



Horizons in Nutritional Science

Folic acid supplementation in pregnancy: are there devils in the detail?

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Abstract

Maternal folic acid (FA) supplementation is well recognised to protect against neural tube defects. Folate is a critical cofactor in one-carbon metabolism involved in the epigenetic regulation of transcription that underpins development. Thus, it is possible that maternal FA supplementation may have additional, unforeseen persistent effects in the offspring. This is supported by the modification by maternal supplementation with one-carbon donors and FA of the epigenetic regulation of offspring phenotype in mutant mice. The present article reviews studies in human subjects and experimental animals of the effect of maternal FA intake and phenotypic outcomes in the offspring. Maternal FA intake was associated with a short-term increased incidence of allergy-related respiratory impairment in children and multi-generational respiratory impairment in rats. Higher maternal folate status during pregnancy was associated positively with insulin resistance in 6-year-olds. In rats, maternal FA supplementation modified hepatic metabolism and vascular function through altered transcription, in some cases underpinned by epigenetic changes. FA supplementation in pregnant rats increased mammary tumorigenesis, but decreased colorectal cancer in the offspring. Maternal FA supplementation decreased a range of congenital cardiac defects in children. These findings support the view that maternal FA supplementation induces persistent changes in a number of phenotypic outcomes in the offspring. However, the number of studies is limited and insufficient to indicate a need to change current recommendations for FA intake in pregnancy. Nevertheless, such effects should be investigated thoroughly in order to support firm conclusions about the risk of unanticipated long-term negative effects of maternal FA supplementation in humans.

Key words: Folic acid; Epigenetics; Disease risk

Folates are a group of B-vitamins differing in oxidation state, addition of one-carbon moieties and length of the poly-glutamate chain; they are found predominantly in fruits and vegetables. Folic acid (FA) is the synthetic oxidised monoglutamyl form of folate that is widely used in vitamin supplements and in the fortification of foods. Within cells, folates act as cofactors in numerous reactions related to growth and repair requiring the transfer of one-carbon units, principally methyl or formyl groups. These include purine and pyrimidine biosynthesis, and via the homocysteine–methionine methylation cycle, phosphatidylcholine biosynthesis by phosphatidylethanolamine *N*-methylation and the epigenetic control of gene transcription by DNA and histone methylation.

Much of the interest in FA lies in the impact of dietary supplementation and fortification strategies in reducing the

incidence of neural tube defects (NTD)⁽¹⁾. NTD, the most common congenital malformation, are caused by the failure of the neural tube to close during embryogenesis between post-conception days 21 and 28, resulting in fatal anencephaly or spina bifida with varying levels of disability. The estimated global incidence of NTD is about >300 000 new cases per year with an estimated 41 000 deaths⁽²⁾. To date, the precise cause of NTD is not known, but is thought to represent the interaction between genetic disposition and environmental factors, including nutrition⁽¹⁾.

FA was first proposed as a means of preventing NTD in 1964⁽³⁾. In four out of five subsequent case–control studies of FA supplementation together with other vitamin supplements and in one non-randomised prospective cohort study in women who did not have a previous NTD-affected

Abbreviations: A^{VY/a}, agouti ‘Viable Yellow’; FA, folic acid; NTD, neural tube defect; PR, protein restricted.

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pregnancy, reductions of 35–75% in the incidence of NTD have been reported^(4–7). Similar positive outcomes have also been reported in non-randomised and randomised recurrence trials^(8–12). Based on this evidence, a number of countries have introduced recommendations for FA supplementation before and during the first trimester at 400 µg/d increasing up to 5 mg/d for women with a previous NTD-affected pregnancy. Unfortunately, the effectiveness of recommendations for FA supplementation in reducing the incidence of NTD has been less than that indicated by the trials⁽¹³⁾. This reflects the findings that, for example, about 50% of pregnancies in the USA are unplanned⁽¹⁴⁾ and less than 25% of women follow the health guidelines^(15,16). Consequently, fifty-three countries have regulations for mandatory fortification of flour with FA (typically between 40 and 220 µg/100 g), although these are not implemented in all nations⁽¹⁷⁾. Implementation of FA fortification has, in general, led to significant increases in folate status and reductions in NTD incidence, although the effectiveness appears to be greatest in populations with the highest incidence of NTD⁽¹⁷⁾.

