**Vitamin B12 Supplementation Influences Methylation of Genes Associated**

**with Type 2 Diabetes and its Intermediate Traits**

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**Abstract**

**Aim:** To investigate the effect of B12 and/or folic acid supplementation on genome-wide DNA methylation.

**Methods:** We performed InfiniumHumanMethylation450 BeadChip Assay in children supplemented with B12 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 post-supplementation in B12 [589 differentially methylated CpGs (DMCpGs) and 2892 regions (DMRs)] and B12+folic acid [169 DMCpGs and 3241 DMRs] groups. Type 2 diabetes (T2D)-associated genes *TCF7L2* and *FTO;* and a microRNA, *miR21* were further investigated in another B12-supplementation cohort. We also demonstrate that methylation influences *miR21* expression and *FTO*, *TCF7L2*, *CREBBP/CBP* and *SIRT1* are direct targets of *miR21-3p*.

**Conclusions:** B12 supplementation influences regulation of several metabolically important T2D-associated genes through methylation of *miR21*. Hence, our study provides a novel epigenetic explanation for the association between disordered one carbon metabolism and risk of adiposity, insulin resistance and diabetes and has translational potential.

**Trial registration number:** ISRCTN59289820; **Date of registration:** Applied on 04/09/2007 and assigned on 07/09/2007; **Retrospectively registered.**

**Key Words -** Vitamin B12, Folic acid, Supplementation, DNA Methylation, Molecular Mechanisms, microRNAs, Type 2 Diabetes.

**Introduction**

Vitamin B12 (B12) is an essential dietary micronutrient for human metabolism. B12 deficiency is classically described in pernicious anaemia [[1](#_ENREF_1)] and is associated with neurological damage [[2](#_ENREF_2)]. B12 deficiency also manifests as hyperhomocysteinemia, which is an important risk marker for cardiovascular disease [[3](#_ENREF_3)], obesity related complexities [[4](#_ENREF_4)], type 2 diabetes mellitus (T2D) [[5](#_ENREF_5)] and metabolic syndrome [[6](#_ENREF_6)]. Vitamin B12 and folic acid play important roles in one-carbon metabolism (OCM); B12 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.

B12 deficiency is common in Indians and is mainly attributed to vegetarian diets, but folate deficiency is relatively rare [[7](#_ENREF_7)]. We have demonstrated associations between maternal plasma B12, folate, and homocysteine concentrations and fetal growth [[8](#_ENREF_8)], and childhood neurocognitive function [[9](#_ENREF_9)], adiposity and insulin resistance [[10](#_ENREF_10), [11](#_ENREF_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](#_ENREF_8)]. Given the potential public health importance of these findings for fetal growth and programming of non-communicable disorders, we performed a pilot trial of oral B12 and folic acid supplementation in children and adults (registration number:ISRCTN59289820) which demonstrated a significant lowering of homocysteine concentrations with physiological doses of B12 supplementation but not with folic acid alone [[12](#_ENREF_12)]. In the present study, we investigated molecular changes associated with B12 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 B12 supplementation either alone or with folic acid. We further demonstrate that B12 supplementation, through methylation of a specific micro RNA, influences regulation of several T2D associated and metabolically important genes.

**Subjects and 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](#_ENREF_13)]. The study design of the B12/folic acid intervention in the extended PMNS cohort has been described earlier [[12](#_ENREF_12)]. In brief, 119 families (parents and children trios) from the extended PMNS were randomized in groups and supplemented daily for 12 months with B12 (10 µg) and/or folic acid (200 µg), to investigate their effect on plasma homocysteine levels. We randomly selected 12 children (out of total 15-17 children per group) from each of 4 supplementation groups - placebo (B0F0), folic acid (B0F200), B12 (B10F0), B12+folic acid (B10F200) and compared their methylome before and after supplementation (Figure 1). Detailed physical and biochemical measurements (B12, 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](#_ENREF_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 B12 intervention trial in school children (Chikki Trial). The 3 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), B12 with multiple micronutrients (MMN), or only B12 (B12) (Figure 1). Haemoglobin, white blood cell counts, plasma B12, folate and homocysteine concentrations were measured at baseline and post-intervention using standard methods. Out of 178 children screened, 14 were excluded because of a low B12 (≤100 pmol/L) or low haemoglobin concentration (Hb<10 g/dl). The remaining 164 children [placebo (n=55), B12 (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 Table S1.

