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Folate and choline: does it take two to tango in early programming of disease?

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Short Title: Folate and choline in early programming

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Abstract

Background: The early life period marks a critical time during which the health trajectory of offspring can be shaped by external influences including maternal nutrition. Folate and choline are water-soluble micronutrients important for fetal development and involved in one-carbon metabolism. Intakes above and below the recommendations commonly occur for both of these nutrients including over-consumption of synthetic folic acid due to widespread vitamin supplement uses and discretionary fortification practices, whereas choline is under-consumed by a majority of the populations including pregnant women. Despite these intake patterns, long-term impact on offspring health is largely unknown. Moreover, limited attention has been on the combined effects of folate and choline despite being metabolically interrelated as methyl nutrients. This review summarizes evidence from animal models and human studies investigating the role of inadequate or supplemental maternal intakes of folic acid, choline and combined effects of folic acid and choline as modulators of health and disease in offspring. With the recent rise in the prevalence of obesity and metabolic diseases, our primary measures of interest were metabolic outcomes.

Summary: Studies examining the role of maternal folate and/or choline in metabolic phenotypes of offspring have mostly been conducted in animal models with a limited number of reports that consider folate and choline together. Interdependent relationship between folate and choline has been demonstrated in studies where a deficiency in one leads to metabolic aberrations in another. Both deficient and excess maternal intakes of folic acid (in varying doses) have been shown to increase risk of obesity and characteristics of the metabolic syndrome in offspring but these findings were restricted to animal studies. Potential metabolic benefits of choline have been suggested in the presence of obesogenic environment but human data were sparse. An imbalanced intake of high folic acid and inadequate choline in the gestational diet created adverse consequences consistent with the obesogenic phenotypes whereas narrowing this imbalance with high choline blocked these effects. Mechanisms by which maternal folate and/or choline influence offspring outcomes may involve epigenetic modification of gene expression with DNA methylation that can be altered globally and gene-specifically. However, the effects of epigenetic programming were inconsistent, as compensatory changes in metabolic products may occur and other contributors including the gut microbiota may provide additional insights into the mechanisms.

Key Messages: Folate and/or choline can impact offspring long-term health, with metabolic consequences that may arise from imbalances between folic acid and choline intakes. However, there is a paucity of mechanistic understanding as various contributors influence programming effects including those beyond epigenetics. As folate and choline are metabolically interrelated, future studies need to consider both nutrients to better elucidate metabolic programming of health and disease.

Introduction

Non-communicable diseases have a tendency to manifest in the later part of life, but their origin may be much earlier. The conceptual framework of "fetal programming" has been recognized to describe that external influences during critical windows of development have a long-lasting impact on the adult phenotype. Initial work described associations between low birth weight and greater risk of cardiovascular disease, type 2 diabetes and metabolic syndrome [1-3]. It was suggested that poor intrauterine conditions that reduced fetal growth can lead to permanent changes in the structure, physiology and metabolism, thereby increasing susceptibility to chronic diseases in adulthood [4]. The wider concept now encompasses an array of variations in early life exposure [5], including over- and under-consumption of nutrients, that may influence disease risk without any impact on birth weight. Among the nutrients, folate and choline are of interest as they are required for early development and inter-related in the common one-carbon metabolic network.

Folate is best known for its role in preventing neural tube defects (NTDs) [6, 7], although inadequate choline intake/status is also associated with the risk of NTDs [8]. The introduction of mandatory fortification with folic acid (the synthetic, oxidized form of folate) in the United States, Canada and others is considered a successful health initiative that dramatically decreased the rates of NTDs [9, 10]. However, widespread vitamin supplement uses and discretionary fortification have resulted in women consuming folic acid at doses 2.5X to even higher than 10X the recommended dietary allowance, and exceeding the Tolerable Upper Intake Level [11, 12]. In contrast to folic acid, intake levels of most of the populations including pregnant women fall below the Adequate Intake for choline [13-15, 12]. Potential reasons for low intakes may involve less consumption of protein-containing foods including eggs and animal products being the richest sources of dietary choline [16], and that choline is absent or in small amounts in prenatal supplements. Despite these intake patterns, their impact on offspring health remains largely unexplored. Recent discussions on potential adverse effects of excess folate/folic acid [17, 18] and benefits of supplemental choline [19] have emerged but the focus has primarily been on a singular micronutrient. As folate and choline are metabolically related where disturbances of one can influence the other, their interactions in modulating offspring phenotypes need further attention.

The purpose of this review is to highlight studies investigating the role of inadequate or supplemental maternal intakes of folic acid, choline and combined effects of folic acid and choline in shaping health trajectories of offspring. Our focus was on metabolic health because the prevalence of obesity and associated co-morbidities in the populations has increased in the recent years [20], with concomitant altered intake patterns of folic acid and choline. First, we will provide an overview of the role of folate and choline in one-carbon metabolism and interdependent relationship. The subsequent sections will summarize evidence from animal and human studies of programming effects by folic acid and/or choline, followed by discussion of the potential mechanisms underlying altered health and disease outcomes in offspring.

