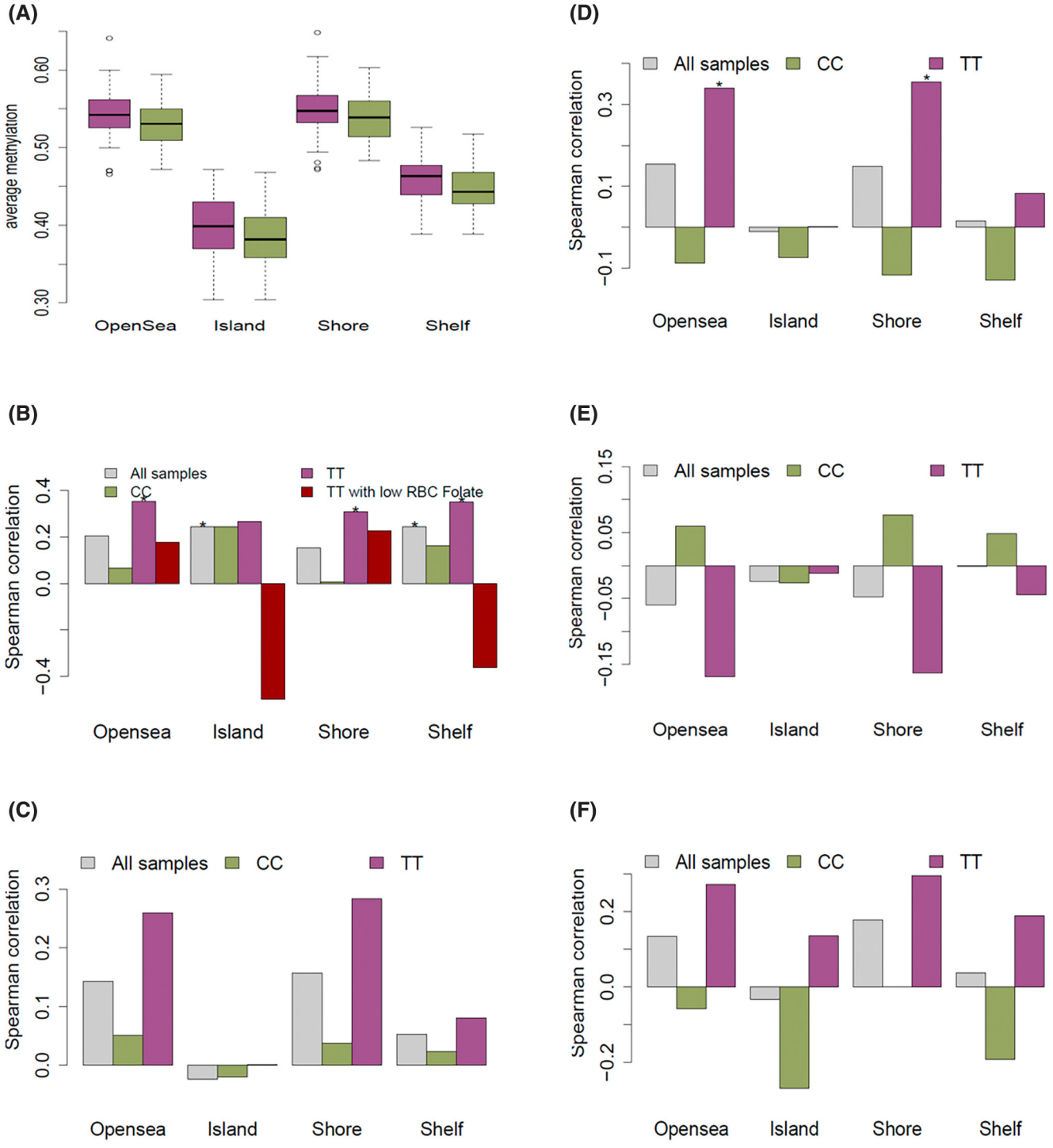

FIGURE 3 Overall DNA methylation patterns in the CpG regions divided by genotype. DNA methylation levels of several CpG regions included in the epigenome-wide analyses were significantly associated with RBC folate levels (B) and SAM:SAH (E). None of the other variates, i.e. Genotype (A), SAM (C), SAH (D), or vitamin B12 (F) were significantly associated with region-specific CpG DNA methylation patterns in the placenta.

none of the loci reached statistical significance after correcting for multiple testing. Among the strongest associations were loci located in the CpG islands of the genes *GRM7*, *RNF222*, and *SV2B* (Figure 3B), genes involved in the circadian entrainment, protein ubiquitination, and uptake and actions of bacterial toxins, respectively.

