Impact of *in utero* folate exposure on DNA methylation and its potential relevance for later-life health – evidence from mouse models translated to human cohorts.

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Abbreviations; Randomised Control Trial – RCT; Developmental Origins of Health and Disease - DOHaD; Methylated DNA immunoprecipitation – MeDIP; Gene ontology – GO; Kyoto Encyclopedia of Genes and Genomes - KEGG; Epigenome-wide association studies – EWAS; Aberdeen Folic Acid Supplementation Trial - AFAST

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Abstract

Scope: Persistent DNA methylation changes may mediate the effects of early-life exposures on later-life health. However, the human lifespan is challenging for prospective studies, therefore data from longitudinal studies are limited. Projecting data from mouse models of early-life exposure to existing human studies offers a potential tool to address this challenge. Methods and Results: C57BL/6J mice were fed low or normal folate diets before and during pregnancy and lactation. Genome-wide promoter methylation was measured in male offspring livers at 17.5 days gestation and 28 weeks. Eight promoters were concurrently hypermethylated by folate depletion in fetuses and adults (>1.10 fold-change;$p<0.05$). Processes/pathways potentially influenced by global changes, and function of these 8 genes, suggest neurocognitive effects. Human observational and randomized controlled trial data were interrogated for translational findings. Methylation at birth was inversely associated with maternal plasma folate in 6 of the genes (-1.15% to-0.16%/nmol/l;$p<0.05$), whilst maternal folic acid supplementation was associated with differential methylation of 4 of these genes in adulthood. Three CpGs were persistently hypermethylated with lower maternal folate ($p=0.04$). Conclusion: Some persistent folate-induced methylation changes observed in mice were mirrored in humans. This demonstrates utility of mouse data in identifying human loci for interrogation as biomarkers of later-life health.

Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis argues for a causal relationship between early-life environment and disease risk in later-life [1]. The majority of studies in the field have provided evidence for associations between early-life factors and cardiometabolic outcomes [2, 3], but the early-life environment may also shape other health outcomes [4-6].

Currently the mechanisms responsible for the observed relationships between early-life environment and later health outcomes are poorly understood. However, modulation of epigenetic marks, including DNA methylation, is a plausible mechanism mediating these relationships [7]. DNA methylation usually refers to the presence of a methyl group at a cytosine residue followed by a guanine in the DNA sequence (i.e. a CpG dinucleotide site). DNA methylation is an important mechanism of gene regulation through which methylation of CpG rich regions may inhibit binding of the regulatory machinery for transcription leading to gene silencing [7]. DNA methylation patterns are altered in response to a range of environmental cues [8, 9] and may therefore act as a mediator between environment, cell function and disease risk. Early-life development, from preconception to childhood, is a critical window characterized by DNA methylation changes, pronounced susceptibility to environmental factors and programming of epigenetic marks that may have long-lasting health effects [9-13]. Whilst transient epigenetic changes due to environmental factors during early development are likely to influence long-term health through structural and physiological changes, it is also plausible that epigenetic changes which are persistent across the life-course may be latent, and the impact of these revealed only at a later point in the life course [14]. Therefore, such

persistent epigenetic changes may influence health later in life, when their impact is triggered by other intrinsic or external factors such as the biological changes associated with ageing or due to the gradual biological impact of the accumulation of one or more environmental factors e.g. smoking or diet. Currently, there are few studies where the required longitudinal data and/or biological samples are available to investigate persistent changes in methylation from birth to older adulthood in response to early-life exposures, and subsequent influence on health. Therefore, novel approaches must be developed to address these knowledge gaps.

Adequate maternal folate consumption during pregnancy is essential for overall healthy fetal development. Folate depletion peri-conceptually and during early pregnancy is associated with increased risk of neural tube defects [15], where the brain and spinal cord fail to develop correctly, as well as isolated orofacial clefts and neurodevelopmental disorders [16]. Whilst maternal folate intake during pregnancy has been putatively associated with some early outcomes, i.e. child BMI [17], asthma[18, 19], and cognitive or neurodevelopmental outcomes in both childhood [20] and early adulthood [21-23], overall the data are limited. Moreover, there is a further lack of understanding of how maternal folate status during pregnancy may affect offspring health in later life.

Since folate is a key source of methyl groups for synthesis of S-adenosyl methionine (SAM) – the universal methyl donor – it is a plausible candidate nutrient for the modulation of DNA methylation [9]. Indeed, many studies have demonstrated that maternal folate intake during pregnancy influences the offspring methylome [24-28]. However, to the best of our knowledge, no study has used genome-wide assessment to investigate if observed changes in methylation as a result of maternal folate depletion alone during pregnancy are likely to persist from development into adulthood, and therefore with potential to influence later-life health.