Folate and choline interdependent metabolism

As part of one-carbon metabolism, folic acid is reduced to produce tetrahydrofolate (THF), and an addition of a methylene group with vitamin B6 as an essential co-enzyme forms 5,10-methylene-THF (shown in Fig. 1). In the presence of riboflavin, 5,10-methylene-THF is reduced to 5-methyl THF. At the crosspoint between the folate and methionine cycles, 5-methyl THF provides a methyl group for the transmethylation of homocysteine to produce methionine in a vitamin B12-dependent reaction. Methionine is the precursor of S-adenosylmethionine (SAM), a universal methyl donor for DNA methylation, which is a well-established epigenetic mechanism that regulates gene expression [21]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group from SAM to the fifth carbon of cytosine. However, demands for SAM cannot be met by methionine biosynthesis alone and require exogenous labile methyl groups including that of betaine (through choline). SAM is demethylated to S-adenosylhomocysteine (SAH), which is hydrolyzed to

homocysteine in a reversible reaction. The methylation of phosphatidylethanolamine to phosphatidylcholine requires SAM catalyzed by the enzyme phosphatidylethanolamine methyltransferase.

The interdependent nature of folate and choline in one-carbon cycle has been reported where a deficiency in one leads to metabolic disturbances in another. Folate deficiency enhances the use of choline as a methyl donor as suggested by depletion of hepatic choline and phosphocholine [22] as well as brain membrane phosphatidylcholine in rats [23]. Moreover, dietary folate restriction in pre-menopausal women reduced plasma phosphatidylcholine levels, which increased after folate treatment [24]. Hepatic steatosis occurred with chronic folate insufficiency associated with greater utilization of betaine for homocysteine remethylation [25], whereas folate had a lipotropic effect in animal models of fatty liver [26]. Similar to dietary folate deficiency, disruption of the gene that codes for methylenetetrahydrofolate reductase (MTHFR) may influence choline metabolism as indicated by lower hepatic concentrations of phosphocholine, glycerophosphocholine and betaine [27]. Men with the *MTHFR* 677TT genotype had lower plasma phosphatidylcholine concentrations [28] that appear to favor the use of choline as a methyl donor [29]. Further, a study that focused on broad patterns of choline dynamics under the influence of genotype revealed that genetic impairments of folate enzymes increase dependence on dietary choline for phosphatidylcholine production [30].

Conversely, choline deficiency leads to an increased demand for folate as indicated by altered folate metabolism. Short-term feeding of a choline-deficient diet in rodents resulted in lower hepatic folate and SAM concentrations [31-33]. Complex interrelationships exist among choline, folate and methotrexate, as choline deficiency in combination with methotrexate perturbed the relative distribution of folate species but not with choline deficiency alone [33]. Reversible changes in hepatic folate concentrations were achieved with choline repletion and discontinuing methotrexate [34]. With 12 months of choline deficiency, the distribution of folate species with derivatives of longer chain lengths differed reflecting increased hepatic residence time of the folate molecule [35]. However, the total folate levels did not change [35], suggesting reduced folate turnover rates that may act as a compensatory mechanism.

Programming by folate

Evidence from animal models demonstrates that a range of maternal folic acid intakes from deficient to excess (non-toxic) amounts can alter metabolic phenotypes of offspring (shown in Table 1). Studies have shown that maternal folic acid restriction leads to increased body weight at 3 weeks of age in male offspring [36, 37], and continued to 12 months of age with the same folic acid-restricted pup diet [36]. Higher body fat and visceral adiposity in the folic acid-restricted group were consistent with higher activities of hepatic acetyl-CoA-carboxylase and fatty acid synthase, higher plasma cortisol levels and disturbances in lipid and inflammatory cytokine profiles [36].

Feeding a high folic acid diet (10X the recommendation for folic acid) during pregnancy has been shown to produce male adult offspring with higher food intake and body weight and lower glucose response to an insulin load [38]. Similarly, greater weight (birth and post-weaning), blood glucose response, adiposity and lipid variables including cell proliferation and differentiation in male offspring have been reported with gestational intake of high 10X folic acid [39].

Other doses at 2.5X, 5X and 20X also produced greater body weight as well as various indicators of glucose and lipid dysfunctions in male and female offspring in adulthood [40-42], suggesting wide-ranging metabolic consequences across high amounts of folic acid.

Offspring on the same high folic acid diet as dams showed amelioration of the obesogenic effects [38], suggesting that the post-weaning diet composition can modify the programming effects derived prenatally. A study that used a high folic acid post-weaning diet in the offspring of dams fed a high multivitamin diet showed normalization of food intake, weight gain, glucose response [43], emphasizing the role of the folic acid in determining the metabolic phenotypes. The effects of diet

may differ depending on the time of exposure and sex of offspring as demonstrated by contrasting blood glucose and phosphoenolpyruvate carboxykinase responses [44]. Female offspring of dams fed a diet containing 10X folic acid during pregnancy had lower body weight [45] but also metabolic disruptions, higher fat and reduced pancreas weight and lean mass [46, 41] with potential interactions with other factors such as post-weaning Western diet [46].

