Review Article

Molecular and genetic considerations for long-term nutrition interventions

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The potential for new functional foods and substantial modification of traditional foods presents unprecedented opportunities and new challenges to the public health community. The technical advances that enable these possibilities presumably present an efficient, cost-effective, and sustainable means of improving human health and preventing nutrition-related diseases. Directed manipulation of the food supply, however, presents risks that also must be considered and monitored carefully to avoid known adverse consequences associated with elevated nutrient intakes. Additionally, more research is necessary to understand the subtle effects and long-term consequences that result from elevated individual nutrient intakes on human health and disease risk through changes in gene expression and genome stability. Growing awareness that genetic variation influences both nutrient requirements and upper levels of safe intake require that the risks and benefits associated with manipulation of the food supply be considered at the individual and population levels. In the present report current approaches and limitations to the precise manipulation of the food supply are considered in light of recent efforts to prevent neural tube defects by increasing the population's dietary folic acid intake.

Key words: folate, food, genetic variation, nutrition, requirements.

Introduction

The potential for new functional foods and substantial modification of traditional foods presents unprecedented opportunities and new challenges to the public health community. The technological advances that enable these possibilities presumably present efficient, cost-effective, and sustainable means of improving human health and preventing disease. Targeted manipulation of the food supply by introduction of genetically modified traditional and novel foods has the potential to replace nutrient supplementation and/or fortification approaches that have ameliorated or prevented nutrient deficiencies resulting in anaemia, birth defects, cognitive deficits and other ailments in both industrially developed and developing countries.

Targeted manipulation of the food supply also may benefit Western societies that suffer from nutrition-related diseases associated with overnutrition and assist those that seek to optimize human nutrition throughout the life cycle. In 1990 approximately 30% of all preventable mortality in the USAs was attributed to inappropriate diets and physical activity patterns.[1] The three primary non-genetic factors that contributed to more than 70% of preventable mortality were tobacco (which accounted for 37% of preventable deaths), diet/physical activity (which accounted for 28% of preventable deaths) and alcohol (which accounted for 9% of preventable deaths). Moreover, studies of monozygotic twin studies estimate that one-third of human cancers are attributable to diet (e.g. heritable factors account for only 17–35% of colorectal, breast and prostate cancers).[2] The remaining etiologies appear to be environmental in nature with diet being the principal agent. Incidences of nutrition-related chronic diseases (obesity, cardiovascular disease, diabetes, among others) are increasing in nearly all regions of the world and in some cases appear to have reached epidemic levels. Changing demographics are worsening the magnitude of these problems because risk for nutrition-related chronic diseases increases with age.[1] Global transitions to free markets and adaptation to Western-style diets and lifestyles (predominately sedentary activity patterns) appear to promote non-communicable chronic diseases with alarming effectiveness! Annual medical care expenses for nutrition-related disorders are now a major drain on national health-care resources and increasingly so in industrializing countries. Therefore, it is not surprising that the prevention and management of morbidity and mortality associated with nutrition-related diseases are among the highest priorities for public health organizations. Restructuring the wholesomeness of the food supply, and promoting healthy dietary habits and physical activity, increasingly are identified as major public health objectives to secure longer lives and better quality of living throughout the life cycle.

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Directed manipulation of food composition presents one approach to enhance the food supply. This approach, however, also presents risks and challenges that must be considered and monitored to avoid unintended adverse consequences. Experiences from past fortification and supplementation initiatives indicate that nutrient levels must be set and monitored carefully because excess intake of specific nutrients (e.g. iodine, iron and vitamin A derivatives) results in significant toxicities and too little fails to achieve the targeted protection.[3–5] There is also evidence that maternal nutrition has less salient but significant effects on fetal metabolism, physiology and epigenetic regulation that impact on development and long-term disease risk.[6] Moreover, recent studies reveal how genetic variation influences both nutritional requirements[7] and toxic levels, thereby bolstering rationales for considering potential risks and benefits in manipulating food composition. The present report reviews current approaches, limitations and benefits of novel approaches for manipulating the food supply. Recent experiences with dietary folic acid fortification and supplementation for the prevention of neural tube defects (NTD) are used as an illustrative example.

