Review Article

Contemporary Issues Surrounding Folic Acid Fortification Initiatives

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ABSTRACT: The impact of folate on health and disease, particularly pregnancy complications and congenital malformations, has been extensively studied. Mandatory folic acid fortification therefore has been implemented in multiple countries, resulting in a reduction in the occurrence of neural tube defects. However, emerging evidence suggests increased folate intake may also be associated with unexpected adverse effects. This literature review focuses on contemporary issues of concern, and possible underlying mechanisms as well as giving consideration the future direction of mandatory folic acid fortification. Folate fortification has been associated with the presence of unmetabolized folic acid (PteGlu) in blood, masking of vitamin B_{12} deficiency, increased dosage for anti-cancer medication, photo-catalysis of PteGlu leading to potential genotoxicity, and a role in the pathoaetiology of colorectal cancer. Increased folate intake has also been associated with twin birth and insulin resistance in offspring, and altered epigenetic mechanisms of inheritance. Although limited data exists to elucidate potential mechanisms underlying these issues, elevated blood folate level due to the excess use of PteGlu without consideration of an individual's specific phenotypic traits (e.g. genetic background and undiagnosed disease) may be relevant. Additionally, the accumulation of unmetabolized PteGlu may lead to inhibition of dihydrofolate reductase and other enzymes. Concerns notwithstanding, folic acid fortification has achieved enormous advances in public health. It therefore seems prudent to target and carefully monitor high risk groups, and to conduct well focused further research to better understand and to minimize any risk of mandatory folic acid fortification.

Keywords: mandatory folic acid fortification, synthetic folic acid, adverse effects, public health

INTRODUCTION

Folate plays an essential role in the human body as a major coenzyme in one-carbon metabolism, including DNA synthesis (dTMP) and methylation. A growing body of literature indicates that these critical roles in cellular homeostasis are associated with altered risk for several diseases including cancer (1-4), Alzheimer's disease (AD) (5,6), thrombogenetic and atherogenetic vascular disease (7-12) including hypertension (13,14). They additionally influence the underlying mechanism which explains the deficiency disease of folic acid-megaloblastic anaemia (15,16). As a low folate status may perturb dTMP and methylation pathways, and as a result, influence pregnancy complications including birth defects such as neural tube defects (NTDs) (15,17-20), the vitamin is clearly important at the reproductive phase of the lifecycle. Periconceptional folic acid supplements have led to a significant reduction in the occurrence of NTDs (21-23), strengthening advocacy for, and implementation of mandatory folic acid fortification in a large number of countries (24-27). However, unexpected and potentially controversial issues have been increasingly reported in relation to mandatory folic acid fortification (28,29). In light of such controversies, the present review focuses on these contemporary issues of concern and their possible relevance to the future direction of mandatory folic acid fortification.

FOLIC ACID ABSORPTION AND DIHYDROFOLATE REDUCTASE

Synthetic folic acid (pteroylmonoglutamic acid, PteGlu)

has a fully oxidised pteridine ring and is a folyl vitamer with only a single glutamate residue conjugated to it. It is therefore very stable under the majority of conditions (i.e, temperature and pH), and is the vitamer used for supplements and food fortification (30). However, when PteGlu is exposed to UV radiation, it breaks down into the photo-scission products *p*-aminobenzoylglutamate (*p*-ABG) and 6-formylpterin (6-FP), the latter of which eventually oxidizes to form pterin-6-carboxylic acid (PCA) (31,32).

PteGlu is absorbed in the proximal jejunum through a saturable, carrier-mediated, pH and energy dependent transport mechanism similar to that required for natural methyl folate (30). However, since PteGlu is not a natural form of folate, it requires additional metabolic steps before it can enter the circulating plasma folate pool as 5-methyltetrahydrofolate (5-CH$_3$H$_4$PteGlu). In order to enter folate metabolism, PteGlu needs to be reduced first to dihydrofolate (H$_2$PteGlu) and then to the active form, tetrahydrofolate (H$_4$PteGlu) which is the methyl group shuttle required for the *de novo* synthesis of purine, thymidylate and methionine. This additional step is exclusively mediated by dihydrofolate reductase (DHFR) (33). The main role of DHFR is to catalyse the reduction of H$_2$PteGlu to H$_4$PteGlu. It is also responsible for the conversion of PteGlu to H$_2$PteGlu, but with a higher K$_m$. In addition, H$_2$PteGlu allosterically modulates the activity of methylenetetrahydrofolate reductase (MTHFR) which is one of the key enzymes of folate metabolism (34). Therefore, DHFR is critical for both the continuous circulation of reduced folate in the body and synthetic PteGlu metabolism (Fig. 1).