Inconsistent findings in human studies exist and may arise due to differences in the background characteristics and diet of the study populations (shown in Table 1). As part of the Amsterdam Born Children and their Development multi-ethnic birth cohort study, low maternal folate levels during pregnancy were associated with higher body mass index (BMI) in offspring, after correcting for multiple confounders [47]. In a cluster-randomized, controlled study in Nepal, 400 µg folic acid daily supplementation from early pregnancy through 3 months postpartum reduced the risk of metabolic syndrome and microalbuminuria, a marker of kidney dysfunction in children of 6-8 years old [48]. When vitamin status was examined, maternal plasma folate levels were not associated with offspring insulin resistance [49]. However, maternal low plasma vitamin B12 status was associated with insulin resistance, with the association restricted to early pregnancy [49]. Low vitamin B12 in conjunction with high folate concentrations may provide the highest degree of insulin resistance as indicated in children of 6 years old in the Pune Maternal Nutrition Study [50]. In the Cambridge Baby Growth Study, no association was found between folic acid supplementation in pregnancy and markers of offspring size at birth or adiposity [51]. It is important to note that observational studies do not have the ability to infer any causal relationships, and self-reporting of folic acid supplementation or missing data may have introduced biases. When maternal micronutrient status is used as a proxy for intake, it may be a marker of other methyl nutrients or dietary factors. Further, differences during the growth period may have been under-reported due to a lack of serial measurements.

Programming by choline

Both low and high choline maternal diets influence offspring phenotypes with most studies limited to male offspring (shown in Table 2). Maternal intake of a choline deficient diet has been shown to reduce body weight at 3 weeks postnatal in male offspring [37]. Metabolic consequences may extend to reduced leptin levels and hypothalamic expression as well as reduced energy expenditure and activity with low 0.5X choline intake [52].

The effects of high choline diet differed depending on the presence of obesogenic environment. Male offspring of dams fed a high choline diet during pregnancy and weaned to a control diet exhibited higher body weight and cumulative food intake in adulthood [52, 53]. Consistent with these obesogenic phenotypes, hypothalamic expression of appetite-stimulating neuropeptide Y was higher and plasma leptin was lower at birth in addition to post-weaning changes including higher plasma leptin and hepatic C16:1 *n*-7/C16:0 ratio that indicates dysregulated fatty acid metabolism [52, 53]. When weaned to a high fat diet, choline appears to provide metabolic benefits as demonstrated by reduced body weight and adiposity as well as improved indicators of glucose and fat metabolism [53]. Another study showed that high maternal intake of choline protects against the effects of high-fat diet with differences observed in post-weaned male offspring [54]. In fetuses from high-fat diet-fed dams, maternal choline supplementation ameliorated excess adiposity and hepatic fat accumulation in addition to downregulation of lipogenic genes [55]. Levels of betaine (oxidized choline) in the fetal liver were higher with choline supplementation regardless of different fat content of the maternal diet but only the high-fat, high-choline group had lower gene expression of betaine-homocysteine S-methyltransferase 1 [55], suggesting altered methyl donor availability.

The effects of maternal choline intake on offspring health have seldom been examined in humans (shown in Table 2). Two prospective cohort studies exist that followed offspring after birth in the United Kingdom, Singapore and the Netherlands [56, 57]. Maternal choline intake was associated with neonatal BMI z score, skinfold thickness and total body fat, indicating greater offspring adiposity at birth but no consistent associations were shown in the first 5 years of life [56]. Moreover, each 1-

µmol/L increase of choline in plasma was associated with a 20-g weight gain in the first year of life and BMI z score at 1-2 years, but not at older ages [57]. These associations suggest that choline is important for early growth but whether these effects persist needs to be confirmed. Limitations can exist where maternal choline intake or plasma concentrations may not represent offspring choline status or transfer/uptake by the fetus. Further, as choline is homeostatically regulated, choline status in the fasted state may not be a reliable marker of changes in dietary choline intake.

Consideration of folic acid and choline

Although a limited number of studies exists, being restricted to animal models, evidence to date emphasizes potential interactive effects of folic acid and choline that underlie metabolic programming of health and disease in offspring (shown in Table 3). Folic acid deficiency with low choline intake during pregnancy did not alter fetal weight nor insulin levels compared to the control group, whereas folic acid deficiency alone resulted in greater fetal weight, insulin and size of kidney and heart as well as reduced liver and lung size [58].