Here we used a novel hypothesis-generating approach, guided by animal experimental data, to investigate the potential impact of altered DNA methylation in response to *in utero* folate exposure

for later-life health. In particular, we identified methylation changes in specific gene promotors that are likely to be persistent across the life-course and explored the biological processes and pathways that are likely to be persistently affected using data from previously established mouse models. We then used this 'discovery' phase to inform a translational 'replication' phase (Figure 1) in which we explored the relevance of the findings in a human setting. To do so, we first analysed and compared data examining the influence of maternal folate depletion on offspring DNA methylation during development and in adulthood in a mouse model to uncover methylation changes, and biological pathways and processes potential affected by those methylation changes, mostly likely to be persistent across the life course. The use of animal models offers the advantage of vastly reducing the likelihood of confounding which is a concern for human (observational) epigenetic studies. However, to address question of translatability in a human context, we further aimed to determine whether findings from our mouse model regarding potentially persistent methylation changes in response to maternal folate are translatable to humans. Thus, in our translation 'replication' phase we examined data from two human studies, a meta-analysis and a randomised controlled trial (RCT), which investigated the relationships between maternal folate status/supplementation during pregnancy and DNA methylation of offspring at birth and in adulthood, respectively.

Experimental Section

Dosage Regimen

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 [25, 26, 29, 30]. 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. Female C57BL/6J mice were allocated at random to either a low folate (0.4mg folic acid/kg diet) or normal folate powdered diet

(2mg folic acid/kg diet) offered ad libitum at a quantity of 6g/d/mouse. This regimen was maintained for 4 weeks prior to mating, during pregnancy and, in the case of adult offspring, during lactation until weaning. Diet compositions were modified from AIN-93G24 and have been described previously [29], with 2mg folic acid representing the standard mouse diet, and 0.4mg representing a depleted diet capable of sustaining pregnancy [31]. L-amino acids were used as a protein source. All ingredients, other than folic acid, were included in both diets at the same concentrations to avoid potential confounding through other dietary factors.

Mouse Tissue Collection

Fetal samples: Animal husbandry, sample collection and confirmation of folate depletion [29] have been detailed previously. Briefly, at 17.5 days gestation, dams were killed for collection of fetal livers which were removed, weighed and snap frozen in liquid nitrogen and stored at -80°C until processed for DNA extraction.

Adult samples: Animal husbandry, sample collection and confirmation of folate depletion [30] have been detailed previously. Briefly, adult offspring were killed at 28 weeks of age for tissue collection. The liver was removed, weighed and snap frozen in liquid nitrogen and stored at -80°C. Prior to extraction, livers were ground under liquid nitrogen to preserve DNA integrity and to ensure homogeneous cell population in subsequently extracted DNA samples.

DNA extraction, methylated DNA immunoprecipitation (MeDIP) and DNA methylation array hybridisation from mouse liver samples

Fetal liver DNA was extracted as described previously [25] from male whole fetal livers using Tri-reagent (Sigma-Aldrich) as per manufacturer's instructions. For 6 litters (n=3 per dietary group,) DNA was pooled for three male fetuses per litter (5 µg/fetus) prior to preparation for MeDIP. Adult liver DNA was extracted from 50 mg ground liver tissue from 24 adult males (n=12 per maternal dietary

group) using the E.Z.N.A.® Tissue DNA Kit in accordance with the manufacturer's instructions. The MeDIP protocol has been described in detail elsewhere [32], and description of the protocol used and subsequent data analysis has previously been published for both fetal [25] and adult [26] samples. Resultant data (i.e. lists of gene promoters reported to have altered methylation in response to low maternal folate intake during early-life) from these publications were used for the subsequent analysis outlined below.

Identification of genes with potentially persistent changes in methylation from development to adulthood in response to maternal folate depletion in the mouse liver

Gene promoters identified as having differential methylation in response to maternal folate depletion in fetal and adult liver were compared using an online list comparison tool (http://jura.wi.mit.edu/bioc/tools/compare.php) with gene symbols as the common identifiers. Hypergeometric tests were carried out using the UCLA online calculator (https://systems.crump.ucla.edu/hypergeometric/index.php) to assess the probability that the observed overlapping changes in methylation were not due to a chance observation ($p<0.05$), with an n of 17,446 as the constant population size, i.e. the number of unique genes for which promoter CpG probes are present on the array.

Gene ontology (GO) and pathway analysis of methylation data derived from mouse liver samples

DAVID Bioinformatics Resources 6.8 [33] Functional Annotation tool was used (May 2020) to carry out gene ontology enrichment analysis and to investigate Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways affected by maternal folate depletion through changes in DNA methylation in the fetal and adult liver, in separate analyses. The threshold for significance was set at $p < 0.05$ (uncorrected) for both Gene Ontology and KEGG pathway enrichment analysis. GO

processes and KEGG pathways below the threshold of significance were compared using an online list comparison tool (http://jura.wi.mit.edu/bioc/tools/compare.php) with GO and KEGG numbers as the common identifiers to find overlapping processes and pathways between fetal and adult samples.