FA is generally considered safe, with a tolerable upper intake level of 1 mg/d and a lowest observed adverse effect level of 5 mg/d⁽¹⁸⁾. Even intakes of 15–100 mg/d are only associated with limited direct toxicity^(19–22). Thus, using FA supplements during pregnancy is viewed as a safe and potentially effective means of reducing the incidence of NTD and the associated cost in terms of human suffering and economic burden. Folate is an essential cofactor for the synthesis of purines and thymidylate, and thus plays an important role in DNA synthesis and replication. Folate also plays a central role in epigenetic processes that regulate the transcription of all genes and, in turn, the phenotype of an organism. Folate restriction and supplementation modify epigenetic marks, in particular DNA methylation, by a mechanism that involves DNA methyltransferase activity but has not been described completely. This raises a potential concern that FA supplementation during pregnancy to reduce the risk of NTD may induce unforeseen changes in the methylome and phenotype of the offspring. The purpose of the present review is to draw on studies in human subjects and in animal models to evaluate the effect of FA supplementation during pregnancy on phenotypic and molecular outcomes in the offspring and to indicate gaps in current knowledge.

Folate and the epigenetic regulation of transcription

Epigenetic processes (DNA methylation, covalent modification of histones and non-coding RNA) regulate gene expression over time scales ranging between minutes for some genes involved in acute energy production⁽²³⁾ to the entire life course for imprinted genes and genes involved in maintaining cell differentiation⁽²⁴⁾. Folate is a critical cofactor for the provision of methyl groups via the homocysteine–methionine methylation cycle for induction and maintenance of both DNA and histone methylation. It is likely, therefore, that any effects of maternal FA supplementation on the phenotype of the offspring would be mediated via an epigenetic mechanism. There is some evidence which shows that variations in

dietary FA can modify the activities of DNA methyltransferases and histone methyltransferases. DNA methylation and histone modification are processes that are intimately linked and while a number of studies have shown that DNA methylation can direct histone modification⁽²⁵⁾, other studies have shown that histone modifications, particularly histone methylation, precede DNA methylation⁽²⁶⁾. To date, studies have focused almost exclusively on the effect of FA supplementation on the DNA methylome, although many of the effects of FA may be initiated through the altered activities of histone methyl transferases and histone methylation. Such studies in animal models, which are summarised below, have shown a causal link between maternal FA supplementation, and the epigenome and phenotype of the offspring. However, at present, only one study has reported the effects of maternal FA supplementation on epigenetic marks in children. Fryer *et al.*⁽²⁷⁾ have shown using an unbiased analysis of 27 000 CpG loci in twelve pregnancies, of which eight out of twelve women took FA supplements, that methylation of specific CpG dinucleotides within LINE-1 elements (a surrogate for genome-wide methylation) was associated with birth weight and cord plasma homocysteine concentration, although the direction of association differed between CpG loci. Of the 540 such loci, sixteen were associated with all three outcomes. These findings support a causal association between maternal FA supplementation, epigenetic regulation and offspring phenotype, although the mechanism that links variation in DNA methylation and birth weight was not investigated. Furthermore, since variation in birth weight has been associated with the differential risk of non-communicable diseases⁽²⁸⁾, these findings point towards a potential association between maternal FA intake and vulnerability to future disease.

Maternal methyl donor supplementation in mutant mice

There are two mouse models that provide clear examples of the effect of maternal methyl donor intake on the phenotype of the offspring. Agouti ‘Viable Yellow’ (A^{VY/a}) and Axin(Fu) bear naturally occurring mutations involving transposons where DNA methylation controls the activity of cryptic promoters linked to the mutant phenotype. A^{VY/a} in which the proximal intracisternal A particle long terminal repeat is hypomethylated have yellow coat colour and an increased tendency towards obesity, some cancers and shorter lifespan compared with black-coated A^{VY/a} mice in which the intracisternal A particle promoter is hypermethylated. Feeding pregnant dams a diet containing increased amounts of methyl donors and FA induces the switching of the offspring phenotype from the yellow- to black-coated phenotype⁽²⁹⁾. Similarly, supplementation of the diet of dams with methyl donors switches the phenotype of the offspring carrying the Axin(Fu) epiallele, reducing the incidence of tail kinking by half⁽³⁰⁾. Although these models represent extreme cases, they point towards the potential for vulnerability due to the rather surprising level of plasticity in the epigenome to nutritional inputs by which methyl donors and cofactors, including FA, in the maternal diet induce persistent changes in the phenotype of the offspring.

