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Altered dietary ratio of folic acid and vitamin B_{12} during pregnancy influences the expression of imprinted H19/IGF2 locus in C57BL/6 mice

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Abstract

Maternal folic acid and vitamin B_{12} (B_{12}) status during pregnancy influence fetal growth. This study elucidated the effect of altered dietary ratio of folic acid and B_{12} on the regulation of H19/IGF2 locus in C57BL/6 mice. Female mice were fed diets with nine combinations of folic acid and B_{12} for 4 weeks. They were mated and the offspring born (F1) were continued on the same diet for 6 weeks post-weaning and were allowed to mate. The placenta and fetal (F2) tissues were collected at day 20 of gestation. H19 overexpression observed under dietary deficiency of folate combined with normal B_{12} (B_{12} normal folic acid-deficient, BNFD) was associated with an increased expression of microRNA-675 (miR-675) in maternal and fetal tissues. Insulin-like growth factor 2 (IGF2) expression was decreased under folic acid-deficient conditions combined with normal, deficient or over-supplemented state of B_{12} (BNFD, BDFD and BOFD) in fetal tissues along with B_{12} deficiency combined with normal folic acid (BDFN) in the placenta. The altered expression of imprinted genes under folic acid-deficient conditions was related to decreased serum levels of folate and body weight (F1). Hypermethylation observed at the H19 differentially methylated region (DMR) (in BNFD) might be responsible for the decreased expression of IGF2 in female fetal tissues. IGF2 DMR2 was found to be hypomethylated and associated with low serum B_{12} levels with B_{12} deficiency in fetal tissues. Results suggest that the altered dietary ratio of folic acid and B_{12} affects the *in utero* development of the fetus in association with altered epigenetic regulation of H19/IGF2 locus.

Key words: Genomic imprinting: Epigenetics: Folic acid: Vitamin B_{12}: Homocysteine: DNA methylation and chromatin immunoprecipitation

As per the Center for Disease Control and Prevention (CDC) guidelines, folic acid supplementation has been recommended daily before and during pregnancy as a preventive measure for neural tube defects[1,2]. Folic acid and vitamin B12 (B12) play an important role in one-carbon metabolism. Folate is a key methyl carrier, whereas vitamin B12 acts as a co-factor for enzyme methionine synthase involved in the conversion of homocysteine (Hcy) to methionine. Vitamin B12 is required for human metabolism as it is an essential dietary micronutrient. B12 deficiency is associated with pernicious anaemia[3] which can further lead to neurological damage[4].

Folic acid fortification policies implemented in different countries have indicated beneficial effects in terms of reduction in plasma Hcy[5–7]. Also, the incidence of deficiency of folic acid is less in the Indian subcontinent; however, the incidence of B_{12} deficiency is very high which is due to a preference towards vegetarian diets[8]. In a developing country like India, guidelines have been formulated to supplement folic acid during pregnancy; however, very little attention has been given to check the B_{12} levels in pregnant women. It is well known that high folate levels mask the deficiency of B_{12} and worsens the condition of anaemia associated with deficiency[9,10]. In recent years, it has been observed that maternal high folate levels with low B_{12} were found to be associated with small-for-gestational age infants and increased insulin resistance in children[11–13]. The same scenario has been seen upon evaluation in rodents[14].

Such associations between maternal folic acid and B_{12} levels and their role in *in utero* development may be mediated by epigenetic marks as both these micronutrients play a role in one-carbon metabolism[15]. Earlier studies, in a yellow agouti mouse model, have shown that methyl donor nutrition during development has a long-lasting effect on epigenetic regulation of genes[16]. Therefore, nutritional deficiencies during *in utero* conditions can influence the epigenetic mechanisms which in turn can affect the state of genomic imprinting. H19/IGF2 is

Abbreviations: BDFD, B_{12}-deficient folic acid-deficient; BDFN, B_{12}-deficient folic acid normal; BDFO, B_{12}-deficient folic acid over-supplemented; BNFD, B_{12} normal folic acid-deficient; BNFN, B_{12} normal folic acid normal; BNFO, B_{12} normal folic acid over-supplemented; BOFN, B_{12} over-supplemented folic acid normal; DMR, differentially methylated region; Hcy, Homocysteine; ICR, imprinting control region; IGF2, insulin-like growth factor 2; miR-675, microRNA-675.

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the most studied imprinted locus due to its role in various cellular processes and involvement in early postnatal growth and development[17].

Expression of H19/IGF2 imprinted locus involves various epigenetic mechanisms, that is, DNA methylation, histone tail modifications, expression of CCCTC-binding factor (CTCF) along with expression of a microRNA-675 (miR-675)[18,19]. DNA methylation at the imprinting control region (ICR) of this locus is required for the establishment of imprinting, which in turn leads to proper transcriptional regulation and parent-of-origin monoallelic expression[20]. Insulin-like growth factor 2 (IGF2) is a paternally expressed gene that regulates embryonic development[21] and promotes fetal and placental growth. H19, a long non-coding RNA, is known to be expressed during embryogenesis[22]. miR-675 lies within the first exon of H19 and is known to regulate placental growth[23]. IGF2 and H19 are closely linked and reciprocally imprinted genes located on the distal end of mouse chromosome 7[24].

Disruption of genomic imprinting leads to reactivation of silent allele, resulting in biallelic expression. Loss of imprinting at H19/IGF2 locus has been associated with several childhood disorders such as Prader–Willi and Angelman syndromes, Silver–Russell syndrome, and Beckwith–Weidemann syndrome[25–27].

Very few studies have been carried out to evaluate the effect of dietary methyl donors on epigenetic regulation of imprinted locus H19/IGF2[28,29]. A study carried out to determine the role of folic acid supplementation during pregnancy has reported loss of imprinting of H19 in the placenta which was not associated with CpG methylation at the H19-ICR. IGF2, however, showed monoallelic expression in the placenta, while a significant loss of imprinting was observed in the cord blood[28]. Another study reported a significant loss of imprinting for IGF2 in the offspring of mothers fed a methyl-donor-deficient diet for 60 d, an effect that persisted for a further 100 d even when offspring were recuperated onto a natural ingredient diet[29]. This study also reported that an overall decrease in IGF2 expression was caused by down-regulation of the paternal allele and suggested that a post-weaning diet can permanently affect the expression of IGF2. A review published recently by Crott[30] suggested the importance of parental folic acid and one-carbon nutrient status in regulating offspring growth and recommended studying the role of imprinted genes therein. However, the literature is silent about the effect of parental dietary manipulations of folic acid and B_{12} on the expression and epigenetics of H19 and IGF2 in maternal and fetal tissues. Therefore, we carried out this study in the C57BL/6 mice model (which is known for its genome similarity to that of humans) to investigate the effect of dietary manipulations of folic acid and B_{12} in maternal/parental diet on the epigenetic regulation of imprinted H19/IGF2 locus in maternal and fetal tissues.

Based on the role of folate and B_{12} in one-carbon metabolism and the previous literature showing deleterious effects of dietary deficiency of maternal folate and B_{12} in the offspring, we expect that dietary imbalance of methyl donors (deficiency of folate/B_{12} combined with either state of another vitamin) *in utero* may influence the methylation patterns of imprinting genes in the offspring in a sex-specific manner. This could disturb the pattern of genomic imprinting such that maternally expressed gene (H19) tends to restrict nutrition to the developing fetus can result in biallelic expression whereas IGF2, a paternally expressed gene associated with fetal development, can be inhibited by high H19 expression by epigenetic regulation which in turn may lead to growth restriction.

Materials and methods

Mice and diets: C57BL/6 male and female mice of about 6 weeks with an average weight of 20 g were obtained from the animal house facility of PGIMER, Chandigarh, India. The animals were acclimatised to normal conditions for 1 week and were housed in a temperature-controlled room with a 12-h light–dark cycle and allowed free access to drinking water and diet according to the groups. This study was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All animal procedures in this study were conducted according to ARRIVE Guidelines for Reporting Animal Research[31]. The protocol was approved by the Institutional Animal Ethics Committee (IAEC Ref No IAEC/465), PGIMER, Chandigarh. Female mice were divided into nine groups (two per cage) based on the different dietary combinations of folic acid and B_{12} and were fed *ad libitum*. The groups included in study were (a) B_{12} normal folic acid normal (BNFN), (b) B_{12} normal folic acid over-supplemented (BNFO), (c) B_{12} normal folic acid-deficient (BNFD), (d) B_{12}-deficient folic acid normal (BDFN), (e) B_{12}-deficient folic acid over-supplemented (BDFO), (f) B_{12}-deficient folic acid-deficient (BDFD), (g) B_{12} over-supplemented folic acid normal (BOFN), (h) B_{12} over-supplemented folic acid over-supplemented (BOFO) and (i) B_{12} over-supplemented folic acid-deficient (BOFD). Each group comprised of twelve female mice and four male mice and diets were fed accordingly for 4 weeks. A total of 36 males and 108 females were included in the study. Male mice were fed a normal diet. Online Supplementary Fig. 1 depicts the workflow of the study.

All diets were prepared according to AIN-93G guidelines[32] and are the same as used in earlier studies[14,33–35] (Table 1).

For normal B_{12} control AIN-93G diet, dietary fibre in form of cellulose was used along with 0·025 mg/kg of added vitamin B_{12}; for B_{12}-deficient AIN-93G diet dietary fibre in form of pectin (citrus peel pectin) with no added vitamin B_{12} and for B_{12} over-supplementation AIN-93G diet, cellulose and 0·1 mg/kg of vitamin B_{12} were used. Mice were given pectin in B_{12}-deficient groups as it leads to lesser availability of B_{12} by binding to intrinsic factor in the intestine[34].

The content of folic acid was 0, 2 and 8 mg/kg diet, in folic acid-deficient, normal and over-supplemented groups, respectively. Succinylsulfathiazole (10 g/kg) was also used in the folic acid-deficient group which inhibits bacterial synthesis of folic acid present in the gut.

Male and female mice were allowed to mate, and the state of pregnancy was confirmed by detecting a vaginal plug in females (A total of six pregnant females per group were used.). Males, as well as remaining females, were re-habilitated. This was denoted with day 0 and the mothers were referred to as F0 generation and the neonates of F0 mothers were denoted as F1 generation fetus.

Table 1. Composition of the diets

	Diets	BNFN (control)	BNFO	BNFD	BDFN	BDFD	BDFO	BOFN	BOFO	BOFD
1.	Corn starch (g/kg) (Sisco Research Laboratories)	398	398	398	398	398	398	398	398	398
2.	Casein (g/kg) (Himedia Labs)	200	200	200	200	200	200	200	200	200
3.	Dextrinised starch (g/kg) (Sisco Research Laboratories)	132	132	132	132	132	132	132	132	132
4.	Sucrose (g/kg) (Himedia Labs,)	100	100	100	100	100	100	100	100	100
5.	Soya bean oil (g/kg) (Millipore Sigma)	70	70	70	70	70	70	70	70	70
6.	Fibre (g/kg)*	50	50	50	50	50	50	50	50	50
7.	Mineral mixture† (g/kg)	35	35	35	35	35	35	35	35	35
8.	Vitamin mixture‡ (g/kg)	10	10	10	10	10	10	10	10	10
9.	Folic acid (mg/kg) (Sisco Research Laboratories)	2	8	0	2	0	8	2	8	0
10.	Vitamin B_{12} (mg/kg) (Sisco Research Laboratories)	0·025	0·025	0·025	0	0	0	0·1	0·1	0·1
11.	Cystine (g/kg) (Himedia Labs)	3	3	3	3	3	3	3	3	3
12.	Choline bitartarate (g/kg) (Himedia Labs)	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5
13.	Tertiary butyl hydroquinone (g/kg) (Himedia Labs)	0·014	0·014	0·014	0·014	0·014	0·014	0·014	0·014	0·014
14.	Total energy (kJ)	1·57	1·57	1·57	1·57	1·57	1·57	1·57	1·57	1·57

BNFN, B_{12} normal folic acid normal; BNFO, B_{12} normal folic acid over-supplemented; BNFD, B_{12} normal folic acid-deficient; BDFN, B_{12}-deficient folic acid normal; BDFD, B_{12}-deficient folic acid-deficient; BDFO, B_{12}-deficient folic acid over-supplemented; BOFN, B_{12} over-supplemented folic acid normal; BOFO, B_{12} over-supplemented folic acid over-supplemented; BOFD, B_{12} over-supplemented folic acid-deficient.

