Factors Influencing the Input Side of Folate Nutrition

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Nutritional Sciences
University of Toronto

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Abstract

Mandatory folic acid fortification of the food supply in Canada has led to a 50% reduction in neural tube defects. However, fortification in addition to folic acid supplement use has led to high blood values in the non-target population. To generate evidence to inform future fortification and supplementation policy, the aim of my thesis was to establish a better understanding of the “input side” of folate nutrition. I investigated the impact of extending folic acid fortification to whole wheat flour and the contribution of colonic bacterial synthesis of folate. Using data from the 2004 Canadian Community Health Survey 2.2 (n = 35,107), a modelling exercise predicted that extending folic acid fortification to whole wheat flour would decrease the prevalence of folate inadequacy in whole wheat consumers while not putting the entire population at additional risks. In the second study, I analyzed colonic evacuant collected from rural Africans (n=20) and African Americans (n=20) consuming their regular diets and after a two-week reciprocal dietary switch. Mean colonic folate content was increased by 40% among African Americans consuming a high fibre diet patterned after a typical rural African diet (43 ± 5g/d of fibre), highlighting folate producing capacity of gut microbiota could be manipulated using dietary approaches. In the third study, I established an approach to detect stable isotopic folate in urine and confirmed pre-formed folate can be absorbed across the human
colon using urinary excretion. While urinary enrichment was detected after administration of single oral and IV dose of $^{13}$C$_5$-5-formyltetrahydrofolate (n=9), it approached the limit of detection and hence was variable. In future studies, inclusion of a pre-study protocol whereby subjects consume a daily folate supplement to saturate tissue stores may be worthwhile. A better understanding of the input side of folate nutrition will help develop dietary strategies that achieve optimal health.
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Published Materials

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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CCHS</td>
<td>Canadian Community Health Survey</td>
</tr>
<tr>
<td>CSFII</td>
<td>Continuing Survey of Food Intakes by Individuals</td>
</tr>
<tr>
<td>DFE</td>
<td>Dietary folate equivalents</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intakes</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated Average Requirement</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GCPII</td>
<td>Glutamate carboxypeptidase II</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography coupled to tandem mass spectrometry</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylenetetrahydrofolate reductase</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-amino-benzoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton-coupled folate transporter</td>
</tr>
<tr>
<td>POFI</td>
<td>Prevalence of folate inadequacy</td>
</tr>
<tr>
<td>PteGlu</td>
<td>Monoglutamyl folic acid</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized clinical trials</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SIDE</td>
<td>Software for Intake Distribution Estimation</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>TMP</td>
<td>Thymidine monophosphate</td>
</tr>
<tr>
<td>UER</td>
<td>Urinary excretion ratio</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine monophosphate</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable upper intake level</td>
</tr>
</tbody>
</table>
1. Introduction

Folate is a generic term for a family of compounds with a pterolylmonoglutamate core that are essential for one-carbon metabolism required for growth and cell division as well as biological methylation reactions (1). Folate mediates one-carbon transfer reactions involved in DNA and RNA biosynthesis as well as amino acid and hence protein synthesis (1). Folate also provides a source of one-carbon units for the generation of S-adenosylmethionine, the universal methyl donor for most biological methylation reactions, including DNA methylation (2, 3). As such, folate plays an important role in health and disease in humans (1). Improved folate intake of women, as a result of folic acid fortification of the food supply in Canada, has led to a 50% reduction in neural tube defects (NTDs) (4). However, it has been suggested that high intakes of folate in the synthetic form (i.e. folic acid) added to foods and found in vitamin supplements may be associated with a number of adverse health outcomes (1, 5, 6). For example, in some human studies, high intakes of folic acid have been associated with masking of vitamin B₁₂ deficiency (1), immune dysfunction (7) and increased risk of various types of cancer (8-10) but others did not find such associations (11, 12). Results from animal studies are more consistent and suggest high folic acid intakes facilitate the progression of neoplastic cells to cancer in models of colorectal cancer (13, 14). In addition, results from studies of both animals and human studies suggest an association between high maternal folate status or high folate intake during pregnancy and a number of adverse outcomes in offspring. These adverse health outcomes include increased risk of developing insulin resistance (5), atopic dermatitis in offspring (15), increased risk of asthma in early childhood (16), higher incidence of childhood retinoblastoma (17) and various undesirable health consequences (18-21) in offspring. Again, other studies examining
the relationship between high maternal folate status and/or folate supplementation on adverse health outcomes in offspring have not found an effect (22, 23).

Like other nutrients, folate homeostasis in the human body is maintained through a balance of input and output of folate. A better understanding of the biology behind the “‘input side’” of folate nutrition will help to set dietary recommendations that strike a right balance between benefits and risks. Folate is available from different sources including naturally occurring folate in foods, and synthetic folic acid that can be obtained from fortified food products and supplements. Folate produced by bacteria residing in colon comprises a significant depot of naturally occurring folate. The overarching goal of this thesis was to investigate various issues related to the “‘input side’” of folate homeostasis. Firstly, in this thesis, I examined how extending folic acid fortification to whole wheat flour in Canada might affect the prevalence of folate inadequacy in the population and the percent of individuals with intakes of synthetic folic acid above the Tolerable Upper Intake Level (UL) (Figure 1). Secondly, I examined how manipulating the provision of fibre in the diet may impact the quantity of bacterially synthesized folate produced in the colon. My final aim of the thesis was to investigate the feasibility of using a non-invasive means of assessing the bacterial contribution of folate produced in the colon by measuring stable isotopes of folate in urine.
Figure 1 Conceptual model of this thesis.

The diagram shows the input side of folate nutrition. The aim of this thesis is to investigate the contribution of fortification/supplements (Study I) and colonic bacterially-synthesized folate (Study II and III) to the input side of folate nutrition.

Mandatory folic acid fortification has been a tremendously successful public health strategy in reducing the risk of infants born with neural tube defects in Canada (5). At present, white wheat flour and cornmeal are mandated to be fortified with folic acid in Canada (24), while non-white wheat flours do not fall under the current mandate. Consumption of whole grain products has been associated with a decreased risk of some chronic diseases (25-28). As a result, Canada’s Food Guide encourages all Canadians to consume whole grains which would not include heavily processed white wheat flour (29). Clearly, there are discrepancies between government of Canada strategy to reduce the risk of NTD through fortification of white wheat flour and that of encouraging the consumptions of whole grain foods to reduce the risk of chronic disease. The impact of extending folic acid fortification to whole wheat flour in Canada is unknown.

Therefore, the aim of Study I of this thesis (Chapter 4) was to evaluate the impact of fortifying whole wheat flour containing foods on the folate intake distribution of Canadians and ensure this would not expose Canadians to excessive levels of folate intake.
Recently, there has been growing interest in investigating the possibility of bacterially-synthesized folate serving as a complementary source of folate for humans. Unlike animals, many bacteria that commonly reside in the human colon can synthesize folate (30-32). The O’Connor lab has previously shown that the amount of folate in the mammalian colon can exceed dietary requirements of humans and is in a form that can be absorbed (33). While recent research suggests that pre-formed folate absorption can occur across the intact mammalian colon (34, 35), very little is known in regard to the quantitative contribution of folate from the bacterial origin to whole body folate homeostasis. Furthermore, it is unknown whether manipulation of the folate producing capacity of bacteria in the colon can impact the amount of folate absorbed across the colon. We and others have previously shown that the inclusion of fermentable substrates, such as fibre or prebiotics, improve folate status of the host or increase the number of Bifidobacteria in the colon (36-38). It is known that specific strains of Bifidobacteria produce folate (30, 32). This thesis reports analyses from a collaborative study where we had access to samples of colonic fluid collected from a group of rural South Africans and African Americans (Study II, Chapter 5). Traditionally, rural South Africans consume a diet that is high in fibre, whereas African Americans consume a diet that is low in fibre (39). During study intervention, rural South Africans were provided with a Western diet containing low fibre, whereas African Americans were provided with a low fat and high fibre diet. We determined whether a reciprocal dietary switch between African Americans and rural South Africans affected the production of colonic folate.

To better understand colonic absorption of folate, we previously conducted studies where a test dose of labelled folate ($^{13}$C$_5$-5-formyltetrahydrofolate) was delivered to the colon of humans during colonoscopy (34) or via enteric caplets designed to disintegrate in the colon (35).
Labelled folate was detected in blood samples, which provided direct evidence that folate can be absorbed across the human colon. In our previous investigations, multiple blood sample collections were involved. Multiple blood sampling is invasive, whereas collection of 24 hr urine samples is not invasive, although the approach might be inconvenient. Sampling of 2 hr urine collections also facilitates a longer follow-up post-dose, which allows for a dosing protocol which facilitates reaching steady-state in the body and enables more time for labelled precursors of folate (e.g. para-amino-benzoic acid [PABA]) to be converted to labelled folate. Therefore, we analyzed folate enrichment using urine samples collected from a previous study where blood enrichment was previously used to establish the colonic absorption of folate (Study III, Chapter 6).

It appears that the relationship between folate intake and optimal health is “U-shaped” where sub-optimal and very high intakes are associated with adverse health outcomes. My thesis contributes to a better understanding of factors regulating the “input side” of folate nutrition through which dietary recommendations and strategies could be developed to achieve optimal health.

This thesis consists of seven chapters. Chapter 2 is a literature review; Chapter 3 contains research rationale, objectives and hypotheses; Chapter 4 presents a study entitled “Modelling demonstrates that folic acid fortification of whole wheat flour would reduce the prevalence of folate inadequacy in Canadian whole wheat consumers” which is referred as Study I. In Study I, I conceptualized and designed the study under the supervision of other senior authors of the manuscript. I also conducted the analyses, interpreted the data and wrote the manuscript; Chapter 5 presents a study entitled “Increasing the consumption of fibre-rich foods in African Americans increases colonic folate contents” which is referred as Study II. In Study II, I
measured folate contents in colonic evacuants, analyzed data, interpreted results and wrote the manuscript; Chapter 6 presents a study entitled “Folate is absorbed across the human colon: evidence by assessment of urinary excretions in response to administration of $^{13}$C$_5$-5-formyltetrahydrofolate” which is referred as Study III. In Study III, I contributed to method development to measure labelled folate in urine using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). I also processed the samples, analyzed data, interpreted results and wrote the manuscript; Chapter 7 outlines an overall discussion and future research directions.
2. Literature review

2.1 Introduction to folate

Section 2.1 was originally published. It is reproduced here with minor modifications.