***DNA Methylation Studies***

*DNA Methylation Profiling, Processing and Quality Control Analysis*

Genomic DNA was isolated from the blood using QIAmp DNA blood midi kit (Qiagen, Germany) and 500 ng was bisulfite converted using EZ-96 DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s instructions. The InfiniumHumanMethylation450 Beadchip Array (Illumina, San Diego, 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](#_ENREF_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](#_ENREF_15)] with noob background correction. Normalized methylation beta values were converted to M values which were used for downstream analyses. Probes on the X, Y chromosomes and cross reactive probes [[16](#_ENREF_16)] were removed from the analysis. Finally, a total of 458,057 probes data from 94 subjects were carried forward for DMCpG and DMR analysis.

*Detection of Differentially Methylation 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](#_ENREF_17)]. In view of cellular heterogeneity [[18](#_ENREF_18)] and strong collinearity in blood cell counts measured using variance inflation factor (VIF) [[19](#_ENREF_19)] principal components (PC) 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 FDR adjusted to p<0.02.

*Detection of Differentially Methylation Regions*

We used the DMRcate Bioconductor R package [[18](#_ENREF_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 modelling the Gaussian kernel smoothing using the Satterthwaite method[50](#ENREF_50) within a predefined bandwidth of λ=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 2 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 beta 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 B12 and non B12 group analysis we defined significant DMRs having BH corrected p<0.05. Finally, 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 and 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, Germany) (Table S2). 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, Germany).

***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 to the frequency of DMCpGs in all annotated probes on the Infinium HumanMethylation450 BeadChip array and on the DMCpGs obtained from the B12 and the B12+folic acid groups.

The Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, 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 microRNA target prediction tools such as miRDB [[20](#_ENREF_20)], miRanda [[21](#_ENREF_21)] and  DIANA-microT [[22](#_ENREF_22)] were used to identify the potential targets of *miR21*.

***Functional Studies***

*Generation of Constructs Related to miR21 DMR*

The *miR21* promoter (*miR21*-Pro-pGL3B, 934bp) and DMR (*miR21*-DMR-pGL3B, 256bp) constructs were generated by cloning them in pGL3 basic vector (pGL3B; Promega, 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 S-adenosyl Methionine for 48 hours at 370C. 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, USA). Methylated and mock methylated DMRs were re-ligated in pGL3B by incubating them for 16 hrs at 160C. The ligated products were gel purified and quantified before transfection in various cell lines. All primers are listed in Table S3.

*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 500bp region of 3’-UTR containing the potential seed sequences into psiCHECKTM-2 dual luciferase reporter vector (Promega, USA) downstream to hRluc gene. For over expressing *miR21-3p* (*miR21-3p*-pmU6), we adopted a method in which 21bp micro RNA sequence was synthesized in a stem loop backbone oligo of 60bp. 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](#_ENREF_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 (Transgene Biotech, China). After PCR amplification, templates were digested with Dpn1 at 370C for 45 minutes and 5 µl of Dpn1 digested amplicons were used for transformation. Sanger sequencing was used to confirm the mutations in the seed sequences (Table S3).

*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 5.0x104 cells per well in 24-well plates, 24 hours 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 PRL control vector using lipofectamine 2000 (Invitrogen). Cell lyses and dual luciferase assays were performed 24 hours after transfection using dual luciferase reporter assay kit (Promega, USA) 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, USA). In brief, an equal amount (1.5 µg) each of methylated and mock methylated *miR21* DMR were incubated with 32P ATP and 10 U polynucleotide kinase in 70 mM Tris-HCL, 10 mM MgCl2 and 5 mM dithiothreitol at room temperature for one hour. 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, USA) and 0.5 µl yeast tRNA (1 mg/ml, Invitrogen, USA) in 20 µl binding buffer (HEPES 20 mM pH 7.9, KCl 150 mM, EDTA 1 mM and Ficoll 8%) on ice for 10 minutes. Subsequently, the binding mix was loaded on 6% native polyacrylamide gel and run at 75 volts at 40C for 12 hours. After the run was over, the gel was exposed to phospho-imager screen (GE Healthcare) for 6 hours followed by scanning by Personal Molecular Imager (PMI, BioRad).