* For normal diets, cellulose was used as dietary fibre whereas for B_{12} deficiency pectin (Sisco Research Laboratories) was used as dietary fibre. and also to induce folate deficiency in folate-deficient groups (succinylsulfathiazole (Millipore Sigma) was given 10 g/kg).

† Mineral mixture (g/kg mixture) (Himedia Labs): calcium carbonate, 357; potassium phosphate, 196; potassium citrate, 70·78; sodium chloride, 78; potassium sulphate, 46·6; magnesium oxide, 24; ferric citrate, 6·06; zinc carbonate, 1·65; manganous carbonate, 0·63; cupric carbonate, 0·3; potassium iodate, 0·01; sodium selenate, 0·01; ammonium paramolybdate, 0·007; sodium metasilicate, 1·45; chromium potassium sulphate, 0·275; lithium chloride, 0·01; boric acid, 0·08; sodium fluoride, 0·06; nickel carbonate, 0·03; ammonium vanadate, 0·006; and sucrose, 221·02.

‡ Vitamin mixture (g/kg mixture) (Himedia Labs): nicotinic acid, 3; calcium pantothenate, 1·6; pyridoxine-HCl, 0·7; thiamin-HCl, 0·6; riboflavin, 0·6; D-biotin, 0·02; vitamin B_{12} (in 0·1 % mannitol), 2·5; vitamin E, 15; vitamin A, 0·8; vitamin D3, 0·25; vitamin K, 0·075; folic acid, 0·2 (control); and sucrose, 974·655 were used to make total weight of the vitamin mixture to 1 kg.

The neonates were weaned until 3–4 weeks of age and then continued for 6 weeks on the same maternal dietary combination of folic acid and B_{12}. Mating was performed among the same dietary group (four males and twelve females in each group), and pregnant females (6 number) were individually housed and sacrificed on day 20 of gestation. The neonates were denoted as F2 generation fetuses. Blood from mothers was collected, serum isolated and stored at -80^0C for biochemical parameters. Placenta (F1) and fetal tissues (F2) were isolated and kept at -80^0C for further use.

Fetal sex determination

DNA was isolated from the tails of fetuses using HiPura Mammalian Genomic DNA purification Kit (Hi-media, cat no. MB506–250PR) as per the manufacturer's instruction for fetal sex determination. Absorbance (A260/280) of 1·8 was taken as an acceptable value. Conventional PCR was carried out for the detection of the SRY gene whose expression is male-specific along with positive control MYOGENIN which is an autosomal gene (online Supplementary Fig. 7). The primers used are shown in Supplementary Table 1. The fetal tissues (F2) were pooled according to sex after sex determination from each female of the group.

Biochemical Assays

Folate levels in the serum were measured by electrochemiluminescence method on Cobas ECLIA e 411 (Roche Diagnostics GmbH, Mannheim, cat no. 07559992190 FOLATE III). Vitamin B_{12} levels in the serum were measured using an ELISA-based kit Mouse vitamin B_{12} kit according to the manufacturer's protocol (CUSABIO, cat no. CSB-E07905m). Hcy levels in the serum were measured on ADVIA Centaur Hcy assay (ADVIA Centaur, Bayer)

mRNA expression studies

RNA isolation from mouse tissues was carried out by using TRIzol reagent (Ambion, Life Technologies Corporation, cat no.15596018). Quantification of RNA was performed spectrophotometrically, and reverse transcription of 1 µg of RNA was carried out using cDNA kit (MBI Fermentas, Life Sciences, cat no. K1621). Endpoint qRT-PCR (quantitative) was carried in Applied Biosystems Real-time PCR (Life Technologies Corporation) on reaction mixture prepared for each sample by mixing SYBR Green master mix (5 µl) with cDNA template (1 µl), 500 nM of primers and 1·5 mM of MgCl2 in a reaction volume of 10 µl. PCR products were amplified using gene-specific primers listed in Supplementary Table 2. mRNA expression was normalised to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase), for which the Ct values were observed to be constant. Relative fold changes in gene expression between different groups were calculated using the comparative threshold cycle or Ct method (ΔΔ CT)[36].

miRNA related to imprinted genes: TRIzol (Ambion, Life Technologies Corporation, cat no.15596018) was used to extract total RNA. cDNA conversion was followed by using mi script II RT kit (Qiagen, cat no. 218161) according to manufactures guidelines. Quantification of miRNA was done by using miRNA-specific primers (online Supplementary Table 3) on a real-time PCR system. The relative expression of each miRNA was normalised against SNORD 70.

Table 2. Body weights of mice in F0 generation (Mean values and standard deviations)

Groups	No. of animals	Mean body weight in grams (initial) Mean	SD	Mean body weight in grams (at mating) Mean	SD
BNFN	Male: 4	20	1·4	36	2·0
	Female: 12	19	1·1	37	1·6
BNFO	Male: 4	19	1·6	35	2·4
	Female: 12	18	1·0	36	2·1
BNFD	Male: 4	20	2·1	33	2·7
	Female: 12	20	1·2	34	1·4
BDFN	Male: 4	21	2·1	33	1·3
	Female: 12	20	1·8	32	1·5*
BDFD	Male: 4	19	1·5	33	1·2
	Female: 12	20	1·3	31	1·4**
BDFO	Male: 4	21	1·5	30	2·1**
	Female: 12	22	1·7	29	1·8***
BOFN	Male: 4	21	2·1	38	1·2
	Female: 12	20	1·8	39	2·1
BOFO	Male: 4	22	1·6	40	1·4
	Female: 12	23	2·1	38	1·9
BOFD	Male: 4	23	1·9	37	2·7
	Female: 12	23	1·7	36	2·5

BNFN, B_{12} normal folic acid normal; BNFO, B_{12} normal folic acid over-supplemented; BNFD, B_{12} normal folic acid-deficient; BDFN, B_{12}-deficient folic acid normal; BDFD, B_{12}-deficient folic acid-deficient; BDFO, B_{12}-deficient folic acid over-supplemented; BOFN, B_{12} over-supplemented folic acid normal; BOFO, B_{12} over-supplemented folic acid over-supplemented; BOFD, B_{12} over-supplemented folic acid-deficient.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ *v.* BNFN.

Table 3. Body weights of mice in F1 generation (Mean values and standard deviations)

Groups	No. of animals	Mean body weight in grams (initial) Mean	SD	Mean body weight in grams (at mating) Mean	SD
BNFN	Male: 4	22	1·6	39·4	1·11
	Female: 12	21	1·4	34·9	0·68
BNFO	Male: 4	23	1·9	37·1	0·87
	Female: 12	24	1·0	35·9	0·85
BNFD	Male: 4	23	2·7	32	0·711**
	Female: 12	21	1·2	31·4	0·91**
BDFN	Male: 4	22	1·1	32·4	0·90**
	Female: 12	20	0·8	29·9	0·66***
BDFD	Male: 4	21	1·7	32·5	1·27**
	Female: 12	19	1·2	29·3	0·92***
BDFO†	Male: 4	20	1·4	26·8	2·17***
	Female: 12	19	1·3	21·8	1·39***
BOFN	Male: 4	23	2·1	38	3·02
	Female: 12	22	1·8	33·6	1·52
BOFO	Male: 4	24	1·6	38·2	2·76
	Female: 12	23	2·1	33·8	1·42
BOFD	Male: 4	22	1·9	39·0	2·08
	Female: 12	21	1·7	34·2	1·37

BNFN, B_{12} normal folic acid normal; BNFO, B_{12} normal folic acid over-supplemented; BNFD, B_{12} normal folic acid-deficient; BDFN, B_{12}-deficient folic acid normal; BDFD, B_{12}-deficient folic acid-deficient; BDFO, B_{12}-deficient folic acid over-supplemented; BOFN, B_{12} over-supplemented folic acid normal; BOFO, B_{12} over-supplemented folic acid over-supplemented; BOFD, B_{12} over-supplemented folic acid-deficient.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ *v.* BNFN,. The data are presented as mean ± SD.
† In BDFO group, most of the animals were died (male as well as female).

Gene-specific DNA methylation by high-resolution melting analysis

Genomic DNA isolation was carried out by using DNeasy blood and tissue kit (Qiagen, cat no.69506). Bisulfite treatment using the EpiTect Bisulfite kit (Qiagen, cat no.59104) was given to the isolated DNA. Thereafter, methylation-specific high-resolution melting (MS-HRM) was performed to analyse the methylation at H19[37] and IGF2 DMR2[29,38] by the method as standardised in our lab by Rahat *et al.*[39]. Methylation standards were used in the range from 0 to 100 % for standard curve generation to determine CpG island's percentage methylation in the promoter region. For the 0 % methylation standard, commercially available control DNA from Qiagen (EpiTect® Control DNA) was used, while the enzymatic treatment of genomic DNA was used to synthesise fully methylated DNA (100 % methylation standard) using M.SssI enzyme (CpG Methyltransferase, New England Biolabs). These methylation standards were also subjected to bisulfite conversion. DNA standards of 0 and 100 % methylation were mixed in proper proportion to generate DNA standards of 0, 20, 40, 60, 80 and 100 % methylation standards. High-resolution melting analysis was performed on Applied Biosystems® StepOnePlus™ Real-Time PCR using gene-specific primers (online Supplementary Table 4). The parameters for designing high-resolution melting primers were as described earlier[40,41]. This was followed by the analysis of raw melt curves in MS-HRM software version 3.0.1. from Applied Biosystems, and then finally the exact percentage of methylation of unknown samples was estimated in reference to the methylation standards by polyfit interpolating function within program MatLab (The MathWorks, Inc.).

Histone modification analysis by chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on the placenta and fetal tissues to estimate suppressive (H3K9me3 and H3K27me3) and with active (H3K4me2) histone marks at ICR/promoter region of H19-ICR and IGF2 as previously described[39,42]. Twenty-five mg of tissue was fixed in 1·5 % formaldehyde, followed by its disaggregation to a single cell suspension, which was subjected to sonication for shearing of chromatin. The sheared chromatin lysate was then divided into aliquots to be used for input DNA, immunoprecipitation with anti-trimethyl H3-K9/K27, anti-dimethyl H3K4 and normal rabbit IgG antibodies (Abcam, Cambridge, cat no. ab8898, ab6002, ab7766, respectively). The phenol–chloroform method was used for DNA isolation from the input and immunoprecipitated complexes and then quantified by real-time PCR using gene-specific primers (online Supplementary Table 5). Input DNA was used for normalisation and the fold enrichment method was employed to calculate results.

Statistical analysis

Since the research question is related to the potential effects of dietary combinations of folic acid and B_{12}, it is imperative to check the independent as well as interactive effects of both. Therefore, a general linear model was used with folic acid and B_{12} as well as their two-way interaction as independent variables to discern whether folic acid or B_{12} exert independent effects on the studied parameters regardless of the other nutrient, or they interact with each other to influence the studied

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parameters[43,44]. To study this, statistical analysis was performed by using two-way ANOVA and pairwise comparisons using one-way ANOVA ($\alpha = 0{\cdot}05$) along with Tukey's *post hoc* test was carried out only after confirming the significant interaction of folic acid and B_{12} with the parameters studied. Statistical software used was GraphPad Prism (v.6.0.1) and IBM SPSS program (v.23). For pairwise comparisons, we first stated the effect of B_{12} deficiency with the normal state of folic acid (BDFN *v.* BNFN), folic acid deficiency with either normal or over-supplemented state of B_{12} (BNFD, BOFD *v.* BNFN) and then the effect of combined deficiency of both vitamins (BDFD *v.* BNFN). Thereafter, the comparison of B_{12} over-supplementation with the normal state of folic acid (BOFN *v.* BNFN) and that of folic acid over-supplementation with the normal state of B_{12} (BNFO *v.* BNFN) followed by the combined state of over-supplementation of both vitamins (BOFO *v.* BNFN) were listed. This pattern was followed for all the genes studied. For finding correlation in different parameters, Pearson's correlation analysis was used. Multiple regression analysis was used for studying the effect of epigenetic regulatory mechanisms on mRNA expression. *P*-value < 0·05 was considered as statistically significant, and data were expressed as mean and standard deviation.