Identification of genes with differential DNA methylation associated with maternal folate status in human studies

To investigate the association between maternal folate status and gene-specific DNA methylation in humans, we examined data from two epigenome-wide association studies (EWAS). The first EWAS is a meta-analysis examining the associations between maternal plasma folate status during pregnancy on DNA methylation in cord blood of 1,988 new-borns using the Infinium HumanMethylation450 Beadchip array from two European birth cohorts (the Norwegian Mother and Child Cohort Study (MoBa) and the Generation R study) [24]. The second dataset was from the Aberdeen Folic Acid Supplementation Trial (AFAST), a randomised controlled trial of folic acid supplementation during pregnancy where DNA methylation was measured in 86 adult offspring. The trial, conducted in the late 1960s, used two doses of folic acid (0.2 and 5 mg/day vs placebo) during pregnancy, with intervention starting at antenatal booking at < 30 weeks gestational age [34]. Richmond and colleagues [35] followed up offspring who could be identified and consented at a mean age of 47 years and collected saliva samples for subsequent Infinium HumanMethylation450 Beadchip array analysis. The EWAS analysis investigated associations between exposure to low dose and high dose folic acid supplementation *in utero* in relation to DNA methylation in adulthood. Four models were investigated: Model 1 – intervention (low and high dose) (n=43) vs placebo (n=43); Model 2 – placebo (n=43), low dose (n=20), high dose (n=23) in an ordinal model; Model 3 – low dose (n=20) vs. placebo (n=43); Model 4 – high dose (n=23) vs. placebo (n=43) [35].

Based on the genes identified through the mouse experiments described above, those with known human equivalents were selected for further exploration in the human datasets. We used summary statistic data from these epigenome-wide association studies to investigate the associations between maternal folate status or intake and DNA methylation for CpGs residing within 1500bp upstream of the transcription start site (TSS) of the target genes where data were available in both studies. Methylation differences at CpG sites surpassing $p<0.05$ are highlighted in the results. Hypergeometric tests were carried out using the online calculator to assess the probability that differences in methylation at CpG sites associated with maternal folate status or intake across both studies was not due to chance ($p<0.05$), accounting for the number of CpGs investigated.

Results

Methylation changes in response to maternal folate depletion in murine liver

We previously reported 333 and 201 gene promotors with altered methylation in response to maternal folate depletion in fetal [25] and adult [26] murine liver respectively. Comparison of these altered gene promoters between fetal and adult murine liver resulted in 8 overlapping genes, all of which displayed modest hypermethylation (as shown by a positive fold change in the range of 1.24-1.43 and 1.10-1.25 for fetal and adult murine liver respectively) in response to maternal folate depletion in both fetal and adult mouse tissue (Table 1). A hypergeometric test suggests that this overlap is unlikely to be due to chance (p=0.039).

Gene ontology and KEGG pathways related to methylation changes in response to maternal folate depletion in murine liver

DNA methylation changes in response to maternal folate depletion were found to potentially influence 44 GO processes in the murine fetal liver, and 46 in the adult murine liver (see Supplementary Tables 1 & 2). Of these, six processes were common in both fetal and adult murine liver (Table 2) and five of these processes were interrelated, with 'system processes' as the overall parent term (Figure 2). For all daughter processes, the genes with altered methylation in response to maternal folate in either the fetal or adult murine liver, were all also present in the parent term (see supplementary tables for details of genes affected in each GO term). Moreover, the majority of affected genes in these processes encode olfactory receptors (see supplementary tables). These G-protein coupled receptors[36] drive the changes in the sixth altered process i.e. the G-protein coupled receptor signaling pathway (Table 2). When comparing the fetal and adult murine datasets, two genes were found in common between the processes i.e. *Olfr33* and *Olfr985.*

KEGG pathway analysis highlighted 4 pathways in the murine fetal liver and 5 in the murine adult liver that may have been influenced by methylation changes in response to maternal folate depletion (Tables 3 and 4 respectively) though only 2 and 1, respectively, of these were within the set-imposed threshold for significance in fetal and adult liver respectively. One pathway relating to "Olfactory Transduction", was common to both fetal and adult liver ($p<0.05$ in both), which aligns with the gene ontology analysis. For the murine fetal liver, of the 26 genes related to this pathway, 12 were hypermethylated and 14 hypomethylated, with 25 genes coding for olfactory receptors. Whereas in the murine adult liver, of the 20 genes related to this pathway, 18 were hypermethylated and 2 hypomethylated, with all 20 genes coding for olfactory receptors. The same 2 genes i.e. *Olfr33* and *Olfr985,* that had been identified through the GO analysis were hypermethylated in both murine fetal and adult liver (Table 1).

Influence of maternal folate status on target gene methylation at birth and in adulthood in human studies

To determine whether there was a similar relationship between maternal folate status during pregnancy and gene-specific promoter methylation in humans, we investigated data from two human studies, one in which DNA methylation was measured at birth [24] and one in later adulthood [35] (mean age 47). Seven of the eight genes that had persistent methylation changes in murine liver in response to maternal folate supply have human equivalents or homologues and therefore were assessed in this analysis. Whilst there was no equivalent human gene found for O*lfr33,* the human *OR8D4* gene is 83% homologous with *Olfr985,* and therefore was included in this further investigated. As with the murine study, we investigated putative promoter methylation by focussing on CpGs residing within 1500bp of the transcription start sites (TSS) of the candidate genes (n=118 CpGs in total, see Supplementary Tables 3 and 4 for full list of CpGs and associated data from each study).