Folate and one-carbon metabolism

The term 'folate' refers to a family of structurally related metabolic cofactors that are present in mammalian cells (Fig. 1).[8] Tetrahydrofolate polyglutamate, the fully processed form of the vitamin, is a cofactor that chemically activates and carries one-carbon units at the oxidation levels of formate (in the form of 10-formyltetrahydrofolate), formaldehyde (in the form of methylenetetrahydrofolate) and methanol (in the form of 5-methyltetrahydrofolate). The one-carbon moiety in the form of 10-formyltetrahydrofolate is required for the synthesis of the purine ring, whereas methylenetetrahydrofolate is required for the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). 5-methyltetrahydrofolate is required for the remethylation of homocysteine to methionine.[8] Methionine, in turn, can be converted to s-adenosylmethionine (SAM), a cofactor that methylates various substrates including DNA, RNA, phospholipids and neurotransmitters (Fig. 2).

Cellular folates contain polyglutamate chains that range from two to nine glutamate residues. These variable length chains are required for specific folates to act as effective metabolic cofactors.[9] However, only the monoglutamate forms of the cofactor are transported effectively into cells.[10] Therefore, naturally occurring dietary folates from foods must be converted to monoglutamate forms by the enzyme g-glutamyl hydrolyase prior to their intestinal absorption.[10] Once transported into cells, the polyglutamate chain is restored by folylpolyglutamate synthetase. An increasingly common source of dietary folate is folic acid, an oxidized, synthetic form of the vitamin that often is present in vitamin supplements and fortified foods. Folic acid contains only a single glutamate and exhibits greater bioavailably than naturally occurring folates.[11]

The mechanisms that regulate cellular and whole body folate status are understood poorly. Folate transport, polyglutamylation and catabolism each contribute to the regulation of cellular folate concentrations.[10] Several studies have indicated that under physiologic conditions, all cellular

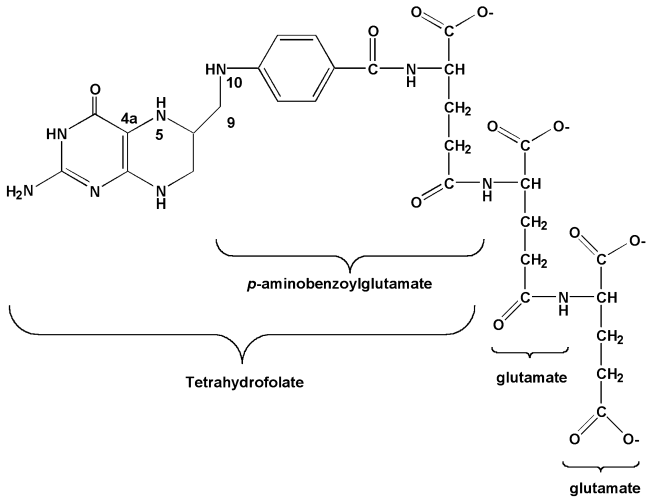

Figure 1. Structure of tetrahydrofolate triglutamate.