Results

Effect of diet on parental body weight

A significant interaction of folic acid and B_{12} was evident in the body weights of mice as analysed by two-way ANOVA ($P < 0{\cdot}001$). The pairwise comparison revealed that body weights of male, as well as female mice (F0 and F1) at the time of mating, were found to be decreased in folic acid and vitamin B_{12}-deficient conditions, that is, BDFN (female 32 ± 1·5), BDFD (female 31 ± 1·4), BDFO (male 30 ± 2·1, female 29 ± 1·8) as compared with controls (male 36 ± 2·0, female 37 ± 1·6) in F0 generation; however, for F1 generation, the decrease in body weight was observed under BNFD (male 32 ± 0·711, female 31·4 ± 0·91), BDFN (male 32·4 ± 0·90, female 29·9 ± 0·66), BDFD (male 32·5 ± 1·27, female 29·3 ± 0·92) and BDFO (male 26·8 ± 2·17, female 21·8 ± 1·39) groups as compared with controls (male 39·4 ± 1·11, female 34·9 ± 0·68) (Tables 2 and 3). A high rate of mortality was observed in the BDFO group in the F1 generation. None of the females in this group was found to be pregnant, so no data related to BDFO are present in the manuscript for the placenta in the F1 and the fetal tissues in the F2 generation. The possible cause of infertility can be due to impaired ovarian function, lower oocyte count or a decrease in follicular number in ovaries. Dietary folate deficiency impairs the folate-metabolising pathway, leading to altered DNA methylation and disrupted DNA integrity, and increased blood Hcy levels. Elevated follicular fluid Hcy levels have been found to be correlated with poor oocyte maturity, reduced fertilisation rates and poor *in vitro* embryo quality[45]. Previous studies have reported that maternal high folate and low B_{12} lead to insulin resistance and are associated with infants born as small-for-gestational age. The condition is also associated with high Hcy levels, high methylmalonic acid concentrations, as well as the elevated prevalence of anaemia and accelerated cognitive decline[46]. Taken together, evidence to date suggests that high folic acid intake exacerbates the detrimental effects of B_{12} insufficiency and if not treated for a long time can lead to significant adverse growth and metabolic events.

Effect of diet on serum folate, vitamin B_{12} and homocysteine levels

As reported in our previously published manuscript[43], pairwise comparison revealed that a significant decrease in serum folate levels was observed in all groups of folic acid deficiency viz. BNFD (6·0 ng/ml), BDFD (5·7 ng/ml) and BOFD (7·7 ng/ml) ($P < 0{\cdot}001$), whereas over-supplementation of folic acid (BOFO (73·4 ng/ml), BDFO (75·9 ng/ml) and BNFO (57·7 ng/ml)) led to an increase in serum folate levels (Fig. 1(a)).

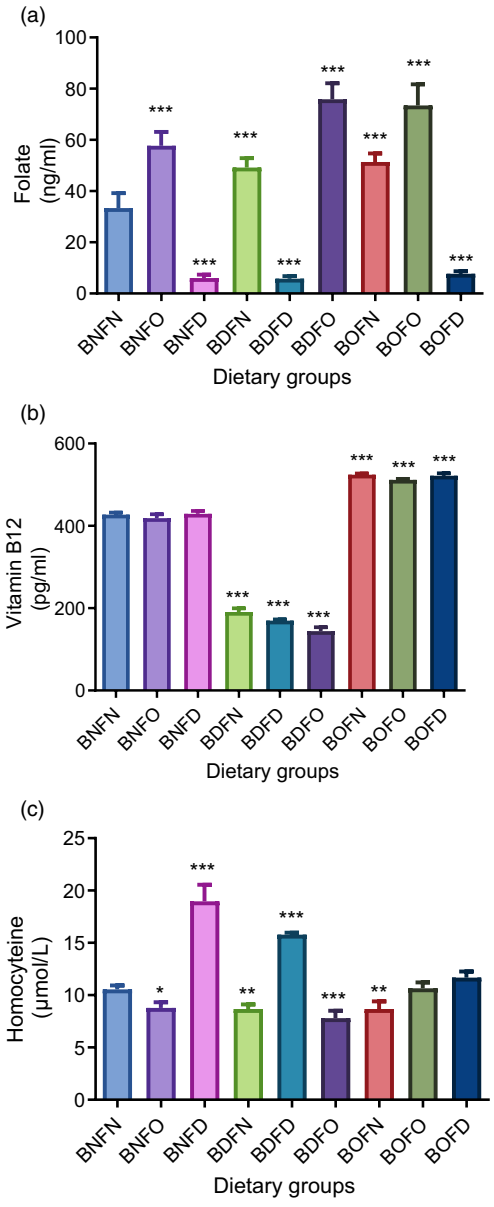

Fig. 1. (a) Serum folate levels, (b) serum vitamin B_{12} levels and (c) serum homocysteine levels in F1 generation mothers.

Similarly, serum B_{12} levels were significantly decreased in animals given vitamin B_{12}-deficient diets viz. BDFO (144·3 pg/ml), BDFN (190·5 pg/ml) and BDFD (169·9 pg/ml) as compared with the normal diet. Moreover, serum B_{12} levels were observed to be significantly increased in animals over supplemented with vitamin B_{12} (Fig. 1(b)). The results demonstrated that the serum levels of folate and vitamin B_{12} were altered in response to dietary intake of folic acid and B_{12}, respectively.

A significant increase in Hcy levels was observed in the serum of the animals given the folic acid-deficient diets. The Hcy level was the highest in BNFD group (18·9 μmol/l) followed by BDFD (15·7 μmol/l) group as compared with the control animals (BNFN) (Fig. 1(c)).

Effect of diet on epigenetic regulation of various imprinted genes

In this study, we had analysed the mRNA expression of H19- and IGF2 imprinted genes in the placenta (F1) and fetal tissues (F2) of mice. It is to note that kidneys were not well defined in fetuses (F2) born to BDFN mothers (F1) and could not be isolated, so data related to fetal kidneys of this group are not present.

Effect of diet on epigenetic regulation of H19 imprinted gene

mRNA expression, DNA methylation and histone modifications analysis at H19-ICR.

Placenta

Folate–B_{12} interactions:

In the placenta, the interaction of folate and B_{12} analysed by two-way ANOVA was found to be significant for mRNA and DNA methylation ($P < 0·001$). H19-ICR histone modification analysis in the placenta revealed a significant interaction between folic acid and B_{12} for H3K9me3, H3K27me3 and H3K4me2. Supplementary Table 6 represents the interaction between folic acid and B_{12} for the placenta.

From mRNA expression results, we observed no changes in BOFN and BOFO groups, so further methylation studies were not performed in these groups. For analysing the histone modification by ChIP, the studied dietary groups in the case of the placenta were BNFD, BDFN, BDFD and BOFD and for fetal tissues (BNFD, BDFD and BOFD). The shortlisting of the groups was done based on previous results keeping in view the limited availability of antibodies and tissue samples.

Pairwise comparisons for B_{12} and folic acid:

mRNA expression

We observed an increase in the expression of H19 under the state of folic acid deficiency in combination with either normal or over-supplemented status of B_{12} (BNFD by 15·9-fold and BOFD by 6·8-fold *v.* BNFN) ($P < 0·001$) (Fig. 2(a)).

DNA methylation

MS-HRM analysis of H19 differentially methylated region (DMR) in the placenta revealed hypermethylation in folic acid-deficient condition combined with the normal state of B_{12} (BNFD, 63·1 % *v.* BNFN) ($P < 0·001$) as well as in the combined deficient state of both vitamins (BDFD, 80·5 % *v.* BNFN) ($P < 0·001$). State of folic acid over-supplementation with B_{12} normal (BNFO *v.* BNFN) also led to an increase in methylation at H19-DMR (77·1 %, $P < 0·001$) (Fig. 2(b)).

Histone modifications

Upon quantifying the levels of histone modifications in the placenta (Fig. 2(c)), it was observed that suppressive H3K9me3 levels in comparison with control (BNFN) were reduced by 2·8-fold under B_{12}-deficient condition in combination with normal folic acid (BDFN *v.* BNFN) ($P < 0·001$) which was also the case under combined deficient states of both vitamins (BDFD) (2·5-fold, $P < 0·001$). However, an overall increase in the suppressive H3K9me3 mark was observed under folic acid deficiency with either state of B_{12} (BNFD (18-fold, $P < 0·01$), BOFD (24·3-fold, $P < 0·001$) *v.* BNFN). Activating mark H3K4me2 was, however, found to be increased with B_{12} deficiency in combination with the normal state of folic acid BDFN (5·6-fold, $P < 0·01$) and also with folic acid deficiency combined with either state of B_{12} (BNFD 18·1-fold, BOFD, 24·4-fold *v.* BNFN) ($P < 0·001$).

Possible epigenetic link:

In the placenta, the observed increased H19 mRNA expression under folic acid-deficient conditions (BNFD and BOFD) could be regulated by an increase in activating histone (H3K4me2) mark (online Supplementary Fig. 2(a)).

Fetal tissues

Folate–B_{12} interactions:

Two-way ANOVA analysis revealed that there was a significant interaction of folic acid and B_{12} for H19 gene expression, methylation and histone modifications in all the fetal tissues studied. Supplementary Tables 7–12 represents the interaction between folic acid and B_{12} for fetal tissues.

Fetal liver (male and female)

Pairwise comparisons for B_{12} and folate:

mRNA expression

In the case of fetal liver, H19 expression was significantly upregulated under folic acid deficiency with the normal state of B_{12} (BNFD *v.* BNFN) in both male (11·3-fold, $P < 0·001$) and female (4·4-fold, $P < 0·01$) fetal tissues. The combined deficient state of both vitamins also led to an increase in expression but only in males (BDFD *v.* BNFN) (3·6-fold, $P < 0·05$) (Fig. 3(a)(i) and (b)(i)).

DNA methylation

Pairwise analysis of the methylation at H19-DMR revealed that B_{12} deficiency in combination with folic acid normal (BDFN *v.* BNFN) ($P < 0·001$) led to a decrease in methylation in fetal tissues of both sexes (males, 37·2 % and females, 16·1 %). On comparing the effect of folic acid deficiency with either state of B_{12} (BNFD, BOFD *v.* BNFN), percent methylation was decreased in males (BNFD, 39·3 % ($P < 0·001$), BOFD, 73·8 % ($P < 0·05$)) whereas in female's methylation was found to be increased (89–90 %) ($P < 0·001$) (Fig. 3(a)(ii) and (b)(ii)). The combined deficient states of both the vitamins led to a decrease in methylation (BDFD *v.* BNFN) (males, 45·9 % ($P < 0·001$) and females, 54·6 % ($P < 0·05$)) along with folic acid over-supplementation combined with B_{12} normal (BNFO, 31–33 %) ($P < 0·001$) in fetal tissues irrespective of their sex.