2.1.1 Folate

Folate is a generic descriptor for a family of biochemically and structurally related compounds that share a common pteroylglutamic acid core and function in the acceptance, redox processing and transfer of one-carbon units (1). Pteroylglutamic acid, or “folic acid”, is seldom found in nature but due to its stability and low cost, is commercially synthesized and used in dietary supplements and as a fortificant in foods. Folic acid consists of a pteridine ring, linked by a methylene bridge to PABA and a single glutamic acid molecule. It is fully oxidized and doesn’t contain a carbon substitution. In contrast, folates found in nature are typically reduced to either dihydro- or tetrahydrofolate and may carry a carbon moiety (i.e. methyl, formyl, methylene or methenyl) *(Figure 2)*. A significant proportion of folates are polyglutamylated; they have several glutamates linked together creating what is commonly referred to as a polyglutamate tail attached to PABA. Folate mediates one-carbon moieties involved in nucleotide biosynthesis (purines and thymidine) and re-methylation of homocysteine to methionine, which can be used for protein synthesis or subsequently converted to S-adenosylmethionine (SAM), the primary methyl donor for over 100 biological methylation reactions (3). Given these roles, the requirement for folate and risk of deficiency are highest during anabolic stages of the life-cycle, in particular during pregnancy, lactation and fetal development.
Folate derivatives

<table>
<thead>
<tr>
<th>Folate derivatives</th>
<th>*R-substitute at N-5</th>
<th>Bridging N-5 and N-10 positions</th>
<th>R-substitute at N-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF^1</td>
<td>-H</td>
<td></td>
<td>-H</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>-CH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,10-methyleneTHF</td>
<td></td>
<td>-CH₂</td>
<td></td>
</tr>
<tr>
<td>5,10-methenylTHF</td>
<td></td>
<td></td>
<td>=CH⁺-</td>
</tr>
<tr>
<td>5-formylTHF</td>
<td>-HCO</td>
<td></td>
<td>-H</td>
</tr>
<tr>
<td>10-formylTHF</td>
<td>-H</td>
<td></td>
<td>-HCO</td>
</tr>
</tbody>
</table>

Figure 2 Chemical structure of folic acid

Other reduced folate forms are denoted by the “*R-substitutions” at the N-5 or N-10 position displayed in the box. ^1THF: Tetrahydrofolate. Adapted from Lindzon and O’Connor (40) and Lakoff (41).
2.1.2 Deficiencies

Folate status is most commonly assessed by measuring blood concentrations of folate and reviewing the size and morphology of blood cells (42). The first sign of suboptimal folate intake is a reduction in serum folate concentration, which is followed by a decrease in red blood cell (RBC) folate concentration and a rise in serum/plasma homocysteine concentration. While serum folate concentrations reflect recent dietary intake, RBC concentrations reflect tissue stores and approximate folate status over the previous 120 days, considering the average life-span of RBC in circulation. Serum/plasma homocysteine concentrations can be a useful functional inverse, albeit non-specific, indicator of folate status; however, serum/plasma homocysteine concentrations can also be elevated for other reasons including vitamin B\textsubscript{12} and vitamin B\textsubscript{6} deficiency (1). Concomitant with blood folate depletion, hypersegmented neutrophils begin to appear in peripheral blood, followed sometime later by the appearance of macrocytic blood cells. Among healthy North Americans post mandatory folic acid fortification of the food supply in 1998, classic folate deficiency manifesting in megaloblastic anemia is rare. The common cut-off values used to define risk of suboptimal folate status are < 7 nmol/L (3 ng/ mL) for serum folate and 317 nmol/L (140 ng/ mL) for RBC folate concentration (43).

A higher RBC folate cut-off (906 nmol/L [400 ng/mL] to 1000 nmol/L [442 ng/mL]) (44, 45) for women capable of becoming pregnant has been proposed to reduce the risk of NTDs (e.g. spina bifida, anencephaly) and perhaps other congenital anomalies (e.g. congenital heart defects, oral left lip and palette) (46). NTDs specifically occur when the neural tube doesn’t close properly in the third and fourth week after conception, often before a woman is aware she is pregnant. While the mechanisms are not completely understood, it is clear from evidence from randomized control trials (RCT) and national population data pre- and post-folic acid fortification of the food
supply that consumption of folic acid during periconception reduces NTD; 25-75% reductions have been observed (4, 47-52). Folate deficiency during pregnancy has also been linked to maternal megaloblastic anemia, low infant birth weight, stillbirth and premature delivery. Suboptimal intakes of folate are also associated with an increased risk of several human cancers including colorectal, prostate and breast cancer (53-56). Mechanisms underpinning this inverse association are likely related to folate’s role in mediating the transfer of one-carbon moieties necessary for DNA synthesis, stability and integrity, and repair (57). In vitro and in vivo evidence suggest folate deficiency may induce DNA strand breaks, chromosomal and genomic instability, uracil misincorporation, impaired DNA repair, and increased mutations, and that folic acid supplementation can correct some of these defects. Other conditions associated with suboptimal folate status, particularly elevated serum/plasma homocysteine concentrations, include cognitive impairment, coronary heart disease and stroke (42). Except for NTDs and macrocytic anemia, the precise nature and magnitude of the purported association between suboptimal folate status and the risk of diseases have not been unequivocally established.

2.1.3 Diet recommendations

The Recommended Dietary Allowance (RDA) for adults for folate is 400 µg/day of dietary folate equivalents (DFEs) (1). For children the RDA (µg DFE) is 150 for 1-3 year olds, 200 for 4-8 year olds, 300 for 9-13 year olds and 400 for 14-18 year olds. The RDA for pregnant and lactating women is 600 and 500 µg/day DFEs, respectively. An adequate intake level of 65 and 80 µg DFE was established for infants 0-6 months and 7-12 months of age, respectively. The concept of DFEs was introduced in the 1998 Dietary Reference Intakes (DRI) for folate as a mechanism to account for the differences in the bioavailability of synthetic folic acid and naturally occurring folate. According to the DRI, synthetic folic acid is 100 % bioavailable
ingested on an empty stomach (1). Folic acid, added to foods as a fortificant, is thought to be ~85% bioavailable and naturally occurring food folates about 50% bioavailable (1). Taken together, the following equation was developed to calculate DFEs: 1 µg of DFEs= 1 µg of naturally-occurring food folate = 0.5 µg folic acid taken in the form of supplements on an empty stomach= 0.6 µg folic acid ingested with foods (1). For women capable of becoming pregnant, the DRI recommends 400 µg of folic acid/day from fortified foods and supplement in addition to natural dietary folates to decrease the risk of NTDs (1). Health Canada recommends women of childbearing age eat folate-rich foods and take a multivitamin supplement containing folic acid (400 µg/day) to further reduce the risk of folate-dependent NTD (46).

2.1.4 Food sources

Excellent sources of naturally-occurring folates are green leafy vegetables, dark green vegetables such as broccoli, and brussel sprouts and orange juice, beans and other legumes (46). As part of a public health strategy to reduce NTD, mandatory folic acid fortification of white wheat flour (140-150 µg/100 g) and grain products labelled enriched on the food package was fully implemented in North America in 1998 (42). Several countries including Chile, Costa Rica, Australia and South Africa have also implemented mandatory fortification policies, while other countries including New Zealand and the United Kingdom have voluntary fortification programs in place. Since folic acid fortification of the food supply in North America, the largest reported component of dietary folate now comes from fortified “grains” instead of fruits and vegetables (58).

2.1.5 Clinical uses of folic acid supplementation

Women who have had a pregnancy affected by a NTD and are planning a pregnancy again may be prescribed higher levels of supplemental folic acid (4000-5000 µg/d) for at least three months
prior to conception through the first trimester of pregnancy (59). Folic acid supplementation is routinely recommended for patients taking medications known to elevate folate requirements and/or interfere with folate metabolism, including anti-folate chemotherapeutic agents (e.g., methotrexate), anti-epileptic medications (e.g., phenytoin, carbamazepine, valproate), sulfa drugs (e.g., sulfasalazine) and treatment for dyslipidemia (e.g., cholestyramine). Folic acid supplementation is also considered in medical conditions that may be associated with folate deficiency, including alcohol abuse, malabsorption from Celiac disease and Crohn’s disease, and gastric bypass surgery.

2.1.6 Tolerable upper intake level
The 1998 DRI reported no adverse effects associated with consumption of folate from foods; hence the UL for folate was based on supplemental folic acid intakes alone and its relationship with masking vitamin B\textsubscript{12} deficiency (1). The Institute of Medicine (IOM) established a UL of 1000 µg/day for folic acid. The UL was calculated using the lowest observed adverse effect level (5000 µg/day) divided by an uncertainty factor of 5 (1). Folate and vitamin B\textsubscript{12} are both co-enzymes involved in the conversion of homocysteine to methionine. According to the IOM, supplementation with $\geq$ 5000 µg/day folic acid can correct the characteristic megaloblastic anemia associated with vitamin B\textsubscript{12} deficiency, thereby potentially obscuring and delaying diagnosis of vitamin B\textsubscript{12} deficiency (1). Untreated vitamin B\textsubscript{12} deficiency can lead to subacute combined degeneration and cognitive decline, and high folic acid intakes may precipitate the neurological complications of vitamin B\textsubscript{12} deficiency while correcting the anemia (5).

2.1.7 Folate and health
While the impact of mandatory folic acid fortification on the reduction of NTDs is undisputed for the target population, there has been significant recent research activity on possible unintended
adverse consequences of high folate status, mainly from folic acid fortification and supplementation. Compared to pre-folic acid fortification of the food supply, serum folate concentrations have doubled and RBC folate concentrations have increased by at least 60% in North America (42). Additionally, post-fortification, unmetabolized folic acid is frequently detected in the circulation of healthy North Americans, even among those not consuming a supplement (60). Recent research suggests it is unlikely that intakes from dietary sources alone (natural folate and folic acid as a fortificant), exceed the UL and hence there is little risk that consumption of folic acid fortified foods on their own present a risk of masking a vitamin B\textsubscript{12} deficiency (42, 61). Use of folic acid supplements, on the other hand, can result in intakes above the UL, particularly among children (62). An emerging body of evidence has suggested that high folate status, primarily from folic acid supplementation, may be associated with adverse health outcomes. For example, several large-scaled, population based studies have suggested that high folate status is a risk factor for developing cognitive impairment in old individuals with low vitamin B\textsubscript{12} (5).

One of the most hotly debated undesirable effects associated with high folate status is potential tumor promotion. As it has been described in section 2.1.2 Deficiencies, adequate folate intake and modest supplemental intakes of folic acid appear to protect against the initiation of neoplastic transformation in normal tissue (57). However, high doses of folic acid supplementation provided post-initiation of neoplastic lesions has been shown to promote cancer development and progression in animal models (14). Available data in humans are mixed but one can perhaps divide those mixed results into two categories: effects of supplemented folic acid and effects of folic acid fortification. Firstly, the Asprin/Folic acid Colon Polyp Prevention Trial, a randomized, placebo-controlled, multicenter clinical trial showed that folic acid
supplementation was associated with a 67% increased risk of recurrent advanced polyps and multiplicity of polyps in the colon (8). In a recent meta-analyses of 10 RCTs, Wien et al. reported that there was no benefit of folic acid supplementation (≥ 400 µg/day) but a borderline significant increase 1.07 (95% CI 1.00 to 1.14) in incidence of overall cancer in the folic acid group compared to controls (9). Further, meta-analyses of six RCTs reporting prostate cancer incidence showed an RR of prostate cancer of 1.24 (95% CI 1.03 to 1.49) for the men receiving folic acid compared to controls. Similarly, a meta-analysis involving data on 50,000 individuals from 13 clinical trial reported that folic acid supplementation showed a tendency towards an increased risk of overall and site-specific cancer incidence (RR 1.06, 95% CI 0.99 to 1.13) (10).

Secondly, when total folate intake including naturally occurring folate from foods and folic acid from fortification and from supplements was considered, Stevens et al. reported that among 43,512 American men and 56,011 American women in the Cancer Prevention Study II Nutrition Cohort, total folate intake was significantly associated with lower risk of colorectal cancer even after folic acid fortification of the food supply (RRQ5vsQ1=0.81; 95% CI: 0.66-0.99; p=.047) (12). Ecological analyses examining the temporal association between folic acid fortification and colorectal cancer rates in the United States, Canada and Chile suggest that folic acid fortification may be at least in part responsible for the increase in cancer rates in these countries in the post-fortification era (63, 64). It should be noted that ecological analyses alone cannot support causality. Additional research is needed to elucidate the relationship between folate intake and cancer, in particular the potential tumor promoting effect of folic acid.