The activity of DHFR differs between species and individuals (35,36). DHFR activity in human hepatic tissue is significantly less than in other mammals and shows an inferior capacity to reduce PteGlu. Additionally, a 5-fold variation between individuals in DHFR activity occurs (35). Genetic variation may also affect the activity of DHFR. Of the known DHFR polymorphisms, a 19 base pair insertion/deletion located in intron 1 (19 bp del) (37-39), a C238T transition in exon 3 (40) and a A458T transition in exon 5 (41) have been thoroughly investigated, and shown to be associated with several disorders, including spina bifida, megaloblastic anaemia, and neurologic disease (37-41).

BACKGROUND TO THE IMPLEMENTATION OF MANDATORY FOLIC ACID FORTIFICATION

Adequate folate consumption is critical for women of child bearing age. Neural tube closure occurs in the early stages of pregnancy (within 28 days) before most women recognize that they are pregnant (42). This becomes even more relevant when one considers that over half of all pregnancies are unplanned (43). It is generally accepted that insufficient maternal folate status is the major risk factor for NTD, along with other genetic, geographic or socioeconomic causes (44).

Early work by Hibbard suggested a putative association between folate deficiency and various kinds of pregnancy complications and congenital disorders early (15).
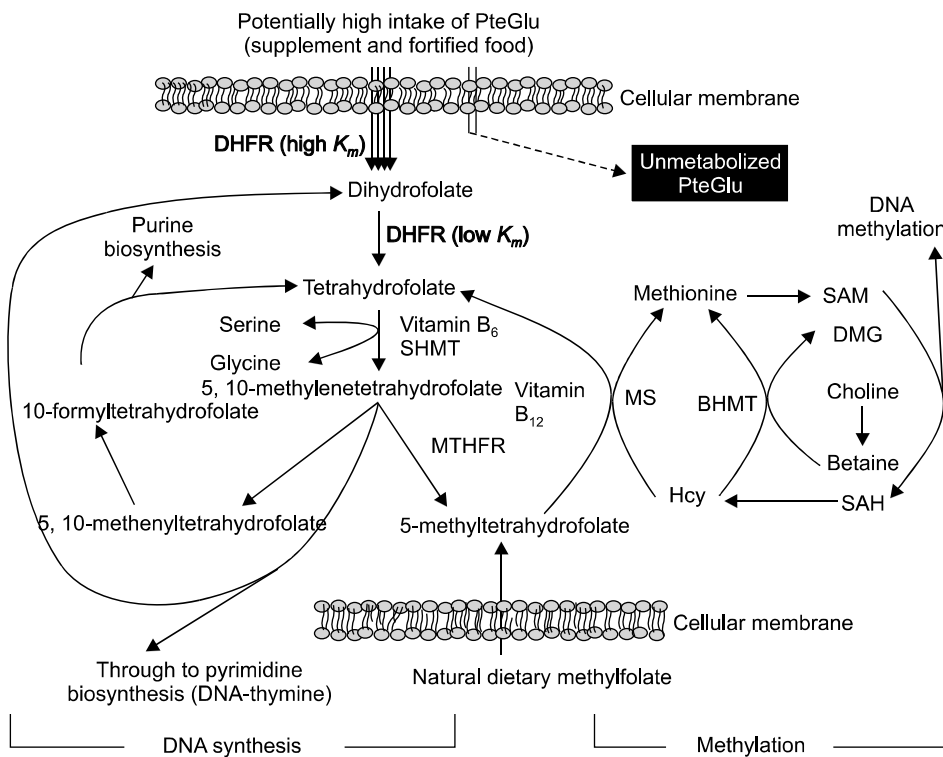

Fig. 1. Entry of synthetic PteGlu into folate metabolism. PteGlu, pteroylmonoglutamate or folic acid; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; DMG, dimethylglycine; BHMT, betaine-homocysteine methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcy, homocysteine.

Subsequent studies have shown the role of folate in preventing NTD (45,46). A major randomized control trial conducted by the Vitamin Study Research Group of the Medical Research Council confirmed the effect of folic acid supplementation in the prevention of NTD, providing significant experimental evidence for implementation of government-mandated folic acid fortification (23). In this study, women that previously experienced pregnancy affected by NTD received 4 mg of folic acid daily from the time they planned their another pregnancy until 12 weeks into it. After their delivery, the NTD recurrence rate of the group which received folic acid supplementation was 1%, but the recurrence rate of those in the non-folic acid group was 3.5% (23). This reduction of NTD rate by folic acid supplements was confirmed in an Asian population (22) and was consistent with results observed in other studies utilising periconceptional multivitamin supplements containing folic acid (21,47).