Histone modifications

Suppressive histone mark (H3K9me3) under folic acid-deficient conditions combined with either state of B_{12} (BNFD, BOFD *v.* BNFN) was found to be increased in males (BOFD)

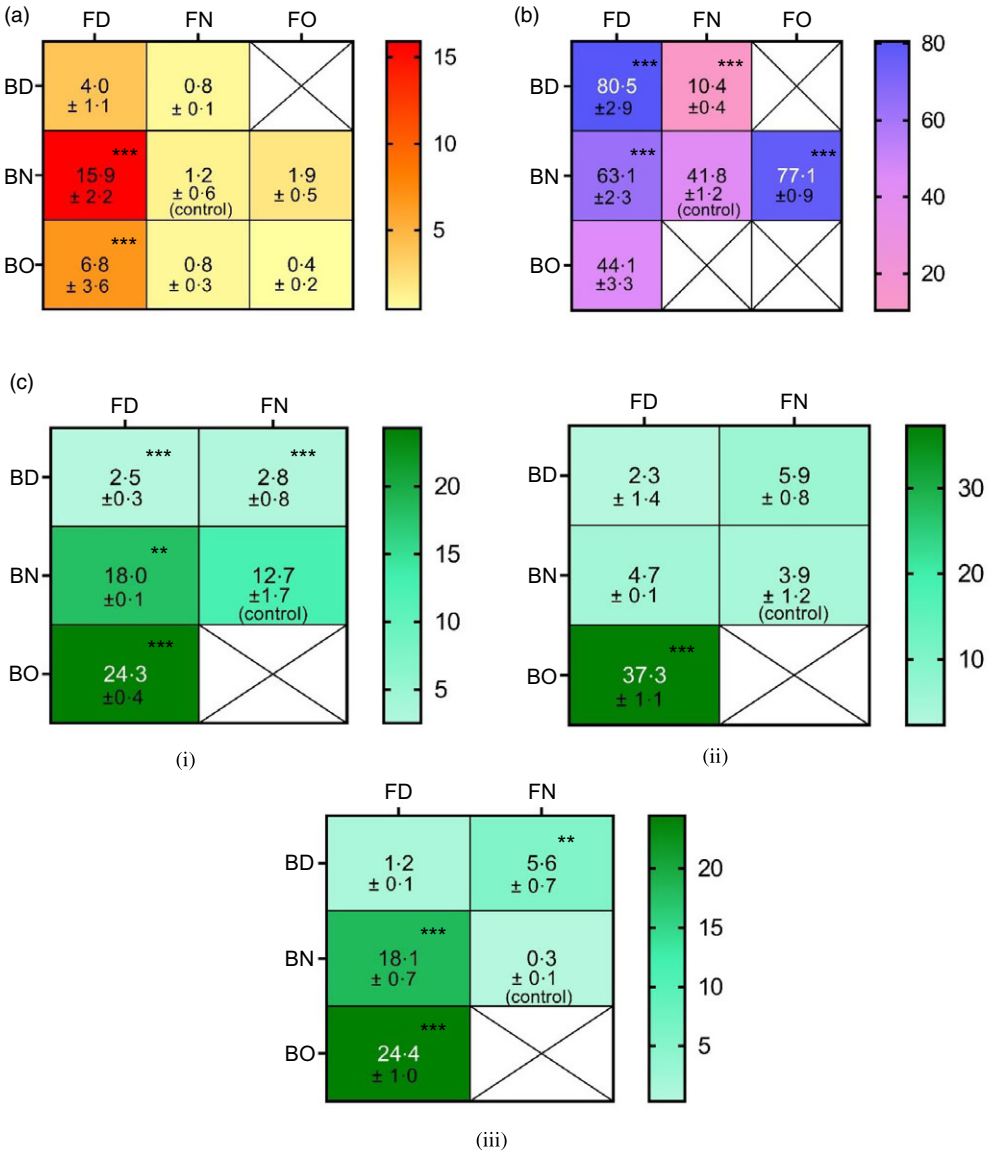

Fig. 2. (a) mRNA expression (fold change) of H19 imprinted gene in maternal placenta, (b) % CpG methylation, and (c) quantification of (i) H3K9me3, (ii) H3K27me3 and (iii) H3K4me2 at DMR of H19-ICR imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; ICR, imprinting control region.

(88·6-fold, $P < 0{\cdot}001$), whereas in females it was decreased by 3·2-fold and 0·3-fold ($P < 0{\cdot}01$–$0{\cdot}001$), respectively. In males, combined deficiency of both vitamins also led to a decrease in histone mark (BDFD) (0·3-fold, $P < 0{\cdot}01$). Suppressive H3K27me3 marks were found to be increased with deficient conditions of folic acid (BNFD, BOFD *v.* BNFN) in males (by 6·7-fold and 94-fold) as well as in females by 19·5-fold (BNFD) ($P < 0{\cdot}01$–$0{\cdot}001$); however, combined deficient condition of both vitamins (BDFD *v.* BNFN) led to a decrease in occupancy in males (0·3-fold) and an increase in females (5·3-fold) ($P < 0{\cdot}05$). The occupancy of activating H3K4me2 mark in females was found to be decreased under folic acid deficiency with over-supplementation of B_{12} (BOFD) (0·1-fold, $P < 0{\cdot}05$), whereas it was increased with folic acid deficiency combined with the normal state of B_{12} (BNFD, 11·4-fold) along with combined deficiency of both vitamins (BDFD, 27·6-fold) ($P < 0{\cdot}001$) (Fig. 3(a)(iii) and (b)(iii)).

Possible epigenetic link:

In the fetal liver, H19 expression was found to be up-regulated under folic acid-deficient condition (BNFD) in male as well as female fetal tissues, which in males could be regulated by a decrease in DNA methylation whereas in females, by a decrease in suppressive H3K9me3 along with an increase in activating histone mark (H3K4me2). Also, the increase in expression observed under combined deficient states of both the vitamins (BDFD) in males could be epigenetically regulated (by a decrease in DNA methylation along with a decrease in suppressive histone marks) (online Supplementary Fig. 2(b)).

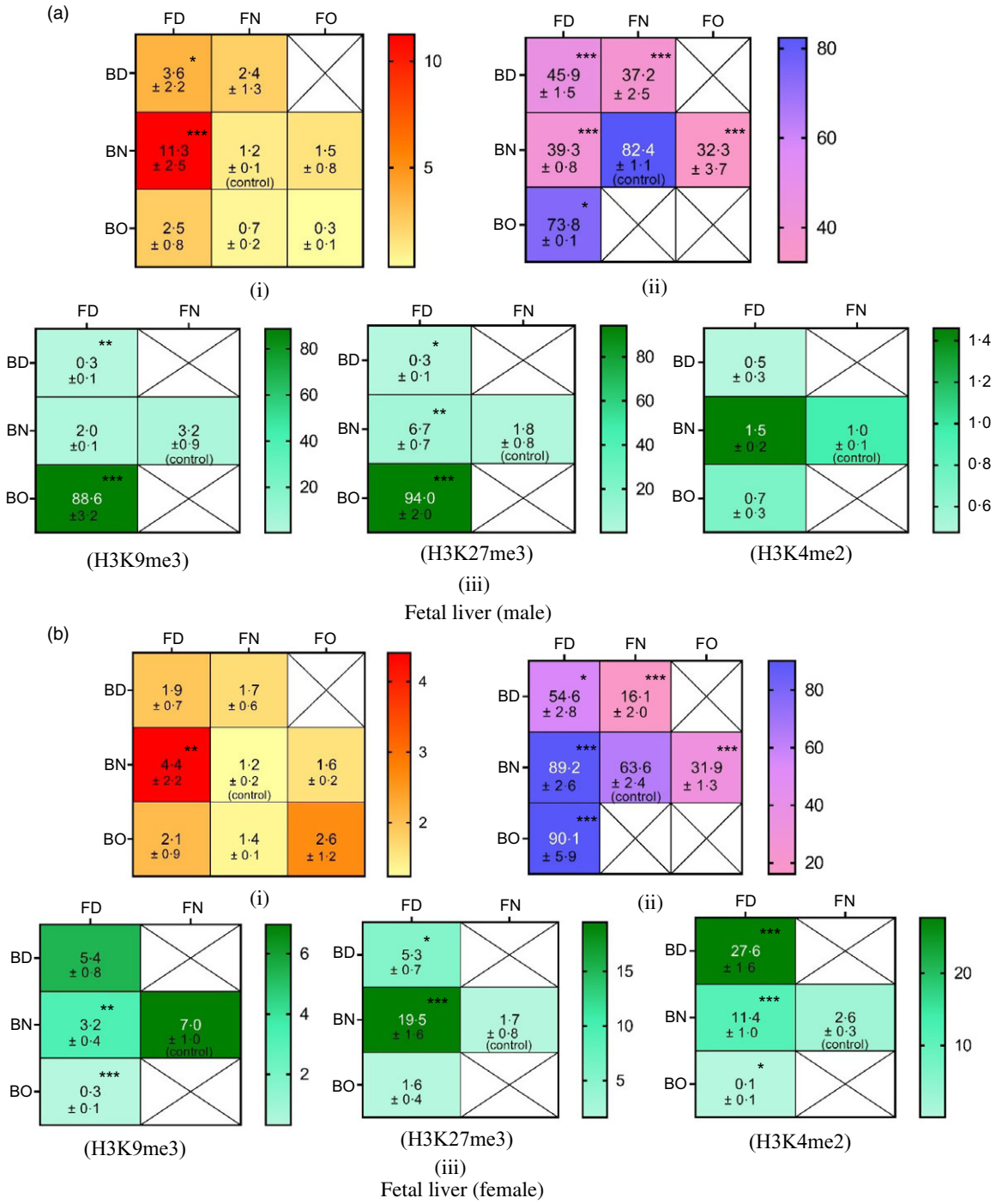

Fig. 3. Fetal liver (a) male and (b) female (i) mRNA expression (fold change) of H19 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of H19-ICR imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA.*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; ICR, imprinting control region.

Fetal kidney (male and female)

Pairwise comparisons for B_{12} and folate:

mRNA

Pairwise comparisons revealed that under a folic acid-deficient condition with normal B_{12} (BNFD *v.* BNFN), the expression of H19 was increased in both male (7·9-fold, $P < 0{\cdot}001$) and female (3·9-fold, $P < 0{\cdot}01$) fetal tissues (Fig. 4(a)(i) and (b)(i)).

DNA methylation

DNA methylation, under folic acid deficiency with B_{12} over-supplementation (BOFD *v.* BNFN) was reduced in both male

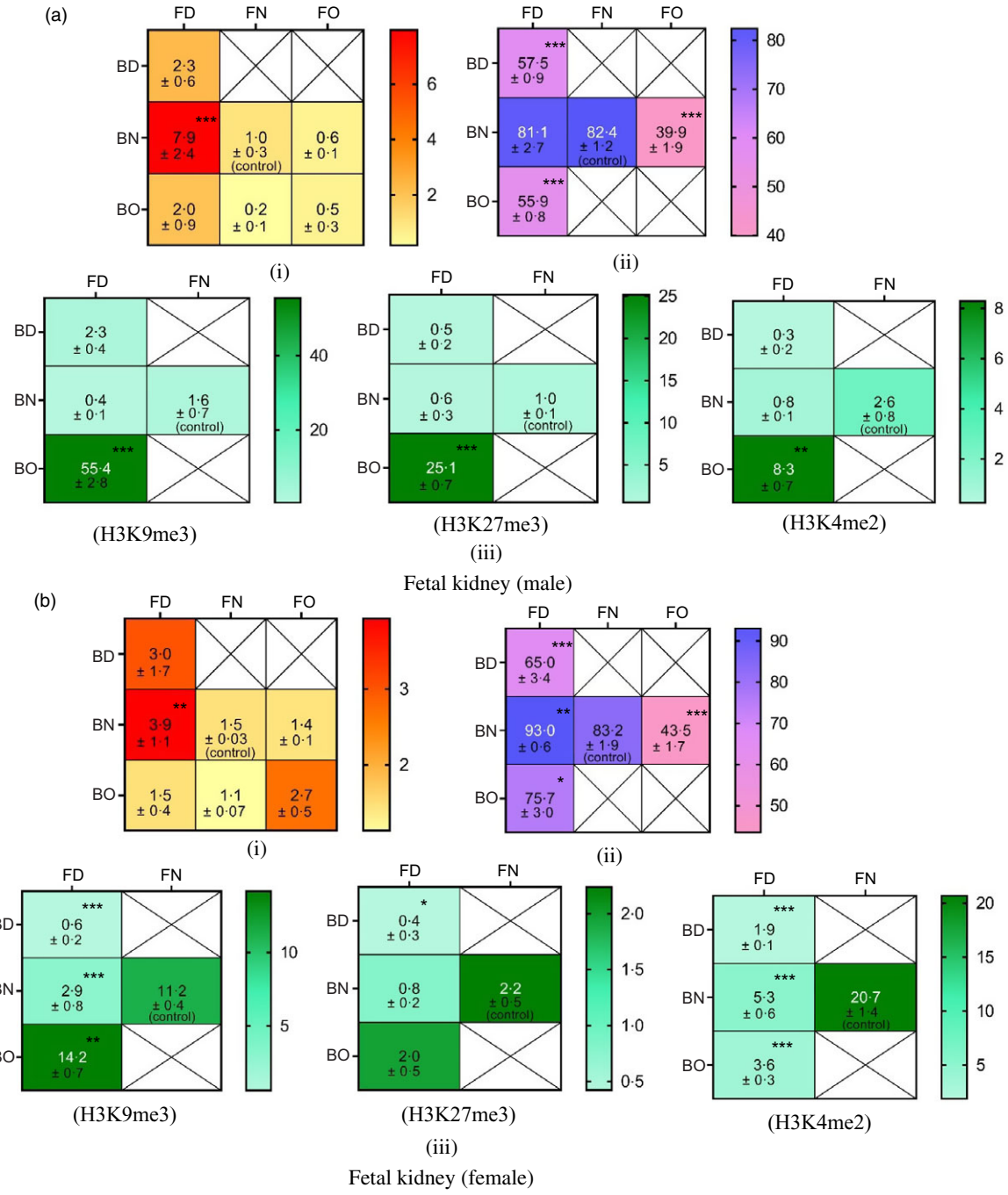

Fig. 4. Fetal kidney (a) male and (b) female (i) mRNA expression (fold change) of H19 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of H19-ICR imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA.*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; ICR, imprinting control region; ICR, imprinting control region.