***Statistical Analysis***

Biochemical data were analyzed using SPSS software (v 17.0; SPSS, Inc., Chicago, IL, USA). Demographic and biochemical values were reported as median and interquartile range. To investigate the significance of change in the values, a non-parametric Mann-Whitney test was conducted for across group (placebo vs other groups) and a paired t-test for within group (baseline vs one 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 was 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 principal components 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](#_ENREF_24)] and compared at baseline (Pre) and after supplementation (Post) using paired student t-tests using Graphpad prism (v 6.0, GraphPad Software, Inc., La Jolla, CA 92037 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±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 Pune Maternal Nutrition Study (PMNS) (Discovery Cohort) at baseline and after intervention are presented in Table 1. This population has a low B12 but adequate folate status, and hyperhomocysteinemia is common. Supplementation for 12 months resulted in higher concentrations of B12 in the B12 (B10F0) and the B12+folic acid (B10F200) groups (by 139.5 pmol/L 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 B12+folic acid and folic acid groups (by 5.4 nmol/L and 16.4 nmol/L respectively; p<0.01 both) but decreased in the B12 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 B12 and B12+folic acid groups (by 3.1 µmol/L and 2.7 µmol/L; p<0.01 both). Placebo group (B0F0) did not show any change in plasma B12 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 B12 concentrations increased with supplementation (along with multiple micronutrients-MMN and B12 alone -B12) by 91.0 pmol/L 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 B12 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 and Their Biological Relevance**

In the PMNS cohort, we compared methylation levels at ~483,000 loci measured on the InfiniumHumanMethylation450 BeadChip Array, pre- and post-supplementation in each group and across groups. Considering the small sample size, we used a false discovery rate (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 differentially methylated CpGs (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 B12 alone and that with folic acid showed many more DMCpGs; 589 and 169 DMCpGs in the B12 and B12+folic acid groups respectively (Figure 2A-D; Table 3) (Table S4 and S5). On comparing the groups that received B12 (B12 and B12+folic acid groups) and those that did not (placebo and folic acid groups), we observed that the group which received B12 had 8609 DMCpGs while the group which did not receive B12 had only 519 significant DMCpGs at FDR<0.02 and beta difference >5% (Table 3, Tables S6 & S7). These observations clearly indicate that B12 supplementation has a larger impact than folic acid on the methylation status of various genes in this population. Finally, we detected contiguous regions of differential methylation (differentially methylated region; DMR) containing multiple CpGs using the DMRcate algorithm (minFDR<0.02 and beta difference >2%). We observed higher number of DMRs in the B12+folic acid group compared to the B12 group (3241 vs 2891 respectively; Tables S8 & S9) while placebo and folic acid groups had only 18 and 27 DMRs respectively (Table 3). Further, combining the data from both B12 supplementation groups identified more significant DMRs (B12 and B12+folic acid; n=3911) in comparison to the two groups that did not receive B12 (placebo and folic acid, n=1725) (Table 3, Tables S10 & S11).

