Research Article

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Epigenomics

Vitamin B_{12} supplementation influences methylation of genes associated with Type 2 diabetes and its intermediate traits

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Aim: To investigate the effect of B_{12} and/or folic acid supplementation on genome-wide DNA methylation. **Methods:** We performed Infinium HumanMethylation450 BeadChip (Zymo Research, CA, USA) assay in children supplemented with B_{12} and/or folic acid (n = 12 in each group) and investigated the functional mechanism of selected differentially methylated loci. **Results:** We noted significant methylation changes postsupplementation in B_{12} (589 differentially methylated CpGs and 2892 regions) and B_{12} + folic acid (169 differentially methylated CpGs and 3241 regions) groups. Type 2 diabetes-associated genes *TCF7L2* and *FTO;* and a miRNA, *miR21* were further investigated in another B_{12}-supplementation cohort. We also demonstrate that methylation influences *miR21* expression and *FTO*, *TCF7L2*, *CREBBP*/*CBP* and *SIRT1* are direct targets of *miR21-3p*. **Conclusion:** B_{12} supplementation influences regulation of several metabolically important Type 2 diabetes-associated genes through methylation of *miR21*. Hence, our study provides novel epigenetic explanation for the association between disordered one carbon metabolism and risk of adiposity, insulin resistance and diabetes and has translational potential.

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Keywords: DNA methylation • folic acid • miRNAs • molecular mechanisms • supplementation • Type 2 diabetes • vitamin B_{12}

Vitamin B_{12} (B_{12}) is an essential dietary micronutrient for human metabolism. B_{12} deficiency is classically described in pernicious anemia [1] and is associated with neurological damage [2]. B_{12} deficiency also manifests as hyperhomocysteinemia, which is an important risk marker for cardiovascular disease [3], obesity related complexities [4], Type 2 diabetes mellitus (T2D) [5] and metabolic syndrome [6]. Vitamin B_{12} and folic acid play important roles in one-carbon metabolism (OCM); B_{12} functions as an essential coenzyme for methionine synthase, which catalyzes methylation of homocysteine to methionine in the presence of the folic acid metabolite, 5-methyl tetrahydrofolate. This is an important step in generating S-adenosyl methionine (SAM), the universal methyl donor in OCM, which plays an important role in transmethylation reactions and epigenetic regulation.

B_{12} deficiency is common in Indians and is mainly attributed to vegetarian diets, but folate deficiency is relatively rare [7]. We have demonstrated associations between maternal plasma B_{12}, folate and homocysteine concentrations

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and fetal growth [8], and childhood neurocognitive function [9], adiposity and insulin resistance [10,11]. A Mendelian randomization analysis using a maternal methylene tetrahydrofolate reductase C677T variant, suggested a causal role for maternal homocysteine concentrations in influencing fetal growth [8]. Given the potential public health importance of these findings for fetal growth and programming of noncommunicable disorders, we performed a pilot trial of oral B_{12} and folic acid supplementation in children and adults (registration number: ISRCTN59289820) which demonstrated a significant lowering of homocysteine concentrations with physiological doses of B_{12} supplementation but not with folic acid alone [12]. In the present study, we investigated molecular changes associated with B_{12} and folic acid supplementation by comparing genome-wide DNA methylation changes in the children in this trial. We identified differential methylation of several genes associated with T2D and related intermediate traits in the groups which received B_{12} supplementation either alone or with folic acid. We further demonstrate that B_{12} supplementation, through methylation of a specific miRNA, influences regulation of several T2D-associated and metabolically important genes.

Subjects & methods

Characteristics of the study populations

Discovery cohort

The present study included children from the Pune Maternal Nutrition Study (PMNS), which was established to examine the relationship between maternal nutrition status, fetal growth and long-term outcomes in the children [13]. The study design of the B_{12}/folic acid intervention in the extended PMNS cohort has been described earlier [12]. In brief, 119 families (parents and children trios) from the extended PMNS were randomized in groups and supplemented daily for 12 months with B_{12} (10 μg) and/or folic acid (200 μg), to investigate their effect on plasma homocysteine levels. We randomly selected 12 children (out of a total 15–17 children per group) from each of the four supplementation groups – placebo (B0F0), folic acid (B0F200), B_{12} (B10F0), B_{12} + folic acid (B10F200), and compared their methylome before and after supplementation (Figure 1). Detailed physical and biochemical measurements (B_{12}, folate and homocysteine concentrations) and white blood cell counts at baseline and one-year follow-up were measured using standard techniques as described earlier [12]. Mean compliance in the study at 12 months was over 80%. Informed consent was obtained from the parents and the Institutional Ethics Committee of the King Edward Memorial Hospital Research Centre approved the study following established guidelines for human research by Indian Council of Medical Research, Ministry of Health, Government of India.

Replication cohort

Selected hits identified from the discovery study were investigated in another B_{12} intervention trial in school children (Chikki trial). The three intervention groups received daily nutrient bars for a period of 120 days under direct observation, and compliance was >95%. The bars were fortified with nothing (placebo), B_{12} with multiple micronutrients (MMN), or only B_{12} (B_{12}; Figure 1). Hemoglobin, white blood cell counts, plasma B_{12}, folate and homocysteine concentrations were measured at baseline and postintervention using standard methods. Out of 178 children screened, 14 were excluded because of a low B_{12} (≤100 pmol/l) or low hemoglobin concentration (Hb <10 g/dl). The remaining 164 children (placebo [n = 55], B_{12} [n = 54] and MMN [n = 55]) were randomized. Mean compliance was similar in all three groups (94.5%). The detailed composition of the nutrient bar is given in Supplementary Table 1.

DNA methylation studies

DNA methylation profiling, processing & quality control analysis

Genomic DNA was isolated from the blood using QIAmp DNA blood midi kit (Qiagen, Hilden, Germany) and 500 ng was bisulfite converted using EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturer's instructions. The Infinium HumanMethylation450 Beadchip Array (Illumina, CA, USA) was used for generation of methylation profiles as per the manufactures' protocols. The arrays were scanned on an Illumina iScan scanner and all quality control probes were analyzed from the control dashboard using Illumina's Genome Studio (v2011.1) methylation module (v1.9.0) with default settings and HumanMethylation450_15017482_v1.1 manifest file. The .idat files obtained from the iScan were imported into the R environment (version 3.3.0) and preprocessed using minfi package [14]. The methylation value (β) represents the proportion of methylation and is calculated as the ratio between methylated probe intensity and total probe intensity (range: 0–1). The normalization was done using the 'funnorm' [15] with noob background correction. Normalized methylation β values were con-

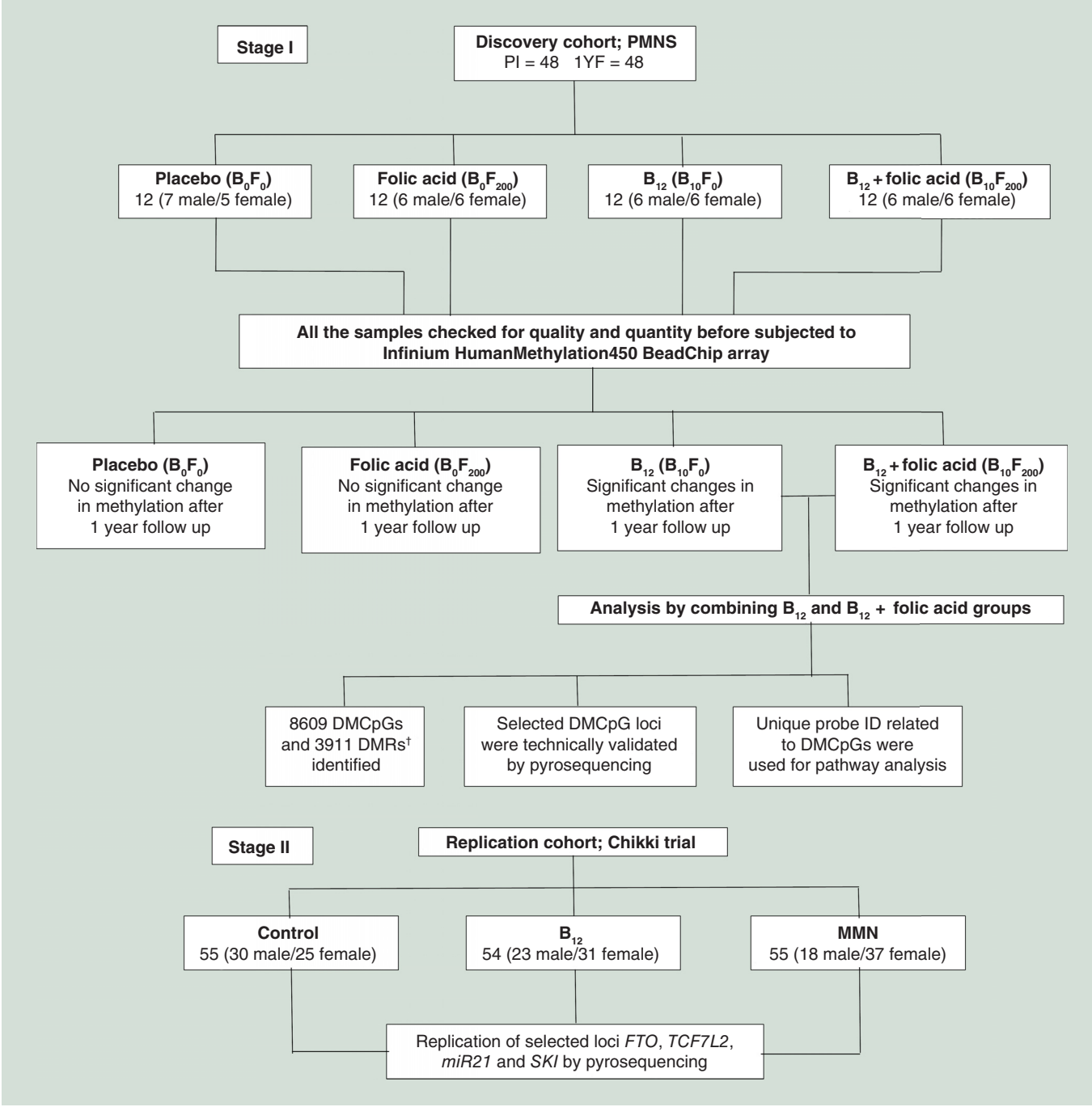

Figure 1. Overview of the study. Stage I – B_0F_0/Placebo: No intervention; B_0F_{200}: No B_{12}, folic acid 200 μg; $B_{10}F_0$: B_{12} 10 μg, no folic acid; $B_{10}F_{200}$: B_{12} 10 μg, folic acid 200 μg. **Stage II** – Control: No intervention; MMN: Multiple micronutrients (B_{12} 1.8 μg, folic acid 300 μg); B_{12}: B_{12} 2 μg; DMCpG: Differentially methylated CpG; DMR: Differentially methylated region.
† for DMR analysis FDR ≤0.02 and mean methylation difference ≥2% was used.
1 YF: One year follow-up; FDR: False discovery rate; PI: Pre-intervention; PMNS: Pune Maternal Nutrition Study.

verted to M values which were used for downstream analyses. Probes on the X, Y chromosomes and cross-reactive probes [16] were removed from the analysis. At last, a total of 458,057 probes' data from 94 subjects were carried forward for differentially methylated CpG (DMCpG) and region (DMR) analysis.

Detection of differentially methylated sites

To identify supplementation-mediated DMCpGs, we tested methylation levels at baseline (pre) against those after 1 year follow-up (post) using linear regression. The regression analysis and empirical Bayes approach were performed using linear models for microarray data (limma) and the subjects as covariates, in order to account for the paired design [17]. In view of cellular heterogeneity [18] and strong collinearity in blood cell counts measured using variance inflation factor [19], principal components (PCs) were derived for the empirical cell counts and these were used in the linear model for adjustment. For genome-wide significance, we set the threshold for false discovery rate (FDR) adjusted to $p < 0.02$.

Detection of differentially methylated regions

We used the DMRcate Bioconductor R package [18] for identification of supplementation-mediated regional methylation differences. Briefly, DMRcate functions to calculate test statistics for each CpG probe using the limma empirical Bayes t-moderated statistic as mentioned in DMCpG analysis. DMRcate further re-calculates p-values at individual CpGs after modeling the Gaussian kernel smoothing using the Satterthwaite method50 within a predefined bandwidth of $\lambda = 1000$ bp and scaling factor $C = 2$. The computed p-values were adjusted for multiple testing using a Benjamin–Hochberg (BH) FDR threshold of 0.05 and the combined information from nearby significant CpGs within the bandwidth. DMRs were constructed by grouping FDR significant sites, which lie at maximum of 1000 bp from each other and contain at least two or more CpGs. minFDR (minimum BH adjusted value) within a DMR is representative of the statistical inference for that region and the mean fold change (meanbetafc) is the mean β-fold change within the region. DMRcate analysis was performed to compare pre- and post-supplementation groups, where we defined significant DMRs having BH corrected $p < 0.2$, and for the combined B_{12} and non-B_{12} group analysis we defined significant DMRs having BH corrected $p < 0.05$. At last, on obtaining the DMRs for each analysis, we termed those DMRs significant which contained minFDR < 0.02 and a meanbetafc of 2% in the final results output.

Pyrosequencing & genotyping

Selected hits from the Infinium HumanMethylation450 BeadChip Array data were technically validated on the same individuals used in discovery experiment ($n = 12$), but freshly bisulfite converted DNA samples were used. Similar approach was used for the replication analysis on individuals in the Chikki Trial ($n = 54$–55 in each group; Figure 1). Primers and assay files were designed using PyroMark Assay Design Software 2.0 (Qiagen; Supplementary Table 2). Pyrosequencing was performed using PyroMark ID 24 according to the manufacturer's instructions and the data were analyzed with the PyroMark Q24 software program (Qiagen).

Bioinformatics analysis

Genome coordinates provided by Illumina were used to annotate the significantly enriched DMCpGs and DMRs. Probes lacking an annotated gene identity and duplicate gene entries were removed and annotated genes (as per official UCSC reference), CpG islands, enhancers and promoter regions were analyzed further. We performed an enrichment analysis to examine whether the significant DMCpGs (FDR < 0.02; absolute beta difference >0.05) were over- or under-represented in different biological features from the Infinium HumanMethylation450 BeadChip annotation file. Annotated probes were tested for enrichment of DMCpGs using a two-tailed Fisher's exact test, compared with the frequency of DMCpGs in all annotated probes on the Infinium HumanMethylation450 BeadChip array and on the DMCpGs obtained from the B_{12} and the B_{12} + folic acid groups.

The Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Inc, CA, USA) contains probe identification information of the Infinium HumanMethylation450 BeadChip. The unique probe identifiers for DMCpGs obtained from each of the intervention groups (FDR < 0.02; beta difference <0.05) were set up as 'Probe List' and were uploaded into the IPA. We used the 'Core Analysis' module to identify the canonical pathways and biological networks altered/regulated due to intervention in the groups studied and reported -10 logarithms of Fisher's exact test p-values in canonical pathway analysis by IPA. Biological functions which were significant at Fischer's exact test were then assigned to network by determining a p-value for the enrichment of the genes in the network for such functions compared with the whole Ingenuity Pathway Knowledge Base as a reference set. Online databases and miRNA target prediction tools such as miRDB [20], miRanda [21] and DIANA-microT [22] were used to identify the potential targets of *miR21*.

Functional studies

Generation of constructs related to miR21 differentially methylated region

The *miR21* promoter (*miR21*-Pro-pGL3B, 934 bp) and DMR (*miR21*-DMR-pGL3B, 256 bp) constructs were generated by cloning them in pGL3 basic vector (pGL3B; Promega, WI, USA) and sequences were verified by Sanger sequencing. Methylated *miR21* DMR was generated by *in vitro* methylation of the DMR with CpG methyl transferase M.SssI in the presence of 32 mM SAM for 48 h at 37°C. Mock methylation was performed in similar way but without M.SssI methyltransferase. Methylation status was verified by digesting the methylated and mock methylated DMR with methylation sensitive BceA1 enzyme (NEB, MA, USA). Methylated and mock methylated DMRs were re-ligated in pGL3B by incubating them for 16 h at 16°C. The ligated products were gel purified and quantified before transfection in various cell lines. All primers are listed in Supplementary Table 3.

Generation of the reporter constructs for validation of miR21-3p targets

We generated reporter constructs of the predicted targets of *miR21-3p* (target-psiCHECK) by cloning the 500 bp region of 3′-UTR containing the potential seed sequences into psiCHECKTM-2 dual luciferase reporter vector (Promega) downstream to hRluc gene. For overexpressing *miR21-3p* (*miR21-3p*-pmU6), we adopted a method in which 21 bp miRNA sequence was synthesized in a stem loop backbone oligo of 60 bp. The forward and reverse strands were synthesized in such way that on annealing, Bbs1 and Xba1 restriction sites were generated. The hybridized oligos were cloned into pmU6 vector having U6 promoter [23]. The same method was used to generate a control for *miR21-3p* (control-pmU6). We mutated seed sequences of target constructs by site-directed mutagenesis where the target constructs were amplified using primers containing the mutation and 2X Trans Taq High Fidelity (HiFi) PCR SuperMix according to manufacturers' protocols (Transgen Biotech, Beijing, China). After PCR amplification, templates were digested with Dpn1 at 37°C for 45 min and 5 µl of Dpn1-digested amplicons were used for transformation. Sanger sequencing was used to confirm the mutations in the seed sequences (Supplementary Table 3).

Dual luciferase reporter assay

We performed dual luciferase reporter assay to evaluate the promoter activity of the *miR21* DMR and the effect of methylation on promoter activity. We used three cell lines, HEK293, HepG2 and MIN6 cell lines and seeded individual cell lines at a density of 5.0×10^4 cells per well in 24-well plates, 24 h before transfection. Cells were co-transfected with different constructs (100 ng of each *miR21*DMR-pGL3B or *miR21*Pro-pGL3B constructs and 400 ng of methylated or mock methylated construct per well) along with 1 ng of *Renilla* Luciferase (pRL) control vector using lipofectamine 2000 (Invitrogen, CA, USA). Cell lyses and dual luciferase assays were performed 24 h after transfection using dual luciferase reporter assay kit (Promega) on the Perkin Elmer multimode plate reader according to the manufacturer's instructions. Firefly luciferase activity was normalized by *Renilla* luciferase activity. For validation of *miR21-3p* targets, each target-psiCHECK construct (100 ng), and either 300 ng of pmU6-*miR21-3p* or pmU6-control were co-transfected in the cell line. Luciferase assay was performed as described above except that on this occasion, the *Renilla* luciferase activity was normalized relative to the firefly luciferase activity.

Electrophoretic mobility shift assay

Methylated and mock methylated *miR21* DMRs were radiolabeled using protocols described by the manufacturer (NEB). In brief, an equal amount (1.5 µg) each of methylated and mock methylated *miR21* DMR were incubated with 32 P ATP and 10 U polynucleotide kinase in 70 mM Tris-HCL, 10 mM $MgCl_2$ and 5 mM dithiothreitol at room temperature for 1 h. The labeled probes were purified using a sepharose bead column and eluted in 100 µl of TE. The radiolabeled methylated and mock methylated DMR probes were incubated with 1.5 µl of HEK293 cell nuclear extract (5 mg/ml), 0.5 µl polydIdC (1 mg/ml, Sigma-Aldrich, MO, USA) and 0.5 µl yeast tRNA (1 mg/ml, Invitrogen) in 20 µl binding buffer (HEPES 20 mM pH 7.9, KCl 150 mM, EDTA 1 mM and Ficoll 8%) on ice for 10 min. Subsequently, the binding mix was loaded on 6% native polyacrylamide gel and run at 75 V at 4°C for 12 h. After the run was over, the gel was exposed to phospho-imager screen (GE Healthcare, IL, USA) for 6 h followed by scanning by Personal Molecular Imager (BioRad, CA, USA).

Statistical analysis

Biochemical data were analyzed using SPSS software (v 17.0; SPSS, Inc, IL, USA). Demographic and biochemical values were reported as median and interquartile range. To investigate the significance of change in the values, a

nonparametric Mann–Whitney test was conducted for across group (placebo vs other groups) and a paired *t*-test for within group (baseline vs 1 year follow-up) comparisons. All statistical analysis for the Infinium HumanMethylation450 BeadChip Array data was performed using 'R' as stated above. Methylation data from pyrosequencing were extracted using the PyroMark Q24 software (v.2.0.6) and CpG sites that 'failed' at the PyroMark software were excluded from the analysis. Variance inflation factor and PCs were calculated using R scripts. All methylation data were then adjusted for age, gender and blood count using linear regression in 'R'. The adjusted methylation values were generated using the Kobor method [24] and compared at baseline (pre) and after supplementation (post) using paired student *t*-tests using Graphpad prism (v6.0, GraphPad Software, Inc, CA, USA). Median methylation differences were analyzed with the Mann–Whitney U test for between-group comparisons (placebo vs supplementation subjects). Additionally, to analyze gender specific methylation changes, methylation data were stratified based on gender and methylation values were compared between the genders as mentioned above. Data in graphs were shown as mean ± standard error of mean (mean ± SEM) and results were considered significant at $p < 0.05$. All luciferase assays were performed in triplicate and repeated at least thrice. The data were normalized with the co-transfected vector and unpaired student *t*-test was performed to evaluate the significance level ($p < 0.05$).

Results

Cohort characteristics

Characteristics of the children in the PMNS (discovery cohort) at baseline and after intervention are presented in Table 1. This population has a low B_{12} but adequate folate status, and hyperhomocysteinemia is common. Supplementation for 12 months resulted in higher concentrations of B_{12} in the B_{12} (B10F0) and the B_{12} + folic acid (B10F200) groups (by 139.5 and 130.5 pmol/l, respectively; $p < 0.01$ and $p < 0.05$, respectively) but it remained unchanged in the folic acid (B0F200) group. Folate levels increased in both the B_{12} + folic acid and folic acid groups (by 5.4 and 16.4 nmol/l, respectively; $p < 0.01$ both) but decreased in the B_{12} group (by 4.5 nmol/l; $p < 0.05$). Plasma homocysteine levels did not change in the folic acid alone group but showed a reduction in both the B_{12} and B_{12} + folic acid groups (by 3.1 and 2.7 μmol/l; $p < 0.01$ both). Placebo group (B0F0) did not show any change in plasma B_{12} and folate concentrations but plasma homocysteine levels increased (by 2.5 μmol/l; $p < 0.01$). In the Chikki Trial (replication cohort; Table 2), plasma B_{12} concentrations increased with supplementation (along with multiple micronutrients [MMN] and B_{12} alone [B_{12}]) by 91.0 and 82.0 pmol/l, respectively ($p < 0.0001$ for both), while no significant change was seen in the placebo group. Plasma folate concentrations increased by 27.8 nmol/l ($p < 0.001$) and 1.3 nmol/l ($p < 0.05$), respectively in the MMN and B_{12} groups, but remained unchanged in the placebo group. Both supplementation groups showed a reduction in plasma homocysteine concentrations (3.8 and 1.4 μmol/l, respectively; $p < 0.0001$) but the levels increased in the placebo group (1.9 μmol/l; $p < 0.0001$). A comparison of change in anthropometric measurements across the groups did not show any significant differences after supplementation indicating no additional effect of supplementation.

Differentially methylated loci & their biological relevance

In the PMNS cohort, we compared methylation levels at approximately 483,000 loci measured on the Infinium HumanMethylation450 BeadChip Array, pre- and post-supplementation in each group and across groups. Considering the small sample size, we used an FDR adjustment and a stringent FDR adjusted $p < 0.02$ for the analysis. At baseline, there were no significant differences in DNA methylation levels (FDR < 0.02 and difference between group average% methylation levels >5%) among the four groups in the PMNS. On comparison of the pre- and post-supplementation methylation data, 12 DMCpGs were detected in the placebo group, presumably representing background change over time and noise in the data. The folic acid group showed 19 DMCpGs, while the groups receiving B_{12} alone and that with folic acid showed many more DMCpGs; 589 and 169 DMCpGs in the B_{12} and B_{12} + folic acid groups, respectively (Figure 2A–D & Table 3; Supplementary Tables 4 & 5). On comparing the groups that received B_{12} (B_{12} and B_{12} + folic acid groups) and those that did not (placebo and folic acid groups), we observed that the group which received B_{12} had 8609 DMCpGs while the group which did not receive B_{12} had only 519 significant DMCpGs at FDR < 0.02 and beta difference >5% (Table 3; Supplementary Tables 6 & 7). These observations clearly indicate that B_{12} supplementation has a larger impact than folic acid on the methylation status of various genes in this population. At last, we detected contiguous regions of differential methylation (DMR) containing multiple CpGs using the DMRcate algorithm (minFDR < 0.02 and beta difference >2%). We observed higher number of DMRs in the B_{12} + folic acid group compared with the B_{12} group (3241 vs 2891, respectively; Supplementary Tables 8 & 9) while placebo and folic acid groups had only 18 and 27 DMRs, respectively (Table 3).

Table 1. Clinical and biochemical characteristics of the discovery cohort (Pune Maternal Nutrition Study).

Groups	Placebo (B0F0)	Folic acid (B0F200)	B_{12} (B10F0)	B_{12} + folic acid (B10F200)
Parameters				
N (Male/female)	12 (7 M/5 F)	12 (6 M/6 F)	12 (6 M/6 F)	12 (6 M/6 F)
Age (years)				
Baseline	9.1 (8.9–9.2)	9.0 (8.8–9.2)	8.9 (8.7–9.1)	8.9 (8.9–9.2)
Supplementation	10.7 (10.7–10.9)	10.7 (10.5–10.8)	10.8 (10.5–10.9)	10.7 (10.5–10.9)
Change	1.7 (1.6–1.8)	1.7 (1.6–1.8)	1.8 (1.8–1.9)	1.8 (1.6–1.8)
Height (cm)				
Baseline	124.9 (123.2–127.1)	127.8 (122.6–133.5)	126.5 (122.5–128.5)	126.3 (119.8–129.4)
Supplementation	133.6 (130.9–139.5)	135.8 (132.7–143.7)	136.6 (131.1–138.6)	134.1 (131.1–141.2)
Change	9.7 (7.9–12.3)	10.0 (8.5–10.8)	9.9 (8.9–10.8)	9.2 (8.3–11.1)
Weight (kg)				
Baseline	21.8 (20.1–23.2)	22.3 (20.8–24.3)	21.5 (18.8–22.3)	22.1 (19.4–23.5)
Supplementation	24.4 (23.6–30.1)	26.6 (24.8–30.2)	25.6 (24.1–27.3)	25.2 (24.3–28.7)
Change	4.1 (3.2–4.5)	4.8 (3.3–5.4)	4.6 (3.5–6.0)	4.3 (2.8–6.1)
BMI (kg/m^2)				
Baseline	13.9 (13.2–14.5)	13.8 (12.8–14.8)	13.1 (12.7–14.5)	13.9 (13.4–14.2)
Supplementation	14.2 (13.5–15.7)	15.0 (13.5–15.8)	14.3 (13.5–14.8)	14.2 (13.4–15.0)
Change	0.5 (0.1–0.7)	0.8 (0.2–1.3)	1.0 (0.2–1.8)	0.4 (-0.1–1.2)
B_{12} (pmol/l)				
Baseline	217.0 (157.0–269.0)	193.5 (161.8–301.8)	177.0 (137.5–196.5)	155.0(117.0–231.8)
Supplementation	197.5 (168.8–224.8)	212.0(177.8–247.5)	328.5 (238.0–362.5)	307.5 (238.5–413.0)
Change	9.0 (-40.0–33.0)	-9.5 (-49.0–46.0)	139.5 (68.3–209.8)† **	130.5 (66.0–194.0)† *
Folate (nmol/l)				
Baseline	17.5 (13.8–24.6)	20.9 (13.5–26.0)	19.3 (13.5–23.1)	19.7 (15.0–22.5)
Supplementation	21.0 (15.9–23.1)	41.0 (26.7–49.1)	13.8 (10.9–17.2)	25.7 (17.8–30.9)
Change	1.6 (-4.4–9.5)	16.4 (5.4–29.6)† **	-4.5 (-7.7–0.4)† *	5.4 (0.1–12.6)**
Homocysteine (μmol/l)				
Baseline	9.4 (7.3–11.1)	9.7 (7.9–11.4)	11.3 (9.1–15.1)	10.6 (9.0–12.3)
Supplementation	12.6 (9.8–16.0)	9.8 (8.1–15.4)	7.6 (6.3–9.2)	7.8 (6.1–9.0)
Change	2.5 (0.9–4.6)**	0.5 (-0.3–3.9)	-3.1 (-4.9–2.0)† **	-2.7 (-4.5–1.6)† **

† Significant difference when compared with the placebo group.
B0F0/Placebo: No supplementation; B0F200: No B_{12}, folic acid 200 μg; B10F0: B_{12} 10 μg, no folic acid; B10F200: B_{12} 10 μg, folic acid 200 μg. N: Number; All values are median and IQR (interquartile range); Significance of change within the group (*$p \leq 0.05$; **$p \leq 0.01$).

Further, combining the data from both B_{12} supplementation groups identified more significant DMRs (B_{12} and B_{12} + folic acid; n = 3911) in comparison to the two groups that did not receive B_{12} (placebo and folic acid, n = 1725; Table 3; Supplementary Tables 10 & 11).