(55·9 %, $P < 0{\cdot}001$) and female (75·7 %, $P < 0{\cdot}05$) fetal tissues; however, folic acid deficiency with B_{12} normal led to hypermethylation at H19-DMR in females (BNFD *v.* BNFN) (93 %, $P < 0{\cdot}01$). The combined deficiency of both the vitamins (BDFD *v.* BNFN) ($P < 0{\cdot}001$) overall led to reduced methylation (57–65 %) in fetal tissues irrespective of their sex (Fig. 4(a)(ii) and (b)(ii)). Over-supplementation of folic acid in combination with B_{12} normal (BNFO) ($P < 0{\cdot}001$) also followed a trend of reduced DNA methylation (39–44 %) in fetal tissues.

Histone modifications

Suppressive histone methylation mark, H3K9me3 and H3K27me3, showed an increase in occupancy under folic acid

deficiency combined with over-supplementation of B_{12} (BOFD *v.* BNFN) in both male (55·4-fold and 25·1-fold, $P < 0·001$) and female (H3K9me3, 14·2-fold) ($P < 0·01$) fetal tissues; however, reduced occupancy of suppressive marks was observed under dietary combination of folic acid deficiency with normal B_{12} (BNFD, 2·9-fold for H3K9me3 *v.* BNFN) along with combined deficiency of both the vitamins (BDFD *v.* BNFN) (0·6-fold and 0·4-fold in females ($P < 0·001$)). Activating mark, H3K4me2 was found to be increased under folic acid deficiency with over-supplementation of B_{12} (BOFD) (8·3-fold, $P < 0·01$) in males; however, in females, deficiency of folic acid overall led to reduced occupancy (1·9–5·3-fold) of histone mark ($P < 0·001$) (Fig. 4(a)(iii) and (b)(iii)).

Possible epigenetic link:

In the fetal kidney, in females, up-regulation of H19 expression observed under folic acid deficiency with the normal state of B_{12} (BNFD) could be regulated by a decrease in suppressive histone modification (H3K9me3) (online Supplementary Fig. 2(c)).

Fetal brain (male and female)

Pairwise comparisons for B_{12} and folate:

mRNA expression

Pairwise comparisons revealed that folic acid deficiency with a normal state of B_{12} (BNFD *v.* BNFN) led to an increase in the expression of H19 in fetal tissues (males by 46·2-fold and females by 25·6-fold) ($P < 0·001$). Similarly, in females, an increase in expression of H19 was also evident under another folic acid-deficient condition combined with over-supplementation of B_{12} (BOFD *v.* BNFN) (18·9-fold, $P < 0·01$) which was also the case under the combined deficient state of both the vitamins (BDFD) (40·6-fold, $P < 0·001$) (Fig. 5(a)(i) and (b)(i)).

DNA methylation

DNA methylation at H19-DMR, under B_{12} deficiency with a normal state of folic acid (BDFN *v.* BNFN) was, however, found to be reduced in male (79·8 %) as well as female (49·5 %) fetal tissues ($P < 0·01$). Folic acid deficiency with either state of B_{12} (BNFD, BOFD *v.* BNFN) led to a change in the DNA methylation in a sex-dependent manner which was decreased in males (23–84 %) ($P < 0·05$–$0·001$) and increased in females (83–85 %) ($P < 0·001$) (Fig. 5(a)(ii) and (b)(ii)). The combined deficient state (BDFD) of both the vitamins also showed a reduction in DNA methylation at H19-DMR in males (69·4 %, $P < 0·01$) which was also the case under folic acid supplementation in combination with a normal state of B_{12} (BNFO *v.* BNFN) (36–42 %) ($P < 0·001$) in the fetal tissues of both sexes.

Histone modification

Suppressive histone methylation mark, H3K9me3, showed reduced occupancy under folic acid deficiency with the normal state of B_{12} (BNFD) in males (1·9-fold, $P < 0·001$) which, however, was found to be increased in females (7·9-fold, $P < 0·01$) along with an increase in said histone mark under BOFD condition in fetal tissues irrespective of their sex (24·7-fold and 11·4-fold, $P < 0·05$–$0·001$). Suppressive H3K27me3 histone mark was found to be decreased in females under folic acid-deficient conditions (BNFD, BOFD *v.* BNFN) (1·9-fold and 2·5-fold, $P < 0·05$–$0·01$), whereas in males it was found to be increased by 45·4-fold (BNFD) ($P < 0·001$). Combined deficiency (BDFD) of both the vitamins led to reduced occupancy of H3K9me3 and H3K27me3 in fetal tissues (0·4–2·5-fold, $P < 0·05$–$0·001$) except for BDFD (female) where it was increased by 22·4-fold (H3K27me3). Activating H3K4me2 was however found to be decreased in males, 0·5–2-fold ($P < 0·001$) under all the deficient states of folic acid, whereas in female's occupancy was increased only under folic acid deficiency with over-supplementation of B_{12} (BOFD, 28·3-fold) ($P < 0·001$) (Fig. 5(a)(iii) and (b)(iii)).

Possible epigenetic link:

The data suggest that in fetal brain, increase in expression of H19 observed under folic acid-deficient conditions, BNFD in males could be epigenetically regulated by a decrease in DNA methylation along with a decrease in suppressive H3K9me3 whereas with folic acid deficiency (BNFD and BOFD) conditions, in females, the increase in expression could be regulated by a decrease in suppressive H3K27me3 mark and also with an increase in activating H3K4me2 (only in BOFD). Also, the combined deficiency of both the vitamins led to an increase in the expression of H19, which could be regulated by a decrease in suppressive H3K9me3 (online Supplementary Fig. 2(d)).

Effect of diet on epigenetic regulation of insulin-like growth factor 2 imprinted gene

mRNA expression, DNA methylation and histone modifications analysis of insulin-like growth factor 2. Placenta

Folate–B_{12} interactions:

Studying the expression of IGF2 by two-way ANOVA revealed that interaction between folic acid and B_{12} was found to be significant for mRNA in the placenta ($P < 0·001$). The interaction between folic acid and B_{12} in case of IGF2 DMR2 methylation was not found to be significant in the placenta ($P = 0·47$); therefore, independent effects of folate and B_{12} on DNA methylation were studied. Supplementary Table 13 represents the interaction between folic acid and B_{12} for the placenta. In the case of histone modifications, the interaction between folic acid and B_{12} was found to be significant for H3K9me3 and H3K4me2 in the placenta ($P < 0·01$), whereas for H3K27me3 it was not significant ($P = 0·112$). Thereafter, independent effects of folic acid and B_{12} were studied for H3K27me3. Pairwise comparisons were only performed after confirming the independent effects of folic acid and B_{12}.

Pairwise comparisons for B_{12} and folic acid:

mRNA

IGF2 expression, under B_{12}-deficient condition with the normal state of folic acid (BDFN *v.* BNFN) was found to be reduced (0·05-fold $P < 0·01$), whereas folic acid over-supplementation with normal B_{12} (BNFO) led to an increase in the expression of IGF2 (5·8-fold, $P < 0·001$) (Fig. 6(a)).

DNA methylation

DNA methylation was also found to be increased under folic acid over-supplementation state with B_{12} normal (BNFO *v.* BNFN) (55·7 %, $P < 0·001$) (Fig. 6(b)).

Histone modifications

The suppressive histone marks, H3K9me3 and H3K27me3, however, showed an increase in occupancy under B_{12}-deficient state combined with normal folic acid (BDFN) (3·3–3·8-fold, $P < 0·001$) which was also the case with folic acid-deficient condition (BOFD) (4·2–8·4-fold, $P < 0·001$) along with combined

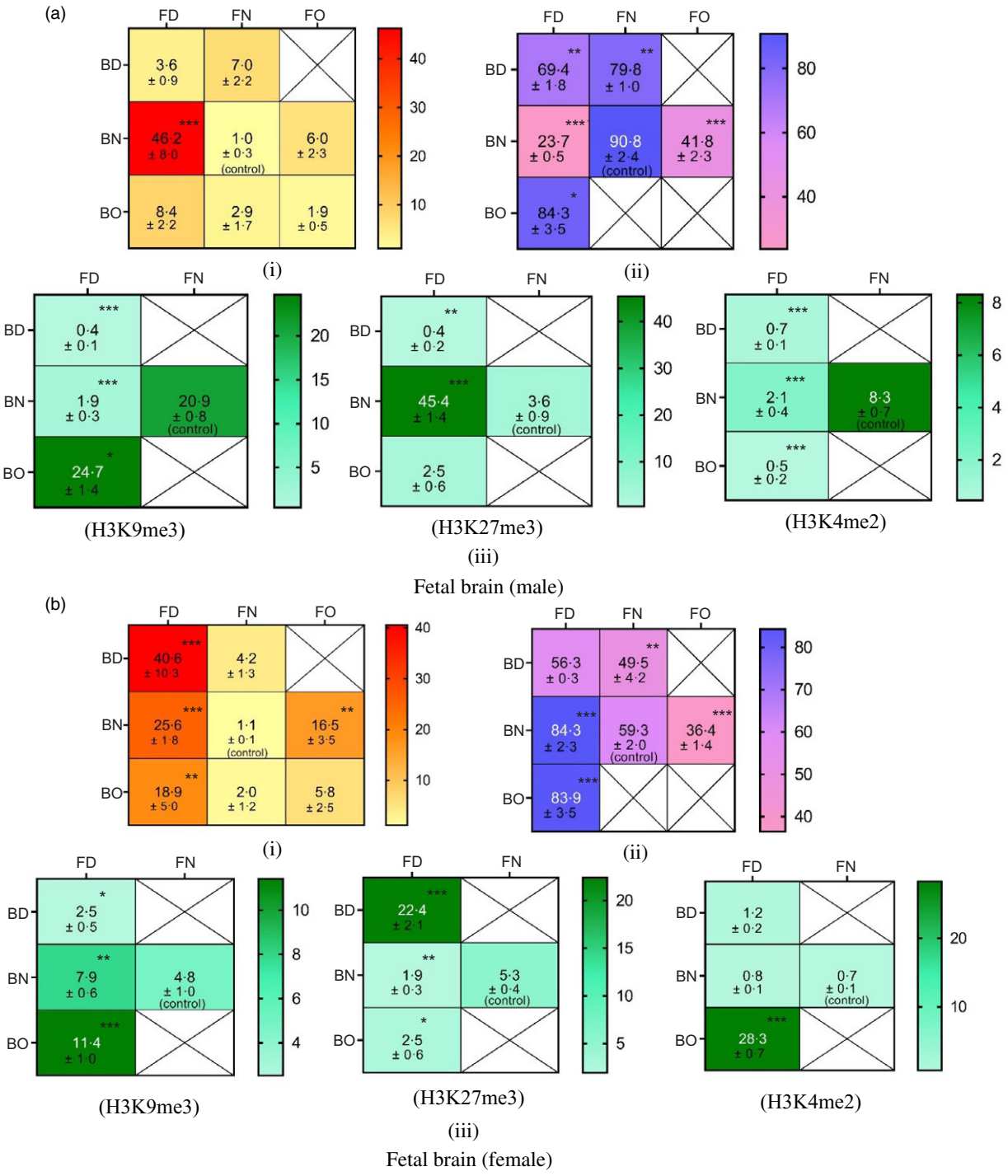

Fig. 5. Fetal brain (a) male and (b) female (i) mRNA expression (fold change) of H19 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of H19-ICR imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA.*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; ICR, imprinting control region.

deficiency of both the vitamins (BDFD) (2·8–5·5-fold, $P < 0·001$). Activating H3K4me2 was overall found to be reduced under all the conditions of deficiency of folic acid as well as B_{12} (0·1–4·0-fold, $P < 0·001$) (Fig. 6(c)).