Two studies reported changes in the post-weaning measures that suggest choline may alter the effect of the high folic acid gestational diet [59, 60]. A diet containing 5X folic acid and 0.5X choline consumed during pregnancy produced male offspring with greater weight gain and food intake in peri-adolescence but these effects became less apparent in adulthood [59]. In a different study, a 10X folic acid gestational diet without choline resulted in phenotypes of obesity including higher body weight, food intake, glucose response and body fat in male and female adult offspring [60]. It is possible that the magnitude of imbalances in the folic acid and choline amounts is an important determinant of metabolic consequences with a greater difference creating more adverse effects. Moreover, sex-specific responses to high folic acid gestational diet occurred only in the presence of choline [60]. Gestational intake of a high 10X folic acid diet with recommended choline produced male offspring with higher body weight, food intake, glucose response and body fat, but in contrast, female offspring had lower body weight without differences in food intake, glucose response and body fat [60]. Lower activity was observed with the obesogenic phenotypes as well as those with lower body weight [60], which indicates that different aspects of energy balance regulation may be influenced by other cues. The modulatory role of choline has been shown with high 2.5X choline, which blocked the expected increase in body weight and food intake associated with high 5X folic acid gestational diet in male offspring up to 20 weeks [59]. While studies to date suggest that folate and choline may interact to influence the metabolic phenotypes, different forms of folate (folic acid or methyl folate) and choline (free choline, fat-soluble choline or oxidized choline) should be considered when examining their programming effects. Comparisons across folate and choline forms are lacking, but different effects can be suggested from the observation that gestational intake of [6S]-5-methyltetrahydrofolic acid compared to folic acid at 5X requirements favoured increased food intake and body weight of mature female offspring [61].

Mechanisms

Variations in phenotypes may arise due to long-term programming of gene function independent of the DNA sequence, as termed epigenetics [62]. Among the epigenetic modifications that have been identified, the most well-established is DNA methylation that is amenable to maternal nutrition [63]. Folate deficiency has been shown to reduce global DNA methylation in the liver of offspring at weaning, but not at post-weaning, though folic acid supplementation at post-weaning led to lower global DNA methylation [64]. With folic acid supplementation during pregnancy, global DNA methylation was reduced in the brain of offspring at birth [65]. In a study that normalized the folic acid content of 10X multivitamin mixture, long interspersed nuclear element-1 methylation (marker of global DNA methylation) was lower, indicating the role of other nutrients in hypomethylation [66]. With gestational choline deficiency, global DNA methylation was higher in the fetal liver whereas choline supplementation resulted in lower methylation in the brain, which may have been due to a

compensatory response of DNMT1 [67]. Maternal choline supplementation with high-fat feeding yielded higher global DNA methylation and *Dnmt1* expression in fetal liver and brain, with modulation in a time-specific manner [68]. However, whether the combined effects of folate and choline on offspring leads to large-scale alterations in DNA methylation has not been examined. Changes in gene-specific methylation allow focused analyses of epigenetic marks that provide insights into disease risk. In the Wistar rat model, feeding a gestational diet with folic acid at recommended quantities and 10X all other vitamins contributed to higher DNA methylation specific to pro-opiomelanocortin (POMC) and serotonin receptor (5-Htr) 2a genes in the adult hypothalamus [66]. These offspring had corresponding higher *Pomc* gene expression, although not to the control level, and lower *5-Htr2a* gene expression, suggesting that folic acid partly accounts for characteristics of the metabolic syndrome [66]. Continuing high folic acid diet post-weaning in offspring of folic acid-fed dams resulted in lower *Pomc* methylation consistent with higher *Pomc* gene expression and amelioration of the obesogenic phenotypes [38]. Thus, DNA methylation may be active postnatally and of dynamic regulation. In an epigenome-wide study in humans, associations have been found between maternal plasma folate and DNA methylation in newborn cord blood at 443 CpGs, with many of the implicated genes related to birth defects, neurological functions or aspects of embryonic development [69], suggesting diverse impacts outside of folate biology Another study identified that in utero folate exposure is linked to hypomethylation upstream of the gene *ZFP57* involved in the establishment and maintenance of genomic imprinting, with markers of increased transcriptional activity [70]. Differentially methylated regions regulate expression of the imprinted genes, the two widely-studied being insulin-like growth factor and H19, but both hypomethylation and hypermethylation have been found with maternal folic acid/folate [71-73] or choline [67]. Although folate is known to be a carrier of methyl groups that is expected to increase DNA methylation, previous findings indicate that folic acid over-consumption leads to pseudo-MTHFR deficiency with reduced 5-methyl THF, SAM and SAM/SAH ratio [74, 75]. Lower SAM production may also occur with inadequate choline because of greater utilization of the cytidine diphosphate choline pathway [76]. While not directly explored, the commonly observed intake patterns of high folic acid and inadequate choline may contribute to reduced DNA methylation potential with unintended metabolic consequences. It is important to recognize that DNA methylation can be subjected to differences in methyl donor availability due to the activities of the gut microbiota involved in substrate utilization and synthesis [77]. As the link between gut microbiota and health is being uncovered [78], the end-products of microbial metabolism are increasingly appreciated as determinants of host health. Recent work demonstrates that altered the gut microbiota composition and gut-derived profiles of short-chain fatty acids and serotonin of offspring of dams fed excess vitamins or imbalanced folate-choline may account for the obesogenic phenotypes [60, 79]. Lastly, sexually dimorphic responses to gestational intakes of folic acid [80, 81] or choline [82] have been observed that reflect biased DNA methylation patterns. It is suggested that the uterine environment may be modified by nutrient levels, with fine-tuning of selective events via DNA methylation in a sex-specific manner [80].

Conclusion

Maternal intakes of folic acid and/or choline may influence risk of metabolic disease in adult offspring, with imbalances between folic acid and choline that determine metabolic outcomes. However, a paucity of mechanistic understanding highlights the need to consider various contributors to programming effects including those beyond epigenetic modulation. As folate and choline are interdependent in one-carbon metabolism, future studies that incorporate the impact of both nutrients will help delineate the complex interplay between maternal nutrition and long-term programming of offspring health.