As a result of these early studies, the first government-mandated folic acid fortification programme was implemented in the United States (US) beginning in 1998, followed by multiple countries, such as Canada, Chile, and Australia, as a population health measure (48). Food fortification is the preferable strategy as it has low risk, but leads to a large effect on NTD prevalence, compared to supplementation for high-risk women alone (49). The level of fortification differs between countries, but in all cases it is designed to reduce the prevalence of NTD pregnancy. It is generally agreed that folic acid supplementation in the US and Canada was successful because population folate status was enhanced (50) and this translated into a reduction in NTD rates (25,27,51,52), although the result varied depending on ethnicity (53).

MANDATORY FOLIC ACID FORTIFICATION POLICIES IN SELECTED COUNTRIES

The folic acid fortification level in four countries, including the US, Canada, Australia, and Chile are presented in Table 1 (54-57). Cereals and flour for baking bread are the main vehicles for fortification because bread and cereal are commonly consumed by the target population: women of child bearing age (16~44 years) (58). Australia decided to administer mandatory fortification of flour (excluding flour in organic bread) from September, 2009. New Zealand deferred the implementation of mandatory folic acid fortification for 3 years and decided to keep fortification voluntary in August, 2012 (imposing a maximum level of 250 µg folic acid/100 g flour) (59).

IMPROVED BLOOD FOLATE LEVEL AND DECREASED OCCURRENCE OF NEURAL TUBE DEFECTS

The mean concentration of serum and erythrocyte folate levels in pre- and post-folic acid fortification periods and the NTD reduction rates in four countries are shown in Table 2. From the US data (NHANES), compared to the pre-fortification (1988~1994) period (60), serum and erythrocyte folate concentrations in post-fortification (1999~2010) periods have increased dramatically, resulting in a 31% reduction in the occurrence of NTD. This remarkable increase of serum and erythrocyte folate level and decreased prevalence of NTD were also detected in Canada (50,52) and Chile (24,26), providing clear evidence that mandatory fortification was an effective process in preventing the occurrence of NTD. No post-mandatory fortification NTD occurrence data is available as yet in Australia. However, since voluntary folic acid fortification of food was introduced in 1995, the total dietary intake in the population has been augmented (pre-fortification: 102 µg/day, post-fortification: 261 µg/day) (61), and as a consequence, the prevalence of NTD has been reduced by approximately 30%, except in the indigenous population (62,63).

THE CONTROVERSY OF POTENTIAL ADVERSE EFFECTS OF EXCESS FOLIC ACID

Since the adoption of mandatory folic acid fortification by many countries, the main goal of reducing the preva-

Table 1. Recommendations for folic acid intake, folic acid fortification policies and reduction of NTDs in four countries

Country	Folate RDI	Folic acid fortification policy	Year implemented
US	400 µg/day 600 µg/day–pregnancy 500 µg/day–lactation	140 µg folic acid/100 g grain product (54)	1998
Canada	US requirements	150 µg folic acid/100 g flour & 200 µg folic acid/100 g pasta (55)	1998
Chile	Ambiguous	220 µg folic acid/100 g flour (56)	2000
Australia	400 µg/day 600 µg/day–pregnancy 500 µg/day–lactation	135 µg folic acid/100 g flour (57)	2009

RDI: Reference daily intake.

Table 2. Mean concentrations of serum and erythrocyte folate for two periods in four selected countries

	Folate concentration (nM)	Pre-fortification	Post-fortification	NTD reduction
US[1] (60)	serum	16.7±0.5	41.0±0.3	19% (25)
	erythrocyte	747±10	1120±7	31% (51)
Canada[2] (64)	serum	18.5 (18.1~18.9)	27.1 (26.8~27.5)	46% (52)
	erythrocyte	680.3 (668.9~691.9)	851.6 (841.2~862.0)	
Chile[3] (24)	serum	9.7±4.3	37.2±9.5	51% (26)
	erythrocyte	290±102	707±179	
Australia[4] (65)	serum	17.7	23.1	N/A
	erythrocyte	881	1071	

[1]Mean±standard error (SE).
[2]Mean±95% confidence interval (CI).
[3]Mean±standard deviation (SD).
[4]No SE, CI, or SD available.

lence of NTD has been achieved. Other additional improvements in homocysteine (Hcy) level and neuropsychiatric symptoms had been reported/expected. However, since virtually all members in society are now exposed to plentiful folate, unexpected adverse phenomena are increasingly being reported (66,67). The precise mechanisms for these adverse effects are not always clear, but it raises questions that need to be addressed so as to plot the future direction of folic acid fortification.

