Maternal folate depletion during early development and high fat feeding from weaning elicit similar changes in gene expression, but not in DNA methylation, in adult offspring

Jill A. McKay[1], Long Xie[2], Michiel Adriaens[3], Chris T. Evelo[4], Dianne Ford[5], John C. Mathers[2].

Human Nutrition Research Centre, [1]Institute for Health and Society, [2]Institute of Cellular Medicine, and [3]Institute for Cell and Molecular Biosciences, Newcastle University, UK; [3]Maastricht Centre for Systems Biology, Maastricht, The Netherlands; [4]Department of Bioinformatics – BiGCaT, Maastricht University, Maastricht, The Netherlands, [5]Faculty of Health and Life Sciences, Northumbria University, UK

Corresponding author: Prof. John C. Mathers. Human Nutrition Research Centre, Institute of Cellular Medicine, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne NE4 5PL, UK, Tel. +44 (0) 1912081133; Fax: +44 (0) 1912081101; e-mail: john.mathers@ncl.ac.uk

Abbreviations: Developmental origins of health and disease (DOHaD);

Key words; Early life nutrition; folate depletion; high fat diet; liver; gene expression; DNA methylation

Abstract

Scope: The 'Predictive Adaptive Response' hypothesis suggests the *in utero* environment when mismatched with the post-natal environment can influence later life health. Underlying mechanisms are poorly understood, but may involve gene transcription changes, regulated via epigenetic mechanisms.

Methods and Results: In a 2x2 factorial design**,** female C57Bl/6 mice were randomised to low or normal folate diets (0.4mg/2mg folic acid/kg diet) prior to and during pregnancy and lactation with offspring randomised to high or low fat diets at weaning. Genome-wide gene expression and promoter DNA methylation were measured using microarrays in adult male livers. Maternal folate depletion and high fat intake post-weaning influenced gene expression (1859 and 1532 genes respectively) and promoter DNA methylation (201 and 324 loci respectively) but changes in expression and methylation were poorly matched for both dietary interventions. Expression of 642 genes was altered in response to both maternal folate depletion and post-weaning high fat feeding, treatments imposed separately. In addition, there was evidence that the combined dietary insult (i.e. maternal folate depletion followed by high fat post-weaning) caused the largest expression change for most genes.

Conclusion: Our observations align with, and provide evidence in support of, a potential underlying mechanism for, the 'Predictive Adaptive Response' hypothesis.

Introduction

There is substantial and growing evidence which suggests that exposures during early life modulate the risk of developing non-communicable diseases in adulthood. This supposition, termed the developmental origins of health and disease (DOHaD) hypothesis, has been substantiated through numerous studies including those showing associations between low birth weight (frequently due to poor nutrition *in utero*) and increased risk of cardiometabolic diseases such as type 2 diabetes, coronary heart disease and hypertension [1]. Such observations indicate a degree of plasticity during *in utero* development through which environmental signals may alter the offspring phenotype [2] in order to prepare it for the anticipated post-natal environment [3]. It has been further proposed that 'mismatches' between the anticipated and actual post-natal environment predispose the offspring to the early development of disease [4-6]. This 'Predictive Adaptive Response' hypothesis suggests that the adverse consequences of inadequate nutrient supply during early developmental may be exacerbated by over-nutrition postnatally.

Many studies report detrimental effects of low folate status before and during pregnancy on offspring health. In rodents, folate deficiency can cause spontaneous abortion, teratogenic effects in offspring, reduced litter numbers, and altered offspring body weight [7, 8]. Evidence from human studies demonstrate that low folate status increases risk of neural tube defects [9-12], other congenital defects [13], adverse pregnancy outcomes [14], and low birth weight [15, 16] - the latter is a strong predictor of cardiometabolic disease in later life [17]). Furthermore, maternal folate intake may influence neurodevelopment [18], language development [19], risk of autism [20, 21] incidence of some cancers (leukaemia [22-24], brain tumours [25, 26] and neuroblastoma [27])[28], development of the metabolic syndrome [29], adiposity and insulin resistance [30] in children.
We have reported that offspring born to folate-depleted, but not folate-adequate, dams had 30% higher TAG concentration when fed a high-fat diet from weaning ($p = 0.005$ for interaction), which

suggests that a maternal nutritional insult during early development may increase susceptibility to the adverse effects of nutritional insults in adulthood [31]. This concept is supported by the observation that maternal folate supplementation during pregnancy in rats fed a low protein diet, reduced plasma TAG concentration in offspring that had been fed a high-fat diet post-weaning [32, 33]. These data provide evidence for a role of maternal folate intake in the 'Predictive Adaptive Response' to the post-natal environment, with implications for long term offspring health.

The mechanisms underlying the 'Predictive Adaptive Response' hypothesis are poorly understood but it is likely that both short and long-term changes in gene expression will play a role. Responsive changes in gene expression could be facilitated by altered epigenetic marks, including DNA methylation and histone modifications, since patterns of epigenetic marks are copied from one cell generation to the next and regulate gene expression [34, 35]. Environmental factors including diet modify epigenetic patterns, particularly DNA methylation, and these observations suggest that epigenetic processes are a mechanism which mediates effects of environmental factors on transcription[36], cell function and health. Folate is a major dietary source of one-carbon moieties used in formation of S-adenosyl-methionine, the universal methyl donor for methylation of biological molecules including DNA, lipids and proteins. Whilst the influence of dietary folate intake on DNA methylation patterns has been reported widely [37-46], few studies have examined the effects of these methylation changes on gene transcription.

We hypothesised that maternal low folate supply during pregnancy and lactation has long-term effects on gene transcription in the offspring and that these effects may be mediated by epigenetic mechanisms. In addition, we hypothesised that a second, and later, nutritional insult would exacerbate these effects. To test these hypotheses, using a 2x2 factorial study design we quantified hepatic genome-wide changes in gene transcription and in promoter DNA methylation in adult mice born to mothers given low or adequate folate intake during pregnancy and lactation and which had

been fed a high or low fat diet from weaning. We undertook our investigations in the liver since this organ is responsible for managing nutrient flows and for defending the body against multiple insults.

Materials and Methods

Animal husbandry and experimental diets

All animal procedures were approved by the Newcastle University Ethics Review Committee and the UK Home Office (Project licence number 60/3979) and have been described previously [31]. Animals were housed in the Comparative Biology Centre (Newcastle University) at 20-22°C and with 12h light and dark cycles. Fresh water was available *ad libitum.* The study followed a 2x2 factorial design with 2 levels of maternal folate supply (low and adequate (described herein as "Normal")) and 2 levels of dietary fat (high and low) fed to the offspring from weaning. Female C57BL/6J mice were allocated randomly to a low folate (0.4mg folic acid/kg diet) or normal folate (2mg folic acid/kg diet) diet (6g/day) (described previously [44]), and maintained on this diet for 4 weeks prior to mating. Mating trios (two females; one male) were provided with 6g/day/mouse of females' allocated diet. Upon presence of a vaginal plug, pregnant females were re-caged and provided with 10g/d of allocated diet throughout pregnancy, with diet quantity increased to 20g/dam/day at 2 weeks post-partum. On the Low and Normal folate diets, 12 and 15 dams, respectively, completed pregnancy and lactation successfully yielding 62 and 83 offspring at weaning, respectively.