Recently, much attention has also focused on maternal folate status and its impact on disease susceptibility in offspring. In animal models, it has been shown that maternal folic acid supplementation changed the coat color in the agouti mice offspring and tail kink in the
AxinFused mice via changes in CpG DNA methylation (65, 66). In humans, high maternal circulating folate concentrations were found to be associated with an increased risk of developing insulin resistance (5) and atopic dermatitis in offspring (15). In addition, folic acid supplementation during pregnancy has been linked with an increased risk of asthma in early childhood (16). Periconceptional use of maternal folic acid supplements has been shown to be associated with a higher incidence of childhood retinoblastoma (17) but other studies have shown a decreased risk of neuroblastoma and leukemia (67, 68). Animal studies have shown that maternal folic acid supplementation during pregnancy decreased the risk of colorectal cancer (69) but increased the risk of mammary tumors (18) in the offspring. Animal work also suggests feeding high levels of folate to rat dams results in increased obesity in their pups weaned to diets containing normal amounts of folate (19-21). These effects seem to be mediated by epigenetic changes in the hypothalamus and regulation of food intake (19-21). However, a definitive conclusion about the aforementioned associations requires confirmation in future human studies with attention to how folate forms (e.g. folates vs folic acid) and dose play a role in adverse health outcomes.

2.1.8 Animal models of folate-related NTDs

As it has been described previously in this thesis, folate appears to possess a protective effect against the development of NTDs, as evidenced by human observational and randomized clinical trials (70-76). Animal models have also been developed to elucidate the specific mechanisms for folate prevention of NTDs (77). Among the many animals models employed, the mouse model is used most frequently (77). Mouse models can provide single-gene or multigene changes thought related to NTDs in humans; mouse model can also provide a uniform genetic background to investigate the effects of environmental factors, such as varying folate intake on
the incidence of NTDs (77). One example of single-gene change model would be the Cart 1 knockout mouse model resulting in null mutation in transcription factor, cartilage homeoprotein 1. Homozygous Cart1 knockout mice develop NTDs and only survive for a short time after birth (78). However, the incidence of NTDs in Cart1 knockout mice can be reduced by 60% through supplementing folic acid during first half of the gestation, therefore suggesting that the Cart 1 mutation involves folate metabolism (78). Secondly, the Alx4/Cart1 model is an example of a complex genetic model, in that the delayed formation of the parietal bone occurs in homozygous Alx4 knockout mice, whereas the single-gene homozygous mutant Cart1 knockout caused NTD (78, 79). While the effect of folate supplementation on the defects in the Alx4 mutant mouse has not been studied, evidence suggests the phenotype of mutant Cart1 is modified by the presence of the Alx4 mutation, indicating gene interactions may affect the development of NTDs (80).

Thirdly, mouse models also provide opportunities to evaluate the effects of environmental factors on folate-related NTDs, in a context of uniform genetic background. For example, folate deficient mice fed with a diet containing less than 906 nmol/kg diet did not appear to result in NTDs, in wild type mice (81). However, the authors comment that since the incidence of spontaneous NTDs is low in normal mice, normal mice may not be the optimal model to detect the effect of folate on neural tube closure (81). In summary, mouse models are useful in investigating the pathogenesis and prevention of folate-related NTDs because animal models allow one to address a single factor under controlled conditions. Furthermore, animal models also provide opportunities to study gene-gene and gene-environment interactions related to the development of folate-related NTDs. However, extrapolating results obtained from animal work to human may require extra caution.
2.2 Folic acid fortification

2.2.1 History of folic acid and prevention of NTDs

A role for folate in the prevention of NTDs was first suggested by Hibbard in 1964 (82, 83). This was later supported by several observational studies showing both periconceptional high dietary folate intake and folic acid supplements were associated with a lower occurrence of NTDs (84-88). The protective effect of periconceptional supplementation of folic acid or multivitamins containing folic acid on NTDs was further explored in non-randomized (70-72) or randomized interventions (73-76), many of which examined women with a previous NTD-affected pregnancy. The most robust and conclusive evidence of folic acid supplementation for prevention of NTDs came from two randomized placebo-controlled trials published in the early 1990’s. The first was supported by the British Medical Research Council (74) and the second, was conducted in Hungary by Czeizel et al. (76). These two studies are described below.

In 1991, the British Medical Research Council published their randomized double-blind trial involving 33 centers in seven countries. Pregnant women (n=1817) with a history of a NTD-affected pregnancy were randomized to one of four treatment groups: 4000 µg/d of folic acid; a multivitamin containing 4000 µg/d of folic acid; a multivitamin without folic acid; and placebo. Folic acid supplementation yielded a 72% reduction in NTD reoccurrence (74). Later, Czeizel et al. conducted a randomized controlled trial that recruited women with no prior NTD-affected pregnancy (76). Subjects (n=4573) were randomly assigned to receive a daily folic acid (800 µg/d) containing multivitamin supplement or a supplement only containing trace elements. There were six NTDs cases among 2056 pregnancies where mother’s consumed no folic acid compared to 0 NTDs case in 2014 pregnancies supplemented with folic acid. On the basis of these findings, several public health campaigns were initiated with the aim of improving the
folate status of women of childbearing age. In 1991, the Centers for Disease Control and Prevention issued a recommendation that all women with a history of an NTD-affected pregnancy should consume 4,000 µg of folic acid daily to prevent the recurrence of NTDs (89). In 1992, soon after the British Medical Research Council study was published, the US Public Health Services recommended all women capable of becoming pregnant should consume 400 µg of folic acid daily to reduce the risk of first occurrence of NTDs (90). In Canada, the Society of Obstetrics and Gynaecologists of Canada issued a similar recommendation that women of reproductive age should consume 400 µg of supplemental folic acid per day in addition to their dietary folate intake (91). In 1998, after reviewing available evidence and literature, IOM similarly recommended women capable of becoming pregnant consume 400 µg of folic acid per day from fortified foods and supplement in addition to usual natural dietary sources of folate (1). Educational campaigns to encourage periconceptional folic acid supplementation in women at reproductive age, however, were deemed not to be satisfactory on their own as the neural tube closes before many women realize they are pregnant and up to 50% of pregnancies are unplanned (88-92). As a result, the United States in 1998 made the decision to fortify wheat flour and a select list of other grain products with folic acid to combat the occurrence of NTDs (93). Canada adopted a similar mandatory folic acid fortification strategy later that year (24).

2.2.2 Fortification
The World Health Organization defines fortification as “the practice of deliberately increasing the content of an essential micronutrient, i.e. vitamins and minerals (including trace elements) in a food, so as to improve the nutritional quality of the food supply and provide a public health benefit with minimal risk to health” (94). Food fortification can be used as a strategy to address widespread nutrient inadequacies and correct the associated risks (95). For example, iodine fortification of salt and vitamin D fortification of milk have led to reductions in the prevalence of
goiter (96) and rickets (97), respectively. Food fortification usually involves restoring the nutrients lost during food processing and manufacturing, such as adding thiamin, niacin riboflavin, folic acid and iron back to refined grains. In some cases, nutrients that are not naturally present in the food can be added, e.g., calcium can be added to orange juice (97). In Canada, food fortification policy has been in place since the early 1900s and has prevented or corrected a number of micronutrient deficiencies, including vitamin A, some B vitamins, vitamin D, iodine and iron (95, 98). Fortification is a complex intervention and needs to strike the right balance between addressing inadequacies while avoiding excessive intakes of the population. In the 1980s, Food and Drug Administration (FDA) regulated the following measures be taken in a fortification strategy: “start with a demonstrated need; consider the unintended consequences; determine the proper fortification vehicle or vehicles and technical feasibility issues; study the potential impact on intakes/status for all age/sex groups; carry out monitoring post-fortification” (97).

2.2.2.1 Food vehicles that deliver folic acid and determination of fortification levels
Grains, specifically wheat flours, were chosen as the appropriate vehicle for folic acid fortification in the food supply in many countries, in part, due to their availability (99). In addition, milk, margarine and rice have also been selected as vehicles for folic acid in some countries (100, 101). While fortification is recognised to be an effective strategy to reach the general population, it can also be directed to a targeted segment of the population through fortifying specific food products that are consumed by the particular subgroup. Furthermore, it has been shown fortifying more than one food vehicle is more effective to reach a larger number of individuals while posing the lower risk of excessive intakes as compared to the use of single food vehicle (97, 102-105).
The level of folic acid to be used to fortify the vehicle, e.g. wheat flour is based on the estimate of per capita consumption of the vehicle in conjunction with the consideration of safety, technological and cost constraints (106). In 2006, the World Health Organization released a guideline for procedures that could be used to determine fortification levels including Feasible Fortification Level, Target Fortification Level, Minimum Fortification Level, Maximum Fortification Level, Legal Minimum Level and Maximum Tolerable Level when planning a mass fortification programme (106). Modelling exercises that simulate intake based on proposed fortification scenarios are valuable in estimating the impact of fortification on a population in reducing the prevalence of inadequacy and minimizing the risk of excessive intakes (93, 107).

2.2.2.2 Modelling exercises
Several folic acid fortification modelling exercises have been conducted in the United States, Canada and elsewhere are presented in Table 1. Food vehicles include wheat flour (61, 107-109), corn masa flour (110-112) and ready-to-eat cereals (107, 108). Several fortification levels were included in the models to determine the impact on folate intakes at the population level. It has been consistently shown in these modelling exercises that folic acid fortification significantly increases the folate intakes at the population level (107, 108) while supplement use is the primary factor that drives intakes above the UL (61, 108, 109, 111). Modelling exercises also provide data on whether a fortification strategy reaches targeted subgroups (110, 112) while avoiding putting the non-targeted population at risk of excessive intakes (111).

2.2.3 Folic acid fortification programs
2.2.3.1 The folic acid fortification program in the United States
In 1996, the FDA mandated that all wheat flour and other grain products labelled enriched be fortified with folic acid at 140 µg per 100 g of flour as of January 1, 1998 (93). Enriched grain
products include flour, rice, breads, rolls, buns, pasta and corn meal. In order to determine the proper fortification level, FDA used the 1987 to 1988 national food consumption data by the US Department of Agriculture to simulate the usual intake of folate with fortification of cereal-grain products, dairy products and juice. The proposed fortification levels considered were 70, 140, 350 µg of folic acid per 100 g of foods. Since grain cereals were consumed by more than 90% of women of childbearing age on a daily basis, it was a logical choice to serve as the primary food fortification vehicle (107). The FDA set the fortification level of 140 µg of folic acid per 100 g of cereal-grain products along with fortification of ready-to-eat cereals up to 100 µg of folic acid per serving. The fortification level was projected to increase folic acid intakes by approximately 100 µg per day in women of reproductive age while not exposing the non-targeted population to folic acid intakes exceeding the UL (93). Fortification of dairy products and juice, even with the lowest fortification level, together with the consumption of already fortified grain-cereal products, ready-to-eat cereals as well as folic acid supplements led some groups in the modelling to excessive intakes, i.e. > 1000 µg of folic acid (93). Therefore, dairy products and juice were not selected as food vehicles to deliver folic acid in the United States.

2.2.3.2 The folic acid fortification program in Canada

Given the well-established protective effect against NTDs and the necessity to meet the requirements of the new United States folic acid fortification program for Canadian exports to the United States, mandatory folic acid fortification commenced in Canada as of November 1998 (24). White wheat flour and cornmeal are mandated to be fortified at 150 µg folic acid per 100 g, and enriched pasta are mandated to be fortified at 200 to 270 µg folic acid per 100 g to compensate for the potential losses during cooking (24, 113). In addition, ready-to-eat breakfast cereals are permitted to be fortified at up to 60 µg of folic acid per 100 g (113).
2.2.3.3 Folic acid fortification programs in other countries

Mandatory fortification programs have been implemented in close to 80 countries, including Chile, Costa Rica, South Africa and Australia (114). Wheat flour is the most prevalent food vehicle used to deliver folic acid, followed by maize and rice (114). In Chile, the mandatory fortification program commenced in 2000 with the level of 220 µg of folic acid per 100 g of wheat flour with the goal of achieving folate intakes of 400 µg per day (115). However, since high folate concentrations in serum were found in the elderly population in Chile, the mandated fortification level was reduced to 180 µg of folic acid per 100 g of wheat flour in 2012 (116).