The majority of DMCpGs in the B_{12} (n = 432/589; 73.3%) and B_{12} + folic acid groups (93/169; 55.03%) were hypomethylated. However, while the majority of DMRs in the B_{12} group (n = 1745/2908; 60.0%) were hypomethylated, those in the B_{12} + folic acid group (2331/3267; 71.35%) were mostly hypermethylated. The DMCpGs were unequally distributed with respect to the annotated genic features in both B_{12} and B_{12} + folic acid groups (Supplementary Table 12). The 589 DMCpG probes in the B_{12} group were located in 424 unique genes of which 75 were promoter-associated, 245 were enhancer-associated and 83 were in CpG islands. Similarly, in the B_{12} + folic acid group, 169 DMCpG probes were distributed in 129 unique genes, 53 of which were in the promoter region, 50 and 61 were enhancer- and island-associated, respectively. Enrichment analysis demonstrated that DMCpGs were under-represented in TS1500, 1st Exon, CpG island, S-shore, promoter and unclassified regulatory regions in the B_{12} group (p-range: $0.01–9.5 \times 10^{-14}$) and over-represented in the enhancer and DNAse hypersensitive regions (p range: $0.05–1.7 \times 10^{-15}$]. However, in the B_{12} + folic acid group, DMCpGs were underrepresented in the gene body, S-shelf and S-shore (p range: 0.03–0.01) and over-represented in TS200, nongene and promoter-associated regulatory regions (p range: $1.6 \times 10^{-3}–1.5 \times 10^{-4}$; Figure 2E–H; Supplementary

Table 2. Clinical and biochemical characteristics of the replication cohort (Chikki Trial).

Groups	Control	B_{12}	MMN
Parameters			
N (Male/female)	55 (30 M/25 F)	54 (23 M/31 F)	55 (18 M/37 F)
Age (years)	11.3 (11.0–12.0)	11.4 (10.9–11.8)	11.4 (10.9–12.2)
Height (cm)			
Baseline	140.6 (135.0–145.5)	140.3 (134.0–144.6)	138.1 (135.0–143.0)
Supplementation	145.6 (139.0–150.0)	144.9 (138.1–149.0)	143.8 (140.0–147.8)
Change	4.3 (3.6–5.2)	4.1 (3.3–5.0)	4.5 (3.9–5.3)
Weight (kg)			
Baseline	28.2 (25.0–34.0)	30.0 (25.8–33.1)	28.8 (25.0–33.4)
Supplementation	30.6 (27.3–39.1)	32.5 (28.2–36.6)	32.7 (27.4–37.3)
Change	2.7 (1.9–4.4)	2.9 (2.0–4.2)	3.1 (2.0–4.8)
BMI (kg/m^2)			
Baseline	14.6 (13.6–16.6)	14.9 (14.1–16.5)	15.0 (13.8–16.3)
Supplementation	15.1 (14.0–17.4)	15.3 (14.6–17.2)	15.3 (14.3–17.2)
Change	0.4 (0.1–1.1)	0.5 (0.2–0.9)	0.7 (0.2–1.1)
B_{12} (pmol/l)			
Baseline	183.0 (141.0–230.0)	187.5 (156.0–226.5)	173.0 (132.0–224.0)
Supplementation	192.0 (135.5–276.5)	289.0 (212.0–383.5)	251.0 (210.0–310.0)
Change	5.0 (-26.5–44.5)	91.0 (47.0–167.0)†****	82.0 (33.0–129.0)†****
Folate (nmol/l)			
Baseline	20.8 (15.6–27.3)	19.6 (16.1–22.8)	19.0 (15.7–24.8)
Supplementation	21.4 (17.0–28.2)	20.7 (16.0–32.0)	45.8 (35.0–58.3)
Change	0.8 (-2.0–5.1)	1.3 (-1.8–8.5)*	27.8 (18.4–37.4)†****
Homocysteine (μmol/l)			
Baseline	16.4 (14.3–21.3)	15.7 (12.6–20.7)	17.0 (13.1–20.6)
Supplementation	18.9 (15.6–24.1)	14.2 (12.5–16.3)	12.7 (10.6–14.4)
Change	1.9 (0.2–4.2)****	-1.4 (-5.0–0.1)†****	-3.8 (-8.1–1.7)†****

All values are median and IQR (interquartile range). Significance of change within the group (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).
† Significant difference when compared with the placebo group.
B_{12}: B_{12} 2 μg; Control: No supplementation; MMN: Multiple micronutrient (B_{12} 1.8 μg, folic acid 300 μg); N: Number.

Table 3. List of differentially methylated CpGs and regions in different supplementation and analysis groups in the Pune Maternal Nutrition Study cohort.

Groups	DMCpGs	DMRs
	FDR < 0.02, Beta_diff >5%	min FDR < 0.02, meanbetafc >2%
Placebo (B0F0)	12	18
Folic acid (B0F200)	19	27
B_{12} (B10F0)	589	2891
B_{12} + folic acid (B10F200)	169	3241
Without_B_{12} (B0F200 + B0F0)	519	1725
Pooled_B_{12} (B10F200 + B10F0)	8609	3911

B0F200: No B_{12}, folic acid 200 μg; B10F0: B_{12} 10 μg, no folic acid; B10F200: B_{12} 10 μg, folic acid 200 μg; B0F0/Placebo: No supplementation; Beta_diff: Absolute difference; DMCpG: Differentially methylated CpG; DMR: Differentially methylated region; FDR: False discovery rate; Meanbetafc: Mean beta fold change; min FDR: Minimum FDR; Pooled_B_{12} (B10F0 + B10F200): B_{12} and B_{12} + folic acid groups combined; Without_B_{12} (B0F0 + B0F200): Placebo and folic acid groups combined.

Table 13). IPA software was used to perform pathways analysis of the genes containing the DMCpGs identified above. We observed significant enrichment of canonical pathways reportedly having a role in T2D, such as estrogen receptor signaling, adipogenesis pathways, glycogen biosynthesis II in the B_{12} group along with other important pathways related to molecular and cellular function, physiology and development, cardiotoxicity, hepatotoxicity and nephrotoxicity (Supplementary Table 14A). Similarly, pathways significantly enriched in the B_{12} + folic acid

group were Cell Cycle – G2/M DNA Damage Checkpoint Regulation, and Wnt/-catenin Signaling. Several other pathways related to molecular and cellular function, physiology and development, cardiotoxicity, hepatotoxicity and nephrotoxicity were also enriched in the B_{12} + folic acid group (Supplementary Table 14B). This indicates that the identified DMCpGs are enriched in pathways related to regulation of development and glucose and lipid metabolism.

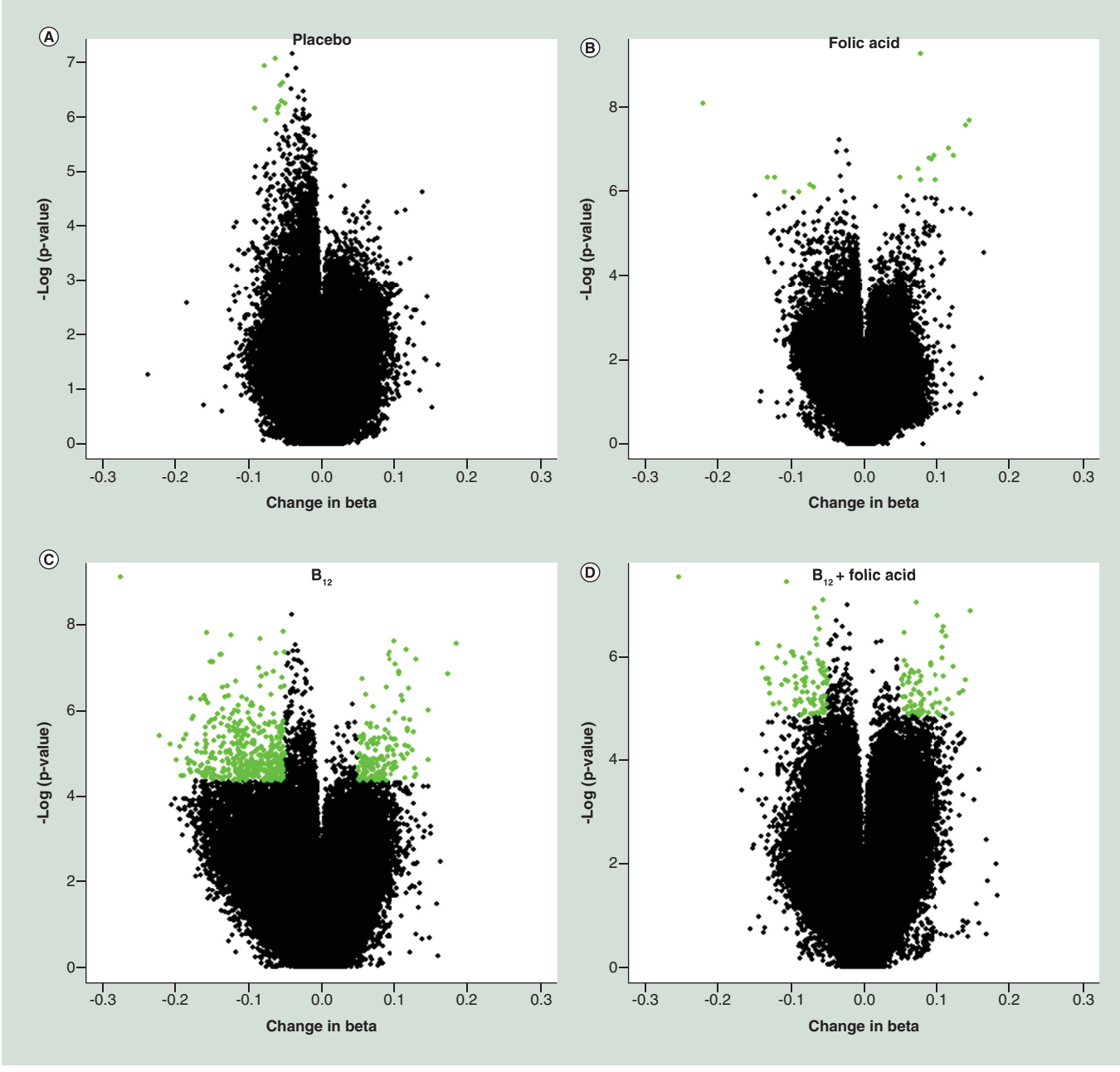

Figure 2. Differentially methylated positions in four supplementation groups and their distribution across different genomic features. Volcano plots show the changes in DNA methylation after supplementation with **(A)** placebo **(B)** folic acid **(C)** B_{12} and **(D)** B_{12} + folic acid. The black dots represent all the probes investigated and the green dots represent beta differences of 5% (methylation changes) and adjusted p-value < 0.02. A total of 589 significant probes in B_{12} group and 169 probes in B_{12} + folic acid group were analyzed and plotted for their distribution across **(E)** genic features, **(F)** CpG island features, **(G)** regulatory features and **(H)** gene and nongenic features, respectively.

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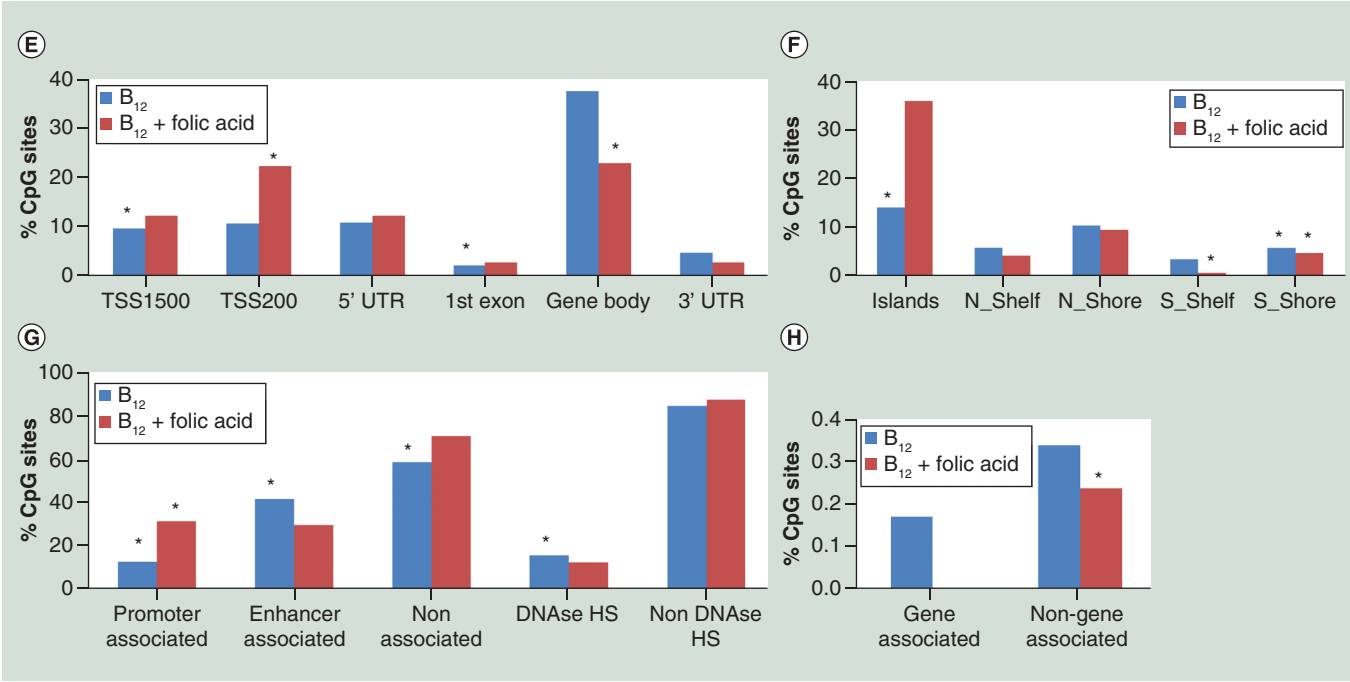

Figure 2. Differentially methylated positions in four supplementation groups and their distribution across different genomic features (cont.).
Volcano plots show the changes in DNA methylation after supplementation with **(A)** placebo **(B)** folic acid **(C)** B_{12} and **(D)** B_{12} + folic acid. The black dots represent all the probes investigated and the green dots represent beta differences of 5% (methylation changes) and adjusted p-value < 0.02. A total of 589 significant probes in B_{12} group and 169 probes in B_{12} + folic acid group were analyzed and plotted for their distribution across **(E)** genic features, **(F)** CpG island features, **(G)** regulatory features and **(H)** gene and nongenic features, respectively.

Table 4. Status of selected differentially methylated CpGs and regions in various supplementation and analysis groups in the Pune Maternal Nutrition Study cohort.