At birth, we found that methylation at 11 CpGs for the evaluated genes in cord blood (at least one in 6 of the 7 gene promoters tested) was modestly associated with maternal plasma folate concentration during pregnancy (Table 5) at $p<0.05$. Each one nmol/l increase in maternal plasma folate concentration was associated with 0.16 – 1.15% reduction in methylation at these CpG sites (Table 5). This emulates the patterns observed in our animal model where lower maternal folate status was associated with higher offspring methylation at all 11 CpGs.

Using data from the Aberdeen Folic Acid Supplementation Trial (AFAST) [35] which investigated DNA methylation in adult offspring of mothers who received folic acid supplementation (0.2 and 5 mg folic acid or placebo) during pregnancy, we found modest effects for 9 CpGs across 4 of the target gene promoters with significantly altered methylation ($p<0.05$) in at least one of the four models (Table 6). Six of the nine CpG sites were found to be associated with high dose folic acid (5mg) vs placebo. Seven of the nine CpGs were hypomethylated in response to supplementation in at least one model (Model 1-0.02 to – 1.66%; Model 2 -0.05 to -2.85%; Model 3 -0.02 to -3.34%; Model 4 -0.09 to -4.55%). These findings mirror the direction of methylation change observed in both our mouse model and in human new-borns associated with maternal folate status that are reported above.

Three of these CpGs (Table 7: cg01678833;*TSPO*, cg13160331;*TSPO*, cg09857513;*WNT16*) were hypomethylated in both new-borns and adults in relation to increased maternal folate status or intake, respectively, in pregnancy (Table 5 and Table 6). We carried out a hypergeometric test to ascertain if the overlap between concordant methylation changes observed at birth and in adulthood were likely to be due to chance. With a total of 118 CpGs investigated, finding 11

significant CpGs in one study and 9 significant CpGs in the second study, an overlap of one CpG between studies would be expected by chance. Our finding of 3 CpGs in common between studies suggests that this overlap is not due to chance (p=0.038 for hypergeometric test).

Discussion

Here we used a novel hypothesis-generating approach to investigate whether DNA methylation changes associated with maternal folate status or folic acid intake during development are likely to persist into adulthood. In addition, we investigated the potential for these changes to have relevance to, or be implicated in biological pathways related to, later-life health. As such, we hypothesise that such epigenetic changes may be latent, and only manifest influencing health in later life in response to additional biological triggers as a result of ageing, or external factors such as environmental cues. Whilst many studies have demonstrated the influence of maternal folate status alone during pregnancy on DNA methylation in the offspring [24-28], to the best of our knowledge, none have used genome-wide analysis to investigate potentially persistent changes across the life course. Here we utilised data from a mouse model to uncover 8 genes which were modestly hypermethylated in both the fetal and adult liver in response to maternal folate depletion during pregnancy. Secondly, we found that the promotor regions of 6 of these genes were similarly hypermethylated in offspring at birth from human mothers with low folate status. Thirdly, at least one CpG site within four (*TSPO, RPS16, WNT16, ART3*) of the seven gene promoters was also modestly hypermethylated in the adult offspring of mothers in the placebo group compared with those given folate supplementation during pregnancy in the AFAST study. Furthermore, 3 CpGs in the promoters of two of the seven investigated genes (*TSPO* and *WNT16*) were modestly hypermethylated with lower folate exposure across both human studies i.e. at birth and in adulthood. This is the same direction of change that was observed in the mouse study in response to lower folate exposure. Hypergeometric tests suggest this finding is unlikely to be due to chance,

demonstrating that, whilst effect sizes were modest, maternal folate intake during pregnancy is likely to provoke persistent change in methylation across the life course, and thus may be a latent mechanism influencing health in later life. We therefore suggest that our findings on concomitantly directional methylation changes that were persistent across the life course in both mouse and human studies demonstrate the robustness of the relationship between maternal folate exposure and methylation of these loci, principally *TSPO* and *WNT16*. Thus, we propose that our novel approach is successful in identifying translatable findings from animal studies to human populations in the context of early life exposures.

To investigate the plausible long-term health impacts of these potentially persistent methylation changes associated with maternal folate, we examined i) the biological processes and pathways predicted to be affected by overall promoter methylation changes in our mouse model and ii) the specific functions of the genes with persistent methylation changes in both the mouse model and human studies. Neurological system processes, sensory perception and sensory perception of smell were persistent GO processes affected by folate status or intake, whilst the only persistent KEGG pathway related to olfactory transduction. Previously dysregulation of olfactory receptors and olfactory dysfunction have been associated with dementia or dementia risk in models of Alzheimer's disease (AD) and human studies[37-41]. Interestingly, although outside of the scope of our workflow, several other olfactory receptor genes have altered promotor methylation at birth in response to maternal folate levels in the previous EWAS meta-analysis at FDR significance [24]. However, these genes did not have altered methylation in adulthood in response to maternal folate in the RCT [35], suggesting that methylation changes in these genes are unlikely to be persistent. This inference should be interpreted cautiously because the RCT may have been underpowered to detect DNA methylation changes in adulthood. Therefore, we suggest that the role of maternal

folate intake during pregnancy on later life epigenetic profiles of genes encoding olfactory receptors warrants further investigation.