The majority of DMCpGs in the B12 (n=432/589; 73.3%) and B12+folic acid groups (93/169; 55.03%) were hypomethylated. However, while the majority of DMRs in the B12 group (n=1745/2908; 60.0%) were hypomethylated, those in the B12+folic acid group (2331/3267; 71.35%) were mostly hypermethylated. The DMCpGs were unequally distributed with respect to the annotated genic features in both B12 and B12+folic acid groups (Table S12). The 589 DMCpG probes in the B12 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 B12+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 underrepresented in TS1500, 1st Exon, CpG island, S-shore, promoter and unclassified regulatory regions in the B12 group (p range, 0.01-9.5x10-14) and overrepresented in the enhancer and DNAse hypersensitive regions [p range, 0.05-1.7x10-15]. However, in the B12+folic acid group, DMCpGs were underrepresented in the gene body, S-shelf and S-shore (p range, 0.03-0.01) and overrepresented in TS200, non-gene and promoter associated regulatory regions (p range, 1.6x10-3-1.5x10-4) (Figure 2E-H; Table S13).

Ingenuity Pathway Analysis (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 B12 group along with other important pathways related to molecular and cellular function, physiology and development, cardio-toxicity, hepatotoxicity and nephrotoxicity (Table S14A). Similarly, pathways significantly enriched in the B12+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, cardio-toxicity, hepatotoxicity and nephrotoxicity were also enriched in the B12+folic acid group (Table S14B). This indicates that the identified DMCpGs are enriched in pathways related to regulation of development and glucose and lipid metabolism.

**Technical Validation and Investigation of Selected DMCpGs and DMRs 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 B12 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 type 2 diabetes associated gene such as transcription factor 7-like2 (*TCF7L2*), fat mass and obesity-associated(*FTO*)*,* peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*),polypeptide N-acetylgalactosaminyltransferase 2(*GALNT2*), insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2)*, potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*), etc. passed the FDR<0.02 (Table S15). Four DMCpGs, located within *FTO* (cg26580413)*, TCF7L2* (cg03683087), *PPARGC1B* (cg08928958)and *GALNT2* (cg00589617) genes and two DMRs (consisting of multiple consecutive positions) located within micro RNA 21 (*miR21*) and *SKI*. Mean methylation difference at these loci within groups with B12 supplementation ranged from 4.20% to 7.60% (Table 4). Firstly, we performed technicalvalidation 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 (Table S16). 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 B12 group (Figure 3A and 3B) but no statistically significant methylation differences were noted in the MMN group (p>0.05), compared to the placebo group. Further, a stratified analysis by sex revealed a median 1.5% increase in methylation at *TCF7L2* in males compared to females in the B12 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 (TSS) and identified 2.60% lower mean methylation levels (p=0.004) in the B12 group compared to the placebo group (Figure 3C). Similarly, the mean percentage of methylation of two CpG analyzed in the *SKI* DMR was 1.96% lower (p=0.058) in the B12 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 B12 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](#_ENREF_25), [26](#_ENREF_26)]. Similarly, *miR21* has a regulatory role in one-carbon metabolism and complex metabolic diseases [[27-29](#_ENREF_27)] and *SKI* is known to influence diet-induced obesity, body composition and lipid metabolism [[30](#_ENREF_30), [31](#_ENREF_31)]. These observations suggest that B12 supplementation influences methylation at the above loci associated with T2D and related intermediate traits.

**The *miR21* DMR 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 (-344bp to +590bp; *miR21\_*Pro\_pGL3B) and a 246 bp region covering only the CpG1-4 in *miR21* DMR (+344bp to +590bp with respect to the TSS; *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 to 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 (EMSA) 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, non-specific 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 T2D and Related Traits are Direct Targets of *miR21-3p***

We used three different micro RNA 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)* andSirtuin1 (*SIRT1*) were selected for further study. The targets were selected based on i) prediction by more than one tool, ii) conserved binding sequences for *miR21-3p* and iii) biological significance, especially with relevance to T2D and related intermediate traits. Co-transfection of *FTO, TCF7L2, CREBBP* and *SIRT1* target constructs and *miR21-3p* over expressing constructs in HepG2 cell line followed by reporter assays showed that over expression 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* over-expression-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; 1) vitamin B12 deficiency is very common in Indians [[7](#_ENREF_7)], and 2) it may be associated with an increased risk of diabetes and cardiovascular disease in an intergenerational manner (fetal programming) [[10](#_ENREF_10), [11](#_ENREF_11)]. We investigated the molecular changes associated with B12 supplementation, alone and with folic acid, in adolescent subjects from a B12 deficient but folate sufficient population and made several important observations. Firstly, B12 supplementation (alone and with folic acid) but not folic acid supplementation alone, led to DNA methylation changes throughout the genome. Secondly, supplementation influenced the methylation levels in several metabolically important genes or their regulators. An exciting finding was that one of the differentially methylated regions, identified within *miR21* regulates the expression of many genes implicated in T2D such as *TCF7L2* and *FTO* which were hypermethylated upon B12 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 B12 nutrition and the associated OCM with risk of T2D and adiposity.