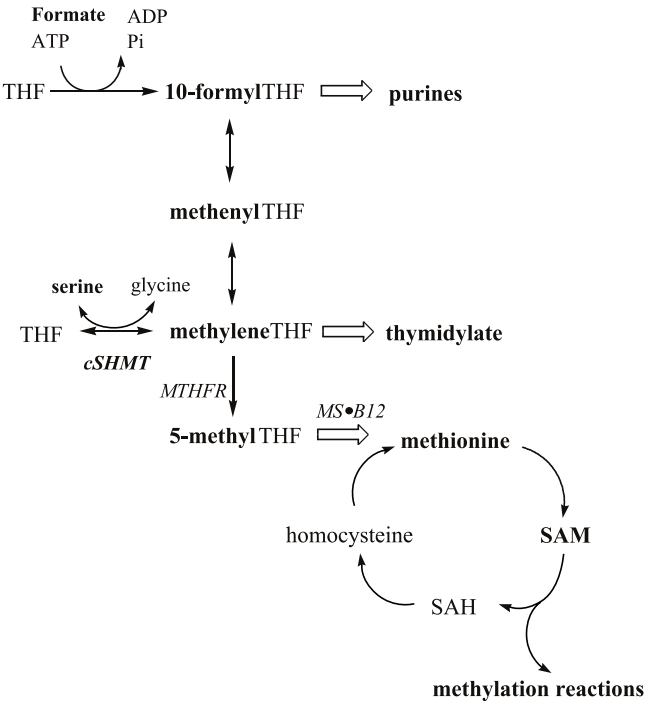

Figure 2. Folate metabolism is required for purine and thymidylate biosynthesis and for homocysteine remethylation to methionine in the cytoplasm. Methionine is converted to S-adenosylmethionine, a cofactor that donates methyl groups for numerous other one-carbon transfer reactions. Mitochrondrial folate metabolism generates formate, a source of one-carbon units for cytoplasmic metabolism. THF, tetrahydrofolate; SAM, S-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MTHFR, methylenetetrahydrofolate reductase; MS-B12, methionine synthase, a vitamin B12-dependent enzyme (MS) requires the enzyme methionine synthase reductase (MSR) for physiological activity.

folate-binding sites are not saturable, indicating that cellular folate concentrations usually are limiting in folate-dependent anabolic pathways and that folate-dependent pathways compete with one another for folate cofactors.[10] In fact, several studies have demonstrated that homocysteine remethylation and dTMP synthesis compete for methylenetetrahydrofolate, a principal folate cofactor (Fig. 2).[12–14] Thus, both methionine and dTMP synthesis are sensitive to folate deficiency. Marginal folate deficiency results in increased extra- and intracellular homocysteine concentrations, and depressed deoxythymidine triphosphate (dTTP) synthesis resulting in DNA with elevated uracil content in DNA due to the misincorporation of uracil during DNA replication.[15,16] Elevated serum homocysteine and/or *S*-adenosylhomocysteine concentration, increased uracil content in DNA, DNA hypomethylation, elevated formininoglutamate in urine, megaloblastic anemia and neutrophil hypersegmentation are thus biomarkers of folate deficiency.[17,18]

Impairments of folate metabolism also can occur in the absence of primary or secondary folate deficiency. Pharmaceutical therapies, certain physiological (e.g. pregnancy) and pathologic (e.g. cancer) states, and single nucleotide polymorphisms (SNP) in genes that encode folate-dependent enzymes (including methylenetetrahydrofolate reductase (MTHFR),[19] methionine synthase (MS),[20] and methionine synthase reductase (MSR)[21,22]) can result in 'conditional' folate deficiencies that become symptomatic in the absence of low intracellular or extracellular folate concentrations.[7] For example, humans with a common polymorphism in the *MTHFR* gene (C677T) have depressed MTHFR enzyme activity. This in turn impairs 5-methytetrahydrofolate synthesis and subsequently reduces the capacity to remethylate homocysteine and generate SAM (Fig. 2). Some SNP in genes that encode MS and MSR also result in states that inhibit homocysteine remethylation.[22] Deficiencies in other vitamins that serve as cofactors in folate metabolism (e.g. vitamins B6 and B12) also present symptoms of folate deficiency.[17] Patients with iron deficiency also display symptomatic folate deficiency without evidence for abnormally low folate intakes or serum folate concentrations.[18] Recent studies have demonstrated that the heavy-chain subunit of ferritin, the iron storage protein, regulates the expression of the gene that encodes serine hydroxymethyltransferase, an enzyme that regulates both the dTMP synthesis and homocysteine remethylation pathways.[23] The effect of ferritin on serine hydroxymethyltransferase gene expression may account for the coupling of iron–folate status.[23] Collectively, these observations indicate that the regulation of folate metabolism is complex, and affected by the availability of several nutrients (folate, iron, vitamins B6 and B12) and several prevalent, well-characterized gene variants.[7]