Next we consider the biological roles of the proteins encoded by the genes that were hypermethylated with lower folate status in mouse and human studies (i.e. *TSPO*, *RPS16*, *WNT16*, *ART3*, *PCDHB6* and *PDLIM3*). Disruption of cell adhesion pathways involving *Pcdhb6* occur in Ms5Yah mice which exhibit motor coordination deficits, and spatial learning and memory impairments [42]. In addition, there is evidence that Wnt16 is a key substrate through which cannabis extracts preserve memory and reduce learning impairment in a mouse model of Alzheimer's disease [43]. Furthermore, evidence suggests that *PDLIM3* is a genetic modifier of age at onset in Alzheimer's disease [44], whilst the 18kDa translocator protein (i.e. TSPO, a marker of neuroinflammation), may be a predictive marker of amyloid pathology linked to Alzheimer's disease [45]. We therefore postulate that the persistent methylation changes observed in our study may plausibly influence neurocognitive outcomes in later life via latent epigenetic marks. We hypothesise that the biological impact of these epigenetic marks may have delayed manifestation in response to subsequent triggers that may include the gradual biological impact of the accumulation of one or more environmental factors (e.g. smoking or diet) or due to other biological changes associated with ageing. Indeed, epigenetic factors have been proposed as one of the key mechanisms in the 'Latent Early-life Associated Regulation' (LEARn) model of Alzheimer disease and dementia development [46] in which maternal nutrition plays a fundamental role in shaping the epigenetic landscape for cognitive function [47]. Therefore, we propose methylation of the genes that we have identified may serve as long term proxy markers of maternal folate status and, given the potential role of the identified genes in neurological processes, should be prioritised for investigation in relation to cognitive function in older adults. However, as far as we are aware, the required longitudinal data (i.e. maternal folate status, epigenetic data at birth and in later life and later life cognitive function)

that would be needed to test the hypothesis that maternal folate affects later life cognition through effects on DNA methylation that persist into adulthood, do not exist. Therefore, novel methodologies will be required to investigate the potential impact of maternal folate status on DNA methylation and cognitive function of offspring in later life. Having uncovered methylation of these loci as a highly plausible maternal folate-related biomarker, it should be feasible to test hypotheses about the relationship between methylation of these genes and cognitive function in older adults using a modified 'meet in the middle' approach[48]. Furthermore, two step Mendelian Randomisation[49], using genetic information as a surrogate for maternal folate-related methylation, could also be used to investigate if maternal folate influences later-life cognitive function via methylation of these loci.

Here we have illustrated how focused examinations of a single nutrient exposure, which is possible in an animal model, may inform more targeted investigations in a human setting. In human studies, this approach may help to reduce 'noise' from confounding factors that have potential to result in false positive associations. Similarly, in accordance with the Bradford-Hill criteria in assessing causal relationships, the coherence of findings between experimental and epidemiological studies increases the likelihood of an effect, and therefore may aid identification of false positives. Additionally, targeted studies that investigate a limited number of key loci reduces the requirement for stringent statistical rationalisation to account for multiple testing that, in epigenome-wide studies, may mask true positive associations. Therefore, a significant advantage of this approach is in increasing the plausibility in uncovering true associations for further study.

One of the key outcomes of this study is proof-of-concept for the utility of mining data and samples collected from animal studies in informing targeted analyses in human cohorts. More specifically, this approach has the advantage of being able to being to address knowledge gaps relating to the

persistence of methylation change in response to early-life exposures for which the necessary human longitudinal data and biological samples are unavailable. Further strengths of this approach are in the ability to generate new hypotheses from previously generated data sets, whilst simultaneously identifying potentially key biomarkers for further study. Moreover, such secondary analyses of data from animal studies adds value to prior research and is in line with the 3Rs.

It is pertinent to note the design of the animal study precluded the ability to assess pups from the same litter over time. Likewise, data from human studies are from different cohorts and therefore do not allow direct longitudinal measurement of persistent methylation change. Hence, here we refer to concomitant changes as 'potentially persistent'. A further limitation of the study was the inability to associate findings with any potential health related outcomes. Use of such retrospectively collected data from mouse models means that new hypotheses cannot be tested in the mice from which data were initially generated. Similarly, for human studies, relevant outcome data sets may not be collected or be publicly available to access.

To conclude, the methodologies employed here maximised the utility of previously generated data, in line with the ethos of both the 3Rs and adding value in research, whilst simultaneously uncovering concomitant methylation changes between species in response to maternal folate during pregnancy that are likely to be persistent across the life-course and to influence health. As such, this approach facilitates research in areas where there are major knowledge gaps and for which the relevant data from longitudinal studies in humans are unavailable. Consequently, this work has generated novel hypotheses to be tested in future studies with the potential to provide predictive biomarkers related to health. The robustness of these findings suggest that this innovative approach could be used in other research contexts in future.