**Methylation Changes Occur with B12 Supplementation and not with Folic Acid Alone**

Vitamins B12 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 SAH levels and their deficiency is known to influence the SAM/SAH ratio [[32](#_ENREF_32), [33](#_ENREF_33)], which is often used as an indicator for cellular methylation potential [[34-36](#_ENREF_34)]. Several studies have reported individual effects of folic acid and B12 supplementation on genome-wide DNA methylation [[37](#_ENREF_37), [38](#_ENREF_38)]. Hence, it was interesting to note that B12 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 B12 deficient individuals did not show any significant methylation changes on supplementation with folic acid [[39](#_ENREF_39)]. Many other recent studies have also failed to demonstrate any effect of folic acid supplementation on DNA methylation in moderately hyperhomocysteinemic subjects [[40](#_ENREF_40), [41](#_ENREF_41)]. As mentioned earlier, the Indian population has high homocysteine levels predominantly due to low B12 status, which may explain the lack of genome-wide significant methylation changes with only folic acid supplementation in our study.

**B12 Supplementation Influences Methylation of T2D Candidate Genes**

The methylation changes in the B12 supplementation groups (B12 alone and B12+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](#_ENREF_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](#_ENREF_25), [26](#_ENREF_26)]. This observation is in line with earlier findings showing an association between B12 deficiency and lipid metabolism [[43](#_ENREF_43)], and glucose [[44](#_ENREF_44)] and cholesterol biosynthesis [[45](#_ENREF_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 non-diabetic individuals [[46](#_ENREF_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*, *PPAR*, *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](#_ENREF_47)]. Thus, these findings provide a potential link between B12-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](#_ENREF_48)].

**B12 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 B12 supplementation both alone and with folic acid. While the role of microRNAs in cancer has been extensively studied, recent studies propose an important role of miRNAs in beta cell function, T2D and cardiovascular disorders [[49](#_ENREF_49)]. Several studies using diet and methyl donor supplementation in animals have reported modulation of miRNA expression in liver and adipose tissue [[29](#_ENREF_29), [50](#_ENREF_50)]. A recent study reported hypomethylation of the same set of CpGs (cg16936953, cg12054453, cg01409343, cg02782634) at the *miR21* locus in Crohn’s disease, and increased expression of *miR21* in the peripheral blood [[51](#_ENREF_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](#_ENREF_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 beta cell function, insulin production, processing and secretion [[52](#_ENREF_52)], the significance of *FTO* in appetite, dietary habits and obesity induction is well known [[53](#_ENREF_53)]. Similarly, the CREB binding protein regulates insulin mediated hepatic gluconeogenesis, insulin resistance in obesity and insulin sensitivity [[54-56](#_ENREF_54)] and *SIRT1* is known to influence glucose/ lipid metabolism, insulin secretion, signaling, inflammation and oxidative stress [[57](#_ENREF_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*. Thissuggests a novel regulatory role for *miR21* methylation in T2D pathways and associated intermediate traits like obesity, insulin resistance and secretion.