Possible epigenetic link:

The decrease in expression caused in the BDFN group could be regulated by an increase in suppressive histone marks (H3K9me3 and H3K27me3) (online Supplementary Fig. 3(a)).

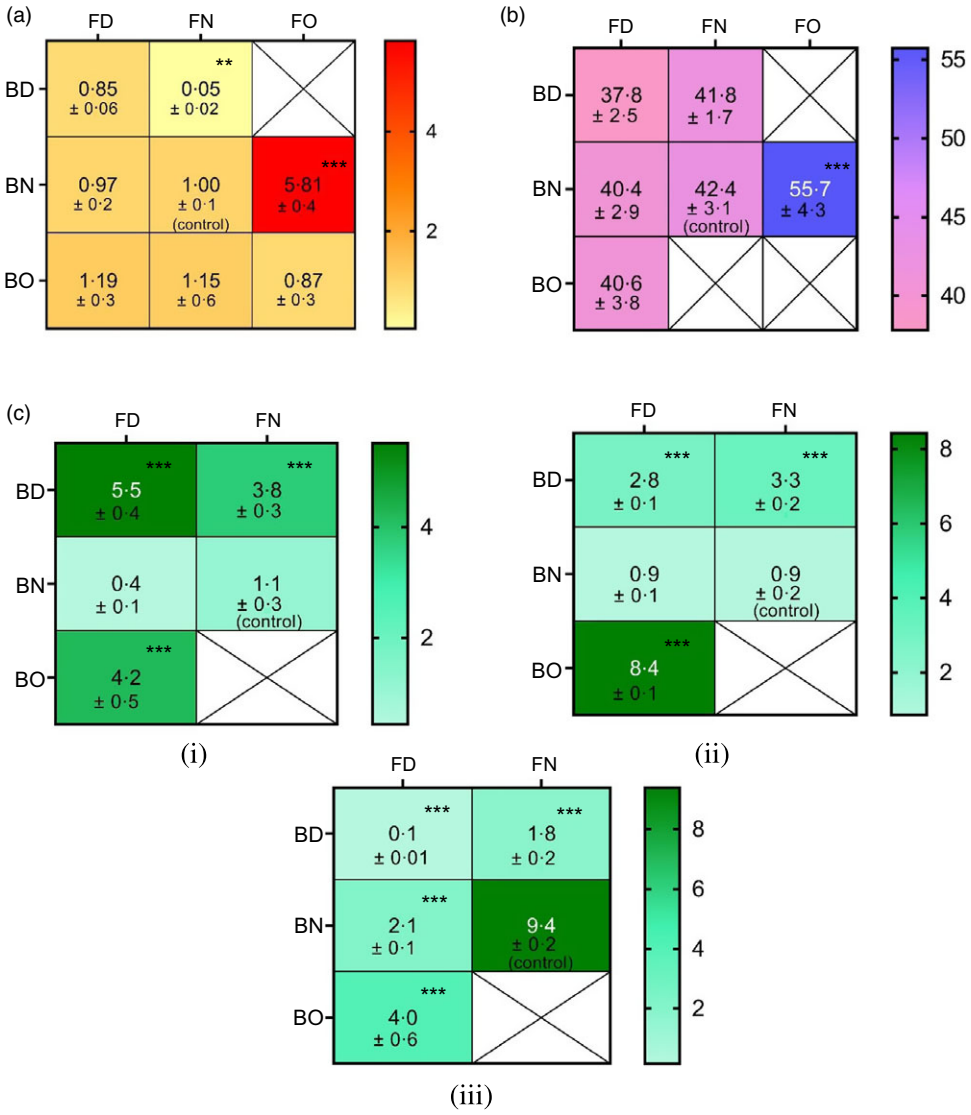

Fig. 6. (a) mRNA expression (fold change) of IGF2 imprinted gene in maternal placenta, (b) % CpG methylation, and (c) quantification of (i) H3K9me3, (ii) H3K27me3 and (iii) H3K4me2 at DMR of IGF2 imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; IGF2, Insulin-like growth factor 2.

Fetal tissues

Two-way ANOVA analysis revealed that there was a significant interaction of folic acid and B_{12} for H19 gene expression, methylation and histone modifications in all the fetal tissues studied. Supplementary Tables 14–19 represent the interaction between folic acid and B_{12} for fetal tissues.

Fetal liver (male and female)

Pairwise comparisons for B12 and folate:

mRNA expression

IGF2 expression in fetal liver was found to be increased with folic acid deficiency combined with over-supplementation of B_{12} (BOFD *v.* BNFN) in the males (3·3-fold, $P < 0{\cdot}001$); however, combined deficiency of both the vitamins (BDFD *v.* BNFN) led to an increase in the expression of IGF2 only in females (2·3-fold, $P < 0{\cdot}01$). Over-supplementation of B_{12} with the normal state of folic acid (BOFN) led to an increase in the expression of IGF2 in fetal tissues of both sexes (2·9–5·2-fold, $P < 0{\cdot}001$) (Fig. 7(a)(i) and (b)(i)).

DNA methylation

B_{12} deficiency with the normal state of folic acid (BDFN *v.* BNFN) led to a reduction in DNA methylation at IGF2 DMR (44–46·8 %, $P < 0{\cdot}01$–$0{\cdot}001$), whereas folic acid deficiency with an over-supplemented state of B_{12} (BOFD *v.* BNFN) led to a sex-specific change in the methylation of IGF2, which was found to be decreased in males (65·4 % ($P < 0{\cdot}05$)) and increased in females (78 % ($P < 0{\cdot}001$)). The combined deficient state of both the vitamins (BDFD *v.* BNFN) overall led to reduced methylation in fetal tissues of both sexes (27–43 %, $P < 0{\cdot}001$) (Fig. 7(a)(ii) and (b)(ii)). Folic acid over-supplementation with the normal state of B_{12} (BNFO)

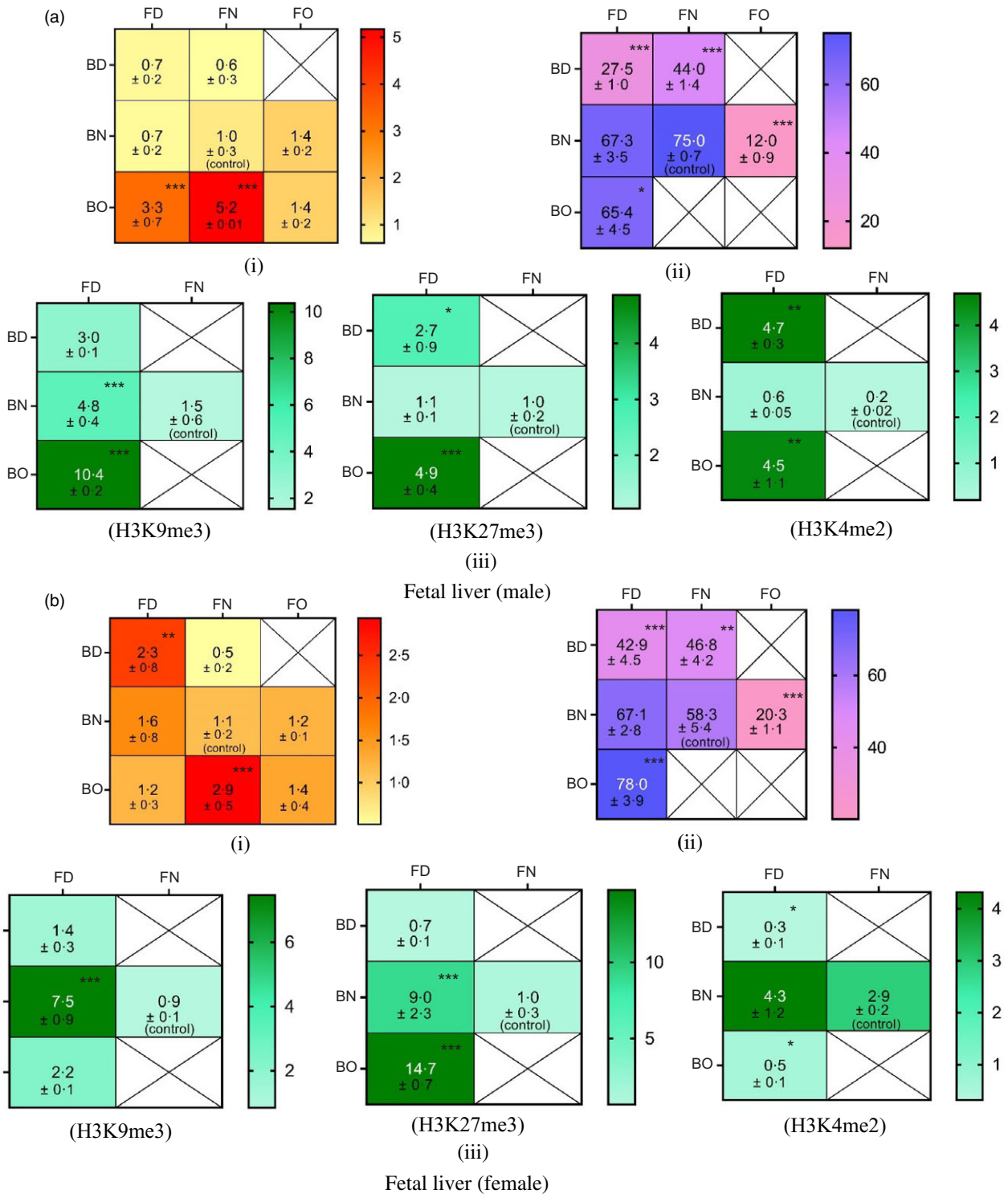

Fig. 7. Fetal liver (a) male and (b) female (i) mRNA expression (fold change) of IGF2 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of IGF2 imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; IGF2, Insulin-like growth factor 2.

showed reduced methylation status of IGF2 DMR regardless of sex (12–20 %, $P < 0{\cdot}001$).

Histone modifications

Suppressive H3K9me3 and H3K27me3 were overall found to be increased with deficiency of folic acid (BNFD, BOFD *v.* BNFN) (4–15-fold, $P < 0{\cdot}001$) in fetal tissues regardless of sex as well as under combined deficient state of both the vitamins (BDFD) (2·7-fold, $P < 0{\cdot}05$), but only in males (H3K27me3). Activating, H3K4me2 in comparison with control showed higher occupancy in males ($P < 0{\cdot}01$) under folic acid deficiency (BOFD, 4·5-fold) as well as combined deficiency of both the vitamins (BDFD, 4·7-fold), whereas, in females, the mark was found to be reduced (0·3–0·5-fold, $P < 0{\cdot}05$) (Fig. 7(a)(iii) and (b)(iii)).

Possible epigenetic link:

The increase in the expression caused under folic acid deficiency with over-supplementation of B_{12} (BOFD), in males, could be epigenetically regulated by a decrease in DNA methylation along with an increase in activating histone mark (H3K4me2). Also, the increase in the expression caused under dietary deficiency of both the vitamins (BDFD) in females could be due to a decrease in DNA methylation observed (online Supplementary Fig. 3(b)).