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Author Contributions

J.A.M., D.E.K. and R.C.R. analysed data. J.A.M., D.E.K. and R.C.R. wrote the manuscript with contributions from M.A., C.T.E., N.R., D.F., and J.C.M. J.A.M. had primary responsibility for final content. All authors have read and approved the final manuscript.

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Conflict of Interest Statement

All authors confirm no conflicts of interest.

Table 1. Genes with altered methylation in both fetal and adult murine liver in response to maternal folate depletion during early development.

	Fetal liver		Adult liver	
Gene symbol	P-val	Fold Change*	P-val	Fold Change*
Art3	0.020	1.34	0.013	1.13
Olfr33	0.039	1.31	0.024	1.25
Olfr985	0.035	1.24	0.015	1.15
Pcdhb6	0.006	1.43	0.036	1.13
Pdlim3	0.019	1.41	0.044	1.10
Rps16	0.037	1.30	0.005	1.11
Tspo	0.016	1.31	0.033	1.15
Wnt16	0.003	1.34	0.027	1.16

*Differential DNA methylation fold change in response to low maternal folate compared to normal maternal folate

Table 2. Gene Ontology processes predicted to be altered due to DNA methylation changes in response to maternal folate depletion in both the fetal and adult murine liver

		Fetal liver		**Adult liver**	
GO ID	GO Process	Number of genes altered/number of genes in process	P-val	Number of genes altered/ number of genes in process	P-val
GO:0003008	system process	44/2696	0.036	45/2696	5.79E-06

GO:0007186	G-protein coupled receptor signaling pathway	41/1919	4.76E-04	32/1919	2.44E-04
GO:0007600	sensory perception	30/1691	0.037	35/1691	1.09E-06
GO:0007606	sensory perception of chemical stimulus	25/1281	0.023	24/1281	4.13E-04
GO:0007608	sensory perception of smell	25/1143	0.006	23/1143	2.15E-04
GO:0050877	neurological system process	35/2077	0.044	40/2077	8.04E-07

Table 3. KEGG pathways predicted to be altered due to DNA methylation changes in response to maternal folate depletion in the fetal murine liver

KEGG ID	KEGG Pathway	Number of genes altered / no of genes in pathway	P-val	Genes altered
mmu04740	**Olfactory transduction**	**26 / 1080**	**0.016**	***OLFR985, OLFR963, OLFR130, PRKG1, OLFR784, OLFR692, OLFR656, OLFR800, OLFR722, OLFR557, OLFR558, OLFR531, OLFR341, OLFR1367, OLFR146, OLFR1308, OLFR598, OLFR1009, OLFR370, OLFR76, OLFR59, OLFR33, OLFR397, OLFR1023, OLFR878, OLFR53***
mmu04713	Circadian entrainment	5 / 98	0.059	*GNGT2, GNAO1, ADCY6, GUCY1B3, PRKG1*
mmu04730	Long-term depression	4 / 61	0.063	*PPP2R1A, GNAO1, GUCY1B3, PRKG1*
mmu04062	Chemokine signaling pathway	7 / 196	0.073	*CCL11, CCL12, GNGT2, NCF1, ADCY6, CX3CR1, CRK*

Table 4. KEGG pathways predicted to be altered due to DNA methylation changes in response to maternal folate depletion in the adult murine liver

KEGG ID	KEGG Pathway	Number of genes altered / no of genes in pathway	P-val	Genes altered
mmu04740	**Olfactory transduction**	**20 / 1080**	**0.006**	***OLFR985, OLFR976, OLFR313, OLFR739, OLFR649, OLFR1378, OLFR1500, OLFR1423, OLFR1339, OLFR1377, OLFR1497, OLFR633, OLFR102, OLFR33, OLFR101, OLFR201, OLFR13, OLFR328, OLFR73, OLFR1408***
mmu00980	**Metabolism of xenobiotics by cytochrome P450**	**4 / 64**	**0.023**	***GSTM4, HSD11B1, ADH5, UGT1A1***
mmu04080	Neuroactive ligand-receptor interaction	7 / 285	0.056	*ADRB3, P2RX4, ADRB2, TSPO, AGTR1A, GLRA2, CHRNB4*
mmu05204	Chemical carcinogenesis	4 / 92	0.059	*GSTM4, HSD11B1, ADH5, UGT1A1*
mmu04022	cGMP-PKG signaling pathway	5 / 163	0.072	*ADRB3, ADRB2, AGTR1A, MAP2K2, MYL9*

Table 5. CpG sites within 1500bp of the transcription start sites of 6 target genes at which methylation status in new-borns was associated ($p<0.05$) with maternal plasma folate status during pregnancy (original data from Joubert *et al.,* 2016 [24]).