Groups	Placebo (B0F0)		Folic acid (B0F200)		B_{12} (B10F0)		B_{12} + folic acid (B10F200)		Without_B_{12} (B0F0 and B0F200)		Pooled_B_{12} (B10F0 and B10F200)	
Gene/probe id	FDR (<0.02)	Beta diff (%)	FDR (<0.02)	Beta diff (%)	FDR (<0.02)	Beta diff (%)	FDR (<0.02)	Beta diff (%)	FDR (<0.02)	Beta diff (%)	FDR (<0.02)	Beta diff (%)
TCF7L2/ cg03683087	x	x	x	x	0.016	5.50	X	x	x	x	1.0×10^{-3}	4.30
FTO/ cg26580413	x	x	x	x	x	x	X	x	x	x	1.2×10^{-3}	6.20
GALNT2/ cg00589617	x	x	0.0125	7.80	x	x	0.014	7.10	7.0×10^{-3}	6.50	9.1×10^{-7}	7.60
PPARGC1B/ cg08928958	x	x	x	x	x	x	X	x	x	x	5.0×10^{-3}	7.20
miR21†	x	x	x	x	1.5×10^{-5}	4.30	$3.x10^{-8}$	4.50	1.3×10^{-8}	3.50	8.0×10^{-15}	4.20
SKI†	x	x	x	x	x	x	X	x	x	x	7.0×10^{-4}	4.70

† Represents DMRs where minFDR and mean beta differences are mentioned. The meanbetafc ic calculated from 12 and 4 CpGs in mir21 and SKI genes, respectively.
B0F200: No B_{12}, folic acid 200 μg; B10F0: B_{12} 10 μg, no folic acid; B10F200: B_{12} 10 μg, folic acid 200 μg; B0F0/Placebo: No supplementation; Beta_diff: Absolute difference; DMCpG: Differentially methylated CpG; DMR: Differentially methylated region; FDR: False discovery rate; Meanbetafc: Mean beta fold change; min FDR: Minimum FDR; Pooled_B_{12} (B10F0 + B10F200): B_{12} and B_{12} + folic acid groups combined; Without_B_{12} (B0F0 + B0F200): Placebo and folic acid groups combined; x: Locus/prob that did not pass FDR.

Technical validation & investigation of selected differentially methylated CpGs & regions in the Chikki Trial

Based on the above results, we selected loci for replication analysis; if they had attained FDR < 0.02 in comparative pooled analysis of groups with and without B_{12} supplementation (Table 4), based on their biological relevance and the established genetic association with T2D and associated intermediate phenotypes such as obesity and insulin resistance. Several T2D-associated genes such as *TCF7L2*, *FTO*, *PPARGC1B*, *GALNT2*, *IGF2BP2*, *KCNQ1*, etc. passed the FDR < 0.02 (Supplementary Table 15). Four DMCpGs, located within *FTO* (cg26580413), *TCF7L2*

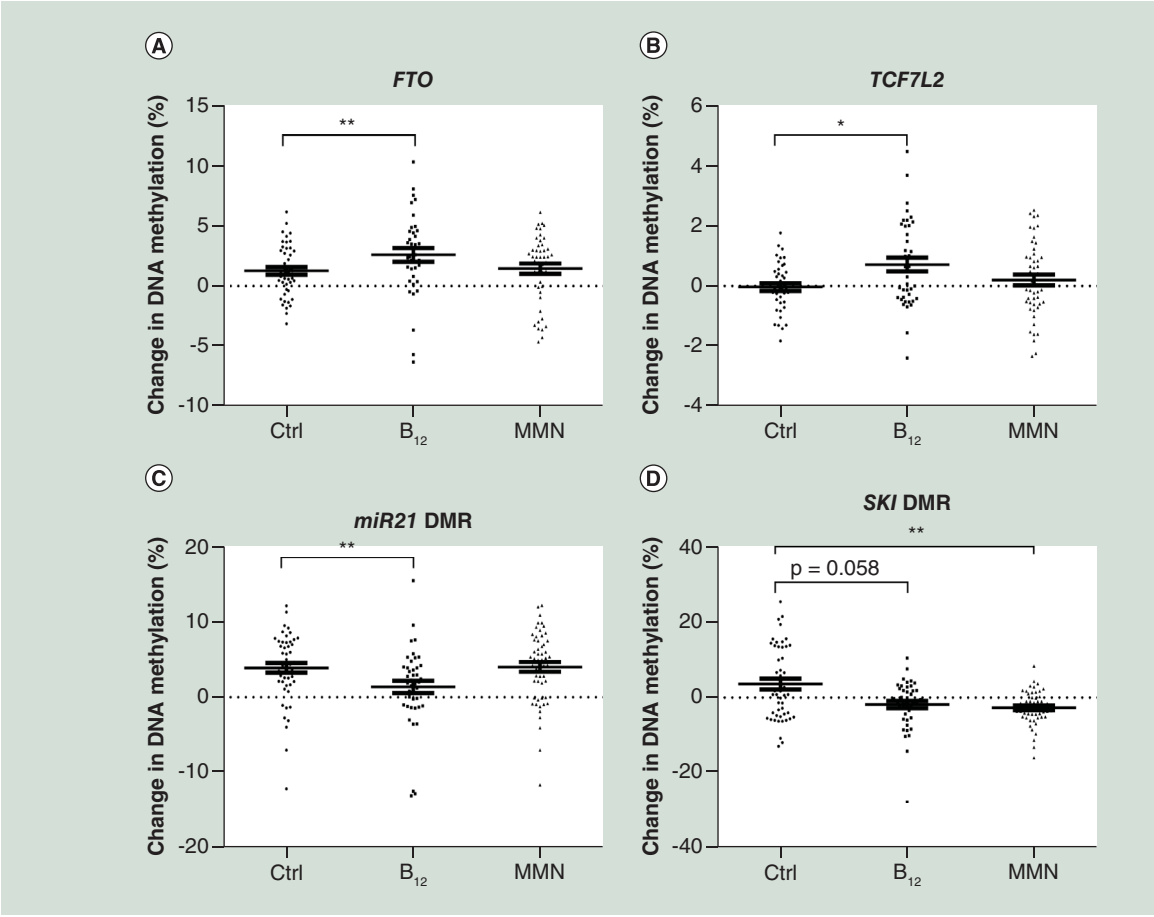

Figure 3. Investigation of selected differentially methylated CpGs and regions by pyrosequencing in the Chikki Trial. Two DMCpG loci (*FTO* and *TCF7L2*) and two DMRs (*miR21* and *SKI*) were selected for replication in the Chikki Trial. Changes in DNA methylation in different groups are shown for **(A)** *FTO*, **(B)** *TCF7L2*, **(C)** *miR21* and **(D)** *SKI*. *$p \leq 0.05$; **$p \leq 0.01$, all data presented as mean ± standard error of the mean.
Ctrl: Control; DMCpG: Differentially methylated CpG; DMR: Differentially methylated region; MMN: Multiple micronutrient.

(cg03683087), *PPARGC1B* (cg08928958) and *GALNT2* (cg00589617) genes and two DMRs (consisting of multiple consecutive positions) located within miRNA 21 (*miR21*) and *SKI*. Mean methylation difference at these loci within groups with B_{12} supplementation ranged from 4.20 to 7.60% (Table 4). First, we performed technical validation of the Infinium HumanMethylation450 BeadChip Array results for four DMCpGs mentioned above. While the finding of significant differential methylation within *FTO* and *TCF7L2* loci was technically validated, no significant methylation differences were observed at the *PPARGC1B* and *GALNT2* loci (Supplementary Figure 1). Association analysis of methylation levels at CpGs in *FTO* and *TCF7L2* with SNPs in a 50 kb region on either side did not show any significant association suggesting no effect of genotype at these CpGs (Supplementary Table 16). Thus, two DMCpGs (*FTO* and *TCF7L2*; both hypomethylated), and two DMRs, *miR21* (hypermethylated) and *SKI* (hypomethylated) were investigated in the replication analysis in the Chikki Trial subjects.

In the Chikki Trial samples, the mean methylation levels increased by 1.92% ($p = 0.004$) at *FTO* (cg26580413) and by 0.58% ($p = 0.012$) at *TCF7L2* (cg03683087) after supplementation in the B_{12} group (Figure 3A & B) but no statistically significant methylation differences were noted in the MMN group ($p > 0.05$), compared with the placebo group. Further, a stratified analysis by sex revealed a median 1.5% increase in methylation at *TCF7L2* in males compared with females in the B_{12} group ($p = 0.0014$) but no significant gender-specific differences were observed in the *FTO* locus (Supplementary Figure 2). Of the 12 CpGs in the *miR21* DMR, we analyzed the region spanning CpGs 1–5 near the transcription start site and identified 2.60% lower mean methylation levels ($p = 0.004$) in the B_{12} group compared with the placebo group (Figure 3C). Similarly, the mean percentage of

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methylation of two CpG analyzed in the *SKI* DMR was 1.96% lower ($p = 0.058$) in the B_{12} group than in the placebo group (Figure 3D). Comparing the placebo and the MMN group, we found no significant methylation changes at *miR21* DMR ($p > 0.05$) but the *SKI* DMR showed a significant methylation difference of 3.31% ($p < 0.006$). Overall, we observed significant hypermethylation in the *FTO* and *TCF7L2* and hypomethylation in the *miR21* and *SKI* DMRs after B_{12} supplementation in the Chikki Trial. While the mean methylation differences were similar at these loci in Chikki trial, the directionality was not same as in the PMNS cohort except for *SKI* locus. *FTO* and *TCF7L2* are established candidate genes for obesity and T2D [25,26]. Similarly, *miR21* has a regulatory role in OCM and complex metabolic diseases [27–29] and *SKI* is known to influence diet-induced obesity, body composition and lipid metabolism [30,31]. These observations suggest that B_{12} supplementation influences methylation at the above loci associated with T2D and related intermediate traits.

The *miR21* differentially methylated region exhibits promoter activity that is suppressed by methylation of CpG1–4 region

In order to investigate the functional importance of differential methylation of CpGs in *miR21* DMR, we generated two reporter gene constructs by cloning the *miR21* promoter region (-344 bp to +590 bp; *miR21*_Pro_pGL3B) and a 246 bp region covering only the CpG1-4 in *miR21* DMR (+344 bp to +590 bp with respect to the transcription start site; *miR21*_DMR_pGL3B) into a luciferase expression plasmid and performed reporter assay after transfecting them into three different cell lines (Figure 4A). Both constructs showed significant promoter-like activity. Compared with the *miR21* promoter, the *miR21* DMR exhibited 45, 47 and 63% promoter activity in HEK293, HepG2 and MIN6 cell lines, respectively (Figure 4B–D). Hence, further experiments on the effect of methylation on promoter activity of *miR21* DMR were conducted by transfecting the methylated and mock methylated *miR21*_DMR_pGL3B construct into the HEK293 cell line. The methylated *miR21* DMR showed significantly less promoter activity than the mock methylated construct in the HEK293 cell line (25% less; $p < 0.001$) (Figure 4E). Furthermore, on electrophoretic mobility shift assay using a methylated and mock methylated DMR sequence and HEK293 nuclear extract, we observed stronger interaction with the methylated DMR, indicating that DNA methylation of *miR21*-DMR affects interactions with putative transcription factor(s) (Figure 4F). In competition assays, nonspecific probes did not affect binding to the DMR (data not shown) suggesting that the interactions between the DMR and putative transcription factor were specific and modulated by methylation.

Genes associated with Type 2 diabetes & related traits are direct targets of *miR21-3p*

We used three different miRNA target prediction databases and tools, *viz.* miRDB, miRanda and DIANA to predict target genes of *miR21*. Of the several hits, four potential targets of *miR21-3p FTO, TCF7L2,* cAMP response element binding protein (CREB) binding protein, *(CREBBP/CBP)* and Sirtuin1 (*SIRT1*) were selected for further study. The targets were selected based on prediction by more than one tool, conserved binding sequences for *miR21-3p* and biological significance, especially with relevance to T2D and related intermediate traits. Co-transfection of *FTO, TCF7L2, CREBBP* and *SIRT1* target constructs and *miR21-3p* overexpressing constructs in HepG2 cell line followed by reporter assays showed that overexpression of *miR21-3p* reduced the reporter activity of *FTO* (48%; $p < 0.0002$), *TCF7L2* (30%; $p < 0.03$), *CREBBP* (40%; $p < 0.01$) and *SIRT1* (44%; $p < 0.0006$), respectively (Figure 5A–D). To further confirm that these genes are direct targets of *miR21-3p*, we mutated the seed sequence of the target construct by site-directed mutagenesis (Figure 5E) and observed that the seed sequence mutation abolished the effect of *miR21-3p* overexpression-dependent reduction of target reporter assay (Figure 5F–I). These observations confirm that *miR21-3p* directly regulates the expression of all four genes, *TCF7L2, FTO, CREBBP* and *SIRT1*.