Effect of maternal folic acid supplementation on offspring allergy disease

A number of studies have investigated the extent to which maternal FA supplementation is associated with the risk of respiratory disease risk or allergy in childhood. Haberg *et al.*⁽³¹⁾ found using self-reported questionnaires in 32 077 subjects that maternal FA supplementation during the first trimester significantly increased the relative risk of wheeze by 6%, of lower respiratory tract infection by 9% and of hospitalisation due to lower respiratory tract infection by 24% up to 18 months of age. Maternal FA supplementation between 30 and 34 weeks of gestation was associated with a 26% increase in the relative risk of physician-diagnosed asthma at 3·5 years among 490 children, but not at 5 years, while there was no association with FA supplementation before 16 weeks of gestation⁽³²⁾. In a study of 628 pregnancies, a supplemental intake of > 500 µg FA/d was associated with an 85% greater risk of allergic disease, mainly eczema, than taking < 200 µg/d⁽³³⁾. High maternal folate status during pregnancy has also been associated with a 30% increase in the risk of childhood atopic dermatitis in a study of 8742 children, although there was no association with FA supplementation⁽³⁴⁾. This suggests that FA rather than folate may be the important factor in determining the risk of allergic disease. However, other studies have not supported these findings. Martinussen *et al.*⁽³⁵⁾ found that FA supplementation during the first trimester of pregnancy in 1499 women had no association with asthma in the children at 6 years of age, which is consistent with the findings of Whitrow *et al.*⁽³²⁾ in 5-year-old children. Magdelijns *et al.*⁽³⁶⁾ also did not find a significant association between maternal FA supplementation and wheeze, lung function or asthma in 2834 children aged 6–7 years. Furthermore, there was no association in a study of 3786 children between maternal FA supplement use during the first trimester and asthma, wheeze, lower respiratory tract infections or eczema at 8 years, although there was a transient association with wheeze (prevalence ratio 1·20) at 12 months⁽³⁷⁾. Serum folate status at 2 years of age has been shown to be related inversely to total serum IgE concentration, atopy and wheeze in a study of 8083 children⁽³⁸⁾. Thus, subsequent folate intake may either modify or supersede the effect of maternal FA intake during early pregnancy on allergic outcomes. Together, these findings suggest that maternal FA supplementation may affect the risk of allergy in young children, but that this effect decreases in older children, possibly reflecting the maturation of the immune system.

Adult F₁ offspring of mice fed a diet with high FA content (17 mg/kg feed) during pregnancy showed greater airway responsiveness to the muscarinic receptor agonist methacholine, which is a characteristic of patients with asthma, compared with the offspring of dams fed a diet with an adequate FA content (2 mg/kg feed)⁽³⁹⁾. Airway challenge with nebulised ovalbumin induced a greater lung allergic response in the offspring of dams fed the high-FA diet than those of dams fed adequate FA. These effects were transmitted to the F₂ generation via the male line, which implies changes in epigenetic marks in germ cells. Increased airway function

responsiveness was associated with hypermethylation of the runt-related transcription factor-3 promoter that suggests a causal mechanism between maternal FA intake and impaired respiratory function⁽³⁹⁾. However, the difference in FA intake between supplemented and control dams was substantially greater in this study than between women who do or do not take FA supplements during pregnancy. This may explain why the effects of FA were persistent in mice, but decreased with increasing time in human subjects. Nevertheless, these findings in mice support an effect of prenatal FA exposure on future respiratory function via a plausible epigenetic mechanism.

Effect of maternal folic acid supplementation on metabolic outcomes in the offspring

In two studies, the relationship between maternal FA intake and metabolic outcomes in children has been examined. No relationship was found between maternal FA intake and body composition in 5783 children aged 9 years⁽⁴⁰⁾ or insulin resistance in 1132 children aged between 6 and 8 years, even in cobalamin-deficient mothers⁽⁴¹⁾. However, maternal erythrocyte folate status at 28 weeks in 700 women was associated positively with insulin resistance in children at 6 years, which the authors suggest reflected adherence to the recommendation that pregnant women in India take 500 µg FA and 60 mg Fe daily from 18 weeks of gestation to prevent anaemia, which was exacerbated by lower cobalamin status⁽⁴²⁾. Hoyo *et al.*⁽⁴³⁾ found in a study of 438 women that methylation of the H19 differentially methylated region, which regulates insulin-like growth factor-2 expression, in cord blood leucocytes was reduced by 2·8% in 428 women who took FA supplements before conception and by 4·9% in 223 women who took FA supplements after conception, compared with those who did not take FA supplements, with a significant offspring sex difference, although phenotypic outcomes have not been reported. These findings support the suggestion that the effects of maternal FA supplementation on the phenotype of the offspring are mediated via epigenetic changes, but the precise nature of the effect may be influenced by the environmental context.