CpG	**Coefficient***	**Standard error**	**P-val#**	**Gene symbol**
cg14530295	-0.0016	6.00E-04	0.007113	*ART3*
cg26866168[α]	-0.0115	0.0027	2.98E-05	*PCDHB6*
cg16622906	-0.005	0.0021	0.01782	*PCDHB6*
cg02515725	-0.0021	8.00E-04	0.0143	*PDLIM3*

cg14632696	-0.0053	0.0025	0.03146	*PDLIM3*
cg20641794	-0.0054	0.0023	0.02195	*RPS16*
cg13160331	-0.0046	0.0013	0.0004358	*TSPO*
cg01678833	-0.0043	0.0016	0.008172	*TSPO*
cg09857513	-0.0031	0.0011	0.006079	*WNT16*
cg16868298	-0.0028	0.001	0.006452	*WNT16*
cg25608490	-0.0026	0.0012	0.02599	*WNT16*

*Coefficient represents methylation beta value

#P-val not adjusted for multiple testing using the false discovery rate (FDR) due to targeted investigation

αSurpasses correction for multiple testing taking into account the 118 CpGs investigated

Table 6. CpG sites within 1500bp of the transcription start sites of 6 target genes at which methylation status in adulthood (mean age 47) was related ($p<0.05$) to maternal folate supplementation during pregnancy (original data from the AFAST study [35]).

	Model 1 - intervention (low and high$^{\phi}$ dose) vs placebo			Model 2 - low dose, high dose and placebo (ordinal model)			Model 3 - low dose vs placebo			Model 4 - hig placebo	
Gene	Coef*	SE	P-val*	Coef*	SE	P-val*	Coef*	SE	P-val*	Coef*	SE
TSPO	-0.0041	0.0072	0.571	-0.0238	0.0119	**0.049**	-0.0084	0.0141	0.552	-0.0455	0.
TSPO	-0.0002	0.0002	0.398	-0.0005	0.0003	0.135	-0.0002	0.0004	0.546	-0.0009	0.
TSPO	-0.0012	0.0036	0.744	-0.0072	0.0060	0.235	-0.0023	0.0073	0.756	-0.0149	0.
RPS16	-0.0034	0.0033	0.308	-0.0098	0.0055	0.079	-0.0033	0.0062	0.596	-0.0158	0.
RPS16	-0.0015	0.0007	**0.029**	-0.0015	0.0012	0.190	-0.0027	0.0013	**0.049**	-0.0009	0.
RPS16	0.0005	0.0014	0.720	0.0024	0.0023	0.310	-0.0006	0.0018	0.764	0.0062	0.
WNT16	0.0042	0.0036	0.250	0.0101	0.0060	0.099	0.0090	0.0075	0.237	0.0155	0.
WNT16	-0.0071	0.0043	0.104	-0.0101	0.0073	0.172	-0.0187	0.0084	**0.029**	-0.0071	0.
ART3	-0.0166	0.0073	**0.025**	-0.0285	0.0123	**0.024**	-0.0334	0.0147	**0.027**	-0.0197	0.

*Coefficient represents methylation beta value. *P-val not FDR corrected due to targeted investigation
#Altered methylation observed in newborns in relation to maternal plasma folate by Joubert et al., 2016[24] (Table 5).
$^{\phi}$ Low dose folate supplementation was 0.2mg per day and high dose 5mg per day.

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Table 7. Genome annotation the 3 CpG sites identified in relation to maternal folate status or intake in new-borns as well as adults

CpG	Chromosome	Position*	Gene	Gene group
cg01678833	22	43547171	*TSPO*	TSS1500
cg13160331	22	43547217	*TSPO*	TSS1500
cg09857513	7	120969044	*WNT16*	TSS200; Body

*based on genome build 37

Figure 1. Study overview. In the initial discovery phase of the study, data generated from our previously published mouse model of folate depletion in pregnancy were used to establish a) genes with altered methylation in response to maternal folate depletion in ***both*** the fetal and adult liver, and therefore most likely to be persistently changed b) potential biological pathways and processes

that may have been influenced by the combination of 'transient' and 'persistent' methylation changes in response to maternal folate depletion in the fetal and adult liver separately and c) which biological pathways and processes are likely to be 'persistently' altered in response to overall methylation changes observed in response to maternal folate depletion. This therefore is the hypothesis-generating phase, whereby investigating the function of the genes with persistently altered methylation coupled with understanding of the probable 'persistent' biological effects offers insight into the potential health outcomes for future investigation. In the second translational 'replication' phase, we investigated if the potentially persistent methylation change observed in our mouse model are relevant in a human context. To do so, we utilised methylation data from a meta-analysis and a randomised controlled trial (RCT), which investigated the relationships between maternal folate status/supplementation during pregnancy and DNA methylation of offspring at birth and in adulthood, respectively.