Interactions between folate metabolism and the genome

Nutrients can reversibly alter chromatin structure and gene expression at the level of transcription through ligand-activated nuclear receptors or by altering DNA methylation status. Nutrients also can regulate mRNA and protein function. Both organic and inorganic nutrients are often important prosthetic groups, cofactors or substrates for proteins and thereby affect their function and stability. Additionally, there are many examples whereby nutrients alter mRNA transcription and translation rates. Nutritional status also can irreversibly influence gene sequence by affecting point mutation rates and genomic stability. During gestation, maternal nutritional status influences embryo and fetal environments, and therefore is one of many selective pressures that determine the fitness of fetal genotypes, especially hapalotypes that influence biochemical pathways.[24] Interestingly, there is increasing evidence that prenatal and postnatal nutritional status can irreversibly imprint or adapt gene expression and disease susceptibility in developing mammals, but the underlying epigenetic mechanisms have yet to be established.[6,25] The interaction of nutrition and the genome is reciprocal; that is, hapalotypes can influence nutritional requirements and thereby the efficiency of metabolic pathways.[7]

Many studies have demonstrated that folate status influences genome stability and gene expression. Folate deficiency in animal models and tissue culture increases chromosomal strand breaks,[26–29] and folate-deficient cells in culture are more susceptible to the effects of radiation and alkylating agents.[26,30] Two general mechanisms have been proposed to account for the many associations between impaired folate metabolism and genome instability: altered DNA methylation patterns and decreased fidelity during DNA synthesis and repair.[27,28] Approximately 4% of cytosine bases within the mammalian genome are modified by methylation. Both genome-wide and allele-specific DNA methylation are influenced by folate metabolism.[31,32] Methylated cytosine residues are generally located in CpG islands, which are dinucleotide repeat sequences commonly found in the 5′ promoter regions of genes. Approximately 90% of cytosine bases are methylated within CpG islands.[33] Recent microarray analyses have indicated that approximately 10% of murine genes are regulated by DNA methylation,[34] and methylation density within the 5′ promoter region generally correlates inversely with levels of gene expression.[33] Thus, DNA hypomethylation, which can be induced by folate deficiency, has two primary effects on the mammalian genome. First, it increases the expression of genes normally modulated by methylation, including tumor suppressor genes.[28,33] Second, hypomethylation relaxes chromatin structure[35] and thereby enhances the accessibility of DNA-damaging agents, resulting in increased genomic mutation, particularly in 'hot spots' associated with cancers.[36] In support of this mechanism, rodents fed diets deficient in folate and other sources of methyl groups were shown to be more susceptible to chemically induced hepatocarcinoma in some but not all studies.[28,37,38]

Folate deficiency also impairs the integrity of DNA synthesis. Folate restriction in cell cultures imbalances deoxyribonucleotide triphosphate (dNTP) pools and increases uracil concentration in DNA.[26,30] In humans and rodents, folate status correlates inversely with uracil content in DNA, presumably resulting from impaired dTMP synthesis and

subsequent elevations in deoxyuridine triphosphate (dUTP) pools.[39] Elevation in dUTP pools results in its misincorporation into DNA during replication. Once uracil is incorporated into DNA, excision repair of uracil from DNA has the potential to result in strand breaks if the residues are in close proximity and are located on opposite strands of the helix.[40] Additionally, imbalances in the dNTP pool may impair the fidelity of DNA polymerases and excision repair enzymes.