Statements

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

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VC and JLS conducted a literature review search, wrote the manuscript and created the tables and figure. CEC conceptualized the content and critically reviewed/edited the manuscript. All authors read and approved the final paper.

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Figures

Fig. 1. The one-carbon pathway methyl nutrients involved in choline metabolism and DNA methylation. Dihydrofolate (DHF); dihydrofolate reductase (DHFR); tetrahydrofolate (THF); serine hydroxymethyltransferase (SHMT); deoxyuridine monophosphate (dUMP); deoxythymidine monophosphate (dTMP); thymidylate synthase (TS); methylenetetrahydrofolate dehydrogenase (MTHFD); methylenetetrahydrofolate reductase (MTHFR); 5-methyltetrahydrofolate (5-mTHF); methyltetra-hydrofolate-homocysteine methyltransferase (MTR); methionine (MET); methionine adenosyltransferase (MAT); S-adenosylmethionine (SAM); DNA methyltransferase (DNMT); phosphatidylethanolamine (PE); phosphatidylcholine (PC); phosphatidylethanolamine N-methyltransferase (PEMT); cytidine diphosphate-choline (CDP-choline); choline dehydrogenase (CHDH); betaine-homocysteine methyltransferase (BHMT); dimethylglycine (DMG); S-adenosylhomocysteine (SAH); S-adenosylhomocysteine hydrolase (SAHH); homocysteine (hCYS); cystathionine-β-synthase (CBS).

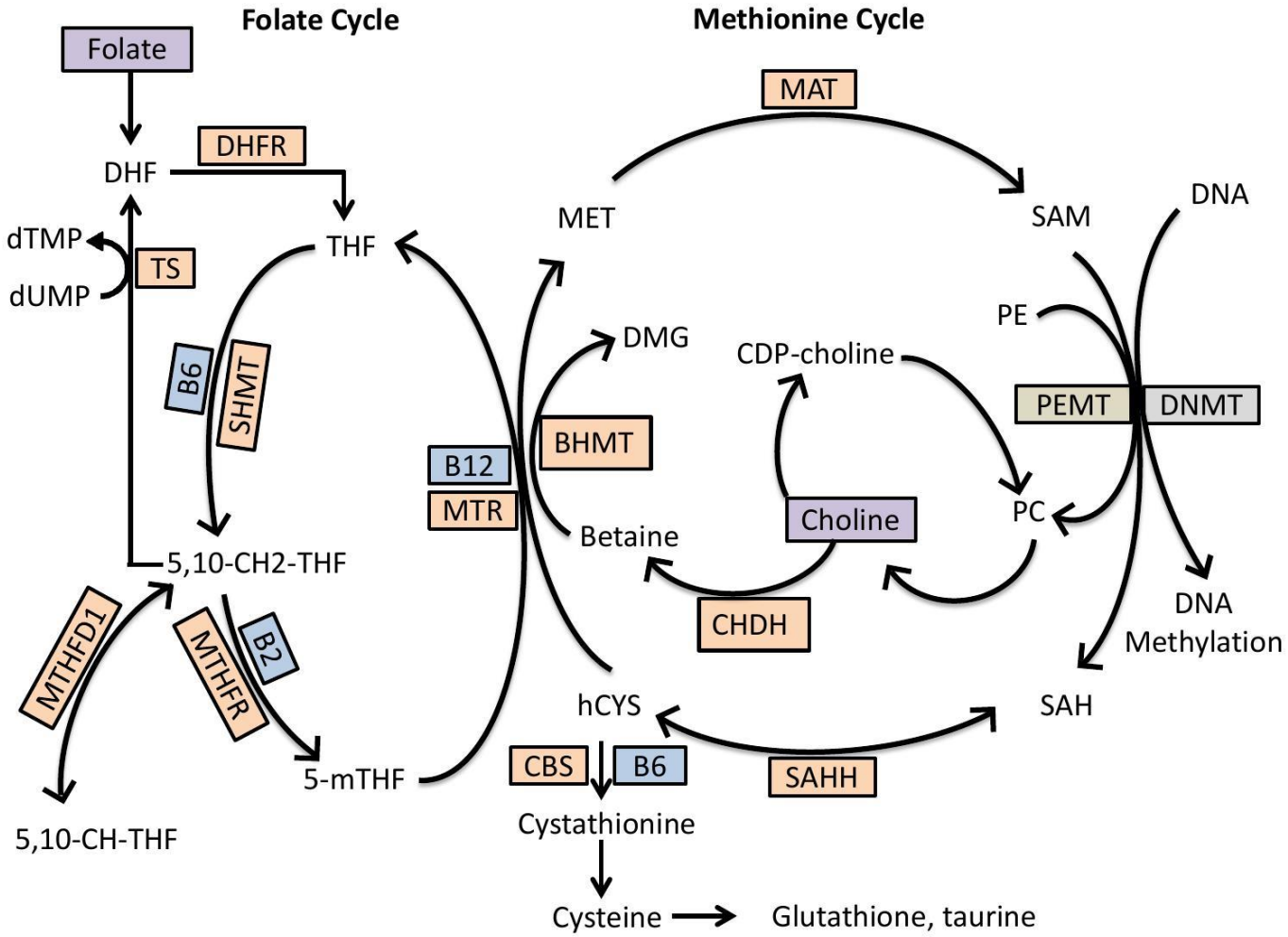

Table 1. Summary of studies investigating effects of maternal folate on offspring metabolic phenotypes