At weaning (mean 22 days post-partum), 105 offspring were re-caged and allocated randomly to a low or high fat diet, details of which have been published previously [31]. Fresh water and allocated diet were available *ad libitum.*

Sample collection

At 28 weeks of age, offspring were anesthetised using gaseous isoflurane, blood was removed by cardiac puncture and animals killed by cervical dislocation. The liver was removed, weighed and snap frozen in liquid nitrogen and stored at -80°C. Livers from a total of 24 male mice were used for

the present study. Each mouse came from a separate litter and was selected as the mouse with the body mass closest to the mean body mass for the litter. This was a 2x2 factorial design study and we had equal numbers of mice from each treatment factor i.e. 12 mice whose dams had been exposed to the Low folate diet and 12 mice whose dams had been exposed to the Normal folate diet (total 24 mice). After randomised at weaning, of these 24 mice, 12 mice received the high fat diet and 12 mice received the low folate diet. Prior to extraction, livers were ground under liquid nitrogen to preserve RNA and DNA integrity and to ensure homogeneous cell population in subsequently extracted RNA and DNA samples. Blood was collected in 1.3mL pediatric blood glucose tubes (NaF/EDTA) (Greiner bio-one, 459085) and centrifuged at 300g for 15 min at 4°C to separate plasma. Plasma and cell fractions were stored separately at −80°C.

Plasma AST and ALT measurements

Plasma aspartate transaminase (AST) and alanine transaminase (ALT) were measured using commercial kits (Roche Diagnostics, Welwyn Garden City, UK) on the Roche P module (Roche Diagnostics, Welwyn Garden City, UK).

RNA Extraction, Gene expression arrays and validation using Real Time PCR

RNA was extracted from 50mg ground liver tissue using the Ambion PureLink RNA mini kit with Trizol according to the manufacturer's instructions. Two micrograms of RNA were incubated at 37°C for 30 min with 2µl DNase (Fermentas) and 2µl DNase buffer in a volume of 20µl, after which 2µl 25nM EDTA were added and reaction incubated at 65°C for 10 min. RNA (15µl ≈ 1.4µg) for each mouse was hybridised to a single array for a total of 24 male mice (n=6 for each dietary regime). Genome-wide transcript abundance was determined by ServiceXS (Plesmanlaan 1/D, 2333 BZ Leiden, Netherlands) on the Affymetrix Gene titan platform using the 3' IVT express labelling method with the Mouse Genome 430 2.0PM array. Before the labelling process, the integrity of all RNA samples

(RIN >8) was confirmed using the Agilent 2100 Bioanalyser. Output data were supplied as Affymetrix CEL files and imported into R (version 2.15.3) using the Affy package [47]. Data were pre-processed separately using gcRMA background correction and quantile normalization [48] to correct for batch effects and other technical confounders. To maximize sensitivity and specificity, updated Entrez gene probe-set annotation was used from the BrainArray project [49] (Version 14.1.0) resulting in 17306 re-annotated probesets mapping to unique transcripts. Statistical analysis comparing the diet groups was performed using the empirical Bayes approach of the limma package [50], using a statistical model with contrasts for the main effect of folate supply, main effect of high fat versus low fat diet, as well as the interaction between folate supply and dietary fat. Genes were considered to be differentially expressed in response to low maternal folate intake if there was a significant ($p < 0.05$) fold change (up or down) of at least 1.2 fold. All raw and processed gene expression microarray data have been deposited in the ArrayExpress database (E-MTAB-4987).

Validation of gene expression changes using real-time PCR

To confirm the gene expression changes observed in the microarray analysis, real-time PCR was performed on RNA samples that were analysed by microarray hybridisation, focusing on 8 gene targets observed to have large fold changes in response to dietary treatments. Gene transcripts analysed were *Lcn2* and *Orm2* (down regulated in response to maternal folate depletion), *Neat1* (up regulated in response to maternal folate depletion), *Sqle* and *Rdh11* (down regulated in response to high fat intake post-weaning), *Ppm1k* (up regulated in response to high fat intake post-weaning), and *Arhgap5* and *Nr1d2* (up regulated in response to both maternal folate depletion and high fat intake post-weaning).

RNA (1µg) was reverse transcribed using Quantitect Reverse Transcription kit (Qiagen, Cat no 205313) according to the manufacturer's instructions. Briefly, 1µg of RNA was incubated with 2µl genomic DNA Wipeout buffer in a reaction volume of 14µl at 42°C for 2 min to remove any genomic DNA. The mixture was then placed immediately on ice. RNA was incubated with 1µl Quantiscript reverse transcriptase, 1µl RT primer mix and 4µl Quantiscript RT buffer for 15 min at 42°C, after which time it was incubated at 95°C for 3 min. The cDNA samples generated were diluted 1:9 with water for use in real time qPCR.

Prior to sample analysis, expression values, linearity and efficiency of each assay were determined through cDNA standard curves completed for each transcript measured. Transcript abundance of the genes of interest was measured on a Roche Lightcycler 480 (Roche Applied Science) in a total reaction volume of 25µl using 2.5 µl Quantitect sybr green transcript specific primers (Qiagen, see Supplementary Table 1 for details of manufacturer's individual catalogue numbers), 12.5µl Quantitect SYBR green mix (Qiagen Cat. No. 204145), 1µl diluted cDNA and 9 µl water and cycling parameters 95°C for 5 min (1 cycle); 95°C for 10 s followed by 60°C for 30 s (40 cycles); followed by a final melt curve analysis and cooling to 40°C. Using the delta CT method, transcript levels of the genes of interest were normalised to *GAPDH* transcript levels which were determined using the procedure and parameters described above.