**Link Between B12-mediated *miR21* Methylation and Regulation of Metabolically Important Genes**

Recent human and animal studies have reported the potential importance of maternal OCM and nutrients regulating it (B12, folate etc) in the programming of metabolic diseases in their offspring [[11](#_ENREF_11), [43](#_ENREF_43)]. Our observation of differential methylation of a set of T2D candidate genes, especially *TCF7L2* and *FTO* and *miR21* by B12 supplementation in a B12 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](#_ENREF_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 and Limitations of the Study**

This is the first study to investigate the effects of B12 and folic acid supplementation on the methylome of adolescents. Our study population is multi-generationally undernourished and suffers from multiple micronutrient deficiencies. The B12 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 (for example pancreatic islet cells) lends support to their importance. We have corrected for principal components 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](#_ENREF_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 B12 group analysis (B12 and B12+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 reports contrasting methylation changes in similar but different supplementation studies both in animals and humans [[59](#_ENREF_59)]. We speculate that these contrasting methylation changes may be due to differences in the structure, design, age, dose and duration of B12 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 1.2kb 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 B12 supplementation, not likely to be chance findings and generates a hypothesis to be tested in future studies.

**Conclusions:** To conclude, we demonstrate that B12 supplementation with and without folic acid in our B12 deficient and folate sufficient population influences the methylation of key genes implicated in the risk of type 2 diabetes and related phenotypes. This may be achieved through regulation of one carbon metabolism *via* *MAT2A* and *MAT2B* by *miR21* which offers a novel epigenetic explanation for the association between one-carbon metabolism and risk of non-communicable diseases. Since B12 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.

**Summary Points:**

* Vitamins B12 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 B12 but not folic acid supplementation in the Pune Maternal Nutrition Study (PMNS).
* In the present study, we have investigated DNA methylation changes in blood samples of children supplemented with different combinations of B12 and folic acid for one year.
* Methylation changes were observed only in the groups which received B12 [589 differentially methylated CpGs (DMCpGs) and 2892 regions (DMRs)] and B12 with folic acid (B12+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.
* Finally, we demonstrated that metabolically important genes like *FTO*, *TCF7L2*, *CREBBP/CBP* and *SIRT1* are direct targets of *miR21-3p*.

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**Ethics approval and consent to participate**

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).

**Authors’ Contributions**

CSY and GRC conceptualized and planned the study with significant intellectual contribution from CHDF and KAL. DKY and SS performed all high throughput and functional experiments and wrote the first draft of the manuscript. HP, JDH and SS performed the analysis of Infinium HumanMethylation450 BeadChip Array data. CVJ performed the statistical analysis of phenotype data from the cohorts. All authors read and provided critical comments on the manuscript. GRC 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.

**Competing interests**

No competing interests declared.

**Availability of data and 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 S4-S11.

**Figure Legends:**

**Figure 1. Overview of the study.** PMNS, Pune Maternal Nutrition Study; PI, pre-intervention; 1YF, one year follow up. **Stage I** - B0F0/Placebo, No intervention; B0F200, No B12, folic acid 200 µg; B10F0, B12 10 µg, no folic acid; B10F200, B12 10 µg, folic acid 200 µg. **Stage II** - Control, No intervention; MMN, Multiple micronutrients (B12 1.8 µg, folic acid 300 µg); B12, B12 2 µg; DMCpG, differentially methylated CpG; DMR, differentially methylated region. #, for DMR analysis FDR≤0.02 and mean methylation difference ≥2% was used.

**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) B12 and (D) B12+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. 589 significant probes in B12 group and 169 probes in B12+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 non-genic features respectively.

**Figure 3. Investigation of selected DMCpGs and DMRs 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≤ 0.05; \*\*, P ≤ 0.01, all data presented as mean±SEM. DMCpGs, differentially methylated CpGs; DMRs, differentially methylated regions.

**Figure 4. Functional characterization of *miR21* DMR.** 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 EMSA, \*\*\*, P ≤ 0.001, all data presented as mean±SEM. DMR, differentially methylated region. EMSA, electrophoretic mobility shift assay.

**Figure 5. Validation of predicted *miR21*-3p targets by luciferase reporter assay**. Over-expression 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* over-expression on (F) *FTO*-mut, (G) *TCF7L2*-mut, (H) *CREBBP*-mut and (I) *SIRT1*-mut in HepG2 cell line. mut, indicates the respective mutated constructs; \*, *P* ≤ 0.05; \*\*, *P* ≤ 0.01, \*\*\* *P* ≤ 0.001; ns, non-significant; all data presented as mean ±SEM.