Folate, developmental anomalies and cancer

Numerous epidemiological studies have revealed associations among folate status, polymorphisms in folate-dependent genes, and risk for disease or developmental anomalies including certain cancers, NTD, and cardiovascular disease.[17,41] However, the biochemical and developmental mechanisms whereby folate prevents the occurrence and recurrence of these disorders are unknown. Ongoing folic acid fortification policies were implemented with the underlying assumption that folate-responsive NTD result from specific metabolic disruptions in either dTMP or methionine (SAM) synthesis, and that these disruptions can be overcome by increasing maternal folate intake. Human SNP in the *MTHFR* gene impair methionine synthesis and increase risk for human NTD, suggesting that impairments in the homocysteine remethylation pathway, or in other affected SAM-dependent methylation reactions for which homocysteine serves as a marker, can impair normal development. However, the NTD–homocysteine association is not supported fully by rodent studies. For some mutant mouse strains, metabolic products of folate metabolism can ameliorate folate-responsive NTD. For example, maternal thymidine supplementation can replace folate supplementation in preventing NTD in the *Pax3* mouse model, whereas maternal methionine supplementation increases risk for NTD in this animal model.[42] Conversely, maternal methionine supplementation in *Axd* mutant mice is protective against NTD.[43] The results from these studies are surprising but not necessarily conflicting. Because dTMP synthesis and homocysteine remethylation are competitive pathways, it is possible that thymidine supplementation of *Pax3* spares methylenetetrahydrofolate for homocysteine remethylation in *Pax3* (Fig. 2). More detailed studies of these and other mouse models of NTD will be required to establish definitive metabolic mechanism(s) that account for the folate–NTD association, which may lead to better targeted intervention strategies.

Folate deficiencies, and polymorphisms in folate dependent enzymes, also are associated with risk for epithelial cancer. It is recognized that 'age is the most potent of all carcinogens'.[44] Cancer risk rises exponentially after the age of 40, driven primarily by an increase in the incidence of epithelial carcinomas.[44] Several mechanisms have been proposed to account for age-related cancer susceptibility, including increased mutational loads and inappropriate epigenetic gene silencing. Mutational load refers to the cumulative acquisition of somatic mutations in DNA that arise from DNA damaging agents, background error rates associated with inaccurate DNA replication and repair, and declining DNA repair efficiency.[44] As mutational load increases, particularly in key loci that encode genes critical for cell-cycle control, cancer is initiated and progresses through the process of clonal selection.[33,45]

Although the biochemical mechanisms that account for association between folate deficiency and cancer risk are not established, current research is focused on folate's effects on DNA mutation rates, DNA stability and gene expression. As described in the previous sections, folate metabolism influences genome stability by at least two distinct mechanisms: misincorporation of uracil into DNA due to impaired dTMP synthesis, and impaired DNA methylation resulting from depressed SAM synthesis. Both uracil misincorporation into DNA and alterations in DNA methylation influence mutation rates, and methylation-induced alterations in chromatin structure also may contribute to the initiation and/or progression of certain cancers.[44] Genome-wide DNA hypomethylation occurs in nearly all cancers and precedes mutational and chromosomal abnormalities that occur as cancer progresses.[28]

Paradoxically, allelic-specific hypermethylation and gene silencing occurs concurrently with genome-wide hypomethylation in cancer.[28,33] Tumor-suppressor genes seem to be particularly sensitive to methylation silencing, and their silencing increases with tumor progression.[33,44,45] The relationship between DNA methylation and cancer susceptibility is supported in murine models. Reduced activity of the cytosine methyltransferase (*Dnmt1*) in these models decreases genome-wide methylation density and reduces the number of age-accumulated adenomas in *Min* (*APC*-deficient) mice.[46,47] These results support human epidemiological studies that link SNP that impair the homocysteine remethylation pathway and decreased risk for colon cancer.[48] Homozygotes for the MTHFR C677T polymorphism have a 40% decreased incidence of colorectal cancer compared to heterozygous (C/T) or normal genotypes (C/C) in the absence of folate deficiency. Therefore, impairment of the homocysteine remethylation pathway by folate depletion is associated with increased cancer risk, while impairment of this pathway by SNP is protective. Thus, the mechanisms that underlie the folate–cancer relationship are not established. Additional research is needed to establish the protective and/or deleterious effects of diverse folate states and various states of DNA methylation on cellular transformations. Once definitive mechanisms are established, long-term nutrition recommendations and/or interventions can be designed more rationally.