DNA extraction, MeDIP and DNA methylation array hybridisation

DNA was extracted from 50 mg ground liver tissue using the E.Z.N.A.® Tissue DNA Kit in accordance with the manufacturer's instructions. The full protocol for immunoprecipitation of methylated DNA (MeDIP) has been described in detail elsewhere [51]. Briefly, 10µg of DNA was incubated at 37°C for 30 min with 20 µl A/T1 RNase (Fermentas) in a 500µl reaction volume. DNA was then sonicated in cold water for 2 min at 20% pulse and 5 Volts of power output with an Ultrasonic Homogenizer 4710

Series (Cole-Parmer Instrument Co.). Extent of sonication was assessed by loading 15µL samples on 1% agarose gel with 0.4µg/mL of Ethidium Bromide. Successfully fragmented DNA samples (between 200-1000bp) were concentrated with silica columns (Zymo Research) to 50µL in TE buffer. To assess the success of the immunoprecipitation, genomic DNA was spiked with positive (i.e. methylated) and negative (i.e. unmethylated) control PCR products derived from lambda phage DNA (described previously [51]). Reaction mixtures were prepared by adding 40 ng of positive control and 40 ng negative control to 4.4µg of sonicated DNA in a total volume of 495 µL of TE buffer. After 10 minutes of denaturation at 95°C, samples were cooled on ice for 10 min and 1/10 of the reaction volume was stored as Input at 4°C. Immunoprecipitation reactions were performed as described previously [51]. PCR amplification of spiked positive and negative controls for both MeDIP and input samples were carried out to confirm methylated controls were present in both MeDIP and input samples, and that unmethylated amplicons were only present in input samples (see Lisanti *et al.* [51] for details). Whole genome amplification of MeDIP and Input samples was carried out using a WGA2 kit (Sigma) following the manufacturer's instructions, with 20ng of template DNA and omitting the fragmentation step. Products of the WGA reactions were purified on silica columns (Qiagen) and elutions were performed with 50µL water. Prior to array hybridisation, quantitative PCR was carried out to confirm the enrichment of methylated DNA in MeDIP compared with corresponding input samples as described previously [51]. Five micrograms of MeDIP and Input DNA were sent to NimbleGen Roche for hybridisation to two-channel MM8_RefSeq_promoter methylation arrays.

All samples passed the quality control analysis performed in the arrayQualityMetrics package [52]. Next, the raw DNA methylation data were normalized using T-quantile normalization applied across samples and separately on the two-channels [53] (corresponding to the MeDIP (Cy5, 635nm) and Input DNA samples (Cy3, 532nm), respectively). Then, for each diet, enrichment scores were calculated, defined as the negative $\log_{10}$-transformed p-value obtained from a Kolmogorov-Smirnov sliding window approach. This approach assesses whether the probe intensities (defined as the $\log_2$-

transformed ratio between the channels, i.e. the MeDIP and Input DNA intensities) observed in a genomic window (for all samples together; n=24) are significantly higher than that expected from the complete intensity distribution of all probes. The genomic window size was set at 750 bp and only genomic windows with at least 4 probes were considered, in accordance with the manufacturer's guidelines. The average probe-spacing for the array is 100 bp, yielding on average 7 measurements per genomic window. Next, for each annotated promoter and for each dietary treatment only the genomic window with the highest enrichment score, in addition to the corresponding genomic window for the contrasting dietary treatment, were considered for further analysis.

Finally, for each sample (n=24) DNA methylation intensity values were calculated based on the mean probe intensity value in each considered genomic window. To assess differences in DNA methylation intensity between diets (n=12 for each dietary factor (maternal folate supply and dietary fat content fed from weaning)), a linear modelling approach implementing heteroscedasticity-consistent standard errors [54] was applied using the *lmtest* and *Sandwich* packages in R, using a statistical model with contrasts for the main effect of folate supply, main effect of high fat versus low fat diet, as well as the interaction between folate supply and dietary fat. For each comparison, this yielded a differential DNA methylation p-value and a differential DNA methylation fold change.

Genes were considered to be differentially methylated in response to low maternal folate supply or high fat intake post-weaning if there was a significant ($p < 0.05$) fold change of -/+1.1. Considering the low fold change threshold, we additionally set an enrichment score difference threshold of -/+ 4 to be more conservative. All raw and processed microarray data have been deposited in the ArrayExpress database (E-MTAB-4990).

Pathway Analysis of Array Data

Pathway analysis was carried out using PathVisio [55], 3.2.0 and the curated pathway collection of WikiPathways [56] (download date: 01-09-2015). For gene expression data, a significant ($p < 0.05$) fold change of at least -/+1.2 fold and imposed Z score of 1.9 for significance was applied to filter for probable changed pathways. For DNA methylation data, a significant ($p < 0.05$) fold change of -/+1.1 and an enrichment score difference of -/+4 and imposed Z score of 1.9 for significance was applied to filter for probable changed pathways.

Due to our focus on pathway-based interpretation, we used uncorrected p-values in selecting differentially expressed or methylated genes within our analyses. We adopted this strategy because of the evidence that being inclusive in the selection of differentially expressed genes for pathway analysis, instead of adhering to conservative statistical cut-offs, leads to a more meaningful biological interpretation [92-94]. In addition, because the number of pathways is much smaller than that of genes [56], multiple testing problems are expected to be alleviated [95].

Positional Gene Enrichment (PGE) analysis

PGE analysis was carried out using the web-based PGE tool (http://homes.esat.kuleuven.be/~bioiuser/pge/index.php) [57] to locate over-represented chromosomal regions for significantly up-regulated, down-regulated, hypermethylated and hypomethyled genes. This tool applies an algorithm using the hypergeometric distribution to test if a chromosomal region is enriched in a given set of genes. A region is determined to be enriched if it contains at least two genes of interest, there is no smaller region containing the same genes of interest, there is no bigger region that included additional genes of interest but the same number of other genes, there is no larger encompassing region with a higher percentage of genes of interest, there is no smaller encompassing region with a better P-value, and it does not contain fewer than the expected number of genes of interest [57]. Resultant data were mapped to chromosomal

locations using Ensembl (www.ensembl.org; Release 67 (Mus Musculus genome build NCBI m37, MM9)).

Statistical Analysis

Statistical analysis of array data is described above. For gene expression data obtained from RT PCR analysis, data distributions were examined by the Kolmogorov-Smirnov test and all data were normally distributed. Analysis of variance (SPSS version 21) was used to examine the effects of diet on plasma AST and ALT and expression data obtained from RT PCR analysis. Linear regression (SPSS version 21) was used to examine the relationship between plasma AST and ALT. $P < 0.05$ was considered statistically significant.

Results

Influence of maternal folate intake during pregnancy and lactation on gene expression in the adult liver

In the adult liver, 1859 genes were differentially expressed in response to maternal folate depletion during pregnancy and lactation with approximately equal numbers of up-regulated (920) and down-regulated (939) genes (see Supplementary Table 2 for full list of genes and Supplementary Figure 5 for Venn diagram). Consistent with other nutritional studies investigating gene expression changes, for a substantial proportion of the genes (95%) expression changes were relatively small (i.e. fold change range of 1.4-2), with the expression of 92 genes changed by greater than two-fold. We also observed that 160 (8.6%) of the differentially expressed genes code for transcription factor proteins

(see Supplementary Table 3 for list of genes). Gene expression changes in response to this maternal folate depletion may influence 23 WikiPathways (pathway analysis using PathVisio) (Table 1).

Influence of maternal folate intake during pregnancy and lactation on DNA methylation in the adult liver

In the adult liver, 201 genes were differentially methylated in response to maternal folate depletion with approximately four times as many hypermethylated (162) as hypomethylated (39) genes (see Supplementary Table 4 for full list of genes and Supplementary Figure 5 for Venn diagram). DNA methylation changes in the adult liver response to maternal folate depletion was found to influence 12 WikiPathways using the chosen criteria (pathway analysis using PathVisio) (Table 2).