**Table 1. Clinical and biochemical characteristics of the discovery cohort (PMNS).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Groups** | **Placebo**  **(B0F0)** | **Folic acid**  **(B0F200)** | **B12**  **(B10F0)** | **B12+folic acid (B10F200)** |
| **Parameters** |  |  |  |  |
| **N (Male/Female)** | 12 (7M/5F) | 12 (6M/6F) | 12 (6M/6F) | 12 (6M/6F) |
| **Age (yrs)** |  |  |  |  |
| 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/m2)** |  |  |  |  |
| 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) |
| **B12 (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)\*\*a | 130.5 (66.0-194.0)\*a |
| **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)\*\*a | -4.5 (-7.7-0.4)\*a | 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)\*\*a | -2.7 (-4.5--1.6)\*\*a |

PMNS, Pune Maternal Nutrition Study; B0F0/Placebo, No supplementation; B0F200, No B12, folic acid 200 µg; B10F0, B12 10 µg, no folic acid; B10F200, B12 10 µg, folic acid 200 µg. N, number; All values are median and IQR (inter-quartile range); Significance of change within the group (\*, P ≤ 0.05; \*\*, P ≤ 0.01); a, significant difference when compared with the placebo group.

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

|  |  |  |  |
| --- | --- | --- | --- |
| **Groups** | **Control** | **B12** | **MMN** |
| **Parameters** |  |  |  |
| **N (Male/Female)** | 55 (30M/25F) | 54 (23M/31F) | 55 (18M/37F) |
| **Age (yrs)** | 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/m2)** |  |  |  |
| 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) |
| **B12 (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)\*\*\*\*a | 82.0 (33.0-129.0)\*\*\*\*a |
| **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)\*\*\*\*a |
| **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)\*\*\*\*a | -3.8 (-8.1--1.7)\*\*\*\*a |

Control, No supplementation; MMN, Multiple micronutrients (B12 1.8 µg, Folic acid 300 µg); B12, B12 2 µg. N, number; All values are median and IQR (inter-quartile range); Significance of change within the group (\*, P ≤ 0.05; \*\*, P ≤ 0.01; \*\*\*, P ≤ 0.001; \*\*\*\*, P ≤ 0.0001); a, significant difference when compared with the placebo group.

**Table 3. List of DMCpGs and DMRs in different supplementation and analysis**

**groups in the PMNS 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 |
| B12 (B10F0) | 589 | 2891 |
| B12+folic acid (B10F200) | 169 | 3241 |
| Without\_B12 (B0F200+B0F0) | 519 | 1725 |
| Pooled\_B12 (B10F200+B10F0) | 8609 | 3911 |

PMNS, Pune Maternal Nutrition Study; B0F0/Placebo, No supplementation; B0F200, No B12, folic acid 200 µg; B10F0, B12 10 µg, no folic acid; B10F200, B12 10 µg, folic acid 200 µg; Without\_B12 (B0F0+B0F200), Placebo and folic acid groups combined; Pooled\_B12 (B10F0+B10F200), B12 and B12+folic acid groups combined; DMCpGs, differentially methylated CpGs; DMRs, differentially methylated regions; FDR, false discovery rate; Beta\_diff, absolute difference; min FDR, minimum FDR; meanbetafc, mean beta fold change

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Groups** | **Placebo**  **(B0F0)** | | **Folic acid**  **(B0F200)** | | **B12**  **(B10F0)** | | **B12+folic acid**  **(B10F200)** | | **Without\_B12**  **(B0F0 and B0F200)** | | **Pooled\_B12**  **(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 | 1.0x10-3 | 4.30 |
| *FTO***/** cg26580413 | x | x | x | x | x | x | X | x | x | x | 1.2x10-3 | 6.20 |
| *GALNT2***/** cg00589617 | x | x | 0.0125 | 7.80 | x | x | 0.014 | 7.10 | 7.0x10-3 | 6.50 | 9.1x10-7 | 7.60 |
| *PPARGC1B***/** cg08928958 | x | x | x | x | x | x | X | x | x | x | 5.0x10-3 | 7.20 |
| *miR21\** | x | x | x | x | 1.5x10-5 | 4.30 | 3.x10-8 | 4.50 | 1.3x10-8 | 3.50 | 8.0x10-15 | 4.20 |
| *SKI\** | x | x | x | x | x | x | X | x | x | x | 7.0x10-4 | 4.70 |