Despite the conflicting findings from human studies, investigations in animal models have shown a consistent effect of increased maternal FA intake on gene expression in the offspring. Moderate restriction of maternal protein intake in rodents has been shown to induce a number of phenotypic changes in the offspring⁽⁴⁴⁾. Increasing FA intake by 5-fold in dams fed a protein-restricted (PR) diet prevented altered promoter methylation and the expression of genes encoding hepatic PPAR α and the glucocorticoid receptor in the juvenile offspring by the PR diet alone⁽⁴⁵⁾. However, sequence analysis of the PPAR α promoter in the liver of the offspring of dams fed the PR diet supplemented with FA showed hypermethylation of specific CpG loci⁽⁴⁶⁾. One possible implication is that some epigenetic changes induced in early life may be cryptic under normal physiological conditions, but may contribute to an altered phenotype when the organism is challenged by environmental stress. If so, this suggests a mechanism by



which epigenetic changes may exert little short-term effect, but may contribute to future changes in phenotype such as altered health status. Furthermore, analysis of the liver transcriptome identified 175 genes which differed between the adult male offspring of dams fed a FA-supplemented diet during pregnancy and controls including the down-regulation of genes in the antioxidant ontology and the up-regulation of genes in the fatty acid metabolism ontology⁽⁴⁷⁾. These findings provide direct evidence for induction by FA exposure during development of persistent changes in hepatic gene activity. Such effects could, for example by reducing antioxidant defence, induce vulnerabilities that are not pathogenic *per se*, but may contribute to increased disease risk. Hoile *et al.*⁽⁴⁸⁾ found that maternal FA supplementation in rats induced altered methylation of specific CpG loci in the hepatic phosphoenolpyruvate carboxykinase promoter in adult female, but not male, rat offspring in a manner which differed directionally between CpG dinucleotides. Chmurzynska *et al.*⁽⁴⁹⁾ found that a 5-fold increase in FA intake during pregnancy decreased body weight at 16 weeks of age, which was accompanied by significant effects on blood lipids and decreased expression of liver PPAR α , PPAR γ and the liver-X-receptor- α , and lower hepatic phosphatidylethanolamine-*N*-methyltransferase, betaine-homocysteine methyltransferase and cystathionine β -synthase⁽⁵⁰⁾.

The effect of FA supplementation on the phenotype of the offspring appears to be modified by the overall nutrient composition of the diet. Feeding pregnant rats a PR diet induced increased systolic blood pressure, impaired acetylcholine-mediated vasodilatation and reduced endothelial NO synthase mRNA expression⁽⁵¹⁾. These changes in vascular function were prevented by supplementation of the maternal PR diet with 5 mg/kg feed of FA. However, there was no effect of FA supplementation on vascular function in the offspring of dams fed a diet with adequate protein content⁽⁵¹⁾. In contrast, the offspring of rats fed a PR diet supplemented with FA also showed reduced post-weaning weight gain, and lower fasting plasma TAG, NEFA, β -hydroxybutyrate and glucose concentrations than the offspring of dams fed the PR diet containing adequate FA⁽⁵²⁾. However, FA supplementation of the control, protein-sufficient diet induced increased fasting plasma lipid and glucose concentrations⁽⁵²⁾. One implication of these findings is that the effects of maternal FA supplementation on the offspring may differ between tissues to the extent of inducing opposing effects that are consistent with differences in the epigenome between cell types. This represents a potential major challenge for making nutritional recommendations to the general public and for the design of studies in human subjects because the relevant tissues may not be readily accessible and thus the effects of maternal FA supplementation may not be detected.

Effect of maternal folic acid supplementation on cancer-related outcomes in the offspring

Although FA fortification has been the focus of investigation with respect to colorectal cancer⁽⁵³⁾, there is limited

information about the effect of maternal FA supplementation on cancer risk in the offspring. A case-control study of the relationship between maternal FA supplementation and the incidence of acute lymphoblastic leukaemia in 393 children up to 14 years of age (1249 controls) found no significant effect of increased FA intake before or during pregnancy⁽⁵⁴⁾. This finding was supported by a meta-analysis of similar studies, although vitamin supplementation, in general, was associated with a significant reduction in risk (OR 0.83). The authors conclude that any effect of multivitamin supplements on acute lymphoblastic leukaemia is unlikely to be due to FA specifically.