Mandatory fortification of wheat flour was implemented in Costa Rica in 1998 at the level of 150 µg of folic acid per 100 g of flour, which was increased to 180 µg of folic acid per 100 g of flour in 1999. In Costa Rica, maize flour and rice is also fortified with folic acid at the level of 180 µg per 100 g and milk is fortified at the level of 40 µg per 100 g (100). Since 2003, it has been mandatory to fortify wheat flour at the level of 150 µg of folic acid per 100 g of flour and maize meal at the level of 221 µg of folic acid per 100 g of maize meal in South Africa (117). In 2009, the mandatory fortification of wheat flour for bread-making at the level of 200-300 µg per 100 g of flour in Australia was initiated in response to the previous planning set out by Food Standards Australia New Zealand (109, 118). Both Australia and New Zealand originally were to begin the folic acid mandatory fortification program in 2009; however, due to the safety concerns of folic acid, New Zealand made a decision to continue with the voluntary folic acid fortification policy rather making it mandatory in 2012 (119). In 2006, the Food Safety Authority in Ireland recommended a mandatory fortification program be implemented. However, in 2009, the authority decided since women of childbearing age were already receiving extra folic acid from voluntary fortification and the incidence of NTDs in Ireland had been declining, the folic acid fortification program remained voluntary (120). In 2009, the European Food Safety Authority
issued a report on the analysis of risks and benefits of mandatory folic acid fortification. In that report, it was concluded that while folic acid might be associated with higher risk of colorectal cancer, more evidence of the association between high folic acid intakes and colorectal cancer is required (121). Currently, none of the European countries have introduced mandatory folic acid fortification while some countries, such as United Kingdom, have had long-standing voluntary policies in place (119).

2.2.3.4 Effects of folic acid fortification programs on NTDs and other health outcomes

Since the initiation of the mandatory fortification program, studies in the United States have shown a decrease in the prevalence of NTDs by 19-32% (47, 49, 52, 122). The reduction in the incidence of both spina bifida and anencephaly have been of a similar magnitude post-fortification, although the reduction of anencephaly took additional years to “catch up” (47, 48, 52). In Canada, a 46% reduction in the prevalence of NTD (from 1.58 cases per total 1000 births pre-fortification to 0.86 cases per total 1000 births post-fortification) was reported using data from seven Canadian provinces (4) since the mandatory fortification was introduced. A greater magnitude of decrease in the NTD rates was observed in Nova Scotia (55%) (incidence) (51) and in Newfoundland (78%) (prevalence) (50) where the baseline prevalence of NTDs was higher compared to other provinces. From pre-fortification to post-fortification, the decline in spina bifida has been greater than that of anencephaly in Canada (4). However, it should be noted that a recent report released by Public Health Agency of Canada indicates that while the prevalence of NTDs had been decreased from 5.3 to 4.4 cases per 10,000 total births between 2001 and 2004, the NTD prevalence increased to 5.7 cases per 10,000 total births in 2010 (123). In particular, a trend towards increased incidence was also seen with spina bifida, specifically from 2008 to 2010 (123). While the etiology of NTDs is no doubt multifactorial, the recent upward
trend in the prevalence of NTDs in Canada might be related to the increased whole grain consumption in the population. Of note, whole grain products currently are not permitted to be fortified with folic acid in Canada. However, unlike white wheat flour, whole grains do provide a source of natural endogenous folate. Other countries with mandatory fortification programs, such as Chile, South Africa, Argentina, Brazil, Costa Rica, Jordan and Iran, have also reported dramatic reductions in the NTD rate, ranging from 19 to 58% since implementation of a folic acid fortification policy (113, 124). The decline in NTD rates varies by country, which are likely due to several factors, such as whether or not population-level data capture early terminations due to a NTD, the baseline birth defect prevalence and baseline folate status of the population (44, 124). A recently published meta-analysis included 123 population-based studies evaluating global birth prevalence of spina bifida by folic acid fortification status (125). The author concluded that the prevalence of spina bifida was lower (33.86 per 100,000 live births) in the countries with a mandatory fortification program than that of a voluntary fortification program (48.35 per 100,000 live births) (125). Overall, mandatory folic acid fortification has been thought to be one of the most successful public health initiatives to combat NTDs related morbidity and mortality worldwide (113).

While the folic acid fortification has successfully reduced the prevalence of NTDs as described above, countries with mandatory folic acid fortification programs seem to have achieved a “floor effect” for folic acid-preventable NTDs (126). It is clear that not all NTD cases can be prevented by increasing folate intake. Indeed, the etiology of NTDs is complex and multifactorial, including genetic variants, maternal status of other nutrients (e.g. vitamin B₁₂) and environmental factors (127). For example, a common polymorphism of methylenetetrahydrofolate reductase (MTHFR, 677C → T) may be associated with increased risk of NTDs as it may lower
availability of folate for the anabolic surge of early pregnancy as evidence by the association of this polymorphism with lower folate status and/or higher homocysteine levels (128, 129). In addition to folate, maternal status of other one-carbon nutrients, including maternal vitamin B$_{12}$ (130, 131) and choline status (132) is also associated with an elevated NTD risk.

Mandatory folic acid fortification and folic acid-containing multivitamins during periconception may also have an impact on the prevalence of congenital heart defects (133). Ionescu-Ittu et al. reported a significant reduction in the prevalence of severe congenital heart defects after the mandatory folic acid fortification program was implemented in Quebec, Canada (134). The decrease in the prevalence of left ventricular outflow tract obstruction, but not overall congenital heart defects was observed post-fortification in Alberta, Canada (135). Similarly, a significant decrease in the prevalence of congenital heart defects was reported in the United States after the mandatory folic acid fortification program was introduced (136). However, others did not find folic acid fortification was related to a reduced risk of congenital heart defects (137, 138).

The decrease in mortality due to stroke among adults was observed in both the United States and Canada between 1998 to 2002, after the mandatory folic acid fortification program was in place (139). The authors speculated that lower homocysteine levels as a result of increased folate status post-fortification might have contributed to the reduction in deaths from stroke in the North American population (139).

### 2.2.3.5 Effects of fortification on folate intake, folate status and other biomarkers

Total folate and folic acid intake in North America has significantly increased since the mandatory fortification program was introduced (140-142). Using data from National Health and Nutrition Examination Survey (NHANES) 2003-2006, Bailey et al. reported that in the post-
fortification era, more than 95% US children aged 1-13 yr met their Estimated Average Requirement (EAR) for folate from food alone (143). In the same cohort, however, more than 50% of the supplement users aged 1-13 yr exceeded the UL (143). In the adult population, analysis on NHANES (2003-2006) revealed that about 20% of women of childbearing age had folate intakes level below their EAR from food alone while up to 5% of the adult population exceeded the UL when folic acid intake from supplement was considered (144). In Canada, Shakur et al. analyzed data from the 2004 Canadian Community Health Survey (CCHS) 2.2 and found that more than 80% of the population met their EAR for folate when the folic acid fortification overage in the food supply was considered (61). The analysis also showed that 5% of the population exceeded the UL due to supplement use (61).

Higher serum and RBC folate concentrations have also been observed in the post-fortification era. Pfeiffer et al. compared serum and RBC folate concentrations pre- (1988-1994) and post- (1999-2004) fortification using national data from the NHANES cohort (43). She and her colleagues reported that compared to the pre-fortification period, serum and RBC folate concentrations were 2.5 and 1.5 times were higher in the post-fortification era, respectively (43). In addition, the prevalence of serum (<10 nmol/L) and RBC folate (< 340 nmol/L) indicative of deficiency was < 1% post-fortification whereas 24% and 3.5% of the population pre-fortification had serum and RBC folate indicative of deficiency, respectively indicative of deficiency (43). In Canada, data from the Canadian Health Measure Survey, a nationally representative survey, show that <1% of the Canadian population is folate deficient while more than 40% of the population demonstrated RBC folate concentrations above 1360 nmol/L, a cut-off that represents the 97th percentile from the NHANES (1999-2004) (145). It appears however, 22% of Canadian women of childbearing age had RBC folate status below 906 nmol/L which is the cut-off that was conventionally thought to be optimal for NTDs protection (45, 145). Unmetabolized folic
acid was detected in serum in older adults (60), adults (146), children (147) and in breast milk (148) post-fortification. It has been reported that increased unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity in postmenopausal women, aged >60 yr in the United States (7).

There is no established cut-off for homocysteine status but it is generally agreed that concentrations above 13 µmol/L are considered high (149) and concentrations below 9 µmol/L (150) are thought to be desirable. Using data from NHANES (1999-2000), Pfeiffer et al. reported 78% of the US population had homocysteine concentrations below 9 µmol/L (151). A total of 95% of the Canadian population had homocysteine concentrations below 13 µmol/L, determined using samples collected post-fortification for the Canadian Health Measures Survey (152).
### Table 1: Modelling exercise that demonstrates the estimate of folic acid fortification scenario on folate intake

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Database</th>
<th>Population</th>
<th>Fortification/modelling scenarios</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crane et al., 1995</td>
<td>USA</td>
<td>The US Department of Agriculture’s 1987-1988 National Food Consumption Survey</td>
<td>General US population</td>
<td>Cereal-grain products: 70, 140 and 350 µg of folic acid per 100 g</td>
<td>The proposed folic acid fortification scenarios would increase folate in the general population as well as women of reproductive age. Further, an increase in folate intake would be seen in the population at the higher end of the folate intake distribution.</td>
</tr>
<tr>
<td>(107)</td>
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<td>Ready-to-eat cereals: 100 or 400 µg of folic acid per serving</td>
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<tr>
<td>Tinker et al., 2012</td>
<td>USA</td>
<td>National Health and Nutrition Examination Survey, 2003-2008</td>
<td>US adults, male and female (non-pregnant), 19yr or older (n=16,665)</td>
<td>Combination of the following scenarios: Enriched cereal grain products: 70, 140, 210, 280, 350 µg of folic acid per 100 g Ready-to-eat cereals: 0, 100, 200, 400 µg of folic acid per serving</td>
<td>The proportion of the US adult population with intakes above the UL would be determined by supplement use. Increase in the fortification level in enriched cereal grain product would not increase the proportion of the population with intakes above the UL but would</td>
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<td>(108)</td>
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<td>Source</td>
<td>Country</td>
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<td>Group Details</td>
<td>Intake Details</td>
<td>Conclusion</td>
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<tr>
<td>Hamner et al., 2013 (110)</td>
<td>USA</td>
<td>National Health and Nutrition Examination Survey, 2001-2008</td>
<td>US non-pregnant, 15-44 yr women (n=5,369)</td>
<td>Corn flour: 140 µg of folic acid per 100 g</td>
<td>A much higher relative percentage change in usual total folic acid intake would be obtained in Spanish-speaking Mexican-American women than that of English-speaking Mexican-American women.</td>
</tr>
<tr>
<td>Hamner et al., 2013 (111)</td>
<td>USA</td>
<td>National Health and Nutrition Examination Survey, 2001-2008</td>
<td>General US population (n=32,590)</td>
<td>Corn flour: 140 µg of folic acid per 100 g</td>
<td>Fortifying corn flour with folic acid would not increase the percentage of the population with intakes above the UL in the entire US population. The proportion of the US adult population with intakes above the UL would be driven by supplement use, not fortification of food supply.</td>
</tr>
<tr>
<td>Study</td>
<td>Country</td>
<td>Survey Description</td>
<td>Population Details</td>
<td>Folic Acid Intake Details</td>
<td></td>
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<tr>
<td>Hamner et al., 2009</td>
<td>USA</td>
<td>National Health and Nutrition Examination Survey, 2001-2008</td>
<td>General US population (n=17,341)</td>
<td>Corn flour: 140 µg of folic acid per 100 g</td>
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<td></td>
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<td></td>
<td>The usual total folic acid intake would be increased by 19.9% in Mexican American women</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>and 4.2% in non-Hispanic white women.</td>
<td></td>
</tr>
<tr>
<td>Shakur et al., 2010</td>
<td>Canada</td>
<td>Canadian Community Health Survey 2.2, 2004</td>
<td>General Canadian population (n=35,107)</td>
<td>Enriched white wheat flour: 150 µg of folic acid per 100 g and select other grain products labelled enriched plus 50% of fortification overage</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td>Supplement: 0, 200, 250, 300, 325, 350, 375, 400, 500, 600, 700, 800, 900, 1000 µg per dose.</td>
<td></td>
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<td></td>
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<td></td>
<td>The prevalence of folate inadequacy would be less than 20% after correction for the overage.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Only 18% of reproductive women consumed &gt;400 µg of folic acid from foods and supplements.</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>The proportion of the population with the intakes above the UL is driven by supplement use, not fortification of the food supply.</td>
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<td></td>
<td></td>
<td>Supplements containing 325-700 µg per dose could provide women of reproductive age with 400 µg/ d while not exceeding the UL.</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Country</td>
<td>Survey Details</td>
<td>Population</td>
<td>Scenario Description</td>
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<tr>
<td>Sacco et al., 2009</td>
<td>Canada</td>
<td>Canadian Community Health Survey 2.2, 2004</td>
<td>General Canadian population (n=35,107)</td>
<td>Assumed full implementation of discretionary fortification that permits a wide range of foods to be fortified. Breakfast cereals The proportion of the population with intake above the UL would range from &lt;5 to up to 24%, depending on the age group; larger percentage exceeding the UL would be seen in children and adolescents.</td>
<td></td>
</tr>
<tr>
<td>Emmett et al., 2011</td>
<td>Australia</td>
<td>National Nutrition Survey for food intake patterns, 1995 Victorian Population Health Survey for supplement use, 2007</td>
<td>Australian women, 16-44 yr (n=3,178)</td>
<td>Scenario 1: folic acid from food fortification (wheat flour for bread making: 200-300 µg of folic acid per 100 g); no one takes supplement Scenario 2: folic acid from food fortification (wheat flour for bread making: 200-300 µg of folic acid per 100 g) and from supplement use Scenario 3: supplement users are also bread consumers Scenario 4: supplement use increased in response to educational campaign while food choice unchanged A total of 3.9, 25, 22 and 30% of the population in scenarios 1-4, respectively would achieve the intake of 400 µg per day of folic acid. The proportion of population with the intakes above the UL would be mainly driven by supplement use.</td>
<td></td>
</tr>
</tbody>
</table>