Integration of gene expression and DNA methylation changes in the adult liver in response to maternal folate depletion during pregnancy and lactation

Of the 1859 genes demonstrating altered expression, and 201 with altered DNA methylation, only 13 genes had both expression and DNA methylation changes in the adult offspring liver in response to maternal folate depletion (Table 3, Figure 1A and Supplementary Figure 6 for Venn diagram). Eight of these genes displayed the expected inverse relationship between changes in gene expression and DNA methylation (Table 3 and Figure 1A).

PGE analysis identified 244, 277, 44 and 5 genomic regions which were over represented as a result of up-regulation, down-regulation, hypermethylation and hypomethylation respectively in the adult liver of animals exposed to maternal folate depletion. Mapping of these regions to chromosomal

locations suggests that, whilst there were some genomic regions in which expression changes were not associated with DNA methylation changes, in the majority of cases DNA methylation and gene expression changes occurred in neighbouring regions (Supplementary Figure 1).

DNA methylation and gene expression were not altered in parallel in any WikiPathways pathway.

Influence of high fat feeding post-weaning on gene expression in the adult liver

In the adult liver, 1532 genes were differentially expressed in response to high fat feeding post-weaning, comprising 861 up-regulated genes and 671 down-regulated genes (see Supplementary Table 5 for full list of genes). For most genes (95%), expression changes were relatively small (i.e. ranging from a fold change of 1.4-2), with the expression of 84 genes changed by greater than two-fold (Supplementary Table 5). We also observed that 102 (6.7%) of the differentially expressed genes code for transcription factors (see Supplementary Table 6 for list of genes). Gene expression changes in response to high fat feeding may influence 18 WikiPathways pathway (Table 4).

Influence of high fat feeding post-weaning on DNA methylation in the adult liver

In the adult liver, 324 genes were differentially methylated in response to post-weaning high fat feeding, with eleven times as many hypomethylated (297) as hypermethylated genes (27) (see Supplementary Table 7 for full list of genes and Supplementary Figure 5 for Venn diagram). Pathway analysis identified 5 WikiPathways pathways with DNA methylation changes in the adult liver in response to high fat feeding post-weaning (Table 5).

Integration of gene expression and DNA methylation changes in response to high fat intake post-weaning in the adult liver

Of the 1532 genes demonstrating altered expression, and the 324 with altered DNA methylation, only 27 genes showed both expression and DNA methylation changes in the adult offspring liver in response to high fat intake post-weaning (Table 6, Figure 1B and Supplementary Figure 7 for Venn diagram). Sixteen of these genes displayed the expected inverse relationship between changes in gene expression and DNA methylation (Table 6 and Figure 1B).

PGE analysis identified 237, 206, 2 and 69 genomic regions which were over represented as a result of up-regulation, down-regulation, hypermethylation and hypomethylation respectively in the adult liver of animals fed high fat diets post weaning. Mapping of these regions to chromosome locations suggests that, whilst there were some genomic regions in which expression changes were not associated with DNA methylation changes, in the majority of cases DNA methylation and gene expression changes occurred in neighbouring regions (Supplementary Figure 2).

High fat feeding post weaning altered both gene expression and DNA methylation in the 'Fatty Acid Biosynthesis' WikiPathways pathway (see Tables 4 and 5 for details).

Comparison of gene expression changes in the adult liver in response to maternal folate depletion and post-weaning high fat intake

Of the 1859 and 1532 genes that were differentially expressed in response to maternal folate depletion and high fat intake post-weaning respectively in the adult liver, there were 642 genes in common (Supplementary Table 8). For almost all of these genes (635), both maternal folate depletion and high fat intake post-weaning had the same effect i.e. the same direction of change in expression in response to each nutritional insult (Figure 2). In addition, 58 (9%) of these 642 differentially expressed genes code for transcription factors (see Supplementary Table 9 for list of

genes). When compared with offspring not exposed to a nutritional insult (i.e. those born to mothers fed an adequate folate diet and who were randomised to the low fat diet at weaning), the largest differences in expression were for offspring exposed to both folate depletion during development and to the high fat diet from weaning for most genes (Figure 3). There were significant interactions between maternal folate supply and fat intake post-weaning for 9 of these 642 genes, 8 of which displayed the same direction of expression change in response to each nutritional insult (Supplementary Figure 3).

Both maternal folate depletion and high fat intake post-weaning altered the same five WikiPathways ('NOD-like Receptor (NLR) Proteins', 'Diurnally Regulated Genes with Circadian Orthologs', 'mRNA processing', 'Pentose Phosphate Pathway', 'Selenium metabolism' and 'Selenoproteins') (see Tables 1 & 4 for details).

Comparison of gene expression changes observed in response to both maternal folate depletion and high fat feeding post-weaning with gene expression profiles associated with liver damage

To test the hypothesis that gene expression changes observed in common in response to both maternal folate depletion and high fat intake from weaning may be involved in the development of liver damage, we compared our list of 642 folate and fat responsive genes with gene expression profiles from rodent models of liver damage. The latter included carbon tetrachloride exposure [58], combined choline and folate deficiency [59], alcohol exposure [60], and a panel of genes whose expression are associated with histopathological toxic liver fibrosis [61]. There was no significant overlap between our folate and fat responsive gene list and those genes differentially expressed in models of liver damage induced by carbon tetrachloride exposure or combined choline and folate deficiency, or genes whose expression are associated with histopathological toxic liver fibrosis (Supplementary Table 10). However, there was a significant overlap (75 and 64 genes, respectively) between our folate and fat responsive gene list and genes which are differentially expressed in response to chronic and intermittent alcohol exposure respectively (Supplementary Table 10).

Of the 75 folate, fat and chronic alcohol responsive genes, 49 changed expression in the same direction in response to all 3 exposures, 25 genes responded in the same direction to folate and fat but in the opposite direction to chronic alcohol exposure and 1 gene had the same directional change to maternal folate depletion and chronic alcohol exposure, but changed in the opposite direction in response to high fat (Supplementary Table 11).

Of the 64 folate, fat and intermittent alcohol responsive genes, 50 changed expression in the same direction in response to all 3 exposures, 12 genes responded in the same direction to folate and fat but in the opposite direction to intermittent alcohol exposure and 2 genes had the same directional change to maternal folate depletion and intermittent alcohol exposure, but changed in the opposite direction in response to high fat (Supplementary Table 12).

Expression of five genes (*Aprt, Ly6e, Strbp, Tpst2, Zbtb44*) differed in response to all four exposures i.e. maternal folate depletion, high fat feeding post weaning, chronic alcohol intake and intermittent alcohol intake.