Folate and genotype rescue

Genetic variation that arises during evolution results from two independent processes: uncorrected genetic mutation that leads to permanent changes in DNA sequences, and selection pressures or random genetic drift that expand mutations within populations.[24] In a recent study, rates of nucleotide base substitution in more than 2000 human/rodent genes were analysed to determine the impact of gene expression patterns on germ line mutation rates and/or selection intensity. This study concluded that silent substitutions

(those that do not change the code) are not subject to selective pressures, but functional mutations in the germ line that change protein sequence and thereby influence biochemistry are influenced by selection in mammals, particularly genes that are expressed narrowly in tissues with the highest environmental exposure. Several genes that encode folate-dependent enzymes for dTMP synthesis and homocysteine remethylation fall into the later category, consistent with the high prevalence of highly penetrant SNP that have been discovered in human populations.

Human NTD are associated with SNP and haplotypes in genes that encode key enzymes in folate metabolism, and these SNP have been demonstrated to inhibit homocysteine remethylation. This selection indicates that DNA mutations that impair homocysteine remethylation or SAM synthesis have selective reproductive advantages in spite of their deleterious effects on neural tube closure. Interestingly, the prevalence of these SNP can vary markedly among different population and ethnic groups,[49] suggesting a role for regional nutritional or other environmental influences in the expansion of the polymorphism or divergent SNP prevalence in the founder populations. Folate supplementation policies have decreased risk for this birth defect from at-risk populations; thereby indicating that folate can rescue developmental anomalies that have a genetic etiology. To date, all identified human SNP that confer risk for NTD are present in genes that encode folate-dependent enzymes and influence the structure, function and activity of encoded proteins, and increased folate intakes can overcome metabolic disruptions associated with the SNP. However, folate's protective effect may not be accounted for fully by metabolic rescue. Experimental animal data indicate that increased maternal folate intake may have a more general effect in rescuing developmental processes that result from genetic mutations, including severe genetic deletions that seemingly are unrelated to folate metabolism. Maternal folic acid supplementation in mice can lower the incidence of NTD resulting from deletion of *Pax3*,[42] the *Cart1*[50] homeobox gene and *Crooked tail*,[51] as well as NTD resulting from maternal hyperthermia.[52] Although mechanisms for such rescues are unclear, folate can be a limiting nutrient for cell growth *in vitro*. Therefore, maternal folate supplementation may be capable of increasing cell proliferation rates and thereby overcoming depressed cell division rates that result from genetic defects or environmental insults.

Human data also support a role for folate in rescuing deleterious genotypes. Since the initiation of folate fortification and maternal folate supplementation, the reported frequency of spontaneous abortions has increased.[53–57] Certain MTHFR polymorphisms and haplotypes are risk factors for decreased fetal viability and recurrent embryo loss in early pregnancy.[58] It has been proposed that elevated maternal folate may prolong these and other pregnancies that otherwise may not undergo implantation or miscarry very early in pregnancy and therefore go undetected.[54,55] One recent study implied that the C677T MTHFR polymorphism is not in Hardy–Weinberg equilibrium, and presents evidence that this MTHFR polymorphism has become more prevalent as a result of maternal folate supplementation.[59] Although the results from this study are equivocal, both the human and murine data indicate that high levels of maternal folate may rescue genotypes that otherwise would be lethal.

Genetics influencing nutrient requirements

The genome–folate relationship is reciprocal; folate status influences genome sequence and expression, and genome sequence can influence optimal folate status. Studies have shown that the C677T MTHFR polymorphism increases folic acid requirements for specific outcomes, and the recent dietary reference intake (DRI) for the B vitamin folate accounts for effects of maternal genotype on nutritional requirements during pregnancy.[60] Therefore, the role of SNP in dictating optimal nutrient intakes is redefining the concept of a universal recommended dietary intake. Historically, the causes of 'heritable' nutrition-related diseases were identified with single-gene defects, for example phenylketonuria. However, most complex diseases including diabetes, cancer, obesity and cardiovascular disease are multifactorial with respect to both the nutritional and genetics components. It is reasonable to assume that as efforts to find and characterize all human SNP progress, some of the genetic contributions associated with these chronic diseases will include the influence of genetic variation on nutrient requirements (and nutrient toxicity levels).