Fetal kidney (male and female)

mRNA expression

In the fetal kidney, expression of IGF2 was found to be reduced under folic acid-deficient conditions (BNFD *v.* BNFN) (0·2–0·4-fold, $P < 0{\cdot}01$) along with combined deficiency of both the vitamins (BDFD) (0·2–0·3-fold, $P < 0{\cdot}01$–$0{\cdot}001$) in both male and female fetal tissues (Fig. 8(a)(i) and (b)(i)). Over-supplementation of folic acid, as well as B_{12} overall, led to an increase in the expression of IGF2 in fetal tissues (2·8–4-fold) ($P < 0{\cdot}001$).

DNA methylation

DNA methylation was found to be reduced under the combined deficient state of both the vitamins (BDFD) (42–44 %, $P < 0{\cdot}001$) in fetal tissues of both sexes, whereas over-supplementation of folic acid with the normal state of B_{12} (BNFO) led to an increase in DNA methylation in males (78·2 %, $P < 0{\cdot}05$) (Fig. 8(a)(ii) and (b)(ii)).

Histone modifications

Suppressive H3K9me3 and H3K27me3 marks showed increased occupancy under folic acid-deficient conditions (BNFD, 10-fold ($P < 0{\cdot}05$), BOFD, 46–74-fold ($P < 0{\cdot}001$) *v.* BNFN) in males, whereas in females only under BNFD (H3K9me3) (twofold, $P < 0{\cdot}01$). Activating mark, H3K4me2 was also found to be increased under folic acid deficiency in males (BOFD, 9·2-fold) ($P < 0{\cdot}001$) in males and in females (BNFD, 2·8-fold) ($P < 0{\cdot}01$) (Fig. 8(a)(iii) and (b)(iii)).

Possible epigenetic link:

The decrease in expression levels of IGF2 in the kidney of both male and female fetal tissues under folic acid deficiency (BNFD) could be epigenetically regulated by an increase in suppressive histone mark (H3K9me3) (online Supplementary Fig. 3(c)).

Fetal brain (male and female)

mRNA expression

Folic acid deficiency in combination with B_{12} over-supplementation (BOFD) and combined deficiency of both the vitamins (BDFD) overall led to reduced expression of IGF2 in both male and female fetal tissues (0·6–0·7-fold) ($P < 0{\cdot}05$) (Fig. 9(a)(i) and (b)(i)).

DNA methylation

DNA methylation was found to be reduced under B_{12} deficiency with the normal state of folic acid (BDFN *v.* BNFN) in fetal tissues (41–56 %, $P < 0{\cdot}01$–$0{\cdot}001$) of both sexes. Hypermethylation at IGF2 DMR was observed in female fetal tissues under folic acid deficiency with either state of B_{12} (BNFD (66·1 %, $P < 0{\cdot}01$), BOFD (74·1 %, $P < 0{\cdot}001$)) (Fig. 9(a)(ii) and (b)(ii)). Combined deficiency of both the vitamins (BDFD) ($P < 0{\cdot}001$) overall led to reduced DNA methylation (22–37 %) in both male and female fetal tissues.

Histone modifications

Suppressive histone marks, H3K9me3, showed an increase in occupancy under folic acid-deficient conditions (BOFD 26·9-fold, BNFD 23·7-fold) ($P < 0{\cdot}001$) along with combined deficiency of both the vitamins (BDFD, 5·7-fold) ($P < 0{\cdot}05$) in males, however, in females, under the same dietary conditions occupancy was found to be reduced (BDFD (1·9-fold, $P < 0{\cdot}001$) and BOFD (5·1-fold, $P < 0{\cdot}05$)). Another suppressive histone mark (H3K27me3) was consistently found to be increased with deficiency of folic acid and B_{12} (BDFD) in fetal tissues regardless of their sex (20–21-fold, $P < 0{\cdot}001$). Activating H3K4me2 showed higher occupancy under folic acid deficiency conditions (BNFD (7·9-fold, $P < 0{\cdot}001$), BOFD (5·4-fold, $P < 0{\cdot}01$)) only in the males (Fig. 9(a)(iii) and (b)(iii)).

Possible epigenetic link:

The decrease in expression of IGF2 observed in the fetal brain under dietary deficiency of both the vitamins (BDFD) could be epigenetically regulated by an increase in suppressive histone modifications. Also, under folic acid deficiency with over-supplementation of B_{12} (BOFD), the observed decrease in expression of IGF2 could be regulated by an increase in DNA methylation in females, whereas in males the possible cause of a decrease in IGF2 expression could be an increase in suppressive histone marks (online Supplementary Fig. 3(d)).

Effect of diet on the expression of miR-675 in relation to H19/IGF2 imprinting locus

miR-675 expression analysis was carried out in the placenta and the fetal liver and kidney.

The interaction of folic acid and B_{12} was found to be significant in the placenta for miR-675 expression ($P < 0{\cdot}001$). Supplementary Table 20 represents the interaction between folic acid and B_{12} for the placenta and fetal tissues.

Placenta

Quantification of miR-675 levels in the placenta revealed increased expression under B_{12} deficiency with the normal state of folic acid (BDFN *v.* BNFN) (3·5-fold, $P < 0{\cdot}01$). Folic acid deficiency combined with the normal state of B_{12} (BNFD *v.* BNFN) (5·1-fold, $P < 0{\cdot}001$) also showed up-regulated expression of miR-675 in the placenta along with combined deficient states of both the vitamins (BDFD) (7·1-fold, $P < 0{\cdot}001$). However, folic acid deficiency with over-supplementation of B_{12} (BOFD) (0·07-fold, $P < 0{\cdot}01$) resulted in reduced expression of miR-675 which was also the case when over-supplementation of B_{12} was combined with either normal folic acid (BOFN) (0·14-fold, $P < 0{\cdot}01$) or over-supplemented state of folic acid (BOFO) (0·06-fold, $P < 0{\cdot}01$) (online Supplementary Fig. 4(a)).

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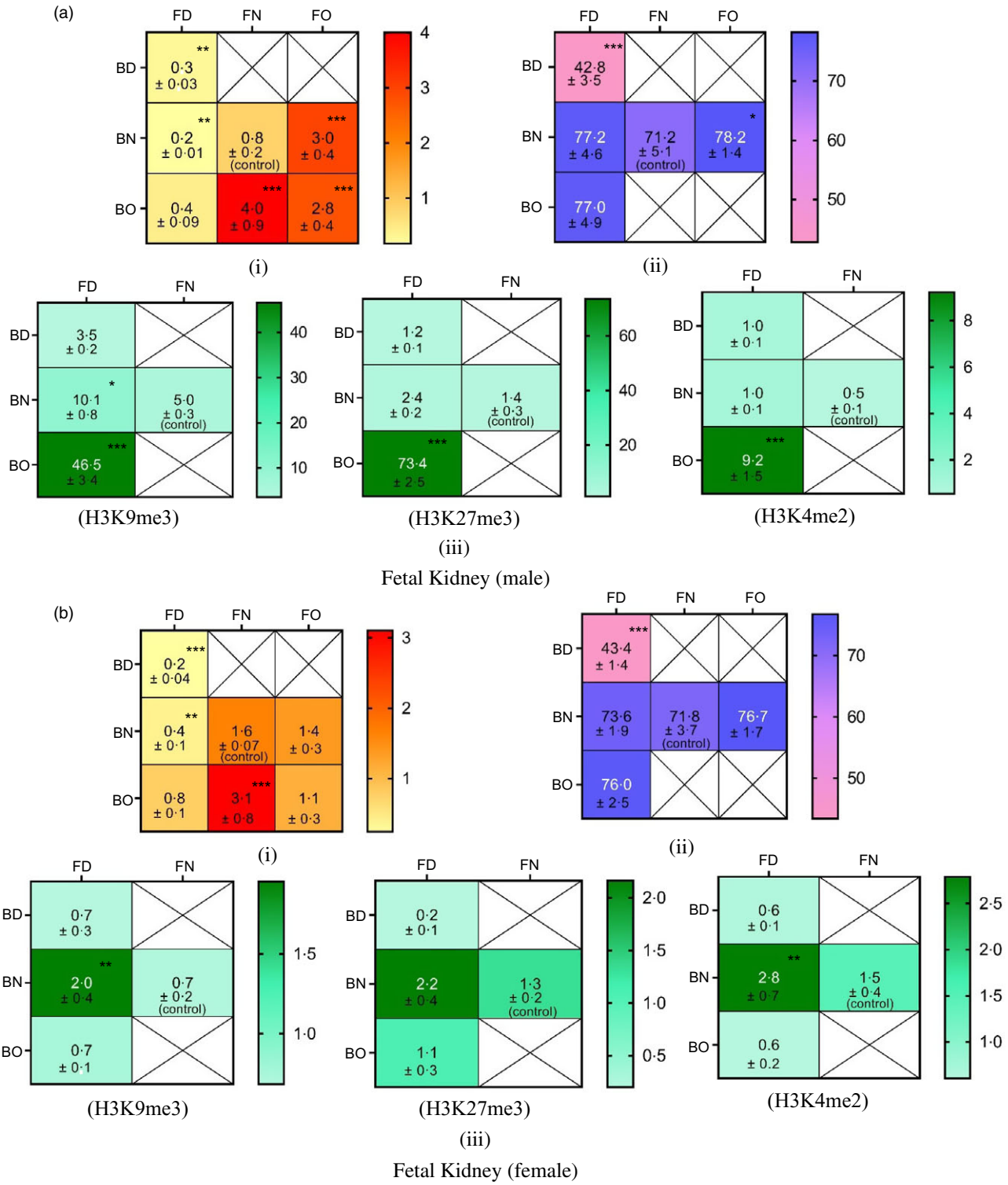

Fig. 8. Fetal kidney (a) male and (b) female (i) mRNA expression (fold change) of IGF2 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of IGF2 imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; IGF2, Insulin-like growth factor 2.