Comparison of DNA methylation changes in the adult liver in response to maternal folate depletion and high fat intake from weaning

Of the 201 and 324 genes that had altered promoter DNA methylation in response to maternal folate depletion and high fat feeding post-weaning respectively, only 5 genes (*Lyar, Ppp2r5e, Tspo, V1ra8, Zfp509*) had altered DNA methylation in response to both exposures. For all 5 of these genes, hypermethylation was observed in response to maternal folate depletion, whereas hypomethylation occurred in response to high fat intake post-weaning. There were no common pathways with DNA methylation changes in response to maternal folate depletion and high fat intake from weaning.

Influence of maternal folate depletion and post-weaning high fat intake on plasma AST and ALT measurements

Neither maternal folate depletion nor post-weaning high fat intake significantly influenced plasma AST or ALT concentrations in adult offspring when data for all mice included in the study or only those mice selected for gene expression and DNA methylation analysis were investigated (Supplementary Table 13 for details). For individual mice, plasma AST and ALT concentrations were highly correlated (Supplementary Figure 4, p=0.001).

Validation of gene expression data from array analysis by RT-qPCR analysis

Data on expression of a panel of genes which showed altered expression in response to maternal folate depletion (*Arhgap5, Neat1, Nr1d2* (up-regulated) and *Lcn2, Orm2* (down-regulated)) or high fat feeding from weaning (*Nr1d2, Ppm1k* (up-regulated) and *Sqle, Rdh11, Arhgap5* (down regulated)) using microarray analysis was validated by RT-qPCR. Expression of 9/10 genes measured by RT-qPCR showed the same direction of change observed in array analysis, with 8/10 of these changes being statistically significant (Supplementary Tables 14 & 15).

Discussion

We hypothesised that i) offspring exposed to folate depletion during pregnancy and lactation would display differential gene expression and DNA methylation patterns in adulthood and ii) feeding a high fat diet from weaning would also modify expression and DNA methylation patterns. In addition, we hypothesised that a nutritional insult during early life development would exacerbate effects of a second (post- natal) nutritional insult i.e. that there would be an interaction between maternal folate supply and fat intake from weaning on these molecular markers.

Maternal folate depletion and post-weaning high fat intake alter gene expression and DNA methylation in the adult liver.

Here we report that both maternal folate depletion and post-weaning high fat intake influenced gene expression and DNA methylation profiles in the adult murine liver. We found a larger number of changes in gene expression in response to both nutritional exposures compared with changes in DNA methylation (i.e. in response to maternal folate depletion, 1859 genes were differentially expressed and 201 gene promotors differentially methylated; in response to high fat intake from weaning, 1532 genes were differentially expressed and 324 gene promotors differentially methylated). We have previously reported that folate depletion during pregnancy evoked approximately 3 times more changes in expression than in DNA methylation (expression of 989 genes v. methylation of 333 gene promoters) in the fetal liver [62, 86]. Others have reported genome-wide, but also relatively small, changes in DNA methylation in the liver of rat offspring from dams fed a high fat diet (45% of energy from fat) throughout gestation and lactation and to the offspring from weaning until 12 weeks of age [87].

Whilst we observed simultaneous changes in gene expression and promoter DNA methylation in response to both nutritional exposures investigated, we found no significant overlap between gene lists for which expression and promoter methylation were altered in response to a given exposure.

These data corroborate our previous findings in the fetal liver in response to maternal folate depletion [62], and data from other studies reporting non-synonymous changes in gene expression and promoter DNA methylation [63-66]. However, as previously explored [62], we used PGE analysis to find regions of the genome enriched for changes in gene expression and promoter DNA methylation in response to maternal low folate and high fat intake post-weaning to test the hypothesis that methylation status of a gene promoter may not influence the expression of that specific gene but could influence expression of a neighbouring gene(s), as has been demonstrated by others [67]. For most regions of promoter methylation change we observed gene expression changes in neighbouring genomic regions which supports our hypothesis that promoter methylation change may regulate gene regulation through cis-acting enhancer mechanisms [62, 67]. However, this contention requires further testing.

In the present study, folate restriction ended at weaning and all mice were provided with adequate folate intake from weaning until sample collection at 28 weeks of age. Despite the similarity in folate intake for the majority of their lives (5 of 6 months of post-natal life), 1859 genes were differentially expressed, and 201 gene promoters differentially methylated in the liver of those adult animals that had been folate depleted during embryonic, fetal and early post-natal life. This suggests that either expression and methylation changes induced by this nutritional insult in early life are sustained into adulthood or that other processes disrupted by early life folate depletion evoke these changes later in adulthood. Supplementation with dietary methyl donors (folic acid, vitamin B_{12}, choline and betaine) of viable yellow agouti (A(vy)) dams results in lifelong changes in methylation of a transposable element in the *Agouti* gene [68]. Within individual animals, the similarity in methylation status of specific CpG sites at this locus in multiple adult tissues from derived from all 3 germ layers provides evidence that, at least for some loci, changes induced early in embryonic development are sustained lifelong.

Our findings support the DOHaD hypothesis that early life nutritional factors alter programming of the offspring with long term molecular consequences that could affect organ function and/or health[69]. In particular our observations provide further evidence of the molecular mechanisms [43, 44, 62, 70, 86] through which maternal folate depletion alters child and adult health [9, 10, 13, 14, 18-28].

In accordance with previous studies, we observed changes in hepatic gene expression in response to high fat feeding [71-73]. Whilst the number of genes exhibiting expression changes that we observed greatly exceeded that of earlier reports, this may be due to differences in study design, diets and type of array used. In addition, different bioinformatic approaches to analyse array data and the thresholds utilised in deeming expression changes significant are likely to contribute to these variances. Despite this heterogeneity, gene expression changes in response to high fat intake appear to modulate similar key pathways/processes involved in lipid and fatty acid synthesis and metabolism in the liver across all studies [71-73].

Whilst there was similarity in the genes showing altered methylation in response to both maternal folate depletion and high fat feeding from weaning, the ratio of hyper to hypomethylation differed radically between dietary insults with ratios of 4:1 and 1:11 in response to maternal folate depletion post-weaning high fat intake, respectively. Hypermethylation was dominant in response to maternal folate depletion whereas hypomethylation was by far the most common response to post-weaning high fat intake. The predominance of hypermethylation in response to maternal folate depletion is in accordance with our previously reported findings in fetal liver [62] and that of other studies in rodents, which have reported inverse associations between intakes of folate and/or other methyl donors and DNA methylation [74, 75]. As previously discussed [62], this paradoxical inverse association between maternal folate intake and DNA hypermethylation in the offspring liver could be due to either compensatory changes in interrelated metabolic pathways (i.e. those involving

choline, methionine, vitamin B_6 and B_{12}) or a tissue specific response to perturbations in folate metabolism.