Considerations for long-term interventions

Numerous epidemiological and clinical studies have demonstrated the potential benefits associated with improving folate status in human populations, and such interventions likely will decrease the incidence of birth defects, epithelial cancers, anemia, and perhaps cardiovascular disease. The literature also indicates that certain genetic groups will benefit more than others, because the risk for folate-related diseases and developmental anomalies includes significant interactive genetic components. Less known are the short- and long-term risks associated with increasing folate levels within populations. At the present time our understanding of folate metabolism, its regulation and interactions with the genome are not sufficient to anticipate long-term benefits or risks related to sustained, long-term nutritional interventions that include unprecedented levels of folate in the food supply. There are three largely unstudied yet important considerations associated with altering folate levels in the food supply: potential adverse reproductive outcomes associated with increasing folate during gestation; health risks associated with manipulation of folate content in the general population; and, optimal approaches to increase folate content of the food supply.

Maternal supplementation with folic acid has both benefits and risks. In general, known benefits far outweigh known risks. The latter include the potential masking of vitamin B12 deficiency and elevated risk of detectable spontaneous abortions. The conservative approach to folate fortification adopted by most governmental agencies (less

than 1 mg/day) is appropriate considering the aforementioned studies concerning genotype rescue that were conducted since the implementation of these policies. However, the long-term risks of elevated maternal folate on the fetus are unknown and unstudied. There is an emerging awareness that gestationally determined epigenetic factors may contribute to disease risk in adulthood. Fetal adaptations to maternal nutritional status, referred to as 'metabolic imprints', may occur within critical developmental windows that include organogenesis and central nervous system (CNS) development.[6] The epigenetic mechanisms that account for such permanent adaptations are not established, but likely involve metabolic or nutrient-induced alterations of genome structure, gene expression or gene mutation. It is likely that maternal folate status influences fetal DNA methylation patterns and DNA mutation rates, events that may induce genomic alterations that maximize embryonic and fetal survival while simultaneously imparting subtle yet lasting effects on disease risk throughout the life cycle. Such potential long-term risks (or benefits) related to disease susceptibility later in life should be investigated carefully in animal models.

The effects of folate status on genome stability and gene methylation also have long-term implications for cancer susceptibility and prevention. Folate is protective against certain epithelial cancers and therefore folate fortification of the food supply may lower cancer incidence.[28] However, growth rates of established tumors can be accelerated by elevated folic acid status because folate can be a growth-limiting nutrient during states of rapid cell division.[61] Therefore, expected benefits in preventing cancer should be established firmly in both human populations and in animals while being attentive to known potential risks.

Finally, the effects of individual genotypes on folate requirements should be quantified. If new risks associated with elevated folate intake are established, our current knowledge of genetic variation indicates that an adequate folate intake for one individual may breach the safe upper limit for another. Therefore, food with elevated folate levels, achieved through novel or genetically engineered methods, appropriately may include labeling information regarding folate content. The effects of genetic variation on bioavailability are also an important consideration for future study. Most folate supplements and fortified foods use synthetic folic acid, not the natural polyglutamate forms of the vitamin. The benefits of increasing food folate by genetic enhancement of crops were reviewed recently.[62] These approaches suffer from the decreased bioavailability of natural food folate, which may be exacerbated by intestinal disorders and SNP in enzymes that promote folate intestinal absorption. Therefore, more study is required to determine the utility of this approach relative to chemical folic acid fortification.[62]

In conclusion, the interactions among nutrients and the genome present challenges for food-based approaches that seek to improve human health. These approaches seek to manipulate gene–environment interactions that have been established and refined throughout evolution by optimizing biochemical processes for survival at least through reproductive stages. Therefore, it should be expected that interventions seeking to affect these pathways, negatively or positively, will elicit both benefits and risks, which likely will differ among individuals. Manipulation of the food system must be informed by appropriately detailed knowledge of the biochemical, metabolic, physiological and epigenetic effects it will elicit in targeted population groups.

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