Changed cellular folate distribution by folic acid fortification; the presence of unmetabolized PteGlu in blood
Interestingly, changed blood folyl vitamer distribution has been observed as a consequence of fortification in the US. A study by Kelly et al. (68) that was carried out before fortification indicated that no PteGlu occurred in fasting blood. However Troen's study conducted after implementation of mandatory folic acid fortification reported that unmetabolized PteGlu was detected in 78% of subjects (69). Kalmbach and colleagues examined the concentration of blood PteGlu before and after folic acid fortification within the Framingham Offspring Cohort (70). The results showed that prior to PteGlu fortification, the proportion of subjects with detectable PteGlu stood at 55% in non-B vitamin supplement users, but after PteGlu fortification this increased to 74.4%. In B-vitamin supplement users, the ratio for subjects with detectable PteGlu also increased from 72.5% to 80.7%. Additionally a study by Obeid et al. (71) found that unmetabolized PteGlu was detected in cord blood from infants independent of maternal periconceptional folic acid supplement intake.

The folate absorption and biotransformation process in humans can be saturated by approximately 400 μg/day of folate (72). Doses of PteGlu at or above this level are transported into the blood in a manner that is directly proportional to the dose taken, without conversion into biologically active 5-CH$_3$H$_4$PteGlu (68,72). Therefore, the habitual intake of a moderately high dose

of PteGlu (mainly from folic acid supplements or fortified food) could result in the chronic appearance of unmetabolized PteGlu in the circulation (68,72,73). In addition, as a blanket intervention, folic acid fortification does not consider each individual's characteristics such as the vitamin requirement and unique permutation of folate-related genetic variants. The excess intake of PteGlu beyond an individual's actual vitamin requirement, and the level of DHFR expression, if low, may lead to the presence of unmetabolized PteGlu in the plasma (35).

It is possible to hypothesize that the presence of unmetabolized PteGlu in blood interferes with folate metabolism, and, further, may be relevant to other adverse effects due to an elevated level of blood folate (74). For example, PteGlu inhibits H$_2$PteGlu reduction by DHFR and hence may act as a competitive inhibitor of this enzyme (75). Therefore, a high level of PteGlu possibly impairs folate related intracellular metabolism (74). Other experimental studies have shown that supplemented PteGlu dysregulates the expression of folate transporters in intestinal and renal epithelial tissues (76) and many other genes in lymphoblast cells (77). However, little clear research evidence exists, and the potential consequences of excess PteGlu remain to be proven.

Folate, the central nervous system and potential adverse phenomena relevant to vitamin B$_{12}$
Since folate is an essential cofactor in the central nervous system (CNS), inappropriate folate nutrition has been associated with many neuropsychiatric disorders (78,79). Various effects have been reported, depending on age. For instance, maternal low folate nutritional status results in the impairment of nervous cell development and proliferation in the foetus. It is also linked to mental retardation, autism spectrum disorder (ASD) and mood disorders (80). Indeed, folate deficiency and hyperhomocysteinemia are commonly observed in psychogeriatric patients. In fact, evidence exists to suggest dementia/AD may occur with homocysteine (Hcy)-related

cerebrovascular health as a component in the disease pathoaetiology in elderly subjects (78). Folate related-mechanisms underlying deficiency have been discussed, with genetic variants in folate and cobalamin metabolism being involved in exacerbating risk (80). Variants of note include MTHFR C677T (81), reduced folate carrier (RFC) A80G (82), DHFR 19 bp del (83) and transcobalamin II G776C (82).

Folate and CNS tissue development and function: Inappropriate folate nutrition may result in impaired DNA development and repair mechanisms in neurons (84). Hippocampus cells cultured in folate deficient medium (deficient in methyl donor) showed increased apoptosis and cell death. This is thought to result from uracil misincorporation and the consequent impaired repair process for amyloid beta peptide (Aβ)-induced oxidative modification of DNA bases. Aβ is a highly multifunctional and abundant protein in brain tissue from AD patients (85).

Additionally, an inappropriate folate level decreases the provision of one-carbon units for methylation (S-adenosylmethionine; SAM). Altered methylation dependant pathways in the CNS, thus may lead to neurological deficiency disorders (80). Methylation of various compounds such as protein, DNA and phosphatidylethanolamine (PE) is critical in functioning of the CNS. Carboxymethylation is significant in functioning of phosphatase 2A protein linked to key CNS proteins in AD pathogenesis (86). Hypermethylation of CpG sites in the brain-derived neurotrophic factor (BDNF) gene causes overexpression of BDNF (87) and it results in short-term memory impairment and learning deficits in a mouse model (88). Methylation of PE generates phosphatidylcholine (PC) which is the endogenous source of the cholinergic neurotransmitter acetylcholine. In a rodent model fed on a folate-deficient diet, PC concentration in brain tissue was significantly lowered and the animal showed impaired memory and learning (89).