Further, the canonical association between DNA methylation and gene silencing depends on co-occurrence with specific histone modifications. The best example is the co-occurrence of DNA methylation and methylation of histone 3 at Lys 9 (H3K9) [88]. In absence of these histone modifications, the relation between DNA methylation and gene expression is much more nuanced than a simple inverse relation. This complexity has been described previously in detail [89, 90]. There is also complex interplay between DNA methylation and transcription factors [91]. Whilst it used to be thought that only proteins with a methyl-CpG binding domain (MBD) could interact with methylated DNA, it is now known that transcription factors (TF) lacking a MBD can also interact with methylated DNA. This suggests that, in specific circumstances, methylated DNA can facilitate binding of activating (as well as silencing) transcriptional machinery [91]. Since we do not have histone modification or TF-binding (ChIP-seq) data in this study, we cannot make the distinction between DNA methylation with canonical gene silencing function/association and DNA methylation with another function/association.

Maternal folate depletion and high fat intake post-weaning elicit similar changes in gene expression in the adult liver

One of the novel and surprising findings of this study was the high similarity in genes which displayed altered expression in response to maternal folate depletion and also in response to high fat feeding from weaning. Moreover, the vast majority (99%) of these genes showed expression changes in the same direction in response to both nutritional insults. This is despite that fact that the nutritional interventions were very different in kind (restriction of a specific B vitamin *v.* high intake of a macronutrient) and were applied at distinctly different stages in the life course. This suggests that the affected genes may be those whose hepatic expression is most labile in response to exogenous insults. Since diets depleted of folate and those with high fat content are used to model

liver damage in rodents [59, 76, 77], we hypothesised that dietary regimes utilised here may have resulted in liver damage. We therefore used two approaches to investigate this hypothesis; firstly we carried out biochemical assessments of liver damage in the plasma of adult mice (i.e. ALT and AST concentrations) and secondly we compared the list of 642 genes with overlapping expression changes in response to both maternal folate depletion and post-weaning high fat diet with reported transcriptional changes in the liver in published models of liver damage [59-61, 78]. We observed no effect of either maternal or post-weaning diet on plasma biochemical measures of liver damage but we found a significant overlap between genes with differential expression in response to alcohol exposure [60] and those we found to be both folate and fat responsive, but not with other models of liver damage i.e. combined folate and choline deficiency [59] or exposure to carbon tetrahydrochloride [58]. These data suggest that the genes with altered expression in response to both maternal folate depletion and high fat intake post-weaning are unlikely to be specific for liver damage as hypothesised. In contrast, it seems that low folate, high fat and alcohol exposures influence hepatic expression of a shared panel of genes that may be involved in the liver's response to these potentially damaging exposures, rather than to overt liver damage per se. In order to further explore these hypothesis, comparisons of the pathways involved in liver damage and early onset of damage with data derived from these models will be of interest.

Our current study cannot address the mechanisms responsible for similarities in differentially expressed genes in the liver in response to low folate, high fat and alcohol exposure, but it is plausible that these exposures trigger changes in one or more transcription factors and/or pathways/processes which lead to the observed changes in gene expression. Indeed, 58 (9%) of the 642 gene differentially expressed in response to both maternal folate and high fat feeding, four (*Clock, Id1, Tfam,* and *Zranb2*) of the 75 and five (*Gtf2h1, Id1, Pias2, Smarcb1,* and *Zhx1*) of the 64 genes associated with chronic and intermittent alcohol exposure respectively have known transcription factor activity.

We hypothesised that maternal folate supply during pregnancy and lactation would exacerbate the effects of a second, and later, nutritional insult. To test this hypothesis, we used a 2*2 factorial study design in which, at weaning, offspring born to dams fed folate depleted and folate adequate diets were randomised to either high or low fat diets. The combined exposure of maternal folate depletion, followed by high fat feeding post-weaning exerted the largest effects on gene expression (Figure 3). Using the same model, we made the novel observation that inadequate maternal folate supply resulted in offspring that were more susceptible to the adverse metabolic effects of high fat feeding [31]. Plasma TAG concentration was increased following the high fat diet only in offspring of mothers that were fed a folate depleted diet but not in those whose mothers were fed a folate adequate diet [31]. Taken together these data provide evidence in support of the 'Predictive Adaptive Response' hypothesis which proposes that adverse consequences of inadequate nutrient supply during early developmental may be exacerbated by over-nutrition post-natally and may predispose to the early development of disease [4-6].

Strengths and Limitations

The main strength of the study described here lies in the overall design, which allowed the investigation of the main effects of maternal and post-weaning diets as well as the interaction between the two. In combination with the integrative analysis of both genome-wide transcription and genome-wide promoter methylation using an array based approach, this allowed us to investigate the underlying mechanisms involved in the 'Predictive Adaptive Response' hypothesis in the context of maternal folate intake which may influence later life health. Here we used the Nimblegen MM8_RefSeq_promoter methylation array platform to interrogate the methylation status of all known promoter regions across the mouse genome. Given the evidence that promoter methylation is associated with gene silencing [79], this enabled us to investigate possible links between promoter methylation and gene transcription. However, growing evidence suggests that DNA methylation at intragenic regions (reviewed in Kulis et al 2013 [80]), CpG island shores [81],

partially methylated domains [82] or long hypomethylated domains [83, 84] can influence gene expression. Since there were not probes for these domains on the array that we used, we were unable to investigate these relationships. In future studies, next generation sequencing technologies may help to elucidate such relationships. To reduce possible variation due to sex effects, the present study investigated livers from male mice only and responses in females remain to be investigated.

Conclusion

We have observed that both maternal folate depletion during pregnancy and lactation and high fat intake post-weaning produce genome-wide changes in gene expression and in promoter DNA methylation in the adult liver. A major finding of this study was that both maternal folate depletion and high fat feeding from weaning alter expression of the same 642 genes. These changes were not associated with liver damage, as we hypothesised initially, but were similar to transcriptional changes seen following exposure to alcohol. The mechanisms underlying these hepatic responses to very different exposures remain to be discovered. Our findings that maternal folate depletion influences transcriptional and DNA methylation patterns in the adult offspring (months after being returned to a normal folate diet) is consistent with the DOHaD hypothesis and suggests plausible mechanisms by which inadequate folate supply during early development may lead to adverse health outcomes in later life. Hepatic expression changes were most pronounced in offspring exposed to the double insult of inadequate folate supply during early development followed by high fat feeding from weaning. This observation supports the 'Predictive Adaptive Response' hypothesis which proposes that a mismatch between the environment anticipated by the fetus, based on early (*in utero*) environmental exposures, and the environment encountered post-natally may predispose to the early development of disease [4-6]. In humans, the adverse effects of poor maternal micronutrient (including folate) status may be exacerbated when children are exposed obesogenic environments and may lead to higher risk of metabolic diseases in adulthood [85].

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Author Contributions

JAM, DF & JCM designed research, JAM and LX conducted research, MA and JAM analyzed the data. JAM, DF and JCM wrote the manuscript with contributions from MA, CE and LX. JAM and JCM had primary responsibility for final content. All authors have read and approved the final manuscript.