Abbreviations: FA: folic acid; HV: high multivitamins; TG: triglyceride; HDL: high-density lipoprotein; TNF-α: tumor necrosis factor- α; MCP-1: monocyte chemoattractant protein-1; IL-6: interleukin-6; TC: total cholesterol; EFA: esterified fatty acid; FFA: free fatty acid; M: male; F: female; Pparγ2: peroxisome proliferator-activated receptor γ2; Mafa: MAF bZIP transcription factor A; Igf1: insulin-like growth factor 1; BMI: body mass index.

Animal Studies

Authors	Model	Maternal Exposure	Duration	Offspring Exposure	Results (specific to FA)
Kumar et al. (2013) [36]	Wistar rats	*Intervention* Restricted FA (0.08 mg/kg) Restricted B12 (0.006 mg/kg) Folate- and B12-deficient (dual deficient) *Comparison group* AIN-76A diet	Pre-pregnancy, pregnancy and lactation	Male offspring Continued the same maternal diets 12 months postnatal	Restricted FA ↑ body weight ↑ body fat and visceral adiposity ↑ plasma total cholesterol, TG, HDL, TNF-α ↓ plasma adiponectin, IL-1β ↑ adipose MCP-1, IL-6, TNF-α, leptin ↑ hepatic acetyl-CoA carboxylase, fatty acid synthase ↑ cortisol
Jadavji et al. (2015) [37]	C57Bl/6 Mthfr+/+ mice	*Intervention* FA deficient (0.3 mg/kg) Choline deficient (0.3 g/kg) *Comparison group* AIN-93G diet	Pre-pregnancy, pregnancy and lactation	Male Mthfr +/+ offspring 3 wk postnatal	*FA deficient* ↑ body weight
Cho et al. (2013) [38]	Wistar rats	*Intervention* High 10X FA (20 mg/kg) *Comparison group* AIN-93G diet	Pregnancy	Male offspring Control or high FA diet 29 wk post-weaning	*Prenatal high FA, post-weaning RV diet* ↑ food intake ↑ body weight ↓ glucose response to insulin load *Prenatal high FA, post-weaning high FA diet* ↓ food intake ↓ body weight Improved glucose response to insulin load ↓ glucose response to glucose load
Xie et al. (2018) [39]	Sprague-Dawley rats	*Intervention* High 10X FA (20 mg/kg) *Comparison group* AIN-93G diet	Pre-pregnancy, pregnancy	Male offspring high fat (60% kcal) diet 17 wk post-weaning	↑ birth weight and body weight 5-17 weeks post-weaning ↑ adiposity index ↑ post loading blood glucose ↑ TC and TG ↑ proliferation of adipose cells ↑ lipid droplets
Huang et al. (2014) [40]	Mouse C57BL/6J	*Intervention* 2.5X FA (5 mg/kg) 10X FA (40 mg/kg) *Comparison group* AIN-93G diet	Pre-pregnancy, pregnancy	Male offspring AIN-93G until 7 wks old, and then high-fat diet until 15 wk postnatal	↑ body weight ↑ adiposity ↑ post-loading blood glucose ↑ insulin resistance ↓ serum adiponectin
Kintaka et al. (2020) [41]	Mouse C57BL/6J	*Intervention* 10X FA (40 mg/kg) *Comparison group*	Pregnancy	Male and female offspring AIN-93G until 53 d postnatal	*FA supplemented* ↑ glucose concentrations at 60 and 120 min after a glucose load (F) ↓ serum fasting and non-fasting insulin (M and F)