Folate is also relevant to neurotransmitter synthesis (84). $5\text{-}CH_3H_4PteGlu$ is involved in the reduction of tetrahydrobiopterin (THB), an essential cofactor for synthesis of monoamine neurotransmitters (dopamine, serotonin and norepinephrine) (90). $5\text{-}CH_3H_4PteGlu$ also supplies methyl groups directly for monoamine transmitters. Insufficient supply of folate or altered folate metabolism by genetic variants may cause dysfunction in these metabolic processes (84,91).

Finally, inappropriate folate nutrition may be a risk factor in cerebrovascular disease, possibly via Hcy-induced cerebro-endothelial dysfunction. Increased Hcy in the brain and cerebrospinal fluid has been found in patients with neurological disorders (92). An elevated synthesis of asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor (nitric oxide induces vas-

odilatation) (93), is suggested to be a significant mediator of Hcy-induced endothelial dysfunction (94). Additionally, Hcy may induce enhanced vascular inflammation, atherogenesis and vulnerability within established atherosclerotic plaques (95), and, have a pro- oxidant effect which leads to oxidative damage to endothelial cells (96,97).

For these reasons, there have been trials carried out to investigate the application of folic acid supplements to improve neuropsychiatric symptoms, although outcomes are not consistent. Daily administration of a large 5 mg folic acid supplement for 4 weeks was not effective in enhancing psychomotor performance in healthy elderly subjects with a normal folate level (98). However, in psychiatric disorder patient with a low blood folate level, 15 mg of daily folic acid intake for 2~3 months improved memory and attention efficiency (99) and recovery from depression or schizophrenia (100) in two independent studies. In line with this, it is possible to speculate that long-term and increased consumption of folate from mandatory folic acid fortification leads to a positive influence on the occurrence of neuropsychiatric disorders with folate deficiency as part of the aetiology. One experimental trial provided evidence supporting relatively low dose (200 μg/day) folic acid supplements as effective in reducing affective morbidity concomitant with lithium therapy (101).

Folate fortification and epilepsy: As described above, folate is generally considered to have a protective effect against neuropsychiatric disorders (102), however, in epilepsy, high dose folate potentially presents significant excitatory effects if the blood-brain barrier mechanism for folate is circumvented (79). Although it is only a putative mechanism, the excitatory properties of the vitamin are mediated by blocking or reversing GABA mediated inhibition (102). The epileptogenic reactions resulting from high levels of folate were observed in animal models (103,104), therefore an increase of folic acid intake via mandatory fortification is an emerging issue in the context epilepsy. In apparent contrast, certain types of anticonvulsants reduce blood folate level (105,106), which is a critical concern for women of childbearing age with epilepsy, with links to birth defects having been reported (107). Further studies with respect to the effects of mandatory fortification on epilepsy, particularly in women of childbearing age are required (107).

Elevated folate level and vitamin B_{12}: How increased folate level influences neurological disorders related to vitamin B_{12} deficiency is a potential issue in relation to mandatory folic acid fortification. Vitamin B_{12} is a critical coenzyme for methionine synthase (MS), and is related to folate metabolism via the action of methionine biosynthesis. Therefore, insufficiency may interrupt the conversion of $5\text{-}CH_3H_4PteGlu$ to $H_4PteGlu$ (folate-trap)

(108). Furthermore, in CNS tissue, MS dependant Hcy-methylation is the sole pathway for methionine production (no betaine is involved in the pathway in this tissue) (109).

The symptoms of vitamin B_{12} deficiency are similar to those of folate. In an early study by Wills et al. (110), two types of anaemia (tropical and pernicious anaemia) responded to crude liver extract containing folate, although pernicious anaemia relapsed during treatment (111). Theoretically, H_4PteGlu could be metabolized during purine and pyrimidine synthesis even with impaired MS, and folate therapy may seem to be effective in improving the symptoms of vitamin B_{12} deficiency, while neurological lesions progress (112). For these reasons, elevated intake of folate (PteGlu) may potentially mask vitamin B_{12} deficiency and prevent early diagnosis of symptoms (megaloblastic anaemia), leading to a late diagnosis when neurologic sequelae have already occurred. This condition -pernicious anaemia- involves demyelinations and is irreversible (111).