**Table 4. Status of selected DMCpGs and DMRs in various supplementation and analysis groups in the PMNS cohort**

PMNS, Pune Maternal Nutrition Study; B0F0/Placebo, No supplementation; B0F200, No B12, folic acid 200 µg; B12 10 µg, folic acid 200 µg; B10F0, B12 10 µg, no folic acid; B10F200, Without\_B12 (B0F0+B0F200), Placebo+folic acid groups combined; Pooled\_B12 (B10F0+B10F200), B12 and B12+folic acid groups combined; DMCpG, differentially methylated CpG; DMR, differentially methylated region; FDR, false discovery rate; Beta\_diff, beta difference, x, locus/probe that did not pass FDR; min FDR, minimum FDR; meanbetafc, mean beta fold change; \*, represents DMRs where minFDR and meanBeta differences are mentioned. The meanbetafc ic calculated from 12 and 4 CpGs in *mir21* and *SKI* genes respectively.

**List of supplementary figures and tables**

Supplementary material includes two figures and 16 tables.

**Supplementary Information File 1 - .docx**

**Supplementary Figure 1 -** Technical validation of selected loci by pyrosequencing.

**Supplementary Figure 2 -** Gender specific methylation changes at *FTO* and *TCF7L2* loci in the Chikki Trial.

**Table S1-** Composition of the nutrient bar used in the Chikki Trial.

**Table S2-** Primer sequences for pyrosequencing.

**Table S3-** Primer sequences for *miR21* functional studies.

**Table S16-** Association analysis of SNPs with *TCF7L2* and *FTO* DMCpG in 50kb region.

**Supplementary Information File 2 .xlsx**

**Table S4 -** List of DMCpGs identified in the B12 (B10F0) group in the PMNS cohort.

**Supplementary Information File 3 .xlsx**

**Table S5 -** List of DMCpGs identified in the B12+folic acid (B10F200) group in the PMNS cohort.

**Supplementary Information File 4 .xlsx**

**Table S6 -** List of DMCpGs identified in the pooled\_B12 group (B10F0+B10F200) in the PMNS cohort

**Supplementary Information File 5 .xlsx**

**Table S7 -** List of DMCpGs identified in the without\_B12 group (B0F0+B0F200) in the PMNS cohort

**Supplementary Information File 6 .xlsx**

**Table S8 -** List of DMRs identified in the B12 (B10F0) group in the PMNS cohort.

**Supplementary Information File 7 .xlsx**

**Table S9 -** List of DMRs identified in the B12+folic acid (B10F200) group in the PMNS cohort.

**Supplementary Information File 8 .xlsx**

**Table S10 -** List of DMRs identified in the pooled\_B12 group (B10F0+B10F200) in the PMNS cohort

**Supplementary Information File 9 .xlsx**

**Table S11 -** List of DMRs identified in the without B12 group (B0F0+B0F200) in the PMNS cohort

**Supplementary Information File 10 .xlsx**

**Table S12 -** DMCpGs distribution across genomic and regulatory features.

**Supplementary Information File 11 .xlsx**

**Table S13 -** DMCpGs enrichment by genomic and regulatory features.

**Supplementary Information File 12 .xlsx**

**Table S14A and S14B -** B12 (B10F0) and B12+folic acid (B10F200) groups IPA analysis results.

**Supplementary Information File 13 .xlsx**

**Table S15 -** Type 2 diabetes and obesity associated genes showing differential methylation after supplementation in the pooled B12 group (B12 and B12+folic acid).