		AIN-93G diet			↓ insulin-positive cells in the pancreas (M and F) ↑ liver triglyceride (F) ↑ liver mRNA expression of Pparγ2 (M and F), Cidec (M)
Liu et al. (2022) [42]	Sprague-Dawley rats	*Intervention* 2.5X FA supplemented (5 mg/kg) 5X FA over supplemented (10 mg/kg) *Comparison group* AIN-93G	Pre-pregnancy, pregnancy	Male offspring Control diet until 16 wk postnatal	*FA supplemented* ↑ fasting glucose ↑ serum TG, visceral fat coefficient ↑ hepatic lipid accumulations ↑ esterified and free C22:4 *FA over supplemented* ↑ body weight ↑ fasting glucose ↑ serum TG, visceral fat coefficient ↑ hepatic lipid accumulations ↑ EFAs (C14:0, C16:0, C24:0); FFAs (C16:0, C18:1, C18:2, γ-C18:3, C20:4)
Cho et al. (2013) [43]	Wistar rats	*Intervention* High 10X multivitamins (HV) *Comparison group* AIN-93G diet	Pregnancy	Male offspring Control, HV or high FA (20 mg/kg) diet 29 wk post-weaning	*Prenatal HV, post-weaning high FA diet* Normalized food intake, weight gain, glucose response compared to control
Hoile et al. (2012) [44]	Wistar rats	*Intervention* FA-supplemented (5 mg/kg) *Comparison group* FA-adequate (1 mg/kg)	Pregnancy	Male and female offspring FA-adequate or FA-supplemented diet for 28 d then FA-adequate diet until 84-90 d	*Maternal folic acid-supplemented* ↑ blood glucose (F) ↑ phosphoenolpyruvate carboxykinase mRNA expression (F) *Post-weaning folic acid-supplemented* ↑ blood glucose (M) ↓ blood glucose (F) ↓ phosphoenolpyruvate carboxykinase mRNA expression (F)
Huot et al. (2013) [45]	Wistar rats	*Intervention* High 10X FA (20 mg/kg) *Comparison group* AIN-93G diet	Pregnancy	Female offspring Control or high FA diet 17 wk post-weaning	Prenatal high FA, postweaning Control diet ↓ body weight Prenatal high FA, postweaning high FA diet Did not normalize body weight
Henderson et al. (2018) [46]	C57BL/6J mice	*Intervention* Supplemental 5X FA (10 mg/kg), no B12 Supplemental FA + adequate B12 *Comparison group* Control FA (2 mg/kg) and B12 (50 µg/kg)	Pre-pregnancy, pregnancy and lactation	Female offspring Control or Western diet (45% kcal fat) 35 wk postweaning	*Supplemental FA + adequate B12* *Control diet PW* ↓ glucose tolerance ↓ pancreas weight ↑ fat mass and ↓ lean mass ↑ gonadal fat pads *Supplemental FA + adequate B12* *Western diet PW* ↓ islet *Mafa* mRNA ↑ liver *Igf1* mRNA

Human Studies

Authors	**Study Design**	**Maternal Exposure**	**Results (specific to FA or folate)**

Krikke et al. (2015) [47]	Prospective cohort; follow-up at 5-6 years The Netherlands	Blood samples collected at the first prenatal visit for maternal folate and vitamin B12 status	*Low maternal folate* ↑ higher BMI
Stewart et al. (2009) [48]	RCT; intervention early pregnancy to 3 months post-partum; follow-up at 6-8 years Nepal	*Intervention* FA (400 µg) FA + iron FA + iron + zinc Multiple micronutrients: FA, iron, zinc, vitamins A, D, E, B1, B2, B3, B6, B12, C, and K *Comparison group* Vitamin A (1000 µg)	*FA (400 µg)* ↓ risk of microalbuminuria ↓ risk of metabolic syndrome *FA + iron + zinc* ↓ risk of microalbuminuria
Stewart et al. (2011) [49]	RCT; intervention early pregnancy to 3 months post-partum; follow-up at 6-8 years Nepal	*Intervention* FA (400 µg) FA + iron FA + iron + zinc Multiple micronutrients: FA, iron, zinc, vitamins A, D, E, B1, B2, B3, B6, B12, C, and K *Comparison group* Vitamin A (1000 µg)	Maternal vitamin B12 deficiency status ↑ insulin resistance
Yajnik et al. (2008) [50]	Prospective cohort; postnatal follow-up every 6 months for 6 years India	Blood samples collected at 18 and 28 weeks of gestation for folate, vitamin B12, total homocysteine and methylmalonic acid concentrations	*High maternal folate* ↑ adiposity ↑ insulin resistance (with high folate and low vitamin B12 being most insulin resistant)
Petry et al. (2021) [51]	Prospective cohort United Kingdom	Self-reported supplement intake questionnaire	No association between folic acid supplementation and size at birth or adiposity in offspring

Table 2. Summary of studies investigating effects of maternal choline on offspring metabolic phenotypes

Abbreviations: Ob-R: leptin receptor; NPY: neuropeptide Y; TG: triglyceride; M: male; Irs1: insulin receptor substrate 1; Bhmt1: betaine-homocysteine *S*-methyltransferase 1; BMI: body mass index.