| Verkaik-Kloosterman et al., 2012 (154) | The Netherlands | Dutch National Consumption Survey-Young Children, 2005/2006 | Dutch children, 2-6yr | Maximum allowed fortification level for folic acid: 100 µg per 100 kcal | In the current market situation, the proportion of population with intakes above the UL would be mainly driven by the supplement use. |
2.3 Folate absorption and metabolism

2.3.1 Folate absorption in the small intestine

Folate absorption in the small intestine usually involves a 2-step process: hydrolysis and absorption. In the small intestine, polyglutamylated folate, naturally abundant in food, needs to be hydrolyzed to the monoglutamyl form, which can be then absorbed. Hydrolysis occurs in the brush border of the small intestines of humans and pigs by glutamate carboxypeptidase II (GCPII) (155, 156). In humans, GCPII is found in the prostate, small intestine, brain, kidney liver, spleen and colon (157). The optimal pH for the complete deconjugation of polyglutamyl folate is around 6.5 (158, 159). Numerous observational studies have reported several polymorphisms (such as 1561C → T and 484A → G) in the gene encoding GCPII that might influence blood levels of folate and homocysteine; however, results have not been consistent (157, 160-164). In vitro evidence using COS-7 cells suggests the 1561C → T mutant variant demonstrates 53% lower GCPII enzyme activity compared the wild type (157).

Subsequent to hydrolysis, monoglutamylated folate may be absorbed across the apical side of the enterocyte through nonsaturable or saturable processes. At a high intraluminal concentrations of folate (>10 µmol/L) as the result of pharmacological intake levels, monoglutamylated folate is transported across cell through a nonsaturable passive diffusion process. Folate can also be transported by a saturable mechanism which is a pH, energy dependent and carrier-mediated process at a lower concentrations of folate (<10 µmol/L). Two transmembrane transporters, reduced folate carrier (RFC) and the recently discovered proton-coupled folate transporter (PCFT) are believed to be primarily responsible for transport of folate at physiological concentrations (156). The characteristics of RFC and PCFT are summarized in Table 2.
Table 2 Characteristics of reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RFC</th>
<th>PCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized</td>
<td>Small intestine, colon, kidney, choroid plexus, liver</td>
<td>Small intestine, colon, kidney, brain, liver, retina, placenta</td>
</tr>
<tr>
<td>distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>Brush border membrane of small intestine and colon</td>
<td>Brush border membranes of small intestine and colon (small &gt; colon)</td>
</tr>
<tr>
<td>expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding affinity for reduced folates</td>
<td>Km 1-10 µM</td>
<td>Km 0.5-2 µM</td>
</tr>
<tr>
<td>Binding affinity for folic acid</td>
<td>Km 200-400 µM</td>
<td>Km 0.5-2 µM</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>7.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Gene locus</td>
<td>21q22.3</td>
<td>17q11.2</td>
</tr>
</tbody>
</table>

Adapted from Lakoff (41)

RFC (official gene symbol SLC19A1) is expressed in the intestine, kidney, liver, choroid plexus and colon (156, 165). RFC has a high affinity for reduced forms of folate (Km 1-10 µM) and a very low affinity for folic acid (Km 200-400 µM) (166). RFC was once thought to be the major route of delivery of folates to cells under the physiological pH. While SLC19A1 (-/-) is embryonic lethal, when the RFC-null mice from pregnant females are supplemented with folate, they can survive but have a variety of congenital malformations (167). The pH optimum for the RFC is about 7.4 and the transport activity is negligible when the pH falls under 6-6.5 (156). As the absorptive surface of the small intestine is an acidic microenvironment, the long accepted
role of RFC in intestinal folate absorption may be questionable. In contrast, the in vivo evidence suggests that PCFT activity increases as pH decreases with the pH optimum of ~5.5. PCFT (SLC46A1) is found in the intestine, kidney, liver, placenta and brain (156). Along the intestine, PCFT can be found in the apical brush border of the duodenum and jejunum. In addition, PCFT appears to have a high and similar affinity for reduced folates and folic acid (Km 0.5-2 µM) (166). Taken together, while both RFC and PCFT are expressed on the apical membrane of proximal small intestine, the characteristics of PCFT, including the pH optimum and the similar affinity for reduced folates and folic acid suggest PCFT likely plays a primary role for folate transport in the small intestine. Severe systemic and neural folate deficiency resulting from hereditary folate malabsorption in humans with a genetic mutation in PCFT (PCFT -/-) and the recently developed PCFT-null (PCFT-/-) mouse model provide additional evidence that PCFT plays a predominant role in folate absorption (165, 168).

Folate intake and status likely influence folate absorption and uptake via RFC and PCFT. Steady-state mRNA and protein expression levels of mRNA increased in the small intestine when mice were fed a folate-deficient diet (169, 170). Similarly, higher PCFT mRNA expression levels were observed in the proximal small intestine of mice fed a folate-deficient diet compared with the ones that were fed a folate-replete diet (171). In contrast, over-supplementation with folic acid may down-regulate folate uptake. Using media designed to mimic folate over-supplementation, Ashokkumar et al. reported a reduction in ³H-folic acid uptake by Caco-2 cells (172). This reduction of folate uptake was associated with a decline in RFC protein expression and in mRNA expression levels of RFC and PCFT. Similarly, Dev et al. demonstrated a decline in intestinal uptake of folic acid in brush-border membrane vesicles harvested from the small intestine of rats over-supplemented with folic acid (173). This was
accompanied by a decrease in protein, but not mRNA, expression levels of both RFC and PCFT in the small intestine. House et al. reported that although mRNA expression levels for PCFT and RFC remain unchanged in laying hens fed 0, 10, and 100 mg/kg of folic acid, mucosal to serosal uptake of folic acid in the duodenum decreased with increasing levels of folic acid (174-176).

Folate receptors (FRs), α, β and γ, are high affinity folate-binding proteins that are found in some epithelial cells but are only expressed very low levels in gut mucosal cells (177). FR are not believed to play a major role in folate absorption. FR- α is found at the apical surface of some tissues, including kidney proximal tubules, ovaries, fallopian tubes while the protein is expressed on the basolateral membrane of retinal pigment epithelium (177). FR- β can be found in the placenta, spleen and thymus. FRs shows a higher affinity to folic acid compared to reduced folate (156, 166). FR- α (-/-) in mice leads to embryonic lethality and the surviving embryos develop NTDs. FR-β-null mice do not experience either embryonic lethality and develop normally (178).

2.3.2 Folate absorption in the large intestine

Mechanisms for folate absorption across the colon are not well understood but it has been hypothesized it may be very similar to how folate is absorbed in the small intestine. Data from organ-cultured endoscopic biopsies of human proximal colon and distal sigmoid mucosa show folic acid uptake by diffusion mediated by a low affinity carrier (179). Studies using purified colonic apical membrane vesicles (180) and a normal human colonic epithelial NCM460 cell line (181) suggest a carrier-mediated, pH dependent folate transport mechanism under the regulation of a protein kinase and a cAMP-mediated pathway. One study that investigated transport of folate across colonic apical membrane, found high activity at pH 5.5 (180). It also found higher
maximum velocity of folate uptake in the colon than in the small intestine, with the highest rate in the proximal colon and slightly lower rate in the distal colon (180). Data from purified human colonic basolateral membrane vesicles also suggest that folate exits the colonocytes through a carrier-mediated, pH dependent system through an electroneutral process (182). The expression of RFC was found in both colonic apical and basolateral membranes (182). In intestine, the PCFT is much less expressed (as determined by the mRNA level) in the colon than in the small intestine (183). It has been recently questioned whether PCFT found in human colon has appreciable activity given the luminal pH of human colon (165). PCFT has a pH optimum of 5.5 whereas pH in the colon ranges from 6.37-7.04 (184) and pH in the colonic mucosa ranges from 7.05-7.46 (185), depending on the regions of the colon.

Results from early studies in rats indicate that intestinal biosynthesis of folate in the colon is sufficient to support normal growth (186). As rats are known to practice coprophagy, it was assumed that bacterially synthesized folates of the colonic origin were made available for tissue incorporation by the rats consuming their folate-containing feces, which is then absorbed across the small intestine (187, 188). More recently, it was demonstrated that folate is also absorbed across the colon of mammals. In an animal study by our research group, pigs were injected with \(^{3}\)H-folic acid into the cecum. Subsequent analysis revealed that 82.1, 12.3, 3.9 and 1.7% of recovered \(^{3}\)H-folate was found in the feces, liver, urine and kidneys, respectively (189). The liver and kidneys are thought to contain 40 to 50% of total body folate. In the same study, pigs were also injected with \(^{3}\)H-PABA (precursor of bacterial folate biosynthesis), and the amount of \(^{3}\)H-folate recovered in the feces, liver and urine was 85.1, 0.4 and 14.6%, respectively (189). Since the injected labelled dose of PABA was later recovered as \(^{3}\)H-folate in tissues, this study illustrates that bacterially synthesized folate can be absorbed across the colon in pigs.
Aufreiter et al. conducted the first study to provide direct evidence that folate can be absorbed across the intact colon of humans (34). During colonoscopy, a physiological dose (648 nmol) of naturally occurring folate ($^{13}$C$_5$-5-formyltetrahydrofolate) was infused into the cecum of adults. Study results showed that $^{13}$C$_5$-5-methyltetrahydrofolate appeared in plasma after cecal infusion at a rate of $0.6 \pm 0.2$ nmol/h compared to $7 \pm 1.2$ nmol/h after intravenous (IV) injection of $^{13}$C$_5$-5-formyltetrahydrofolate. It is known that 5-formyltetrahydrofolate is converted to methyltetrahydrofolate in the small intestine intracellularly prior to entering the circulation. While $^{13}$C$_5$-5-methyltetrahydrofolate cleared within 4 hours after IV injection, it continued to rise over the same time period after cecal infusion of the test dose suggesting that while absorption may be slow, it likely occurs along the entire length of the colon (transit time 24-72 hours).