Discussion

The study was driven by two important considerations: vitamin B_{12} deficiency is very common in Indians [7], and it may be associated with an increased risk of diabetes and cardiovascular disease in an intergenerational manner (fetal programming) [10,11]. We investigated the molecular changes associated with B_{12} supplementation, alone and with folic acid, in adolescent subjects from a B_{12}-deficient but folate-sufficient population and made several important observations. First, B_{12} supplementation (alone and with folic acid) but not folic acid supplementation alone, led to DNA methylation changes throughout the genome. Second, supplementation influenced the methylation levels in several metabolically important genes or their regulators. An exciting finding was that one of the DMRs identified within *miR21* regulates the expression of many genes implicated in T2D such as *TCF7L2* and *FTO* which were

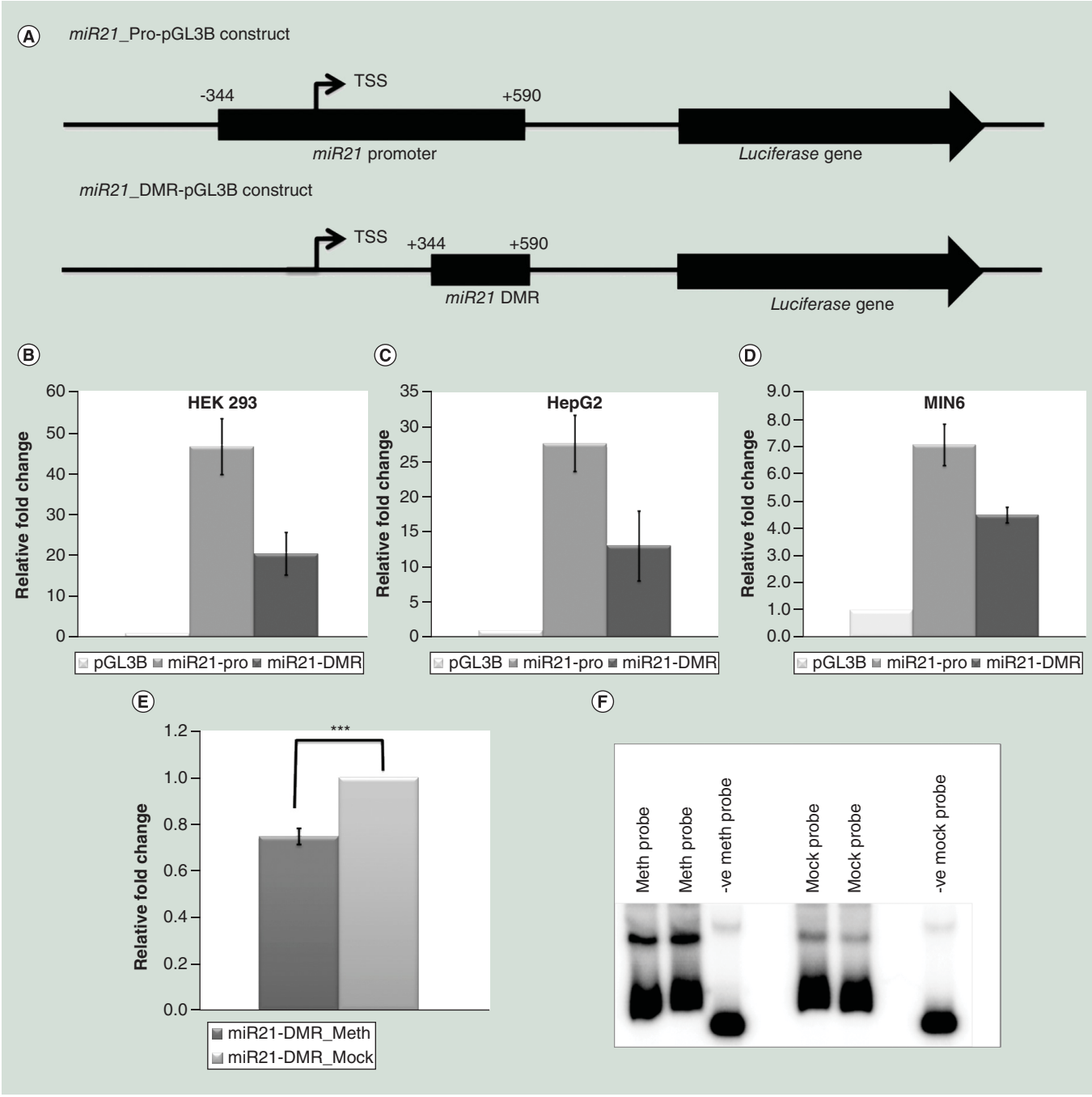

Figure 4. Functional characterization of *miR21* differentially methylated region. Schematic representation of *miR21* promoter and *miR21* DMR clones, in basic luciferase reporter vector (pGL3B) **(A)**. Relative promoter activity of *miR21*-DMR in **(B)** HEK 293, **(C)** HepG2 and **(D)** MIN6 cell lines. **(E)** Effect of methylation on promoter activity of *miR21*-DMR. **(F)** Differential interaction of putative transcription factor(s) with methylated and mock methylated *miR21*-DMR assessed by electrophoretic mobility shift assay.
***$p \leq 0.001$, all data presented as mean $\pm$ standard error of the mean.
DMR: Differentially methylated region.

hypermethylated upon B_{12} supplementation in both the PMNS and Chikki cohorts. Thus, we have identified a novel epigenetic mechanism mediated by *miR21* that may be a link between B_{12} nutrition and the associated OCM with risk of T2D and adiposity.

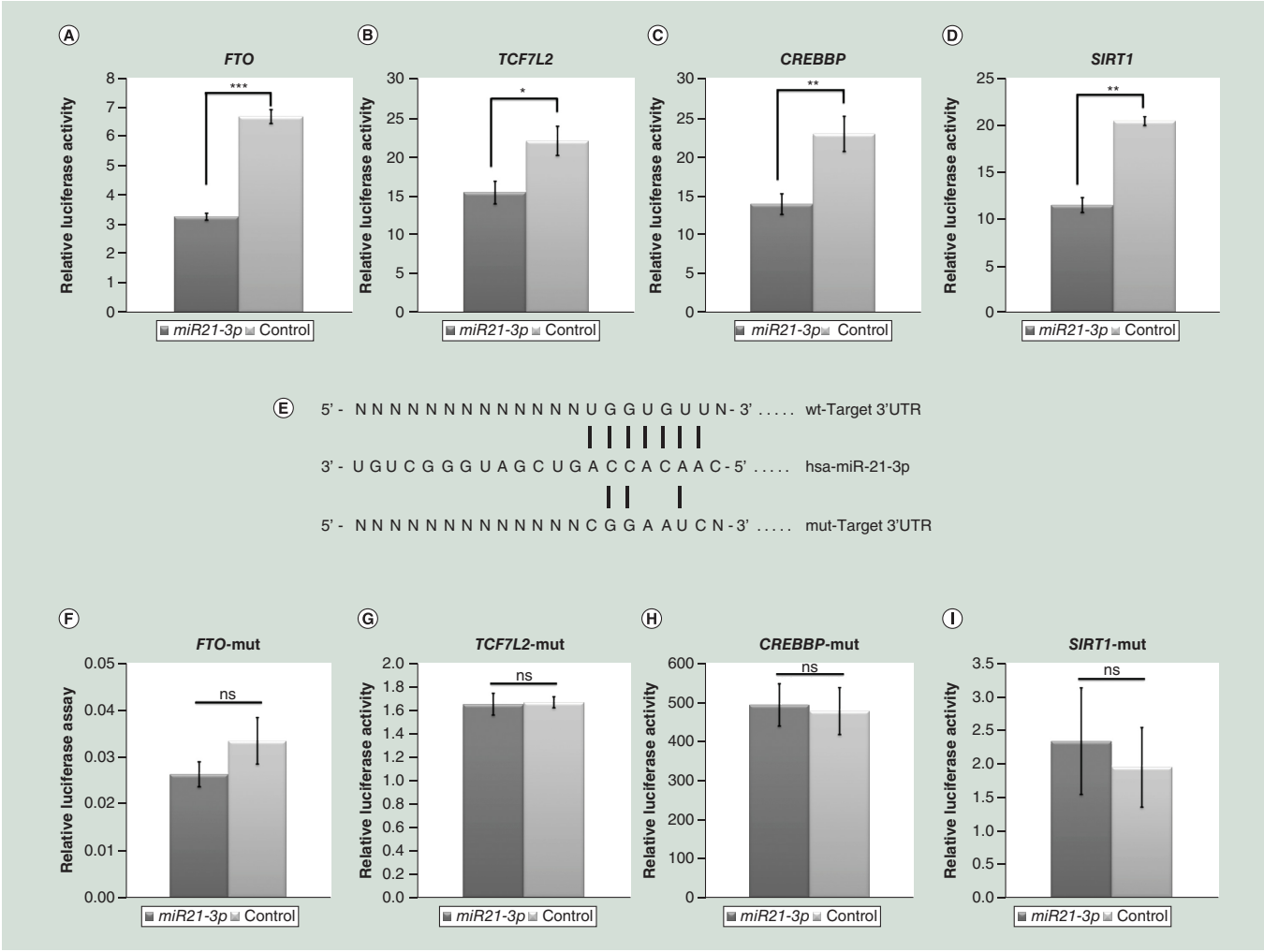

Figure 5. Validation of predicted *miR21*-3p targets by luciferase reporter assay. Overexpression of *miR21-3p* (*miR21-3p*–pmU6) significantly reduces relative expression of reporter gene containing putative binding sequence of predicted targets in comparison to control (control-pmU6) **(A)** *FTO*, **(B)** *TCF7L2*, **(C)** *CREBBP* and **(D)** *SIRT1*. **(E)** Schematic representation of putative binding site for *miR21*-3p in 3′-UTR of the target genes (top), mutation of the seed sequences of target genes (bottom). Mutation of seed sequences abolishes the effect of *miR21-3p* overexpression on **(F)** *FTO*-mut, **(G)** *TCF7L2*-mut, **(H)** *CREBBP*-mut and **(I)** *SIRT1*-mut in HepG2 cell line. mut, indicates the respective mutated constructs. All data presented as mean ± standard error of the mean.
*p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001.
ns: Nonsignificant.

Methylation changes occur with B_{12} supplementation & not with folic acid alone

Vitamins B_{12} and folic acid regulate the one-carbon metabolic pathway by acting on the same enzyme (methionine synthase). Both play a role in determining SAM and S-adenosyl homocysteine (SAH) levels, and their deficiency is known to influence the SAM/SAH ratio [32,33], which is often used as an indicator for cellular methylation potential [34–36]. Several studies have reported individual effects of folic acid and B_{12} supplementation on genome-wide DNA methylation [37,38]. Hence, it was interesting to note that B_{12} supplementation significantly influenced DNA methylation, both alone and when given with folic acid. Folic acid alone only induced changes in a small number of DMCpGs, a similar number to that observed in the placebo group suggesting that these may reflect change over time or noise. An earlier study in young Australian adults also reported that folate-sufficient and marginally B_{12}-deficient individuals did not show any significant methylation changes on supplementation with folic acid [39]. Many other recent studies have also failed to demonstrate any effect of folic acid supplementation on DNA methylation in moderately hyperhomocysteinemic subjects [40,41]. As mentioned earlier, the Indian

population has high homocysteine levels predominantly due to low B_{12} status, which may explain the lack of genome-wide significant methylation changes with only folic acid supplementation in our study.

B_{12} supplementation influences methylation of Type 2 diabetes candidate genes

The methylation changes in the B_{12} supplementation groups (B_{12} alone and B_{12} + folic acid) were widespread across the genome, including the gene body, intergenic regions, CpG islands, enhancer and promoter-associated features, which corroborates earlier observations that methylation changes are not restricted to any specific region of the genome [42]. Interestingly, the genes that showed differential DNA methylation patterns were related to glucose and lipid metabolism, as exemplified by differential methylation of *TCF7L2* and *FTO*, the strongest candidate genes for T2D and obesity, respectively and many others [25,26]. This observation is in line with earlier findings showing an association between B_{12} deficiency and lipid metabolism [43], and glucose [44] and cholesterol biosynthesis [45]. An earlier study by Dayeh *et al.* has reported differential methylation of several T2D loci including *FTO* and *TCF7L2* in pancreatic islets of diabetic and nondiabetic individuals [46]. We observed differential methylation of the same CpG site cg26982104 in *FTO*, which was reported to be differentially methylated in pancreatic islets. Similar methylation differences were also noted in our study for several T2D loci such as *ADCY5*, *PPARγ*, *IGF2BP2*, *KCNQ1,* etc. that were reported by Dayeh *et al.* Another study based on T2D candidate genes has reported increased DNA methylation at *FTO* obesity susceptibility haplotype in females susceptible to T2D [47]. Thus, these findings provide a potential link between B_{12}-mediated differential methylation of *FTO* and its association with T2D. It was interesting to note gender-specific differences in *TCF7L2* methylation, which need to be studied further in view of recent studies that have shown sex-specific differences in both DNA methylation and expression contributing to altered insulin secretion in human islets [48].

B_{12} supplementation regulates metabolically important genes by influencing methylation of *miR21*

In addition to the differential methylation of *FTO* and *TCF7L2*, this study for the first time identified hypermethylation of a genomic stretch near the promoter region of *miR21*, as a result of B_{12} supplementation both alone and with folic acid. While the role of miRNAs in cancer has been extensively studied, recent studies propose an important role of miRNAs in β-cell function, T2D and cardiovascular disorders [49]. Several studies using diet and methyl donor supplementation in animals have reported modulation of miRNA expression in liver and adipose tissue [29,50]. A recent study reported hypomethylation of the same set of CpGs (cg16936953, cg12054453, cg01409343 and cg02782634) at the *miR21* locus in Crohn's disease, and increased expression of *miR21* in the peripheral blood [51]. This is in agreement with our '*in vitro*' results, which demonstrate reduced expression of *miR21* due to hypermethylation and increased interaction between the methylated *miR21*-DMR and a putative transcription factor. Although earlier studies had suggested that DNA methylation blocked transcription factor binding, a recent study has shown that DNA methylation can promote transcription factor binding and does so for about a third of transcription factors [15]. Since *miR21* methylation suppressed promoter activity, the putative transcription factor could be a transcriptional repressor, whose identification needs further investigation. We validated four target genes of *miR21-3p, TCF7L2, FTO, CREBBP* and *SIRT1*, which have established importance in obesity, insulin function, glucose and lipid metabolism. While genetic variants in *TCF7L2* are strongly associated with T2D and it acts as the master regulator of β-cell function, insulin production, processing and secretion [52], the significance of *FTO* in appetite, dietary habits and obesity induction is well-known [53]. Similarly, the CREB binding protein regulates insulin-mediated hepatic gluconeogenesis, insulin resistance in obesity and insulin sensitivity [54–56] and *SIRT1* is known to influence glucose/ lipid metabolism, insulin secretion, signaling, inflammation and oxidative stress [57]. It is worth noting that all four target genes showed significant methylation differences upon supplementation at FDR < 0.02 in the pooled group analysis; the effect size was smaller for *CREBBP* and *SIRT1*. This suggests a novel regulatory role for *miR21* methylation in T2D pathways and associated intermediate traits like obesity, insulin resistance and secretion.

Link between B_{12}-mediated *miR21* methylation & regulation of metabolically important genes

Recent human and animal studies have reported the potential importance of maternal OCM and nutrients regulating it (B_{12}, folate, etc.) in the programming of metabolic diseases in their offspring [11,43]. Our observation of differential methylation of a set of T2D candidate genes, especially *TCF7L2* and *FTO* and *miR21* by B_{12} supplementation in a B_{12}-deficient population suggests a possible molecular mechanism for these observations. We can only speculate about the possible mechanism/pathway that could link the methylation changes observed in the

above loci. The SAM/SAH ratio determines the methylation potential of the cell, and conversion of methionine to SAM is regulated by two key enzymes, methionine adenosyltransferase 2A and 2B (MAT2A and MAT2B), which are established targets of *miR21-3p* [28]. Thus, differential methylation and expression of metabolically important genes like *TCF7L2*, *FTO, CREBPB* and *SIRT1* could occur as an effect of *miR21* on *MAT2A* and *MAT2B*, which regulate the SAM levels. This indicates that *miR21* may be the key regulator functioning in a two-pronged way, orchestrating the methylation potential of the cell as well as regulating expression of key genes of metabolic importance.