Statistically significant associations were observed between SAM levels and placental DNA methylation of two loci located in the bodies of the genes *LOXL3* and *GNL1* ($p \leq 4.67$ E-07), genes that are important for the formation of crosslinks in collagens and elastin and *GTP binding*, while several other loci were close to reaching statistical

significance, such as loci located in the bodies of the genes *DEMMD1A*, *RNF5*, and *DMP1* (Figure 3C). SAH was significantly associated with DNA methylation of one CpG locus located in the CpG island *ZC3H12D* ($p = 5.13$ E-07), a tumor suppressor gene, while several other CpG loci were close to reaching statistical significance. One gene, *FAM109B*, important for endocytic trafficking, even had 3 CpG loci close to reaching statistical significance (Figure 3D).

The SAM:SAH ratio was significantly associated with the placental DNA methylation of several loci. These loci were located in the genes *RAD54L2*, *ETS2*, *ZNF836*, and *ABAT* ($p \leq 7.41$ E-07). *RAD54L2* modulates androgen receptor-dependent transactivation, *ETS2* regulates genes involved in development and apoptosis, *ZNF836* is a *DNA binding transcription factor activity and ABAT* is involved in transmission across chemical synapses and the neurotransmitter release cycle. Several other loci were close to reaching statistical significance (Figure 3E). None of the top loci associated with SAM, SAH, or SAM:SAH overlapped.

Placental DNA methylation of none of the loci included on the microarray was significantly associated with levels of vitamin B12, although some loci were close to reaching significance. Three of these loci were located in the CpG island of the same gene, namely *ZFP42* (Figure 3F), a gene involved in *DNA binding transcription factor activity* and *sequence-specific DNA binding*.

This suggests that DNA methylation of only a few loci was significantly affected by MTHFR genotype, SAM, SAH, or SAM:SAH levels, and we did not observe widespread methylation changes with variation in the folate biosynthesis pathway. Furthermore, none of the investigated variables affected DNA methylation patterns of multiple loci located in the same gene.

3.4 | Low RBC folate and MTHFR TT genotype

We hypothesized that maintaining sufficient levels of RBC folate was most important in individuals carrying the MTHFR C677T variant. To investigate this hypothesis, the association between placental DNA methylation and RBC folate levels in participants carrying the MTFHR C677T variant was examined, by performing analyses using both genotype and RBC folate tertile (lowest tertile vs. two higher tertiles) as indicators. The RBC values were split into tertiles with the following cutoffs; 0–607; 607–758 and 758–1025 ng/g HB. This gives a split into 30, 29, and 29 samples, respectively (the RBC value was unknown for 2 participants). Of the participants in the lowest tertile group, 14 carried the MTHFR C677T variant.

Relatively large differences were observed for several loci included in the microarray. For example, differential DNA methylation greater than 10% was observed for loci in the gene bodies of the *PCDHB1*, *CNTN3*, and *CNTNNA2* genes. Although none of these loci reached statistical significance after correcting for multiple testing, two loci located in the gene bodies of *CCDC88B* and *LYSMD3* were close (Figure 4).

These results suggest that patterns of DNA methylation are not significantly affected in participants who are carrying the MTHFR C677T variant and have low levels of RBC folate.