A decline in cognition in the elderly is another potential issue of vitamin B_{12} related to mandatory folic acid fortification (113). Morris et al. (114) suggested that participants with a high intake of folate combined with low blood vitamin B_{12} showed faster cognitive decline, compared to a group with high folate intake and high total vitamin B_{12} intake. A report containing three Australian cohorts also suggested impaired cognitive performance in the elderly group with combined low serum vitamin B_{12} and high red cell folate (115).

10 to 15% of the elderly population (over 60 years) are not taking sufficient vitamin B_{12} (116), and the low level of blood vitamin possibly stems from many reasons including, lack of intrinsic factor, atrophic gastritis and other gastrointestinal issues (Crohn's and celiac disease) as well as drug and alcohol consumption (102). The prevalence of low serum vitamin B_{12} in the absence of anaemia and macrocytosis has not changed since the implementation of mandatory folic acid fortification in the US (117). However, in a Canadian study, the prevalence of people who have supraphysiological serum folate levels (45 nmol/L) with vitamin B_{12} deficiency increased from 0.09% (pre-) to 0.61% (post-fortification) (118). As a result, in the US, the Food and Nutrition Board of the Institute of Medicine established a tolerable upper intake level for folate (UL) of <1000 μg per day (111). Continuous monitoring of vitamin B_{12} levels and the balance between this B-vitamin and folate is required.

Increase of colorectal cancer risk
Colorectal cancer (CRC) is the best studied disease for which folate is considered to be an aetiological factor (119). A number of large studies indicated that folate is clearly related to CRC with high folate intake reducing CRC risk by about 40% when compared to low folate intake (3,119-122). The Nurses' Health Study conducted in the US also showed that there was a 75% reduction in CRC risk in women using multivitamin supplements containing 400 μg of PteGlu (2). It concluded that high dose folic acid supplements could reduce CRC risk (123), and that high serum and erythrocyte folate might have a protective effect against CRC (124,125).

Concerns have been raised that increased CRC occurrence has been observed in countries where mandatory folic acid fortification has been implemented, although meta-analyses do not suggest clear trend towards (126,127). Hirsch et al. (128) examined the rates of hospital discharges of patients with colon cancer in Chile. The results suggested, since folic acid fortification was implemented (2001~2004), CRC has increased by 162% in the 45~64 year group and by 192% in the 65~79 year group, compared to the pre-fortification period (1992~1996). An increased CRC incidence after folic acid fortification in Canada and the US was also reported by Mason et al. (129). The absolute occurrence rate of CRC peaked in 1998 (US) and 2000 (Canada), which is independent of an increased rate for colorectal endoscopy.

These contrasting results could form the basis for a number of hypotheses: Firstly, folyl vitamers may have different roles in cellular metabolism. Researchers have already observed differential effects of vitamins due to their source or chemical from (synthetic or natural) in protecting against cancer occurrence (130). In a study of oesophageal cancer cases, intake of methyl folate from food was associated with a reduced risk for oesophageal adenocarcinoma, while a high level of PteGlu from supplements was associated with an elevated risk of pre-cancerous lesions (131). In line with this, natural dietary folate may have protective effects with respect to CRC risk, whereas PteGlu from supplements and fortification may augment disease risk (132).

Another hypothesis is that increased folic acid intake may promote the proliferation of pre-existing neoplasms. As neoplastic cells have much higher rates of proliferation compared to normal tissue (133), supplementary folic acid intake could be a growth factor for neoplastic cells (129). In the Aspirin/Folate Polyp Prevention Study, participants in the group treated with folic acid were administered 1000 μg of PteGlu per day for 5 years. This administration did not decrease the risk of adenoma in the large intestine, and it is possible that the folic acid supplementation may have contributed to the recurrence of colorectal adenomas (134).

Additionally, DNA synthesis, methylation and repair processes may be affected by elevated folic acid intake. It has been suggested that excess vitamin may initiate the

carcinogenic mechanism in normal colonic tissue (29). As a major one-carbon supplier, excess folic acid via mandatory fortification may affect metabolic pathways related to oncogenes or tumour suppressor genes, and consequently promote the development and progression of CRC. An altered DNA repair and methylation pattern is a potential concern, since the effect of such damage is tissue-, site-, and gene-specific (66,135) with such effect possibly remaining dormant (136). Since nation-wide folic acid fortification only began in the late 1990s, there may still be insufficient epidemiological information available for long term effects to be fully verified.