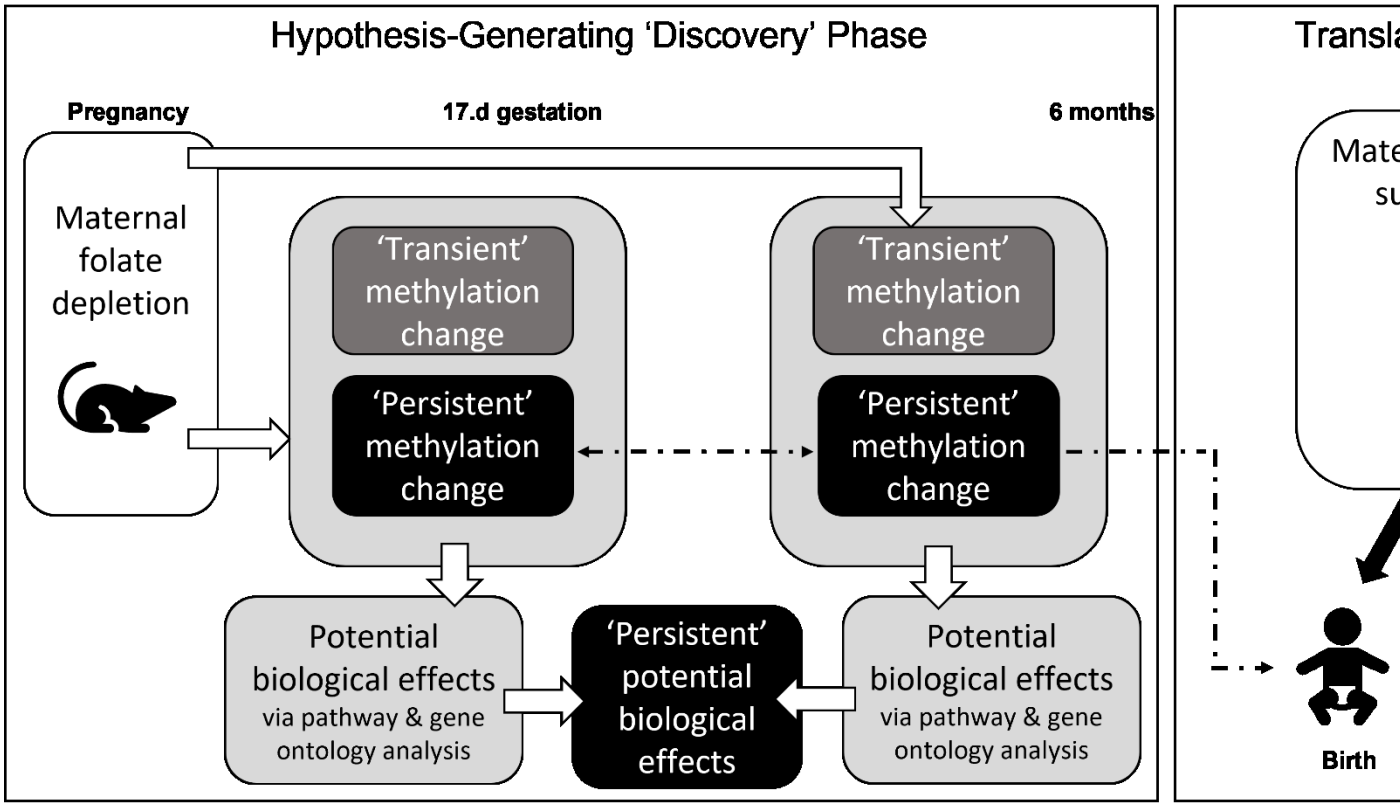
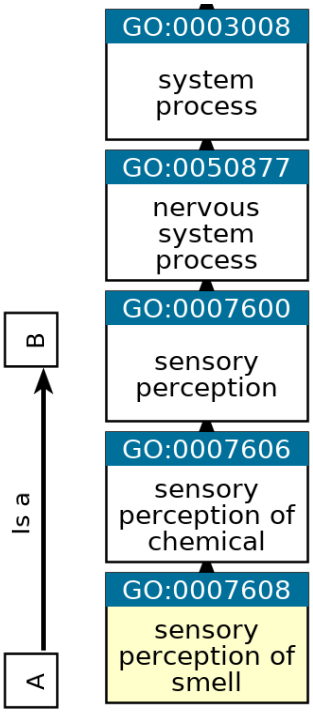


Figure 2. Flow diagram depicting the relationships between overlapping GO processes related to methylation changes in response to maternal folate depletion in murine fetal and adult liver. The boxes marked A and B connected by an arrow represent the relationship between the GO process i.e. in this diagram this represent that GO:0007608 – sensory perception of smell is a GO:0007606 – sensory perception of chemical. Therefore, here the 'parent' term overall is GO:0003008 – system process, with the downstream 'daughter' processes being the next level of depth regarding the exact biological process affected. Modified from QuickGO (https://www.ebi.ac.uk/QuickGO). Whilst all genes altered on daughter processes were also observed on parent processes, parent process had additional altered genes.



Methylation refers to DNA markers responsible for gene-activation. We investigated if methylation changed in response to maternal folate intake during pregnancy. Eight genes had increased (hyper)methylation in fetal and adult mouse liver in response to maternal folate depletion. In humans, two of these genes were consistently hypermethylated with lower folate during pregnancy, the function of which suggests implications for cognition.