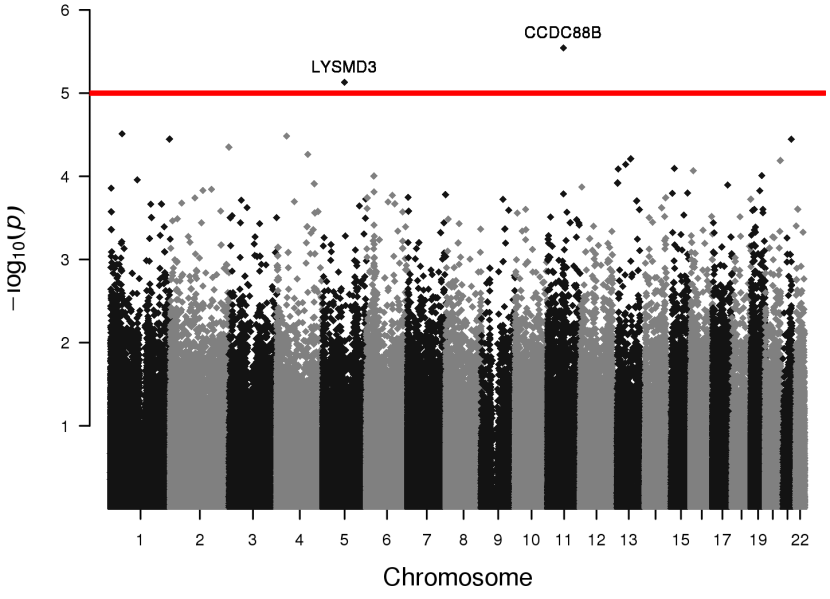

FIGURE 4 Differential DNA methylation in participants carrying the TT genotype and with low levels of RBC folate versus the rest. Manhattan plot to show differential DNA methylation in participants carrying the TT genotype and who had low levels of RBC folate ($n = 14$) versus the rest ($n = 74$). None of the loci reached statistical significance after correction for multiple testing.

4 | DISCUSSION

This study was designed to investigate the effect of some of the major players in the biosynthesis pathway, e.g., RBC folate, SAM, SAH, and vitamin B12 levels, on placental DNA methylation in association with the MTHFR genotype. Several studies have reported an effect of folic acid intake on DNA methylation levels in PBL in women[6,7] and associations between maternal RBC folate and cord blood DNA methylation levels have been suggested.[24] However, the effect of folate on the placental epigenome remains less studied. In vivo experiments have suggested that folate levels increased placental EVT invasion and angiogenesis, both crucial for placental development and regulation of fetoplacental circulation,[25] low folate concentrations were reported to increase placental vascular resistance.[26] Placental DNA methylation plays an important role in the development of both the placenta and the fetus,[12–15] supporting the importance of folate in maintaining placenta function.

We investigated the impact of the MTHFR genotype on placental DNA methylation. No correlations were found between the MTHFR genotype and placental DNA methylation in any of the CpG island relations. However, specific loci located in the *MTHFR*, *GTRAP*, and *ANKRD30BL* genes did reach genome-wide significance. While *MTHFR* is important in the biosynthesis pathway, *GTRAP* plays a role in synaptic transmission and the in vivo function of *ANKRD30BL* remains largely unknown. None of these genes is known for their expression in placental tissues. The association between genotype and these specific loci is probably more likely due to the cross-talk between DNA methylation and genetic variation and not to a specific role in these pathways.

We investigated the hypothesis that remaining sufficient levels of RBC folate were most important in participants carrying the *MTHFR* C677T variant. We observed positive correlations between RBC folate levels and placental DNA methylation, in particular for the group carrying the MTHFR C677T polymorphism. While this was most evident in the average DNA methylation at the different CpG island relations, none of the specific CpG loci reached genome-wide significance. This suggests a general increase of DNA methylation with RBC folate, however, this is not specific to certain genomic locations. Furthermore, we hypothesized that DNA methylation is most sensitive to changes in the biosynthesis pathway for participants carrying the MTFHR C677T polymorphism and who was falling within the lowest RBC tertiles. Epigenome-wide analyses revealed some loci with relatively large DNA methylation differences of 10% or greater compared to participants with high RBC folate levels however, none of these loci reached statistical significance. These observations suggest that low levels of RBC folate in cord blood did not significantly affect placental DNA methylation in our study group.