In the Aufreiter study, the normal colonic microbiota milieu was disturbed due to colonic cleansing in preparation for colonoscopy (34). To investigate colonic absorption of folate with an intact microbiota, a method to deliver folate to the colon was developed and validated by our research team. Specifically, barium sulphate caplets coated with a unique pH dependent acrylic copolymer were developed to non-invasively and quantitatively deliver stable isotopes of folate past the ileocecal junction of adult humans (190). These newly designed pH-dependent release caplets were utilized in a subsequent experiment to deliver an 855 nmol oral dose of $^{13}$C$_5$-5-formyltetrahydrofolate to the colon of human volunteers (35). Their microbiota remained intact and disintegration of the caplets was monitored by fluoroscopy. The apparent absorption rate was determined using the mathematical model described by Wright et al. (191). Study results revealed that the rate of appearance of $^{13}$C$_5$-5-formyltetrahydrofolate was $0.33 \pm 0.09$ nmol/h in response to the caplet dose and the mean apparent bioavailability of folate across the colon was
46%. These findings suggest that a significant amount of folate can be absorbed across the human colon in the presence of an undisturbed microbiota.

2.3.3 Folate distribution, physiological functions and excretion

After absorption, folates are transported to the liver, which accounts for about 50% of the body pool (192, 193). Absorbed folates are converted back to the polyglutamyl form in the liver and are further metabolized or stored. Some liver folate enters the enterohepatic circulation and is excreted into bile (193, 194). It is believed most biliary folate is reabsorbed in the gastrointestinal tract to regulate the meal-to-meal fluctuation of folate supply to cells (194).

Due to the first pass-effect, the liver retains approximately 10-20% of the absorbed folates with the rest entering systemic circulation. As noted in the section 2.1, within cells, tetrahydrofolate (THF) polyglutamates functions as a coenzyme, involved in nucleotide synthesis and methylation cycle by accepting and transferring one-carbon (Figure 3). In the methylation cycle, MTHFR is responsible for the conversion of 5,10-methylene THF to 5-methyl THF, which is the methyl donor for the remethylation of homocysteine to methionine. The MTHFR 677C → T polymorphism converts alanine to valine in the MTHFR enzyme (195) and is associated with reduced thermostability. The overall enzymatic activity of this thermolabile variant is found to be 35-50% of the wild type (C/C). Those homozygous (T/T) for this polymorphism have a greater reduction in MTHFR enzyme activity compared to those who are heterozygous (196, 197). It is expected that the reduced efficiency of converting 5,10-methylene THF to 5-methylTHF decreases the amount of circulating folate (i.e., 5-methylTHF). While the association between RBC folate levels and MTHFR variants is inconclusive (likely due to the limitations in analytical methods) (198), TT genotype has been found to be associated with a 10-
35% reduction in plasma/serum folates. It is believed that folate requirement is higher in TT homozygous individuals (199). Increased homocysteine levels have been reported in CT heterozygous individuals with the presence of heterozygosity for the MTHFR 1298A→C (200).

Folate is excreted in urine and bile with an estimate 0.3-0.8% of the total folate body pool being excreted daily (201). Daily urinary excretion of folate ranges from 3-75 nmol in the form of intact folate (201). Since folate is effectively reabsorbed in the proximal renal tubule through FR-α, less than 5% of ingested folate is excreted in urine following a normal physiological dose (194, 202-204). Folate loss through urinary excretion appears to be intake-dependent. When subjects were supplemented with 454 nmol/d (200 µg/d), 680 nmol/d (300 µg/d) and 907 nmol/d (400 µg/d) of folate for 10 weeks, mean urinary folate was 3, 6 and 25 nmol/d, respectively (205). Higher levels of urinary folate excretion has also been found in alcoholic subjects (206). It should be noted that the majority of excretion products in human urine are folate cleavage products (~330 nmol/d) resulting from the cleavage of C9-N10 bond to yield para-aminobenzoylglutamate and its acetylated derivative para-acetamidobenzoylglutamate (205).

Unlike the excretion of intact folate in urine, urinary excretion of para-aminobenzoylglutamate and para-acetamidobenzoylglutamate is not affected by increasing folate intake from 454 to 907 nmol/d for 10 weeks (205).
Figure 3 Folate-dependent one-carbon metabolism involving methylation and nucleotide synthesis.

DHF: dihydrofolic acid THF: tetrahydrofolic acid; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; TMP: thymidine monophosphate; UMP: uridine monophosphate. Enzymes: 1. thymidylate synthase; 2. serine hydroxymethyltransferase; 3. dihydrofolate reductase; 4. methylenetetrahydrofolate reductase; 5. 5,10-methenyltetrahydrofolate synthetase; 6. methionine synthase; 7. betaine-homocysteine methyltransferase; 8. DNA methyl transferase; 9. SAH hydrolase. Adapted from Lakoff (41)
In humans, the folate content in bile has been estimated to range from 227 to 5300 nmol/d (193, 194). Much of this is reabsorbed in the gastrointestinal tract (194). The colonic folate content measured in colonic evacuants is reported to range from 1494 to 1838 nmol/d (39). The folate content in adult human feces appears to be about 415-454 nmol/d (194, 207). In infants, feces contained about 90 nmol/d of folate; folic acid was detected in 2 out 12 exclusively human fed infants (receiving no folic acid) and 6 out of 10 infant formula fed infants (receiving folic acid from the infant formula) (33).

2.4 Folate bioavailability

The term bioavailability can be defined as the fraction of an ingested nutrient that is absorbed, reaches the targeted tissue and is available for metabolic processes in the body or for storage (208). In pharmacology, bioavailability is determined as the plasma or urine response to an oral dose compared to the plasma or urine response to an IV dose of the similar compound. When a dose is given via the IV route, the bioavailability is considered to be 100%. In the case of folate, since some evidence suggests oral and IV doses of folate (e.g. folic acid) may be handled differently in the body, the classic definition of bioavailability may not be completely suitable (203, 208). For this reason, in this thesis, folate bioavailability can be thought to be equivalent to folate absorption. In some studies, the reference dose (folic acid) is given orally to determine the relative bioavailability of a test dose (different folate forms). Factors affecting folate bioavailability include extrinsic factors, (such as forms of folates, monoglutamyl vs polyglutamyl forms, food matrix) and host-related intrinsic factors, such as disease, age, luminal pH and polymorphisms in GCPII and RFC (Table 3).
Table 3 Factors influencing folate bioavailability

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects on folate bioavailability</th>
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<tbody>
<tr>
<td><strong>Extrinsic factors</strong></td>
<td></td>
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<tr>
<td>Folate form-level of reduction</td>
<td>Reduced folate &lt; folic acid (209)</td>
</tr>
<tr>
<td></td>
<td>Reduced folate = folic acid (210, 211)</td>
</tr>
<tr>
<td>Folate forms-polyglutamate chain</td>
<td>Polyglutamyl folic acid &lt; monoglutamyl (212, 213)</td>
</tr>
<tr>
<td></td>
<td>Polyglutamyl folic acid = monoglutamyl (214, 215)</td>
</tr>
<tr>
<td>Matrix of food</td>
<td>Folic acid &gt; folate from a mixed diet (216)</td>
</tr>
<tr>
<td>Fibre</td>
<td>↓ folate bioavailability</td>
</tr>
<tr>
<td>Tomatoes, orange juice and citrate</td>
<td>↓ folate bioavailability (217, 218)</td>
</tr>
<tr>
<td><strong>Host-related intrinsic factors</strong></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal disease, such as atrophic</td>
<td>↓ folate absorption (219)</td>
</tr>
<tr>
<td>gastritis, celiac disease and Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>GCPII (1561C→T)</td>
<td>↓ folate absorption (157)</td>
</tr>
<tr>
<td>RFC (80A→ G)</td>
<td>↓ folate absorption (220, 221)</td>
</tr>
<tr>
<td>MTHFR (677C→T)</td>
<td>Affects the conversion of folate in the body and may have higher folate requirement (197, 199)</td>
</tr>
<tr>
<td>Age</td>
<td>No effect (222)</td>
</tr>
<tr>
<td></td>
<td>Older &lt; younger (223)</td>
</tr>
</tbody>
</table>

Adapted and updated from Melse-Boonstra 2004 (208).
2.4.1 Quantifying folate bioavailability using acute protocols: plasma response

Folate bioavailability can be evaluated using long term or short term protocols (224). Long term protocols involve investigating the effects of feeding reduced folates or folic acid for extended period of time (normally >4 weeks) on folate status (224). Folate status can be examined using a number of biomarkers, such as plasma/RBC folate concentrations and plasma homocysteine concentrations (224). In this literature review, I will focus my review to description of short term protocols. Short term folate kinetics often have been examined using a single dose of folate administrated intravenously through injection or provided orally as food, fortified foods or specific folate forms. Monitoring of plasma/serum folate responses are often conducted using serial measurements up to 10 hr post-dose. The responses between different test doses can be compared using the size of post-dose area under the curve (AUC) or other kinetics parameters, such as the maximal plasma/serum folate concentration ($C_{\text{max}}$) and the time of maximal concentration ($t_{\text{max}}$). When the data are expressed as relative bioavailability, the plasma/serum AUC corresponding to the oral dose (test dose) is divided by the plasma/serum AUC corresponding to the reference dose (IV or oral). The relative bioavailability can also be used as an indicator to compare bioavailability between treatments. It should be noted that comparing AUC of the test dose against the reference dose is valid only if the assumption is correct that the reference and test dose are metabolized in a similar manner in the body. Traditionally, the test dose has been made up of either folic acid or reduced folates whereas the reference dose was usually folic acid. Wright et al. (2003) (225) reported that reduced folates and folic acid may be
handled differently in the body. As such, they proposed the calculation of the apparent rate of absorption, which was then adopted by some subsequent studies to express relative folate bioavailability. The calculation of apparent absorption rate involves three equations using several kinetic markers, such as labelled folate concentration (C), time to peak labelled folate (t\text{max}), the time during which labelled folate initially remains at baseline (t\text{lag}), volume of plasma (V) and constant of excretion rate (k) (225). Acute studies of folate bioavailability may include the use of either unlabelled or labelled folates as the test dose.

2.4.1.1 Acute studies with unlabelled folates

Acute studies with unlabelled folates administered orally were conducted to compare the bioavailability of different folate forms (Table 4). For example, the bioavailability of 5-methyltetrahydrofolate compared to folic acid was found to be equivalent based on the AUC of plasma folate concentration and the C\text{max} of plasma folate concentration in response to the test doses (226, 227). Folate bioavailability from different foods was also investigated (228, 229). Compared to folic acid, folates from spinach (229) and folates from aleurone flour (228) produced a similar AUC (based on folate concentrations in plasma from serial sampling), indicating similar bioavailability of folate from spinach, aleurone flour relative to the folic acid reference dose. The role of the polyglutamate chain in folate bioavailability was also investigated (214, 230). Using unlabelled monoglutamyl folic acid as a test reference, the bioavailability of heptaglutamyl folic acid (PteGlu\text{7}) was 54-63% relative to monoglutamyl folic acid (PteGlu) (230).