Strengths & limitations of the study

This is the first study to investigate the effects of B_{12} and folic acid supplementation on the methylome of adolescents. Our study population is multigenerationally undernourished and suffers from MMN deficiencies. The B_{12}-deficient and folate-sufficient status is similar to many other vegetarian populations, making our findings important for other similarly affected populations. However, there are a few limitations in the study. Our observations are based on methylation data on genomic DNA from peripheral blood cells, and therefore may not be directly extrapolated to other tissues. However, the commonality of many sites of differential methylation in blood cells and metabolic tissues (e.g., pancreatic islet cells) lends support to their importance. We have corrected for PCs derived from the cell composition to account for differences in methylation due to cellular heterogeneity, collinearity of blood cell counts and consequent inflation in our study. The methylation changes in our study are relatively small but like genetic studies, the contribution of individual methylation differences to complex disease phenotypes is likely to be small [58]. In view of a small discovery cohort, we used a stringent FDR cut-off $p < 0.02$ and analyzed loci which passed the criteria in the combined B_{12} group analysis (B_{12} and B_{12} + folic acid group). One of the differences in the findings in the discovery and validation studies is the contrasting directionality of the DMCpGs and DMRs. Though it is difficult to explain the mechanism, there are several studies which report contrasting methylation changes in similar but different supplementation studies both in animals and humans [59]. We speculate that these contrasting methylation changes may be due to differences in the structure, design, age, dose and duration of B_{12} and folic acid supplementation in two cohorts. We have made similar observations on methylation changes with different dose of folic acid in an independent animal study (data not shown). Since *FTO* and *TCF7L2* are strongly associated with T2D and obesity, there is a possibility that the methylation changes may be the effect of SNPs near the CpGs at the two loci. Comparison of the genotype data from a 1.2 kb flanking region around specific CpGs did not demonstrate any association with variants in this region and CpG methylation. Therefore, this study along with functional results demonstrates that methylation at these loci is sensitive to B_{12} supplementation, not likely to be chance findings and generates a hypothesis to be tested in future studies.

Conclusion

To conclude, we demonstrate that B_{12} supplementation with and without folic acid in our B_{12}-deficient and folate-sufficient population influences the methylation of key genes implicated in the risk of T2D and related phenotypes. This may be achieved through regulation of OCM *via MAT2A* and *MAT2B* by *miR21* which offers a novel epigenetic explanation for the association between OCM and risk of noncommunicable diseases. Since B_{12} deficiency is common in elderly population and other vegetarian population, therefore, our findings could have potential public health significance if confirmed in other populations and nutritional trials.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2017-0102.

Author contributions

CS Yajnik and GR Chandak conceptualized and planned the study with significant intellectual contribution from CHD Fall and KA Lillycrop. DK Yadav and S Shrestha performed all high-throughput and functional experiments and wrote the first draft of the manuscript. H Pan, JD Holbrook and S Shrestha performed the analysis of Infinium HumanMethylation450 BeadChip Array data. CV Joglekar performed the statistical analysis of phenotype data from the cohorts. All authors read and provided critical comments on the manuscript. GR Chandak is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Summary points

- Vitamins B_{12} and folate are critical determinants of one-carbon metabolism necessary for DNA methylation.
- Homocysteine, a summative marker of one-carbon metabolism, is an important risk marker for cardiovascular disease, Type 2 diabetes and metabolic syndrome.
- We have earlier demonstrated significant reduction in plasma homocysteine levels by B_{12} but not folic acid supplementation in the Pune Maternal Nutrition Study.
- In the present study, we have investigated DNA methylation changes in blood samples of children supplemented with different combinations of B_{12} and folic acid for 1 year.
- Methylation changes were observed only in the groups which received B_{12} (589 differentially methylated CpGs [DMCpGs] and 2892 regions [DMRs]) and B_{12} with folic acid (B_{12} + folic acid; 169 DMCpGs and 3241 DMRs).
- We observed significant DMCpGs in Type 2 diabetes-associated genes like *TCF7L2* and *FTO*.
- We also identified a DMR within *miR21* gene upon supplementation.
- Using *in vitro* techniques, we demonstrated that methylation of *miR21* DMR represses its expression by modulating interactions with putative transcription factors.
- At last, we demonstrated that metabolically important genes like *FTO*, *TCF7L2*, *CREBBP*/*CBP* and *SIRT1* are direct targets of *miR21-3p*.

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Availability of data & material

The summary association statistics from the genome-wide methylation data presented in this study will be made available at the institutional website (www.ccmb.res.in). The results of DMCpGs and DMRs identified in this study using Infinium HumanMethylation450 BeadChip are provided in Supplementary Tables 4–11.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study was approved by the KEM Hospital Ethics Committee and informed written consent of the parents and informed written assent of the participants has been taken (ref: KEMHRC/VSP/Dir Off/EC/065; Project No. 067).

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Maternal homocysteine in pregnancy and offspring birthweight: epidemiological associations and Mendelian randomization analysis

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Abstract

Background: Disturbed one-carbon (1-C) metabolism in the mother is associated with poor fetal growth but causality of this relationship has not been established.

Methods: We studied the association between maternal total homocysteine and offspring birthweight in the Pune Maternal Nutrition Study (PMNS, Pune, India) and Parthenon Cohort Study (Mysore, India). We tested for evidence of causality within a Mendelian randomization framework, using a methylenetetrahydrofolatereductase (*MTHFR*) gene variant rs1801133 (earlier known as 677C→T) by instrumental variable and triangulation analysis, separately and using meta-analysis.

Results: Median (IQR) homocysteine concentration and mean (SD) birthweight were 8.6 μmol/l (6.7,10.8) and 2642 g (379) in the PMNS and 6.0 μmol/l (5.1,7.1) and 2871 g (443) in the Parthenon study. Offspring birthweight was inversely related to maternal homocysteine concentration—PMNS: –22 g/SD [95% confidence interval (CI): (–50, 5), adjusted for gestational age and offspring gender]; Parthenon: –57 g (–92, –21); meta-analysis: –40 g (–62, –17)]. Maternal risk genotype at rs1801133 predicted higher homocysteine concentration [PMNS: 0.30 SD/allele (0.14, 0.46); Parthenon: 0.21 SD (0.02, 0.40); meta-analysis: 0.26 SD (0.14, 0.39)]; and lower birthweight [PMNS: –46 g (–102, 11, adjusted for gestational age, offspring gender and rs1801133 genotype); Parthenon: –78 g

(–170, 15); meta-analysis: –61 g (–111, –10)]. Instrumental variable and triangulation analysis supported a causal association between maternal homocysteine concentration and offspring birthweight.

Conclusions: Our findings suggest a causal role for maternal homocysteine (1-C metabolism) in fetal growth. Reducing maternal homocysteine concentrations may improve fetal growth.

Key words: Maternal homocysteine, offspring birthweight, causality, *MTHFR* variant, folate, vitamin B_{12}, Mendelian randomization analysis

Key Messages

- Maternal 1-C metabolism is thought to be important for fetal growth.
- Indian mothers have high homocysteine concentrations due to dietary deficiencies (vitamin B12 and protein).
- Higher maternal homocysteine concentrations predict earlier delivery and lower birthweight.
- Mendelian randomization analysis linked variant rs1801133 of the MTHFR gene with neonatal birthweight.
- This supports causality of the association between maternal 1-C metabolism and fetal growth.
- Reducing maternal homocysteine concentrations may improve fetal growth.

Introduction

Small size at birth is associated with poor perinatal outcomes and an increased risk of type 2 diabetes and cardiovascular disease (CVD) in later life.[1–3] Indian babies are among the smallest in the world and this is attributed to the small size and poor nutrition of their mothers.[4] Studies relating maternal nutrition to newborn size have mostly investigated macronutrients; recent observational and interventional studies have shown a role for maternal micronutrient nutrition.[5–6]

The Pune Maternal Nutrition Study (PMNS) investigated the effect of maternal nutrition on fetal growth and future risk of diabetes and CVD. We found that higher frequency of intake of micronutrient-rich foods and higher circulating folate and vitamin C predicted larger newborn size.[7] In a subset of PMNS participants, we found that mothers of small-for-gestational-age (SGA) babies had higher total homocysteine concentrations compared with those in appropriate-for-gestational-age pregnancies.[6] Homocysteine is a key metabolite in one-carbon (1-C) metabolism, and homocysteine levels influence several cellular processes including DNA methylation and synthesis of nucleic acids and proteins.[8] Homocysteine concentration is influenced by a number of genetic polymorphisms that affect the function of different enzymes regulating 1-C metabolism, and also by dietary intake of 'methyl donors' (folate, vitamin B_{12}, choline and betaine) and other vitamins (B_6 and B_2, co-factors for enzymes regulating the 1-C metabolism). The association of elevated maternal homocysteine with fetal growth restriction suggests a role of maternal 1-C metabolism in fetal growth and development, and a potential for improvement by dietary manipulations.[9–11] However, observational associations may be confounded by various lifestyle factors and may suffer from reverse causality. Causality is best proved by conducting randomized controlled trials which are time- and labour-intensive, expensive and sometimes unethical if they deviate from local established standards of care. Mendelian randomization is an alternative method that uses genetic variants associated with environmental and lifestyle exposures to overcome these limitations. The genotype is randomized at conception and is unaffected by lifestyle confounders, and there cannot be reverse causality. Therefore the 'instrumental component' calculated from genotype can only be causally related. Demonstration of such a relationship, therefore, improves confidence in the causality of associations.[12]

Methylenetetrahydrofolatereductase (encoded by *MTHFR*) is an important enzyme in 1-C metabolism that helps the conversion of homocysteine to methionine by providing the methyl donor tetrahydrofolate.[13] Although several homocysteine-raising variants of *MTHFR* have been identified, the rs1801133 variant (earlier known as 677C→T polymorphism) significantly reduces the enzyme efficiency (by 75%), and individuals carrying the risk genotype 'TT' have ~20% higher circulating homocysteine levels than those with the wild CC genotype.[14] This polymorphism has been used as an instrument for investigating

causality of associations of homocysteine with various cardiometabolic phenotypes,[15,16] and in clinical practice to predict high risk of adverse outcomes in pregnancy (neural tube defects and other congenital anomalies).[17]

We aimed to investigate the association between maternal homocysteine concentration and offspring birthweight in a large number of mother-offspring pairs by combining data from two Indian birth cohorts. We also used the rs1801133 variant for a Mendelian randomization analysis (instrumental variable and triangulation methods) to test the hypothesis that homocysteine concentrations in mothers have a causal role in determining fetal growth and size at birth.

Methods

Participants

The PMNS was started in 1993 in six villages near Pune to study the relationship between maternal nutrition and fetal growth.[7] Women were studied twice during pregnancy (18 6 2 and 28 6 2 weeks of gestation) for nutritional, biochemical and demographic information. Socioeconomic status (SES) was assessed using a standardized questionnaire for rural India.[18] None of the women were receiving vitamin supplements at enrolment. According to national guidelines, the women were supplemented with iron (60 mg) and folic acid (500 μg) tablets, one daily for 100 days from 18 weeks of gestation onwards.

The Parthenon Cohort Study was set up in 1997 to investigate the relationship between maternal glucose tolerance and fetal growth in the antenatal clinic at the Holdsworth Memorial Hospital (HMH), Mysore.[19] Women were studied at 30 6 2 weeks of gestation for anthropometric, biochemical and demographic information. SES was assessed using the Kuppuswamy scoring system for urban India.[20] The number of deliveries reflects only those who delivered at the HMH. Maternal supplement use was recorded when the women were recruited in mid pregnancy, but not at 30 weeks of gestation. At recruitment, 188 women (36.5%) reported taking vitamin supplements containing both vitamin B_{12} and folic acid, and 52 (10%) reported taking folic acid alone though the dosage was not recorded.

The institutional ethics committees at both centres approved the study and informed consent was obtained from all women and their families.

Data collection

In the PMNS, neonatal birthweight was measured to the nearest 50 g using a Salter spring balance (Salter Abbey, Suffolk, UK) within 72 h of birth, and in the Parthenon Cohort Study within 24 h, using a digital weighing scale (Seca, Germany) to the nearest 5 g. In the PMNS, an early morning fasting blood sample was collected at home, transported on ice to the local research centre and centrifuged, and plasma aliquots were transferred to the KEM Hospital Research Centre (KEMHRC) and stored at –80°C until further use. In the Parthenon Cohort study, fasting blood was collected in the clinic, plasma separated within 2 h and aliquots stored at –80°C.

Biochemical and genetic analyses

Red cell folate concentrations in PMNS were measured by radioimmunoassay (Becton Dickinson, Oxford, UK),[7] plasma vitamin B_{12} by microbial assay[21,22] and homocysteine by the fully automated GC-MS method.[23] For the Parthenon study, plasma folate and plasma vitamin B_{12} were measured by microbial assays[21,22,24] and homocysteine by fluorescence polarization immune assay at KEMHRC.[25] Plasma glucose and triglyceride concentrations were measured by standard enzymatic kits using auto analyzer (Abbott VP Super System, Irving, TX). The inter- and intra-assay coefficient of variation was <5% for all measurements.

Genotyping for both studies was carried out at the CSIR-Centre for Cellular and Molecular Biology, Hyderabad. Genomic DNA was isolated from blood samples using the salting-out method and DNA plated in uniform concentrations. We used a Sequenom-based MassARRAY technology to genotype the rs1801133 variant. The genotyping success rate was >95%. We confirmed the genotypes for ~10% of the samples by sequencing the purified polymerase chain reaction (PCR) products on an ABI 3730 Genetic Analyzer. Inconsistency of only 0.003% (1/384) suggested high genotyping accuracy.

Definitions

Low vitamin B_{12} refers to a plasma concentration <150 pmol/l[23] and hyperhomocysteinaemia to a concentration >10 µmol/l.[26] Low folate status was defined as an erythrocyte folate concentration <283 nmol/l and plasma folate concentration <7 nmol/l.[27] Prematurity refers to delivery at <37 weeks of gestation, low birthweight (LBW) as a birthweight <2500 g and SGA as per Oken *et al.*[28]

Statistical methods

Data are presented as mean (SD) for continuous variables, median (25th-75th centiles) for skewed variables and percentages for categorical variables. Vitamin B_{12},

homocysteine and folate concentrations had skewed distributions and were log-transformed for the analyses. They were adjusted for the gestational age at which the sample was taken. Because of differences in methods between the two cohorts, we used study-specific SD scores for folate concentrations and SES scores in the combined analysis. Higher scores refer to better SES. Associations between exposures and birthweight were analysed using multiple linear regression adjusted for gestation and gender. Effect sizes are represented as change in physiological units [95% confidence intervals (CI)] per SD change in the exposure. In the genotype homocysteine association, the effect size is represented as SD change in homocysteine per allele. This aligned the units with those used in the triangulation analysis. Genotype distribution at rs1801133 was tested for compliance with the Hardy-Weinberg equilibrium (HWE). Associations between maternal genotype and continuous variables were examined using an additive model. Since genotype frequency, homocysteine concentrations and birthweight were different between the two centres, we examined associations in each cohort separately and then combined by meta-analysis using a fixed effect inverse variance model after testing for between-study heterogeneity (I^2).