We also investigated the influence of SAM and SAH availability on placental DNA methylation levels. As expected, SAM levels were positively correlated- and SAH levels were negatively correlated to overall DNA methylation, with the strongest association found in individuals carrying the MTHFR C677T polymorphism. Again, even though associations were found with CpG loci regions, only a few individual loci included on the microarray reached statistical significance and none of these loci were associated with multiple covariates. This confirms our previous hypothesis that the biosynthesis pathway acts on DNA methylation in general and not on specific CpG sites.

The lack of site-specific significant associations and differential DNA methylation patterns may be due to the characteristics of our study population. First, this study only included participants that were homozygous for the MTHFR gene, and placental DNA methylation in individuals with the heterozygous MTHFR genotype was not measured. As a result, we do not know how the players of the one-carbon metabolism influence placental DNA methylation in individuals with the heterozygous MTHFR genotype. Second, this study was performed in the United States, where foods are fortified with folic acid.[5] In addition, only 34 of the participating mothers did not take folic acid supplements prior to and/or during pregnancy. This resulted in relatively high levels of RBC folate in our study population and none of our participants were folate deprived. It is well possible that the lack of associations in our study is due to the high levels of RBC folate in our participants and that placental DNA methylation would be affected more strongly in folate-deprived individuals. Another potential explanation for the lack of association may be that the association between placental DNA methylation and folic acid intake is not that strong. Other studies similarly did not find associations between folate levels and DNA methylation. For example, a study by Jung et al. did not observe differences in global DNA methylation in PBL between individuals that received either folic acid or a placebo, regardless of MTHFR genotype.[27] The third plausible explanation may be that placental DNA methylation is relatively stable, regardless of folate or SAM and SAH levels. Placental folate uptake from the maternal circulation is the result of the coordinated action of the folate transporters localized in placental syncytiotrophoblast microvillous membranes. The active nature of these placental folate transporters is reflected by the observations that fetal folate concentrations can be higher in the fetus relative to the mother.[28,29] Giugliani et al. reported in 1985 that the placenta may concentrate and store folate.[30] Thus, we may speculate that maternal folate is primarily used to

maintain sufficient levels of folate in the placenta and the neonate. We measured cord blood RBC folate and SAM levels and thus the levels of both placental folate and SAM in our study population remain unknown. However, positive associations between maternal plasma, cord blood plasma, and placental folate concentrations have been previously reported.[28,30] An ability to maintain relatively stable placental folate levels may result in the ability to maintain stable placental DNA methylation patterns.

In this study, we have focused on investigating the MTHFR C667T polymorphism. It is possible that associations between DNA methylation and folic acid intake can be found at other polymorphisms for this gene (e.g., the A1298C gene polymorphism). We also did not include other genes that may play roles in the modulation of one-carbon metabolism and that may influence the provision of methyl groups for DNA methylation. It is possible that other genes do show stronger associations between placental DNA methylation and folic acid intake and/or SAM levels.

In conclusion, genome-wide placental DNA methylation patterns were observed to be relatively stable and not significantly affected by levels of one-carbon metabolites. MTHFR genotype was observed to be associated with DNA methylation of several loci, including a locus in the *MTHFR* gene. RBC folate, SAM, SAH, or vitamin B12 levels did not significantly affect loci-specific placental DNA methylation. In contrast, RBC folate and particularly the SAM:SAH ratio did affect overall CpG DNA methylation in some CpG regions when the loci were split according to their CpG island relation. This was most evident in participants carrying the MTHFR C677T variant suggesting a stronger influence of the biosynthesis pathway on the overall placental DNA methylation in MTHFR TT individuals than in MTHFR CC individuals.

AUTHOR CONTRIBUTIONS

Karin B. Michels and Sanne D. van Otterdijk designed the research; Sanne D. van Otterdijk performed the research; Hagen Klett and Sanne D. van Otterdijk analyzed the data; all authors have contributed to the final version of the manuscript.

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DISCLOSURES

The authors declare they have no competing financial interests or conflict of interest.

DATA AVAILABILITY STATEMENT

Privacy/ethical restrictions: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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