To obtain a measurable folate response to the test dose in plasma, non-isotopic acute studies often administrated relatively high doses of folate. Once test doses exceed physiological levels,
folates are likely absorbed through passive diffusion (231). As such, the distribution and metabolism of folate at higher doses may not reflect normal physiological conditions and the estimates of folate bioavailability using such a high dose may not represent the amount of folate that is nutritionally relevant (232). In addition, cell culture work showed decreased steady-state mRNA and protein expression levels of RFC and PCFT when cells were cultured at an over-supplementation level of folate as compared to a physiological concentration of folate (172). The study suggests high folate intake may downregulate folate uptake in the small intestinal and the renal epithelial cells (172).

In order to enhance the folate response in plasma and ensure the ingested dose does not go towards a depleted peripheral folate pool, most acute studies include a pre-dose saturation period. In the saturation period, participants are provided with high dose folic acid (>1000 µg) for around 10 days to several weeks prior to the assessment of folate bioavailability. Since the liver storage of folate has been standardized through the saturation process, the subject-to-subject variation can be minimized to improve the sensitivity of the model. However, this approach has also been criticized because such protocols do not necessarily represent normal physiological conditions (208).

2.4.1.2 Acute studies with labelled folates

Investigation of folate bioavailability in acute studies involves using isotopically labelled folates in a single tracer protocol that measures plasma folate response following an oral dose of labelled reduced folates or folic acid (Table 5). For ethical reasons stable isotopes, rather than radioisotopes of folate are used in human studies. These provide high specificity since the labelled folates detected in blood must originate from the ingested dose (232). In isotopic studies
the enrichment of labelled folates to unlabelled folates is measured in the blood, rather than the increase in total folate concentrations, and therefore, a high oral dose is not needed to gain a measureable response in blood and the dose of folate required is closer to physiological levels and much lower than the dose administered in unlabelled studies (208). For in vivo kinetics studies, folates have been labelled either on the glutamate tail (13C), or on the central benzene ring with (2H). Because the natural abundance of isotopes must be taken into consideration, tracers with more than one heavy atom are usually preferable (232).

Early work in this area, required synthesis of labeled folates by investigators, as stable isotopes of folate were not available commercially (233). More recently it has become common to have isotopic folates custom-made commercially, to meet specific standards to fulfill the regulatory requirements for clinical trials. This process tends to be both expensive and lengthy and is currently a significant drawback of stable isotope studies. The molar ratio of labelled–to-unlabelled folates has been measured by gas chromatography/mass spectrometry or LC/MS/MS (233) after correction for the known natural abundance of the isotopes. 2H and 3H have been found to demonstrate isotope effects, i.e., differences in chemical, physical, and metabolic characteristics between labelled and unlabelled forms of the analyte of interest (208, 232). In addition, 2H has been reported to exchange with 1H (unlabelled hydrogen) through proton exchange (234). As a result, newer stable isotope studies chiefly employ the use of labelled carbon or nitrogen. While the introduction of the use of stable isotopes has significantly advanced knowledge of folate kinetics, data on bioavailability of native food folates is rare (202) and requires the use of intrinsically labelled food folates. This presents several technical limitations including the potential lack of specificity of labelling and difficulties with labelling animal sources of food (208). However, the absorption of pre-formed folate across the human
colon has been established, and its bioavailability determined using an acute study protocol with a single oral dose of labelled folate (35). Lakoff et al. (2014) calculated an apparent absorption of 46% following oral administration of (6S)-$^{13}$C$_{5}$-5-formyltetrahydrofolate (320 µg). The apparent absorption in the small intestine had been previously reported in the literature to be 38%, in response to a similar dose of the same folate preparation ((6S)-$^{13}$C$_{5}$-5-formyltetrahydrofolate) (191).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Pre-saturation</th>
<th>Test formulation</th>
<th>Dose (µg)</th>
<th>AUC (h.mol/L) or apparent absorption (%)</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monch et al., 2015 (235)</td>
<td>12 males, 12 females</td>
<td>800 µg folic acid for 2 weeks</td>
<td>Folic acid (oral)</td>
<td>375</td>
<td>113&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Relative bioavailability: Spinach &gt; wheat germ &gt; camembert cheese (relative to folic acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Camembert cheese (200 g)</td>
<td>595</td>
<td>21&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wheat germ (50 g)</td>
<td>245</td>
<td>44&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach (500 g)</td>
<td>545</td>
<td>123&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Pentieva et al., 2004 (226)</td>
<td>13 males</td>
<td>5000 µg folic acid for 1 week</td>
<td>Folic acid (oral)</td>
<td>500</td>
<td>146</td>
<td>Bioavailability: folic acid = (6S)-5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate (oral)</td>
<td>500</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Konings et al., 2002 (214)</td>
<td>6 males, 6 females</td>
<td>No</td>
<td>Folic acid (oral)</td>
<td>500</td>
<td>40</td>
<td>Relative bioavailability: ~80% for both spinach preparations (relative to folic acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach, Mono-polyglutamate=40:60 (307 g)</td>
<td>436</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach, monoglutamate (367 g)</td>
<td>362</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Fenech et al., 1999 (228)</td>
<td>8 males, 8 females</td>
<td>No</td>
<td>Folic acid + Wheat bran (100g) oral</td>
<td>500</td>
<td>49 (males), 37 (females)</td>
<td>Bioavailability: Folic acid = aleurone flour &lt; wheat bran alone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aleurone flour (100 g)</td>
<td>515</td>
<td>38 (males), 46 (females)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wheat bran (100 g)</td>
<td>94</td>
<td>6 (males), 8 (females)</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Gender</td>
<td>Sample Size</td>
<td>Treatment</td>
<td>Folate (µg)</td>
<td>Bioavailability</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------</td>
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<td>----------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-------------</td>
<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td>Prinz-Langenohl et al., 1999 (229)</td>
<td>10 males, 10 females</td>
<td>No</td>
<td>Folic acid (oral)</td>
<td>400</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach (600 g)</td>
<td>480</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach (300 g)</td>
<td>240</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Keagy et al., 1988 (230)</td>
<td>6 males</td>
<td>500 µg of folic acid for 9 days</td>
<td>Folic acid, monoglutamate added to formula</td>
<td>500</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Folic acid, polyglutamate added to formula</td>
<td>500</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Witthoft et al., 2003 (202)</td>
<td>2 males</td>
<td>960 µg of folic acid for 1 week</td>
<td>(6S)-5-CH₃-H₄folate (oral)</td>
<td>380</td>
<td>66²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-5-CH₃-H₄folate (intramuscular)</td>
<td>380</td>
<td>127²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Broccoli</td>
<td>193</td>
<td>34²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strawberries</td>
<td>201</td>
<td>37²</td>
<td></td>
</tr>
<tr>
<td>Witthoft et al., 2006 (236)</td>
<td>8 males, 1 female</td>
<td>960 µg of folic acid for 1 week</td>
<td>Folic acid in bread</td>
<td>216</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-5-CH₃-H₄folate in fermented milk</td>
<td>207</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yeast folate, polyglutamate</td>
<td>71</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

1 Blood sampling duration ranged from 7 to 12 h, except for Keagy et al., 1988 (230). ² Dose-corrected area under the curve (AUC); ³ Differences between treatments were significantly different (p<0.05); ⁴ Subjects were supplemented with the test dose for 9 days. Blood sampling duration was 2 hr; Adapted and modified from (201)
Table 5 Acute studies using plasma response following administration of labelled folates to determine folate bioavailability

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Pre-saturation</th>
<th>Test formulation</th>
<th>Dose (µg)</th>
<th>AUC (h.mol/L) or apparent absorption (%)</th>
<th>Findings^1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AUC (h.mol/L)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AUC of (6S)-13C6-5-HCO-H4folate is 220% relative to the AUC of 13C6-folic acid</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wright et al., 2003 (225)</td>
<td>10</td>
<td>No</td>
<td>13C6-folic acid (oral)</td>
<td>279</td>
<td>19^2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-13C6-5-HCO-H4folate (oral)</td>
<td>230</td>
<td>42^2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13C5-folic acid in bread</td>
<td>198</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13C5-folic acid in breakfast meal</td>
<td>198</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-13C5-5-CH3-H4folate in bread</td>
<td>207</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Ohrvik et al., 2010 (237)</td>
<td>3 males, 5 females</td>
<td>No</td>
<td>13C5-folic acid in bread</td>
<td>198</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13C5-folic acid in breakfast meal</td>
<td>198</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-13C5-5-CH3-H4folate in bread</td>
<td>207</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wright et al., 2005 (191)</td>
<td>14</td>
<td>No</td>
<td>13C6-folic acid (oral)</td>
<td>279</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-13C6-5-HCO-H4folate</td>
<td>230</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach 15N1-7folate</td>
<td>270</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

Apparent absorption (%)

Apparent absorption rate: (6S)-13C6-5-HCO-H4folate =
<table>
<thead>
<tr>
<th>Spinach $^{15}\text{N}_1$-folate $^{13}\text{C}_6$-folate acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Large intestine</td>
<td></td>
</tr>
<tr>
<td>Lakoff et al., 2014 (35)</td>
<td>6 males, 3 females</td>
</tr>
</tbody>
</table>

1 Blood sampling duration ranged from 4 to 12 hr; 2 Dose-corrected area under the curve (AUC); 3 Differences between treatments were significantly different (p<0.05)
2.4.2 Quantifying folate bioavailability using acute protocols: urine response

Quantification of folate bioavailability using urinary excretion of folate in response to ingested and/or a reference dose (IV or oral) may involve use of either unlabelled folates (Table 6) or labelled folates (Table 7-8). The primary indicator of folate bioavailability is calculated using total urinary folate excretion compared to the total amount of ingested folate. Urine collection periods range from 24 to 48 hr post-dose which is much longer than the blood sample collection in acute protocols and therefore potentially captures the continuous metabolic processes.

2.4.2.1 Acute studies with unlabelled folates

In most studies, the bioavailability of different food folates was determined using the total urinary folate excretion against the total ingested folate in foods, with minor variations in assumptions and calculations (Table 6). The use of a short term protocol with administration of a single known dose of unlabelled folate has been used to determine bioavailability of folate originating from different foods, added fortificants (202, 235, 236) and folate forms (e.g. monoglutamyl versus polyglutamyl folic acid) (230, 238). However, as shown in Table 6, as the folate excretion into urine is normally quite low, percent recovery of the test dose is also low, making it difficult to observe the differences in folate bioavailability between foods. A notable exception was in an early study reported by Tamura et al. (238) where the folate bioavailability was calculated using a different equation involving the use of a standard response curve.

Tamura et al. (238) found folates in liver and banana were highly bioavailable (70-96% relative to folic acid) whereas folates from romaine lettuce and orange juice were relatively lower (25-31% relative to folic acid) in bioavailability. Furthermore, the same study also showed a substantial reduction in bioavailability for polyglutamyl folic acid (PteGlu7) supplemented in orange juice when the pH of orange juice was lowered to 3.7. Tamura’s calculation was
however not commonly adopted by subsequent studies. In a later study, Keagy et al. (230) gave monoglutamyl folic acid (PteGlu) or polyglutamyl folic acid (PteGlu$_7$) to subjects and reported that the urinary excretion of polyglutamyl folic acid was only 70% of that of monoglutamyl folic acid, indicating the latter being more bioavailable. As shown in Table 6, only a very small increase in urinary folate excretion is obtained if the ingested dose is under 200-300 µg of folate (202, 236). Therefore, many trials gave a higher folate dose (average of 410 µg, range from 70 to 900 µg) and included a pre-saturation protocol to increase urinary folate excretion and to minimize subject-to-subject variation in folate excretion (Table 6). The drawback of administration of a non-physiological dose of folate and pre-loading subjects with folic acid has been described in the previous section discussing plasma kinetics. Most often when both urinary and plasma response are both available in a trial, urine response was not used as the primary measure for folate bioavailability (202, 230, 236), likely due to low urinary folate excretion and high subject-to-subject variation.