We tested the causality of the association between maternal homocysteine concentration and offspring birthweight by instrumental variable and triangulation analysis.[29] For the instrumental variable analysis, we used a two-stage least-square process. In the first stage, we calculated the SD change in homocysteine concentration per maternal rs1801133 allele in the PMNS and Parthenon cohorts. In the second stage, this instrument was used to estimate the association between maternal homocysteine and offspring birthweight. The difference between estimates from the observed and instrumental variable analyses was tested using the Durbin-Wu-Hausman test. We also performed a meta-analysis of these results.

In the triangulation method (see Supplementary data available at *IJE* online), we calculated the expected effect of maternal homocysteine on offspring birthweight by dividing the observed effect between maternal rs1801133 variant and offspring birthweight, and the observed effect between maternal rs1801133 variant and homocysteine concentration, and compared it with the observed effect.

Results

We restricted the analysis to 526 mother-baby pairs in the PMNS and 515 mother-baby pairs in the Parthenon cohort study, whose maternal homocysteine concentrations, maternal rs1801133 genotype and offspring birthweight data were available. These mothers and babies were no different from others in the original cohorts with respect to maternal age, body mass index (BMI), SES and offspring birthweight (data not shown). Mothers in the PMNS were younger and thinner than Parthenon mothers, and more likely to be vegetarian (Table 1). PMNS babies were lighter at birth than Parthenon babies, and had a higher prevalence of SGA births.

Maternal homocysteine, folate and vitamin B_{12} concentrations

Median maternal homocysteine concentration was 8.6 μmol/l in the PMNS and 6.0 μmol/l in the Parthenon cohort (32.3% and 3.7% had hyperhomocystenaemia, respectively) (Table 1). Low folate was rare in both cohorts but 70% of mothers in the PMNS and 43% in the Parthenon cohort had low vitamin B_{12} concentrations. Both vitamin B_{12} and folate concentrations were inversely related to homocysteine concentrations (PMNS: $r = -0.27$ and -0.39, respectively; Parthenon: $r = -0.24$ and $r = -0.28$, respectively; $P < 0.001$ for all). Maternal BMI was inversely associated with vitamin B_{12} concentrations in the Parthenon cohort (standardized $\beta = -0.4$, $P = 0.02$) but not in the PMNS (standardized $\beta = -0.09$, $P = 0.3$). Maternal folate concentration was directly associated with SES in the Parthenon cohort ($r = 0.3$, $P < 0.001$). Maternal B_{12} was directly associated with SES in the PMNS ($r = 0.1$, $P = 0.04$). Non-vegetarians had lower median homocysteine concentrations than vegetarians in the Parthenon cohort (5.9 vs 6.7 μmol/l, $P < 0.01$).

Maternal homocysteine, folate and vitamin B_{12} concentrations and offspring birthweight

Folate concentrations were positively associated with birthweight (PMNS: $\beta = 22.8$ g/SD, 95% CI: 4.7, 40.9, $P = 0.01$; Parthenon: $\beta = 8.2$ g/SD, 95% CI: −7, 23.8, $P = 0.3$). Vitamin B_{12} concentrations were not associated with birthweight in either cohort (PMNS: $\beta = 5.1$ g/SD, 95% CI: −14.5, 24.6, $P = 0.6$; Parthenon: $\beta = 1.6$ g/SD, 95% CI: −13.9, 17.1, $P = 0.8$). Mothers with higher plasma homocysteine concentrations delivered earlier and had lighter babies (Table 2). One SD increase in plasma homocysteine concentration predicted a 0.1-week earlier delivery ($P = 0.15$) in the PMNS, and a 0.2-week earlier delivery ($P = 0.03$) in the Parthenon cohort. The effect in both cohorts combined, using fixed effects meta-analysis, was 0.1 week/SD ($P = 0.01$), P for heterogeneity = 0.5 and $I^2 = 0\%$. After adjusting for gestational age and gender, one SD increase in plasma homocysteine concentration predicted a 22-g lower birthweight ($P = 0.1$) in the PMNS,

Table 1. Maternal and offspring characteristics in Pune Maternal Nutrition Study (PMNS) and Parthenon Study

Characteristic	PMNS	Parthenon	*P*-value[d]
Number	526	515	
Maternal			
Age (years)[a]	21.5 (3.5)	23.9 (4.1)	<0.001
Primipara[b]	160 (30.4)	256 (49.7)	<0.001
SES score[a]	27.1 (6.6)	34.0 (6.4)	NA
Vegetarian diet[b]	164 (31.6)	44 (9.3)	<0.001
Tobacco use[b]			
Smoking	0 (0)	0 (0)	NA
Chewing	68 (13.0)	0 (0)	0.01
BMI (kg/m^2)[a]	20.4 (1.9)	23.7 (3.6)	<0.001
Gestation at measurement of biomarkers (weeks)	29.4 (1.2)	29.1 (1.6)	0.51
Plasma homocysteine (μmol/l)[c]	8.6 (6.7, 10.8)	6.0 (5.1, 7.1)	<0.001
>10 μmol/l[b]	170 (32.3)	19 (3.7)	<0.001
Erythrocyte folate (nmol/l)[c]	958 (734, 1261)	–	NA
<283 nmol/l[b]	1 (0.2)	–	NA
Plasma folate (nmol/l)[c]	–	34.4 (16.8, 51.2)	NA
<7 nmol/l[b]	–	24 (4.7)	NA
Plasma vitamin B_{12} (pmol/l)[c]	122 (94, 164)	162 (123, 223)	<0.001
<150 pmol/l[b]	368 (70.4)	221 (43.0)	<0.001
Offspring			
Gender (boys)[b]	280 (53.2)	247 (48.0)	0.09
Gestation (weeks)[a]	39.0 (1.7)	39.1 (1.7)	0.93
Preterm (<37 weeks)[b]	47 (8.9)	46 (8.9)	0.99
Birthweight (g)[a]	2642 (379)	2871 (443)	<0.001
Low birthweight (<2500 g)[b]	160 (30.4)	91 (17.7)	<0.001
SGA[b]	356 (67.7)	217 (42.1)	<0.001
Maternal genotype			
rs1081133[b]			
CC	378 (71.9)	415 (80.6)	0.003
CT	132 (25.1)	92 (17.9)	
TT	16 (3.0)	8 (1.5)	
Offspring genotype			
rs1081133[b]			
CC	381 (74.9)	393 (78.9)	0.029
CT	113 (22.2)	101 (20.3)	
TT	15 (2.9)	4 (0.8)	

SES, socioeconomic status; BMI, body mass index; SGA, small-for-gestational-age.

NA refers to measurements which are not comparable; SES can not be compared as the scales of measurements vary according to rural/urban area.

Values are mean (SD)[a] or number (%)[b] or median (25th, 75th centile)[c].

[d]The *P*-value is for the difference between the PMNS and Parthenon cohorts derived using t-tests for continuous and chi square tests for categorical variables.

57 g ($P = 0.002$) in the Parthenon cohort and 40 g ($P < 0.001$) in the meta-analysis, P for heterogeneity = 0.15.

Maternal rs1801133 genotype, homocysteine concentrations and offspring birthweight

The genotype frequencies in the mothers and children did not show evidence of deviating from HWE in both the cohorts ($P > 0.05$). The maternal frequency of the risk allele ('T') at rs1801133 was 15% in the PMNS and 11% in the Parthenon cohort; it was similar in mothers and children (Table 1). The TT genotype was uncommon in both cohorts (≤3%), but was more common in the mothers and babies in the PMNS.

The maternal T allele at rs1801133 was associated with higher maternal plasma homocysteine concentrations and TT mothers had the highest plasma homocysteine concentrations (Table 3). Per allele, there was a 0.30-SD ($P < 0.001$) increase in homocysteine in the PMNS, a 0.21-SD ($P = 0.04$) in the Parthenon cohort and 0.26-SD ($P < 0.001$; P for

Table 2. Association between maternal plasma total homocysteine (tHcy) and offspring birthweight and gestational age at birth

Quintiles of tHcy (μmol/l) (median, range)	Birthweight (g) (mean, SD)	Gestation (weeks) (mean, SD)
PMNS (*N*=526)		
1. 5.44 (1.78–6.34)	2713 (348)	39.4 (1.6)
2. 7.14 (6.38–7.78)	2605 (402)	38.9 (1.7)
3. 8.59 (7.79–9.37)	2638 (392)	39.1 (1.8)
4. 10.38 (9.38–11.38)	2655 (389)	39.1 (1.6)
5. 13.33 (11.41–27.59)	2601 (357)	39.1 (1.7)
P^1	0.02	0.15
$\beta 1$ (95% CI)	–39 (–71, –7)	–0.10 (–0.25, 0.04)
P^2	0.11	0.16
$\beta 2$ (95% CI)	–22 (–50, 5)	–0.10 (–0.25, 0.04)
Parthenon (*N*=515)		
1. 4.49 (2.26–4.85)	2921 (431)	39.3 (1.6)
2. 5.31 (4.87–5.68)	2928 (365)	39.4 (1.3)
3. 6.03 (5.70–6.41)	2909 (405)	39.0 (1.6)
4. 6.82 (6.42–7.47)	2821 (445)	38.8 (1.7)
5. 8.51 (7.48–40.73)	2772 (536)	38.8 (2.2)
P^1	<0.001	0.03
$\beta 1$ (95% CI)	–74 (–112, –36)	–0.17 (–0.32, –0.02)
P^2	0.002	0.02
$\beta 2$ (95% CI)	–57 (–92, –21)	–0.17 (–0.32, –0.02)
Meta-analysis		
P^1	<0.001	0.01
$\beta 1$ (95% CI)	–56 (–81, –32)	–0.14 (–0.24, –0.03)
P for heterogeneity	0.16	0.5
I^2	48.4%	0.0%
P^2	<0.001	0.009
$\beta 2$ (95% CI)	–40 (–62, –17)	–0.14 (–0.24, –0.04)
P for heterogeneity	0.15	0.6
I^2	51.0%	0.0%

tHcy concentrations are shown as quintiles for ease of interpretation. However, in regression analysis tHcy is used as a continuous variable in SD units.

P^1: unadjusted; P^2: adjusted for gender and gestational age as appropriate.

$\beta 1$, $\beta 2$ represent corresponding effect sizes.

heterogeneity = 0.5) in the meta-analysis. The T allele was not associated with other maternal characteristics which influence birthweight (circulating folate, vitamin B_{12}, fasting glucose, triglycerides, BMI and SES) (data not shown).

The maternal T allele at rs1801133 was associated with lower offspring birthweight (Table 3). In the meta-analysis, birthweight decreased by 46 g per allele ($P = 0.051$, adjusted for gestation and gender)and by 61 g ($P = 0.019$) after further adjustment for fetal genotype. Higher maternal folate protected the fetus against the birthweight-lowering effect of the TT genotype. Thus, the mean birthweight of babies born to TT mothers who had folate concentrations in the highest tertile was 2827 g compared with 2266 g among those in the lowest tertile. ($P = 0.047$, in the combined data) (Table 4).

Instrumental variable analysis

We calculated the effect of the rs1801133 genotype on homocysteine concentration, which is the 'gene effect' and therefore the 'instrumental variable' (Table 5). We expressed this as the SD change in homocysteine concentration per allele of the rs1801133 variant. In the second step, we used this variable to predict birthweight. In Pune, birthweight decreased by 84 g ($P = 0.3$, adjusted for gestation and gender) per SD change in tHcy concentration; in the Parthenon cohort by 309 g ($P < 0.001$) and in the meta-analysis by 224 g ($P < 0.001$). The difference between Pune and Parthenon of 231 g [standard error (SE) 130 g] was not statistically significant ($P = 0.08$). These results were unaffected by additional adjustment for the fetal genotype. There was a difference between the estimates from the

Table 3. Association of maternal *MTHFR* rs1801133 genotype with homocysteine, offspring birthweight and gestational age at birth

MTHFR rs1801133 genotype	N	tHcy (μmol/l) (median, 25th, 75th centile)	Birthweight (g) (mean, SD)	Gestation (weeks) (mean, SD)
PMNS	526			
CC	378	8.4 (6.6, 10.4)	2651 (383)	39.1 (1.8)
CT	132	9.3 (7.3, 11.9)	2620 (349)	39.0 (1.6)
TT	16	9.3 (6.9, 13.8)	2615 (530)	39.8 (1.6)
	P^1	<0.001	0.41	0.77
	$\beta 1$ (95% CI)	0.30 (0.14, 0.46)	–26 (–88, 36)	0.04 (–0.23, 0.31)
	P^2		0.28	0.77
	$\beta 2$ (95% CI)		–29 (–81, 23)	0.04 (–0.23, 0.31)
	P^3		0.11	0.72
	$\beta 3$ (95% CI)		–46 (–102, 11)	–0.05 (–0.34, 0.24)
Parthenon	515			
CC	415	6.1 (5.1, 7.1)	2865 (438)	39.0 (1.8)
CT	92	5.8 (4.8, 6.9)	2911 (449)	39.5 (1.5)
TT	8	7.7 (6.5, 10.6)	2679 (610)	39.2 (1.4)
	P^1	0.04	0.56	0.02
	$\beta 1$ (95% CI)	0.21 (0.02, 0.40)	–26 (–113, 61)	0.39 (0.05, 0.72)
	P^2		0.10	0.02
	$\beta 2$ (95% CI)		–67 (–148, 13)	0.39 (0.06, 0.72)
	P^3		0.10	0.05
	$\beta 3$ (95% CI)		–78 (–170, 15)	0.40 (0.01, 0.79)
Meta-analysis				
	P^1	<0.001	0.32	0.09
	$\beta 1$ (95% CI)	0.26 (0.14, 0.39)	–26 (–78, 26)	0.18 (–0.03, 0.39)
	P-heterogeneity, I^2	0.49, 0.0%	0.99, 0.0%	0.10, 60%
	P^2		0.051	0.09
	$\beta 2$ (95% CI)		–46 (–92, 0)	0.18 (–0.03, 0.39)
	P-heterogeneity, I^2		0.40, 0.0%	0.11, 61%
	P^3		0.02	0.32
	β (95% CI)		–61 (–111, –10)	0.12 (–0.12, 0.35)
	P-heterogenity, I^2		0.44, 0.0%	0.07, 71%

tHcy concentrations are adjusted for gestation at measurement.

P^1: derived by regression using genotype as a continuous variable; P^2: additionally adjusted for gender and gestational age as appropriate; P^3: additionally adjusted for offspring *MTHFR* rs1801133 genotype.