Feeding rats a diet containing 5 mg/kg feed of FA before pregnancy and during pregnancy and lactation increased the rate of formation and the number of mammary tumours induced by 7,12-dimethylbenz[*a*]anthracene and was associated with lower global DNA methylation in tumours from the adult offspring at 28 weeks of age compared with the offspring of dams fed a diet containing 2 mg/kg feed⁽⁵⁵⁾. In contrast, FA supplementation during pregnancy and lactation in mice reduced the number of terminal end buds, the structure that gives rise to mammary tumours, which suggests a lower risk of mammary tumours⁽⁵⁶⁾. One important difference between these studies is whether or not a tumour-inducing agent was used. Thus, differences in the effect of maternal FA supplementation on mammary cancer may reflect the latency period before tumour formation and/or the interactions between the methylome and the carcinogen. Using the same dietary design as Ly *et al.*⁽⁵⁵⁾, maternal FA supplementation was also found to decrease the risk of azoxymethane-induced colorectal cancer by 64% in 31-week-old offspring⁽⁵⁷⁾. These findings emphasise the potential for the tissue-specific effect of FA supplementation in humans.

Effect of maternal folic acid supplementation on congenital heart defects

In two studies, it has been investigated whether maternal periconceptional FA supplementation protects against congenital heart malformations. A case-control study of the effect of periconceptional multivitamin supplementation involving 958 cases and 3029 controls showed a 24% reduction in the risk of non-syndromic cardiac defects, a 54% reduction in the risk of outflow tract defects and a 39% reduction in ventricular septal defects⁽⁵⁸⁾. A Hungarian randomised trial of periconceptional multivitamin supplementation also showed a 58% reduction in overall reduction in the risk of congenital heart defects⁽⁵⁹⁾. Although both studies used supplements containing FA, neither was able to investigate the effects of individual components of the supplements. However, a recent case-control study designed to test the specific effect of FA supplementation showed an overall 18% reduction in congenital heart defects, with a 38% reduction in septal defects. Such beneficial effects may be the result of a direct effect of FA on the transcriptome of the developing heart⁽⁶⁰⁾. However, FA supplementation did not reduce the risk of congenital defects in the offspring of diabetic mothers⁽⁶¹⁾.

Conclusions and research needs

The findings of studies of human subjects have demonstrated the associations between maternal FA intake and phenotypic variation in children. These observations are complemented by the findings of studies in animal models that provide a mechanistic basis for these observations via the effects on the epigenome. Since the evaluation of FA toxicity did not include the assessment of epigenetic effects, one implication of these findings is that the assessment of FA safety may need to be revised to take into account epigenetic effects. Together, these findings raise important considerations about induction of adverse health effects in offspring by FA supplementation during pregnancy. First, what is the magnitude of the effect of FA intake during pregnancy on the phenotype of the offspring? There are currently too few data on which to base conclusions and the available estimate differs markedly between health outcomes. If the effect of maternal FA supplementation on the offspring is large, at least for some outcomes, to what extent is this acceptable when balanced against prevention of NTD? Although genetic background may exert an influence on the effect of prenatal FA exposure on health outcomes, because the effect of FA supplementation operates through epigenetic plasticity rather than genetic vulnerability, the potential number of individuals affected is likely to be greater than the number of NTD pregnancies. Again, this would need to be balanced against NTD prevention. The limited evidence reported to date precludes drawing firm conclusions, but is sufficient to raise concern. More studies are needed to address this, preferably randomised controlled trials, although these have ethical implications, into the effects of maternal FA intake on the epigenome and phenotype of the child across a wide age range to assess the stability of such changes in postnatal life. Such investigations need to be underpinned by information about the validity of proxy tissues that can be obtained readily in study cohorts, and experiments in model systems to provide mechanistic links between exposure to FA during development, and epigenetic and phenotypic outcomes. The potential for modulation of the effects of FA on epigenetic processes and phenotypic outcomes by other nutrients, by environmental, genetic and ethnic factors and by metabolic effects, such as capacity for DNA synthesis during development, need to be clarified. Answering these questions will involve considerable technical and financial challenges. However, in the face of the implementation of FA fortification in a number of countries and the ongoing debate about this strategy in others, failure to address the nature and extent of the effects of prenatal FA exposure on the offspring may have negative implications for future public health.

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