Acknowledgements

We thank Sandra Hogg, CBC, Newcastle University for care of the animals and Dr Jelena Mann (Institute of Cellular Medicine, Newcastle University) for advice regarding the assessment of liver damage. This project was funded by NuGO ('The European Nutrigenomics Organisation; linking genomics, nutrition and health research', NuGO; CT-2004-505944) and the BBSRC (BB/G007993/1), with additional salary support from the North of England Children's Cancer Research Fund (NECCR). MA's work at the Maastricht Centre for Systems Biology has been made possible with the support of the Dutch Province of Limburg.

Conflict of Interest

The authors have no conflicts of interest, financial or otherwise.

Table 1. WikiPathways, identified through pathway analysis, which may have been altered due to gene expression changes in the adult liver in response to maternal folate depletion during pregnancy and lactation

WikiPathway	Number of genes altered on Pathway	Number of genes measured on Pathway	Total number of genes on pathway	% affected	Z Score	p-value (permuted)
mRNA processing	82	375	552	21.87%	8.32	<0.001
Diurnally Regulated Genes with Circadian Orthologs	11	39	49	28.21%	3.93	<0.001
Exercise-induced Circadian Regulation	11	40	50	27.50%	3.83	<0.001
Apoptosis	17	82	84	20.73%	3.41	0.001
PluriNetWork	43	277	292	15.52%	3.39	0.002
Phase I biontransformations, non P450	3	6	10	50.00%	3.34	0.003
Proteasome Degradation	11	46	61	23.91%	3.28	0.003
TNF-alpha NF-kB Signaling Pathway	28	166	185	16.87%	3.19	0.002
Pentose Phosphate Pathway	3	7	20	42.86%	2.97	0.004
IL-7 Signaling Pathway	9	42	45	21.43%	2.58	0.012
Complement Activation, Classical Pathway	4	13	19	30.77%	2.57	0.015
Kit Receptor Signaling Pathway	12	64	68	18.75%	2.47	0.01
Eicosanoid Synthesis	5	19	34	26.32%	2.46	0.015
Integrin-mediated Cell Adhesion	16	94	102	17.02%	2.43	0.016
NOD-like receptor (NLR) Proteins	3	9	10	33.33%	2.4	0.022
IL-9 Signaling Pathway	5	21	25	23.81%	2.19	0.035
Macrophage markers	3	10	10	30.00%	2.17	0.032
Selenium metabolism/Selenoproteins	8	41	49	19.51%	2.14	0.027
Insulin Signaling	22	150	160	14.67%	2.1	0.028
G13 Signaling Pathway	7	35	39	20.00%	2.07	0.025
IL-3 Signaling Pathway	15	95	101	15.79%	2.03	0.036
Complement and Coagulation Cascades	10	58	64	17.24%	1.96	0.059
EGFR1 Signaling Pathway	23	163	177	14.11%	1.94	0.06

Table 2. WikiPathways which may have been altered due to promoter DNA methylation changes in the adult liver in response to maternal folate depletion during pregnancy and lactation

WikiPathway	Number of genes altered on Pathway	Number of genes measured on Pathway	Total number of genes on pathway	% affected	Z Score	p-value (permuted)
ACE Inhibitor Pathway	1	8	14	12.50%	3.17	0.007
TCA Cycle	2	31	45	6.45%	2.95	0.015
Monoamine GPCRs	2	31	41	6.45%	2.95	0.012
Type II interferon signaling (IFNG)	2	33	35	6.06%	2.82	0.041
Irinotecan Pathway	1	10	13	10.00%	2.77	0.038
GPCRs, Other	5	152	210	3.29%	2.74	0.007
Estrogen metabolism	1	12	29	8.33%	2.47	0.026
Glucocorticoid & Mineralcorticoid Metabolism	1	12	27	8.33%	2.47	0.025
Glycolysis and Gluconeogenesis	2	48	71	4.17%	2.12	0.022
Oxidative phosphorylation	2	49	65	4.08%	2.08	0.031
Glucuronidation	1	16	33	6.25%	2.04	0.046
Amino Acid metabolism	3	95	206	3.16%	2.02	0.031

Table 3. Genes with changes in both expression and DNA methylation in the adult liver in response to maternal folate depletion during pregnancy and lactation.

Gene symbol	Expression Fold Change	Expression P Value	DNA Methylation Fold Change	DNA Methylation P Value
2310044H10RIK	-1.3	0.009	1.2	0.046
CCDC107	-1.3	0.009	1.1	0.045
CCNL2	2.4	0.003	1.1	0.030
CDK4	-1.2	0.034	1.1	0.019
F2R	1.4	0.007	1.1	0.011
KIF13B	-1.3	0.005	1.1	0.044
LBP	-1.3	0.002	1.2	0.026
LYAR	1.5	0.015	1.1	0.027
MFAP1A	1.5	0.020	1.1	0.012
SCAND1	-1.5	0.015	1.1	0.001
SEPW1	-1.3	0.005	1.1	0.042
TIAL1	1.3	0.030	1.2	0.048
ZFP612	1.3	0.004	-1.3	0.032

Table 4. WikiPathways which may be altered due to gene expression changes in the adult liver in response to high fat feeding post-weaning

WikiPathway	Number of genes altered on Pathway	Number of genes measured on Pathway	Total number of genes on pathway	% affected	Z Score	p-value (permuted)
Cholesterol Biosynthesis	11	12	30	91.67%	9.45	<0.001
Pentose Phosphate Pathway	6	7	20	85.71%	6.69	<0.001
Fatty Acid Biosynthesis	8	22	26	36.36%	4.13	0.001
mRNA processing	60	375	552	16.00%	4.05	<0.001
Nucleotide Metabolism	6	15	36	40.00%	3.88	<0.001
NOD-like receptor (NLR) Proteins	4	9	10	44.44%	3.45	0.004
Mitochondrial Gene Expression	6	18	23	33.33%	3.31	0.005
SREBF and miR33 in cholesterol and lipid homeostasis	4	11	14	36.36%	2.92	0.003
Iron Homeostasis	4	13	16	30.77%	2.5	0.021
Translation Factors	9	42	52	21.43%	2.48	0.017
PPAR signaling pathway	13	70	88	18.57%	2.41	0.02
Fatty Acid Beta Oxidation	7	31	46	22.58%	2.35	0.014
Diurnally Regulated Genes with Circadian Orthologs	8	39	49	20.51%	2.2	0.02
EBV LMP1 signaling	5	21	23	23.81%	2.12	0.03
Glucuronidation	3	10	33	30.00%	2.11	0.043
Selenium metabolism/ Selenoproteins	8	41	49	19.51%	2.04	0.037
Estrogen signalling	12	70	77	17.14%	2.01	0.04
Glutathione metabolism	4	16	38	25.00%	2.01	0.046

Table 5. WikiPathways identified through pathway analysis which may be altered due to promoter DNA methylation changes in the adult liver in response to high fat feeding post-weaning

WikiPathway	Number of genes altered on Pathway	Number of genes measured on Pathway	Total number of genes on pathway	% affected	Z Score	p-value (permuted)
Amino acid conjugation of benzoic acid	1	2	12	50.00%	4.86	0.001
Mismatch repair	2	9	10	22.22%	4.35	0.006
Regulation of Cardiac Hypertrophy by miR-208	1	6	9	16.67%	2.57	0.029
Fatty Acid Biosynthesis	2	22	26	9.09%	2.39	0.027
Acetylcholine Synthesis	1	7	18	14.29%	2.33	0.033

Table 6. Genes with changes in both gene expression and DNA methylation in the adult liver in response to post-weaning high fat intake.