Efficacy of antifolate medication
Antifolate drugs are used in chemotherapy. Due to the similarity in chemical structure between antifolate drugs and folate vitamers, antifolates inhibit target folate enzymes. For instance, methotrexate (MTX), an antifolate, is a widely used drug that can be curative for patients with solid tumours and autoimmune diseases (137). It is metabolized by DHFR and depletes the intracellular activated folate pool (138). It therefore leads to the interruption of purine and thymidylate synthesis, and impedes DNA replication and tissue proliferation, resulting in cell death. For this reason, there has been concern whether elevated blood folate levels due to PteGlu fortification may interfere with the mechanism and efficacy of antifolates chemotherapy.

Arabelovic and his colleagues computed MTX doses per patient per year (139). They compared the overall mean MTX doses before and after 1998 (when mandatory folic acid fortification was instituted) for thirty-six rheumatoid arthritis subjects in the US. Although the study involved a small number of subjects and reported only preliminary data, it determined that the mean annual MTX dose was higher after folic acid fortification.

No precise mechanism between increased dose of MTX and folic acid fortification has been hypothesized as yet. However, one *in vitro* study may provide experimental evidence to support this notion. Intestinal and renal tissues cultured in media containing a high level of PteGlu presented significant down-regulated folate uptake, showing decreased expression of RFC, folate receptor and proton-coupled folate transporter/heme carrier protein 1. This may suggest that chronic exposure to a high level of folate leads to increased folate consumption required to meet the elevated metabolic demand (76).

Little research has been carried out on this issue, therefore careful monitoring of this phenomena related to MTX dose is needed, and should be extended to other antifolates and antimetabolites such as pemetrexed and Fluorouracil (5-FU) as resistance is a critical issue in

therapy using antifolates (140).

Reduction in cytotoxicity of natural killer cells
As alluded to earlier, Troen et al. (69) reported that unmetabolized PteGlu found in the blood circulation after mandatory folic acid fortification was implemented, was associated with decreased natural killer (NK) cell cytotoxicity. The association with PteGlu was also independent of circulating 5-CH₃H₄PteGlu and total folate. However, one *in vitro* experiment suggested that supplemented PteGlu and 5-CH₃H₄PteGlu did not lead to any changes in the NK cell cytotoxicity (141). The influence of PteGlu on NK cell function remains to be proven, further studies to verify this are required.

Photolytic conversion of PteGlu into a potential genotoxic product
The presence of unmetabolized PteGlu resulting from mandatory folate fortification may be an issue of relevance given the potential for *in vivo* photolysis of PteGlu. PteGlu is photostable under anaerobic conditions (142), while, in the presence of oxygen and UV radiation, PteGlu is converted into the photolytic-degradation products PCA and 6-FP which can cause the cellular oxidation of 2'-deoxyguanosine 5'-monophosphate (143), and, further, sequence-specific DNA cleavage (G residue) (144,145). Altered DNA stability due to this oxidation of precursor DNA monomer may be a major risk in carcinogenic mechanisms (145). Over supplementation caused by folic acid fortification in high doses may result in more unmetabolized circulating PteGlu in the blood which has the potential to accelerate DNA break-down if vitamin photolysis occurs. There have in fact been studies looking at the application of this photolytic product (6-FP) in anti-cancer photodynamic therapy (146-148), however any genotoxicity associated with dietary intake level or any disease-specific and metabolic characteristics have not yet been clearly elucidated. Additionally, photo-degradation of PteGlu is accelerated by other photosensitizers such as other unconjugated pterin moieties (142) and riboflavin (149), and the interaction with other nutrients is still unknown. It is, therefore, a putative concern in skin and other cancers in the post-fortification era (31,145).

Increased twin births
A randomized-cohort study in Hungarian and Swedish women showed an approximately 40% increase in twin births in women who took multivitamins, compared with women who took just trace elements (150-152). Increasing incidence of twin births has also been reported in Chile (153) and the US (154-157). Recently it has been suggested that folate fortification might increase the success rate of *in vitro* fertilization (IVF).

Haggarty and colleagues (158) reported that high plasma and erythrocyte folate contribute to increased IVF success. However, it is a complex issue, since twin or multiple pregnancies are a risk factor for maternal and infant morbidity and mortality (159).

Elevated maternal folate may influence fat mass and insulin resistance of offspring

In a six year follow-up study of pregnant Indian women, higher maternal erythrocyte folate levels predicted higher offspring adiposity and higher homeostatic model assessment of insulin resistance. Furthermore, high insulin resistance was observed in the offspring born to mothers with a combination of high folate and low vitamin B_{12} levels. It is hypothesised that folate trapped as 5-CH_3H_4PteGlu (by vitamin B_{12} deficiency) and increased methylmalonyl-CoA could confer elevated lipogenesis (160). Interestingly, a report relating high PteGlu intake and concentration of the vitamin in breast milk has been published: According to Houghton, pregnant women who were administrated a PteGlu supplement during their pregnancy showed the presence of unmetabolized PteGlu in their milk and low milk folate binding protein synthesis (161).