Animal Studies

Authors	Model	Maternal Exposure	Duration	Offspring Exposure	Results (specific to choline)
Jadavji et al. (2015) [37]	C57Bl/6 Mthfr+/+ mice	*Intervention* Choline deficient (0.3 g/kg) FA deficient (0.3 mg/kg) *Comparison group* AIN-93G diet	Pre-pregnancy, pregnancy and lactation	Male Mthfr +/+ offspring 3 wk postnatal	*Choline deficient* ↓ body weight
Hammoud et al. (2020) [52]	Wistar rats	*Intervention* Low 0.5X choline (0.5 g/kg choline) High 2.5X choline (2.5 g/kg choline) *Comparison group* AIN-93G diet	Pregnancy	Male offspring Normal fat diet for 17 wk post-weaning	*Low choline* ↓ hypothalamic Ob-R expression (birth) ↓ plasma leptin (birth) ↓ energy expenditure and total locomotor activity *High choline* ↑ hypothalamic NPY expression (birth) ↓ plasma leptin (birth) ↑ cumulative food intake ↑ body weight gain
Hammoud et al. (2021) [53]	Wistar rats	*Intervention* High 2.5X choline (2.5 g/kg choline) *Comparison group* AIN-93G diet	Pregnancy Switched to D10012G diet during lactation	Male offspring Normal fat or high fat (45% kcal) diet for 17 wk post-weaning	*High choline – normal fat* ↑ cumulative food intake ↑ body weight ↑ plasma leptin ↑ hepatic C16:1 *n*-7/C16:0 *High choline – high fat* ↓ body weight ↓ visceral adiposity ↓ plasma insulin and TG ↓ insulin resistance ↑ hepatic *n*-3 ↓ hepatic *n*-6/*n*-3 and C18:1 *n*-9/C18:0
Korsmo et al. (2020) [54]	C57BL/6J	*Intervention* Normal fat + choline (25 mM added to water) High fat + choline *Comparison group* Normal fat (D12450J diet) High fat (D12492 diet)	Pre-pregnancy, pregnancy	Male and female offspring Fed either normal or high fat diet for 6 wk post-weaning	*Prenatal normal fat + choline, post-weaning high fat* ↓ food intake (M) *Prenatal high fat + choline, post-weaning high fat* Attenuated high fat-induced rise in blood glucose, glucose intolerance, serum leptin (M) ↑ adipose tissue Irs1 mRNA expression

Jack-Roberts et al. (2017) [55]	C57BL/6J	*Intervention* Normal fat + choline (25 mM added to water) High fat + choline *Comparison group* Normal fat (D12450J diet) High fat (D12492 diet)	Pre-pregnancy, pregnancy	Offspring at E17.5	*Normal fat + choline* ↑ liver betaine *High fat + choline* Attenuated high fat-induced rise in percent body fat and hepatic triglyceride content ↑ liver betaine ↓ liver Bhmt1 expression ↓ liver mRNA expression of lipogenic genes

Human Studies

Authors	Study Design	Maternal Exposure	Results (specific to choline)
van Lee et al. (2019) [56]	Prospective cohort; follow-up until 5 years United Kingdom and Singapore	Blood samples collected at 11 weeks (United Kingdom) and 26-28 weeks gestation (Singapore) for choline concentrations	High maternal choline ↑ neonatal BMI z score ↑ neonatal subscapular and triceps skinfold thickness ↑ neonatal total body fat
Moltó-Puigmartí et al. (2021) [57]	Prospective cohort; follow-up until 8 years The Netherlands	Blood samples collected between 34 and 36 weeks of gestation for choline and betaine concentrations	Each 1 µmol/L increase in maternal plasma choline ↑ weight gain in 1 year ↑ BMI z score, odds of BMI z score > 85th percentile at 1-2 years

Table 3. Summary of studies investigating effects of both maternal folate and choline on offspring metabolic phenotypes
Abbreviations: FA: folic acid; F: female; M: male.

Animal Studies

Authors	Model	Maternal Exposure	Duration	Offspring Exposure	Results (specific to FA and choline)
Maloney et al. (2009) [58]	Rowett hooded strain rats	*Intervention – Exp. 1* FA-deficient (FD) FA-deficient with low-methionine (FDLM) FA-deficient with low-choline (FDLC) FA-deficient with low-methionine and low-choline (FDLMLC) *Intervention – Exp. 2* FA-deficient (FD) FA-deficient with low-methionine and low-choline (FDLMLC) *Comparison group* Control diet	Pre-pregnancy, pregnancy	Exp. 1 Fetuses removed at 21 days gestation Exp. 2 Male and female offspring Stock diet 24 wk postnatal	*FD* ↑ fetal weight ↑ fetal insulin ↑ kidney and heart size ↓ liver and lung size *FDLC* Similar fetal weight and insulin to controls
Hammoud et al. (2021) [59]	Wistar rats	*Intervention* Low 0.5X choline (0.5 g/kg choline), high 5X FA (10 mg/kg); (LCHF) Normal 1X choline (1 g/kg choline), high 5X FA (10 mg/kg); (RCHF) High 2.5X choline (2.5 g/kg choline), high 5X FA (10 mg/kg); (HCHF) *Comparison group* AIN-93G diet (RCRF)	Pregnancy	Male offspring AIN-93G diet 20 wk PW	*LCHF* ↑ food intake ↑ weight gain *RCHF* ↑ food intake ↑ weight gain *HCHF* ↓ plasma insulin compared to LCHF ↓ plasma leptin compared to RCHF
Mjaaseth et al. (2021) [60]	Wistar rats	*Intervention* High 10X multivitamins High 10X FA (20 mg/kg) with recommended choline (1 g/kg choline) High 10X FA (20 mg/kg) without choline *Comparison group* AIN-93G diet	Pregnancy	Male and female offspring High fat diet (60% kcal) 12 wk PW	*High FA* ↑ body weight and fat percentage (M) ↓ body weight (F) ↑ food intake (M) ↓ total rearing activity *High FA, no choline* ↑ body weight and fat percentage ↑ food intake ↓ total rearing activity (M) ↑ glucose response