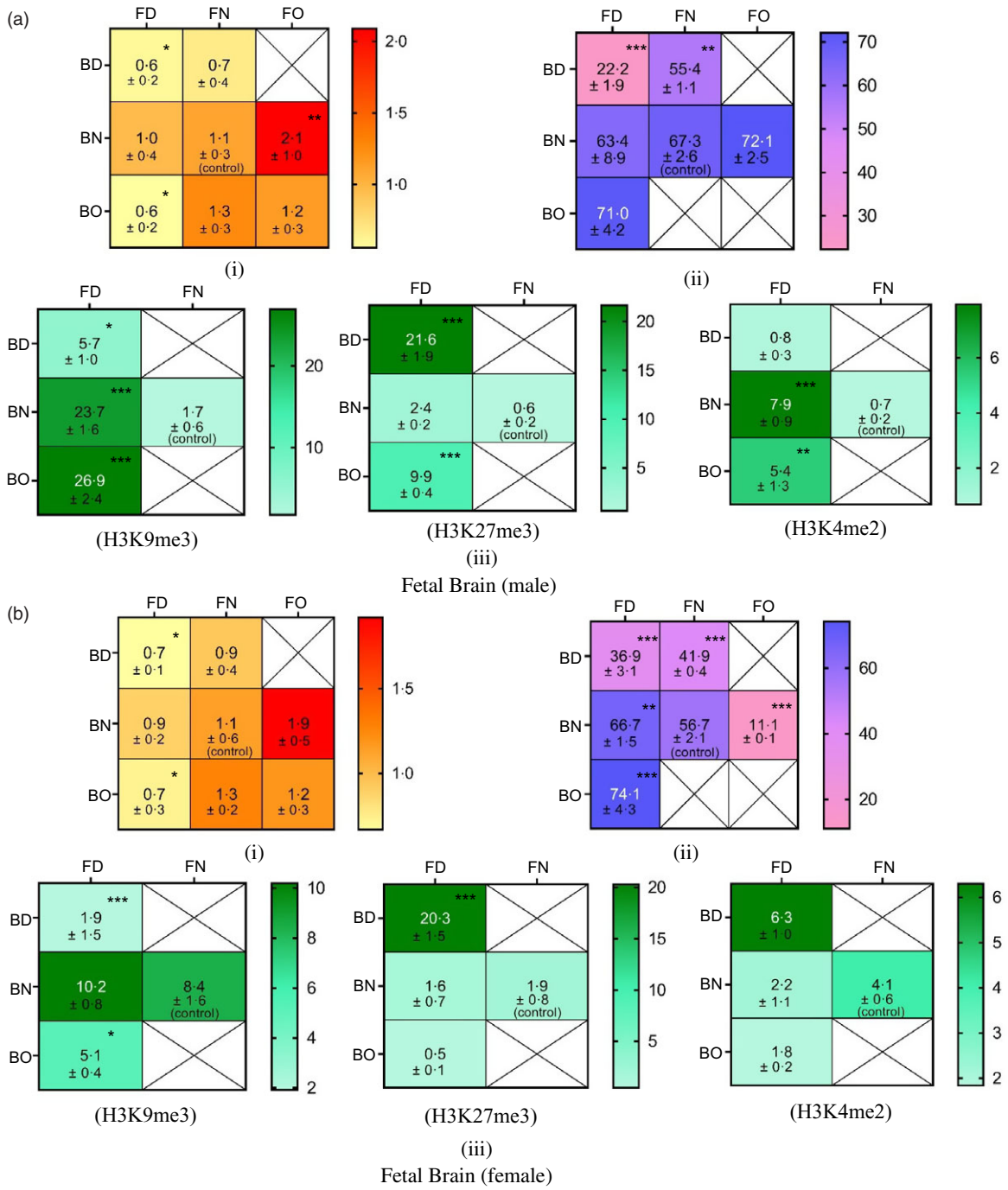

Fig. 9. Fetal brain (a) male and (b) female (i) mRNA expression (fo ld change) of IGF2 imprinted gene, (ii) % CpG methylation, and (iii) quantification of H3K9me3, H3K27me3 and H3K4me2 at DMR of IGF2 imprinted gene. Fold enrichment relative to non-specific IgG acting as negative control and normalised with input DNA. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ *v.* BNFN. The data are presented as mean ± SD. B_{12} normal folic acid normal (BNFN), B_{12} normal folic acid over-supplemented (BNFO), B_{12} normal folic acid-deficient (BNFD), B_{12}-deficient folic acid normal (BDFN), B_{12}-deficient folic acid over-supplemented (BDFO), B_{12}-deficient folic acid-deficient (BDFD), B_{12} over-supplemented folic acid normal (BOFN), B_{12} over-supplemented folic acid over-supplemented (BOFO) and B_{12} over-supplemented folic acid-deficient (BOFD). DMR, differentially methylated region; IGF2, Insulin-like growth factor 2.

Fetal liver (male and female)

The interaction of folic acid and B_{12} in fetal tissues was found to be significant in the kidney and liver of both sexes.

In males, folic acid deficiency with the normal state of B_{12} (BNFD *v.* BNFN) (7·9-fold, $P<0{\cdot}001$) led to an increase in the expression of miR-675. However, in females, B_{12} deficiency (BDFN *v.* BNFN) (0·03-fold, $P<0{\cdot}01$) as well as folic acid deficiency (BNFD, BOFD *v.* BNFN) (0·06–0·08-fold, $P<0{\cdot}01$) combined with either state of the other vitamin overall led to a decrease in expression of miR-675, which was also the case with over-supplemented conditions of folic acid and B_{12} ($P<0{\cdot}01$) (online Supplementary Fig. 4(b)).

Fetal kidney (male and female)

In comparison with control (BNFN), folic acid deficiency combined with either state of B_{12} led to reduced expression of miR-675 in fetal tissues (0·02–0·05-fold, $P < 0·05$–$0·01$); however, combined deficiency of both the vitamins (BDFD *v.* BNFN) (3·8-fold, $P < 0·001$) resulted in increased expression of miR-675 in males (online Supplementary Fig. 4(c)).

Supplementary Fig. 2, 3 and 5 represents the overall summary of the changes observed in expression, DNA methylation and histone modifications of the genes studied.

Supplementary Fig. 6 depicts the overall conclusion of the study.

Discussion

Folic acid and vitamin B_{12} are the key methyl carriers, and their roles have been well implicated in the establishment of methylation during the early stages of life and pregnancy. We for the first time tried to address the effect of different combinations of normal, deficient and over-supplemented folic acid with B_{12} in maternal/parental diet on epigenetic regulation of H19/IGF2 in the placenta and fetal tissues. H19, which is expressed from maternal allele, was significantly increased with dietary deficiency of folic acid and along with all combinations of B_{12}, that is, BNFD, BDFD and BOFD in the placenta as well as fetal tissues irrespective of their sex. miR-675, which lies in the promoter region of H19, was also found to be increased under these conditions. Histone modifications were the major regulatory factor for change in mRNA expression of H19 (suppressive H3K9me3 and H3K27me3 decreased and activating H3K4me2 increased). The underlying regulatory mechanisms for increased H19, however, were found to be sex- and tissue-specific. IGF2, which is expressed from the paternal allele, was significantly found to be decreased in the BDFN group in the placenta, with folic acid-deficient conditions (BNFD and BOFD) along with combined deficiency of both vitamins (BDFD) in the kidney and brain of fetal tissues. The DMR2 region of IGF2 promoter was found to be hypomethylated under combined deficiency of both vitamins (BDFD). Suppressive histone modifications such as H3K9me3 and H3K27me3 overall were found to be increased, whereas activating (H3K4me2) was found to be decreased in the placenta and fetal tissues. A well-known 'parental conflict hypothesis' states that paternally expressed genes tend to be growth-promoting, whereas maternally expressed genes tend to be growth-limiting[47]. From our study, it is clear that the altered dietary ratio of folic acid and B_{12} led to an increased expression of maternally expressed gene H19, whereas the paternally expressed gene was found to be decreased. These data suggest that dietary deficiency of folic acid with either combination of B_{12} (BNFD and BOFD) or combined deficiency of both vitamins (BDFD) plays a key role in regulating the state of genomic imprinting.

In the present study, dietary imbalance of folic acid and B_{12} led to a decrease in the body weights of mice in the F1 generation. The maximum decrease in the body weight was observed in the BDFO group which also showed higher rates of mortality, which is in line with the literature[12,48]. These females might be suffering from some neurological[49] or metabolic disease[50,51] due to excess folic acid and low B_{12}, leading to higher mortality rates. Mice exposed to folic acid and B_{12}-deficient diets led to a decrease in serum folate and B_{12} levels, respectively. Folic acid deficiency significantly led to an increase in Hcy levels with a maximum increase in the BNFD group (Fig. 1(a)–(c)). Folate deficiency is known to cause hyper-homocysteinemia which may lead to various neurological deficits[52].

We observed that folic acid deficiency alone was the independent factor responsible for an increase in H19 expression which either combined with B_{12} deficiency or over-supplementation had a similar effect as that of folic acid deficiency alone in the placenta (Fig. 2(a)) as well as fetal tissues (Figs. 3–5(a)(i) and (b)(i)). Previous studies have shown that H19 overexpression in the tissues such as the brain, where the gene is normally not expressed, led to lethal effects in embryos[53,54]. We also observed the highly overexpressed H19 in the fetal brain in BNFD and BDFD dietary groups. In contrary to our results, an earlier study has suggested that diets supplemented with methyl donors led to a significantly high expression of H19 in IUGR lineage animals[17].

H19 in our study, in general, showed hypermethylation under the conditions of either folic acid deficiency or B_{12} deficiency in the placenta (Fig. 2(b)). Although being methyl donors, deficiency of both vitamins should lead to hypomethylation, but we observed hypermethylation of IGF2R in the placenta, and this could be attributed to compensatory mechanisms mediated via other vitamin or due to compensatory up-regulation of DNMT as reported in the literature[55] which might influence the methylation status. Although folate deficiency leads to global hypomethylation, there have been reports of gene-specific hypermethylation[55,56]. In the fetal tissues (Fig. 3–5(a)(ii) and (b)(ii)), H19 showed differential methylation according to sex under folic acid deficiency conditions where it was decreased in BNFD and BOFD groups in males and increased in females in liver, kidney and brain as compared with controls. The results suggest that methylation at H19 is affected by diet in a sex-specific manner. Sex-specific differences in methylation have been reported previously[57,58], and this could be linked to the process of X-chromosome inactivation caused by methyl donor deficiency. Earlier studies have also found tissue-specific changes in H19-DMR in response to hyper-homocysteinemia, which was associated with higher levels of S-adenosylhomocysteine (SAH) and lower S-adenosylmethionine (SAM)/SAH ratios in the brain and liver in mice[59]. Another study has shown that SAM: SAH ratio was associated with high H19 methylation with individuals having low B_{12} levels[28]. It is possible that, at low B_{12} levels, the concentrations of SAM and SAH become more important determinants of methylation[28,60].

The chromatin composition at H19/IGF2 imprinted domain[61,62] is highly polarised. We observed that in our study, overall suppressive H3K9me3 levels at H19-ICR were found to be decreased, whereas activating mark H3K4me2 was found to be increased under folic acid-deficient conditions in the placenta (Fig. 2(c)) as well as the fetal tissues (Figs. 3–5(a)(iii) and (b)(iii)). The regression analysis has led to an understanding that the increase in mRNA expression of H19 in fetuses was highly regulated by an increase in activating histone modification

and a decrease in suppressive ones (online Supplementary Table 21). However, it was also observed that DNA methylation had an important role to play in regulating mRNA expression in case of the male fetuses, where an increase in mRNA expression was associated with a decrease in the methylation.

miR-675, which lies within the first exon of H19, was found to be increased under folic acid- and B_{12}-deficient conditions combined with either state of another vitamin in the placenta, while reduced under over-supplementation conditions (online Supplementary Fig. 4). In the fetal tissues, however, the expression was sex-specific which was found to be increased with deficiency of folic acid/B_{12} in males and decreased under all conditions in females. A significant positive co-relation of H19 with miR-675 was found in the placenta and the fetal liver (male) (online Supplementary Table 23). Previous studies have established that miR-675 plays an important role in limiting placental overgrowth[23].

Deficient conditions of vitamins (BDFN in the placenta) and particularly folate deficiency with either state of B_{12} in fetal tissues led to a decrease in mRNA levels of IGF2, whereas over-supplementation of either folic acid or B_{12} was associated with an increase in the expression in both the placenta (Fig. 6(a)) and the fetal tissues (Figs. 7–9(a)(i) and (b)(i)). Severe placental and fetal growth restrictions are associated with complete loss of IGF2 expression in the mouse placenta[63]. Studies in mice have shown that placental and fetal growth abnormalities such as pre-eclampsia can arise due to the altered imprinting status of H19, IGF2 and IGF2R which are preceded by placental pathologies[64].

IGF2 DMR2 overall showed hypomethylation under the conditions of B_{12} deficiency combined with either state of folate in fetal tissues irrespective of the sex (Fig. 6(b)). Previously, folic acid deprivation *in utero* has been associated with methylation differences at IGF2 DMR2 which were tissues-specific[65]. The methyl-deficient diet given for 60 d post-weaning also led to hypomethylation at IGF2 DMR[29] in mice.

Quantification of histone marks at IGF2 DMR revealed that deficiency of folic acid and B_{12} led to an increase in suppressive marks (H3K9me3 and H3K27me3) in both the placenta (Fig. 6(c)) and the fetal tissues (Fig. 7–9(a)(iii) and (b)(iii)), whereas activating mark (H3K4me2) was found to be decreased, thus, correlating with the decreased expression of IGF2 (online Supplementary Table 22).

Conclusion

This study led to the conclusion that *in utero* and lifetime exposure to the altered dietary ratio of folic acid and B_{12} affects the epigenetic regulation of imprinted genes which can be passed transgenerationally. This study has a potential limitation as the allele-specific enrichment of epigenetic marks could not be studied. However, this study gives an insight into the total expression of H19/IGF2 under the influence of dietary imbalance of folic acid and B_{12} in maternal/parental diets. Further studies on the allele-specific expression with respect to dietary manipulations of folic acid and B_{12} will be beneficial to confirm the effects on genomic imprinting.

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A. M. and J. K. designed the study and analysed the data. B. R. helped in analysing maternal and fetal tissues. A. M. and D. S. performed the experiments. A. M. and J. K. prepared the manuscript, and all authors critically revised and approved the manuscript.

The authors state that there is no conflict of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114521004220

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