$\beta 1$, $\beta 2$, $\beta 3$ represent corresponding effect sizes per allele change. For homocysteine this is in SD units, for others in physiological units

observed and instrumental variable analysis (test of 'endogenecity'). Thus, the instrumental variable effect was larger than the observed effect. These results suggest that the effect of homocysteine on birthweight is causal. Triangulation analysis also provided a similar conclusion (see Supplementary data, available at *IJE* online).

Discussion

In this study from two Indian birth cohorts, higher maternal homocysteine concentration predicted lower offspring birthweight. This effect was contributed both by a shorter gestation and by growth restriction. The causality of the association is supported by the instrumental variable and triangulation analyses in the Mendelian randomization framework using maternal genotype at the rs1801133 variant of *MTHFR*. This showed that the instrumental variable estimate of the maternal homocysteine effect on offspring birthweight was larger than the observed effect of 28-week homocysteine measurements. The instrumental variable is free of confounding and measurement error, is fixed at birth and therefore represents 'lifetime' exposure of the conceptus to elevated maternal homocysteine concentration from the periconceptional period to the time of birth. It also eliminates the possibility of reverse causality. Thus it suggests a true underlying causal association between the exposure (homocysteine concentration) and the outcome (birthweight). This association was independent of offspring genotype, suggesting that maternal homocysteine metabolism rather than fetal homocysteine metabolism is

responsible. The two Indian cohorts in this study are geographically separated and have demographic and nutritional differences. Moreover, the distribution of the rs1801133 genotype and homocysteine concentrations (exposure) and birthweight (outcome) were different. Such stratification might produce spurious associations. However, the meta-analysis confirms the associations observed in the individual cohorts and there was no evidence of heterogeneity in the results, supporting a true association.

There are only a few human studies which have investigated the role of maternal 1-C metabolism and related nutritional and genetic factors in fetal growth. These are predominantly in well-nourished European populations. The Hordaland study in Norway observed that high maternal plasma homocysteine concentrations (measured many years after the pregnancy) were associated with adverse pregnancy outcomes including low birthweight, and maternal rs1801133 polymorphism increased the risk of intrauterine growth restriction.[30] A study in Newcastle, UK[31] and the Generation R cohort in The Netherlands[32] both demonstrated that lower maternal folate concentrations predicted lower offspring birthweight. The variant rs1801133 of *MTHFR* by itself was unrelated to birthweight, but in combination with low folate predicted lower birthweight. In our study, women with TT genotype gave birth to lighter babies, but higher folate levels provided some protection against this effect. This suggests that both maternal nutrition and her genotype influencing 1-C metabolism have a causal influence on fetal growth and neonatal birthweight.

Mothers in our study are relatively thin, have low intakes of energy, protein and micronutrients and their babies are amongst the smallest in the world. Moreover, mothers in the Indian subcontinent have a different balance between folate and vitamin B_{12} compared with Europeans: adequate in folate due to vegetarian food and supplements but low in B_{12} due to a low intake of foods of animal origin.[7,33] Unlike in Europeans, in whom low folate is a major contributor to hyperhomocysteinaemia, vitamin B_{12} contributed much more to the risk (49%) compared with folate (8%) in our population, similar to the findings in Nepalese pregnant women.[34] Low maternal vitamin B_{12} predicted intrauterine growth restriction in a study in Bangalore, India[35] and in babies of migrant Indians in the UK,[36] whereas high maternal homocysteine and low folate predicted intrauterine growth restriction in Pakistan.[37] These studies support a role for maternal 1-C metabolism in fetal growth and suggest that disturbances in this

Table 4. Mean offspring birthweight (g) according to maternal *MTHFR* rs1801133 genotype by tertiles of folate concentrations in combined data

Maternal *MTHFR* rs1801133 genotype	Tertile of SD score for folate[a]		
	1	2	3
CC	2747	2739	2814
CT	2639	2729	2903
TT	2266	2572	2827

[a]We used SD scores of folate concentrations because of different units of measurements in the two cohorts.

Table 5. Observed and instrumental variable analysis of the maternal homocysteine offspring birthweight association

Analysis	Observed change in birthweight (g) per SD change in total homocysteine			Instrumental variable estimate of change in birthweight (g) per SD change in total homocysteine			P^1
	Effect size (g)	95% CI	*P*	Effect size (g)	95% CI	*P*	
Unadjusted							
PMNS	–39	–71, –7	0.02	–89	–295, 118	0.40	0.63
Parthenon	–74	–112, –36	<0.001	–268	–462, –75	0.007	0.045
Meta-analysis	–56	–81, –32	<0.001	–198	–338, –57	0.006	0.044
Heterogenity *P* (I^2%)	0.16 (48.4)			0.20 (33.5)			
Adjusted for gestational age and gender							
PMNS	–22	–50, 5	0.11	–84	–259, 90	0.34	0.48
Parthenon	–57	–92, –21	0.002	–309	–488, –131	0.001	0.005
Meta-analysis	–40	–62, –17	<0.001	–224	–349, –100	<0.001	0.003
Heterogenity *P* (I^2%)	0.15 (51.0)			0.078 (68.0)			
Additionally adjusted for fetal genotype							
PMNS	–27	–55, 1	0.06	–135	–324, 53	0.16	0.25
Parthenon	–58	–94, –22	0.002	–308	–490, –125	0.001	0.006
Meta-analysis	–43	–65, –20	<0.001	–248	–378, –118	<0.001	0.002
Heterogenity *P* (I^2%)	0.29 (37.9)			0.076 (39.4)			

P^1: *P*-value for the Durbin-Wu-Hausman test for the difference between the estimates from observed and instrumental variable analysis.

pathway may lead to fetal programming, so called 'nutrient-mediated teratogenesis'.[38] On this background, it is surprising that a recent study in Norwegian pregnant women failed to show an association between dietary folate intake, supplemental folic acid, maternal plasma folate, homocysteine concentrations in the second trimester and offspring birth size.[39]

The possible mechanisms of these associations include the role of 1-C (-CH_3) groups in nucleic acid synthesis and methylation of DNA which contributes to regulation of gene expression.[33] In addition, 1-C metabolism provides the essential amino acid methionine for protein synthesis. The importance of dietary methyl donors in fetal programming is well illustrated in a number of animal studies.[40,41] Hyperhomocysteinaemia could also have a direct damaging effect on endothelium by lowering nitric oxide and stimulating pro-inflammatory and oxidative stress pathways, which could impair placental perfusion and function.[42] Recently a number of studies have demonstrated an association between factors influencing fetal growth (nutrition and smoking), birth size and a number of epigenetic (DNA methylation) markers in the cord blood.[43–50]

Our study has several strengths and some limitations. One of the major strengths is the simultaneous measurement of maternal nutritional and genetic markers in relation to fetal growth. Inclusion of two Indian birth cohorts increases the generalizablity of the results within India. The populations studied have a high incidence of micronutrient deficiencies and of low birthweight, increasing the statistical power and relevance of the investigation. Both studies have a comprehensive range of measurements including many well-known confounders and effect modifiers, allowing appropriate statistical adjustments in the analysis. The similarity of the results in the two cohorts, despite many differences in the design and methods, and results of meta-analysis suggests biological consistency. Findings in the instrumental variable and triangulation analysis strengthen the causality of association between maternal homocysteine and offspring birthweight.

One of the limitations was the difference in methods for some of the measurements in the two cohorts. We adjusted for these by using cohort-specific SD scores in the analysis. Another limitation is a relatively small number of mothers with the risk genotype TT because of the low frequency of the risk allele at rs1801133 variant which, however, is consistent with other Indian studies.[51] Given the differences in risk allele frequency and other maternal and neonatal characteristics in the two cohorts, it is difficult to discount an effect of population stratification, but significant results in the meta-analysis argue against stratification. Use of a single genetic instrument, as opposed to multiple instruments that would allow checks for pleiotropy, may also constitute a limitation. However, the rs1801133 variant in *MTHFR* is a well-established instrument for homocysteine levels and has been used in many different studies for association with many diverse traits.[14,15] Potential pleotropic effects cannot be ruled out. In the presence of vertical pleiotropy or pathway-specific effects, this genetic variant will exert its effect on birthweight through a direct pathway. Whereas this is a potentially informative relationship, it is difficult to rule out alternative intermediate effects through DNA methylation and alteration in function of other genes. This could have horizontal pleiotropic effects, leading potentially to widespread biological effects and more global patterns of secondary effect.[52] We acknowledge this biological complexity, but would like to emphasize that we have taken the best measures and their instruments to make appropriate use of the data and investigate biological relationships.

Our finding of an important role of maternal 1-C metabolism in fetal growth is likely to promote interest in public health interventions to improve vitamin B_{12} and folate status in young Indian women. The role of folate in prevention of neural tube defects is well established and it is current standard practice to improve folate intake in the pre- and periconceptional period in developed countries. The role of maternal vitamin B_{12} is only just emerging; it influences fetal growth, neural tube development and neurocognitive development.[53,54] In a recent vitamin B_{12} intervention trial in India, started in the first trimester of pregnancy, there was a borderline reduction in incidence of intrauterine growth retardation.[55] An imbalance with low B_{12} and high folate in the mother predicts insulin resistance in the child, and high folate predicts higher adiposity in the child.[33] Thus, balanced B_{12} and folate nutrition in the mothers, taking care to avoid folate excess, may improve fetal growth and reduce risk of later diabetes and CVD, in line with the Developmental Origins of Health and Disease (DOHaD) theory of 'fetal programming'. Our estimates suggest that if all the mothers had circulating homocysteine concentrations in the lowest quintile (median 4.7 μmol/l) seen in our study, the incidence of low birthweight would fall from 26% to 19%. This would be a substantial public health achievement and should be tested.

Supplementary Data

Supplementary data are available at *IJE* online.

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Commentary: One-carbon metabolism has major implications for fetal growth and development beyond neural tube defects

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Small effect sizes may not be headline grabbing but can nonetheless have important public health implications. The paper by Yajnik *et al.*[1] in the current issue of *IJE* reports on a 56 -g reduction in birthweight of infants per standard deviation (SD) increase in homocysteine levels during pregnancy. However, a comparison of women in the lowest vs highest quartile for homcysteine suggests the potential for much greater effects (differences of 110–150 g). In addition, somewhat unusually, the estimates of the effect of maternal homocysteine on birthweight using a genetic proxy in the study by Yajnik and colleagues show a much stronger association (a reduction of about 250 g per SD of homocysteine) than the observational biomarker-birthweight association. This implies that the 'real' effect of one-carbon metabolism and its related nutrients (in the absence of confounding by other factors) is much greater than the 50 g reported, and is even greater that the difference in birthweight of offspring from smoking and non-smoking mothers (around 150 g to 200 g difference)[2] and suggests a key role of this pathway in fetal growth and development.

The use of Mendelian randomization to identify causal risk factors for adverse fetal outcomes

Adequate nutrition and a 'healthy lifestyle' are known to be important for fetal and maternal health during pregnancy but, due to confounding, the role played by specific nutrients is somewhat elusive despite thousands of studies on the subject. Mendelian randomization was first suggested in this journal in 2003[3] as a principle which can avoid confounding by using genetic variants as surrogates for differential exposure levels. This approach has been extended by using maternal genotypes as indicators of *in utero* environment and has previously also shown that elevated maternal glucose levels during pregnancy leads to offspring with a lower birthweight[4] and has been used as a proof of principle, to add further weight to the evidence that smoking reduces fetal birthweight.[2] In addition, maternal MTHFR C677T genotype has been found to be associated with neural tube defects (NTD) in offspring,[5] supporting the randomized controlled trial evidence that folate is a risk factor for NTDs.

The instrumental variable analysis calculates the unconfounded effect of the exposure on outcome given the effect of the genetic variant on exposure and the effect of the genetic variant on outcome. The instrumental variable analysis by Yajnik *et al.*[1] shows a larger effect than the observed homocysteine-birthweight effect, which suggests that the observed analysis is an underestimate.

Gene-environment interaction shows real importance of pathway

Although maternal MTHFR C677T genotype has been used as a surrogate for circulating homocysteine levels in the study by Yajnik *et al.*,[1] the evidence suggests that actually what may be important is the interaction between maternal genotype and circulating folate levels. The effect of being in the lowest vs the highest quartile for red cell folate concentration was a reduction in birthweight of 561 g among those with TT genotype: a finding which is in accordance with a study by Jacques *et al.*[6] who showed that the effect of genotype was far greater among those with low plasma folate.[6]

In addition, a similar gene-environment interaction was observed for birthweight in a very different population in Newcastle, UK, in 2005.[7] These findings collectively suggest a much more important role of this pathway in birthweight than first glance would indicate.

Birthweight as a surrogate outcome

Birthweight is nearly always recorded for infants, even in less developed countries, and it is a good surrogate for growth during gestation. Barker used associations between birthweight and later adult coronary heart disease to postulate the fetal origins of disease hypothesis.[8] However, it is unlikely that low birthweight causes coronary heart disease in adulthood; it is more likely that the same mechanisms which affect growth in the fetus lead to other perturbations which affect later disease risk. We know this because observations from the Dutch Hunger Winter show that women exposed to famine during mid to late gestation had babies with significantly reduced birthweights, in contrast to those exposed in the first trimester whose birthweight were unaffected.[9] Despite this, children affected by famine in the first trimester still went on to have higher rates of heart disease than the control population who were not exposed to famine.[9]

A pointer towards epigenetic programming

In addition to affecting birthweight, the MTHFR C677T genotype is thought to have wide-ranging effects: from neural tube defects and miscarriage, to vascular disease, cancer, depression and schizophrenia. How can it be the case that a common genetic variant can have such wide-ranging effects? The answer is that the one-carbon pathway is key to two vital cellular processes, DNA synthesis and repair, and DNA methylation. The MTHFR enzyme is important in regulating this pathway and directing folate metabolites between the two processes. Methylation of DNA is an important (if not the most important) mechanism controlling gene expression. Epigenetic marks such as DNA methylation which control gene expression are largely determined *in utero* and can be lifelong and far reaching. Friso *et al.*[10] assessed genomic DNA methylation levels in peripheral blood mononuclear cell DNA from 105 subjects who had the MTHFR TT genotype and 187 with the MTHFR CC genotype. They found dramatic (50%) reductions in global methylation levels in those with the TT genotype, but only among those with low folate.

Conclusion

The findings by Yajnik *et al.*[1] add to a growing body of evidence which points towards a key role of the one-carbon pathway in determining birthweight, with folate being particularly important among those with the TT genotype. Since birthweight may only be a proxy for 'fitness' at birth, it is likely that the effects of folate/homocysteine are much more far reaching. In addition, this adds more weight to the evidence that DNA methylation is the mechanism by which nutrition during pregnancy affects offspring growth and also later disease risk.

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