Excess periconceptional folate may buffer negative effects of deleterious genotypes, and hence lead to gene selection

Although still controversial, infants born in Spain following the recommendation for folate supplement use showed an increase in the frequency of mutant alleles in MTHFR C677T and A1298C (162). Enhanced maternal blood folate may increase neonatal carriage of these mutant alleles which might otherwise (in a folate deplete environment) lead to embryo loss (162). This raises potential public health issues in that these genetic variants have been known to be associated with multiple disorders such as CRC, AD, and cardiovascular disease. In other words, this phenomenon might potentially influence both long term morbidity and mortality. Furthermore, survival of embryos with these MTHFR genotypes may lead to an increased number of women of childbearing aged who possess these genetic variants which have been known to act as risk factors for pregnancy complications including congenital disorders (158,162-164).

Folic acid fortification and autism spectrum disorder

Periconceptional folic acid supplements have generally been considered protective for ASD, although precise causality is ambiguous (165,166). The level of plasma Hcy, adenosine and SAM were significantly elevated in mothers with ASD children (82). Studies in Norway (165) and the US (166) support this finding, providing evidence that maternal use of folic acid supplements de-

creased risk of ASD 49% and 48% in their offspring, respectively.

However, recent studies also suggested that ASD occurrence is increasing with time, and increased intake of maternal folic acid supplements might be partially responsible for it (167,168). Beard et al. (167) suggested that increased prescribed vitamin use containing 1 mg of folic acid supplement was associated with increased ASD occurrence between 1976~1997. Another study in the US analyzed data from the Center for Disease Control for 1994~1999 and also generated information that the use of maternal folic acid supplements increased the risk for ASD by approximately 2.5 times (168).

Many possible theories have been given for the association between increased ASD occurrence and folic acid supplements. Leeming and Lucock (169) have examined some of these in a recent review. Rogers speculated that a high intake of PteGlu increased the number of infants with the MTHFR C677T mutant allele. However, after birth, the high level of folate experienced in utero could not be maintained. As a result of this, methylation pattern and Hcy level may be changed, and thus lead to increased occurrence of ASD (170). Advanced studies proposed underpinning molecular mechanisms. Elevated PteGlu level may dysregulate the expression of multiple genes related with early brain development (fragile X mental retardation 1, G protein-coupled receptor 37 like 1 and testis-specific serine kinase 3) (77). Additionally, elevated PteGlu intake may be relevant to increased expression of GABA (type A) beta 1 receptor, and disrupt inhibitory synaptic transmission of neuron development in the embryo (171). As not much evidence exists to debate the genotoxicity of folic acid supplements in the increased occurrence of ASD, any information and ideas should be interpreted with caution.

Inequality in folate nutrition based on ethnicity/race and economic status

An individual's socioeconomic background has an effect on their health status (172), therefore the elimination of health disparities across different groups within a population is a major concern when formulating public health policies (173). Research evidence suggests that the efficacy of folic acid fortification may be influenced by socioeconomic status and race/ethnicity, and the relative risk is concentrated in disadvantaged groups (174). The absolute risk of low erythrocyte folate was significantly decreased via mandatory fortification programmes, however the relative ratio of low folate status was increased in low income groups and whites (174). In another study, the ratio of low daily folate intake in non-Hispanic white women was significantly lower than other non-Hispanic black and Hispanic women (175). In

addition, in Australia, the decreased NTD occurrence by voluntary fortification was only observed in non-indigenous offspring (62,63). The commencement of mandatory folic acid fortification resulted in an increase of folate nutritional status in all socioeconomic and ethnic groups, however the relative risks between groups remains. This may be worthy of note, because any remaining risks may be arising across groups on a relative scale, even though it only accounts for a very small number of case in the population (176). It is therefore important to be mindful of the optimisation of intervention for targeting high risk groups to reduce the existing disparities (174,177).

CONCLUSION

The potential adverse effects that might arise from folic acid fortification are complex and could lead to a "fear of folate" if not dealt with carefully (178). However, despite this, folic acid fortification has achieved its main goal: the reduction of NTDs along with other health benefits such as decreased mortality rates after stroke (179). In the context of the benefits to public health, the implementation of folate fortification should be evaluated taking account of nutritional condition and genetic background. In particular, in countries which have already commenced fortification, monitoring the dietary intake and blood folate levels to determine the effective and safe level of folic acid fortification, along with further studies to understand the molecular mechanisms underpinning adverse effects, will help to resolve and more clearly elucidate the concerns dealt with in this paper (28).

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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