Gene Symbol	Expression Fold Change	Expression P Value	DNA Methylation Fold Change	DNA Methylation P Value
1300014I06RIK	-1.4	0.003	-1.1	0.020
ACSS2	-2.0	0.000	-1.1	0.019
ALDH1B1	-2.2	0.006	1.1	0.007
ATAD2B	1.8	0.025	-1.1	0.028
ATL2	1.2	0.031	-1.2	0.003
ATP2B2	-1.3	0.000	1.1	0.003
C80913	1.5	0.026	1.2	0.003
CCL9	-2.1	0.001	-1.1	0.028
DIO1	1.3	0.020	-1.2	0.012
DNAJA1	1.3	0.012	-1.1	0.032
DNAJC3	1.6	0.038	-1.1	0.009
GDAP2	-1.5	0.002	-1.2	0.001
GOT1	-1.4	0.002	-1.1	0.014
HECA	1.7	0.029	-1.2	0.005
HECTD2	1.2	0.037	-1.1	0.012
ITM2C	-1.4	0.019	-1.1	0.029
KARS	-1.3	0.050	-1.1	0.032
LYRM5	1.5	0.030	-1.1	0.030
NAP1L1	1.5	0.028	-1.1	0.044
PLSCR2	1.8	0.005	-1.1	0.010
RABGAP1L	1.2	0.001	-1.1	0.012
RSRC2	1.4	0.044	1.1	0.025
SCO1	1.2	0.003	-1.1	0.033
STX8	-1.3	0.049	-1.1	0.015
TSPO	-1.4	0.019	-1.1	0.029
WWP1	1.9	0.026	-1.1	0.047
ZFP260	1.4	0.018	-1.1	0.006

Figure 1. Scatterplot summarising the direction of, and fold change in, expression and DNA methylation of genes with significant changes in both in adult liver in response to A) maternal folate depletion and B) post-weaning high fat intake.

Figure 2. Scatterplot summarising the direction and level of fold change in expression of genes with significant changes in the adult liver in response to maternal folate depletion and high fat intake post-weaning.

Figure 3. Box-and-whisker plot of the absolute fold change for 642 genes found to be differentially expressed in response to both maternal folate depletion and high fat intake post-weaning. Absolute gene expression fold changes for each dietary regime were calculated relative to gene expression in offspring of dams fed the folate adequate diet followed by the low fat diet post-weaning (i.e. absence of nutritional insult). The low maternal folate, high fat post-weaning group displayed the largest overall change across the 642 genes, with significantly larger median fold change compared with the normal folate, high fat group ($p < 2.2 \times 10\text{-}16$) as well as the low folate, low fat group ($p < 2.2 \times 10\text{-}16$).

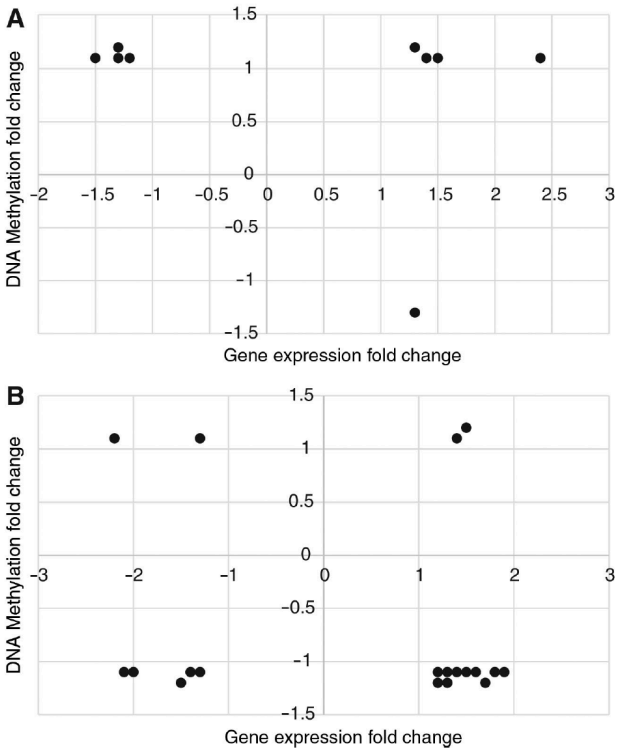

Fig 1

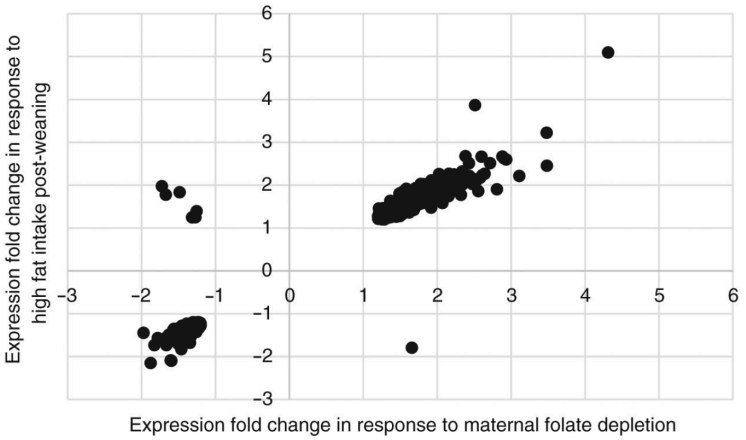

Fig 2

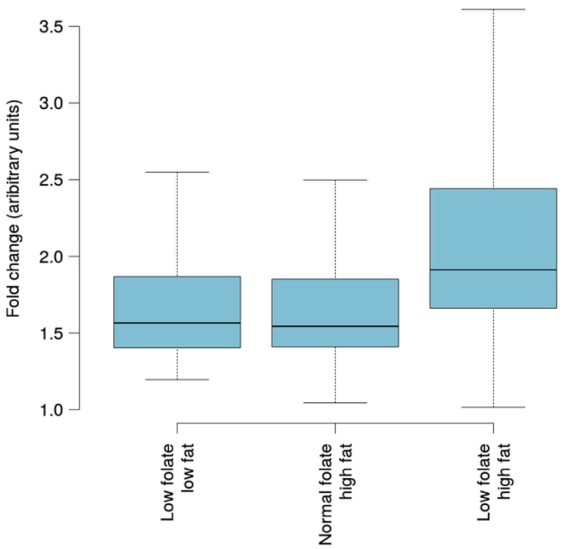

Fig 3