2.4.2.2 Acute studies with labelled folates

Acute studies with labelled folates using urinary isotope excretion to evaluate folate bioavailability have traditionally involved use of two tracers to determine the bioavailability of added folate fortificants in different foods (203, 204, 239) and compare the bioavailability of different forms of folate (209, 212, 215). Acute studies are also useful to validate short term folate kinetics protocols where it is necessary to investigate whether two tracers are metabolized in a similar manner in the body (204, 240). This short-term protocol has been also used to compare folate absorption between individuals with NTD affected pregnancy and normal controls (241, 242). In dual tracer protocols, folate bioavailability may be determined by: (1) administering a test dose of labelled folate orally and a dose of differently labelled folate
intravenously (e.g. deuterium versus carbon-labelled); (2) oral administration of two doses of differently labelled folate. A summary of these studies can be found in Table 7. Only one report compared the urinary isotope excretion after administration of single oral dose of labelled folic acid (Table 8). Folate bioavailability can be assessed by comparing the urinary excretion of isotopically labelled folate from the two oral doses or IV dose in urine. Also, the urinary excretion ratio (UER) may be calculated as follows: Recovery of isotopic oral dose (%) ÷ Recovery of isotope IV dose (%). Since the administration of an IV dose is considered to be 100% bioavailable, the UER is used as the primary index for absolute absorption.

When labelled folate are utilized, the dose of ingested folate provided is usually much lower (range 60-400 µg) than the dose used in protocols with unlabelled folate. Although the sensitivity and specificity have significantly improved with the use of labelled folate as compared to unlabelled folate, sensitivity continues to be an issue when the protocol involves a single physiological dose. This has been addressed by the inclusion of the pre-saturation protocol where subjects ingest supplemental folate for a number of weeks to saturate tissue stores of folate (Table 6-8). The recovery of an oral dose of labelled folate ranges from 0.1-2% without a pre folate-saturation period and 14-60% with a saturation period. Similarly, recovery of an IV dose of folate without a pre folate-saturation protocol ranges from 0.1-1.9% compared to 21-25% with folate-saturation. No doubt the low recovery of orally or IV-administered labelled folates in urine, especially without a pre folate-saturation period, reflects uptake of folate into the splanchnic bed, the liver, and peripheral tissues as well as reabsorption of folate in the kidney. In addition to low enrichment of labelled folate in urine, there is high subject-to-subject variation. While the pre folate saturation period has been shown to increase urine enrichment of labelled folates, it has also been criticized as unphysiological.
The use of UER as the primary index for quantitating relative folate bioavailability is based on the assumption that *in vivo* kinetics, turnover, distribution enterohepatic circulation and clearance is similar for the two test doses (oral or IV) being compared (203). However, many studies using an oral/IV dual-stable isotope protocol consistently reported UER greater than 1, indicating greater urinary excretion of labelled folate derived from the oral dose than from the IV dose *(Table 7)*. Since the IV dose is considered to be 100% bioavailable, this scenario is theoretically impossible. Finglas et al. suggested that this error is introduced when using folic acid as the reference IV dose, which may be metabolized differently than naturally occurring forms of folate and therefore recommended the use of (6S)-5-methyltetrahydrofolate as the reference dose for future studies (239).
Table 6 Acute studies using urine response following administration of unlabelled folates to determine folate bioavailability

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Pre-saturation</th>
<th>Test formulation</th>
<th>Dose (µg)</th>
<th>Bioavailability (%)</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monch et al., 2015 (235)</td>
<td>12 males, 12 females</td>
<td>800 µg of folic acid for 2 weeks</td>
<td>Folic acid (oral)</td>
<td>375</td>
<td>21.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Urine data on bioavailability of food folate (relative to the reference folic acid) is mostly in agreement with plasma data.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Camembert cheese</td>
<td>595</td>
<td>1.7&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wheat germs</td>
<td>245</td>
<td>2.7&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spinach</td>
<td>545</td>
<td>6.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Witthoft et al., 2006 (236)</td>
<td>8 males, 1 female</td>
<td>960 µg of folic acid for 1 week</td>
<td>Folic acid in bread (6S)-5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate in fermented milk</td>
<td>216</td>
<td>8-&lt;5%&lt;sup&gt;1&lt;/sup&gt; (details not reported)</td>
<td>Findings of the study were addressed using plasma results: apparent absorption rate is similar across the three treatments.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yeast folate, polyglutamate</td>
<td>207</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Witthoft et al., 2003 (202)</td>
<td>2 males</td>
<td>960 µg of folic acid for 1 week</td>
<td>(6S)-5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate (oral)</td>
<td>380</td>
<td>1.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Findings of the study were addressed using plasma: relative bioavailability: 47%, 67% for strawberries and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6S)-5-CH&lt;sub&gt;3&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate (intramuscular)</td>
<td>380</td>
<td>2.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Broccoli</td>
<td>193</td>
<td>1.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Strawberries</td>
<td>201</td>
<td>1.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Keagy et al., 1988 (230)(^2)</td>
<td>6 males</td>
<td>500 µg of folic acid for 9 days (during the study)</td>
<td>Folic acid (monoglутamate) added to formula</td>
<td>500</td>
<td>45(^1)</td>
<td>Urinary excretion of folic acid(^7) is 70% of folic acid.</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Folic acid(^7) (polyglutamate) added to formula</td>
<td>500</td>
<td>33(^1)</td>
<td></td>
</tr>
<tr>
<td>Tamura et al., 1973 (238)</td>
<td>10</td>
<td>2x10,000 mg of folic acid 2 days prior to the test day, 5000mg of folic acid 1 day prior to the test day and 2,000mg every other day during the study</td>
<td>Orange juice</td>
<td>840</td>
<td>31(^3)</td>
<td>Folates in banana, liver and beans are more bioavailable than folates in orange juice, romaine lettuce and egg yolk.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Romaine lettuce</td>
<td>750</td>
<td>25(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Egg yolk</td>
<td>400</td>
<td>39(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Banana</td>
<td>250</td>
<td>82(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lima beans (cooked)</td>
<td>240</td>
<td>70(^3)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Liver</td>
<td>840</td>
<td>96(^3)</td>
<td></td>
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</tbody>
</table>

\(^1\) Total folate excreted as % of intake; \(^2\) Subjects were supplemented with the test dose for 9 days; \(^3\) Calculation of bioavailability of food folates: \([\text{A mg-C mg}]÷\text{B mg}\). A: Value obtained from urinary excretion data in reference to the standard curve established by ingestion of folic acid. B: Amount of folates in test foods. C: Amount of folic acid (mg) ingested together with the test foods.
Table 7 Acute studies using urine response following administration of dual stable isotope-labelled folates to determine folate bioavailability

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Presaturation</th>
<th>IV dose (µg)</th>
<th>Oral dose (µg)</th>
<th>Sampling duration (hr)</th>
<th>Treatments</th>
<th>Recovery of IV dose (%)</th>
<th>Recovery of oral dose (%)</th>
<th>UER&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Results&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IV and oral doses administration</strong></td>
<td></td>
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<tr>
<td>Finglas et al., 2002 (239)</td>
<td>15 females</td>
<td>No</td>
<td>$^2$H&lt;sub&gt;4&lt;/sub&gt;-folic acid (100 µg)</td>
<td>$^{13}$C&lt;sub&gt;6&lt;/sub&gt;-folic acid (225 µg)</td>
<td>48</td>
<td>Capsule</td>
<td>0.6</td>
<td>1.3</td>
<td>2.2</td>
<td>UER&gt;1&lt;sup&gt;2&lt;/sup&gt;; Relative to folic acid capsule, wheat bran &lt; bioavailability than bread</td>
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<td></td>
<td>$^2$H&lt;sub&gt;2&lt;/sub&gt;-folic acid (104 µg)</td>
<td>$^{13}$C&lt;sub&gt;5&lt;/sub&gt;-folic acid (98.8 µg)</td>
<td>36</td>
<td>Water</td>
<td>0.1</td>
<td>0.1</td>
<td>0.9</td>
<td>No differences in bioavailability across test foods.</td>
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<td></td>
<td></td>
<td>White bread</td>
<td>0.1</td>
<td>0.2</td>
<td>0.6</td>
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<td></td>
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<td></td>
<td>Wheat bread</td>
<td>0.2</td>
<td>0.2</td>
<td>0.6</td>
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<td></td>
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<td></td>
<td></td>
<td>White rice</td>
<td>0.2</td>
<td>0.1</td>
<td>0.7</td>
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<td></td>
<td></td>
<td></td>
<td>Pasta</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Study</td>
<td>Gender</td>
<td>Treatment</td>
<td>Dose</td>
<td>Time</td>
<td>UER</td>
<td>Differences in Bioavailability in Folate Forms</td>
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<tr>
<td>Rogers et al., 1997</td>
<td>2 males, 2 females</td>
<td>No</td>
<td>$^{13}$C$_5$-folic acid (400 µg)</td>
<td>24</td>
<td>1.4</td>
<td>2.1</td>
<td>3.8</td>
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<td></td>
<td></td>
<td></td>
<td>$^{12}$C$_5$-folic acid (400 µg)</td>
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<td>1.9</td>
<td>2.4</td>
<td>3.8</td>
<td></td>
<td></td>
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<tr>
<td>Gregory et al., 1992</td>
<td>7 males</td>
<td>2000 µg of folic acid for 1 week</td>
<td>$^{2}$H$_2$-folic acid (100 µg)</td>
<td>48</td>
<td>1.3</td>
<td>2.4</td>
<td>6.5 UER $&gt;1^{2/3}$</td>
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<td></td>
<td></td>
<td></td>
<td>$^{13}$C$_5$-folic acid (445 µg)</td>
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<td></td>
<td>$^{2}$H$_2$-folic acid (135 µg)</td>
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<td></td>
<td>$^{2}$H$_2$-5-CHO-H$_4$folate (186 µg)</td>
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<td></td>
<td>$^{2}$H$_2$-10-CHO-H$_4$folate (311 µg)</td>
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<td></td>
<td>$^{2}$H$_4$-folic acid (221 µg)</td>
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<td></td>
<td>$^{2}$H$_2$-5-CH$_3$-H$_4$folate (238 µg)</td>
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<td>$^{2}$H$_4$-folic acid (221 µg)</td>
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<td></td>
<td>$^{2}$H$_4$-folic acid (298 µg)</td>
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<td></td>
</tr>
<tr>
<td>Gregory et al., 1991</td>
<td>7 males</td>
<td>2000 µg of folic acid for 1 week</td>
<td>$^{2}$H$_4$-folic acid (221 µg)</td>
<td>48</td>
<td>25</td>
<td>38</td>
<td>1.45 UER $&gt;1^{2/3}$ for the</td>
<td></td>
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</tr>
<tr>
<td>Reference</td>
<td>Subjects</td>
<td>Pre-saturation</td>
<td>Oral dose 1 (µg)</td>
<td>Oral dose 2 (µg)</td>
<td>Sampling duration (hr)</td>
<td>Recovery of oral dose 1 (%)</td>
<td>Recovery of oral dose 2 (%)</td>
<td>Results</td>
<td></td>
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</tr>
<tr>
<td>Boddie et al., 2000</td>
<td>11 female cases (history with NTD affected pregnancy)</td>
<td>2000 µg of folic acid for 30 days</td>
<td>$^{2}\text{H}_{2}$-folic acid$_5$ (103 µg)</td>
<td>$^{13}\text{C}_{5}$-folic acid (250 µg)</td>
<td>48</td>
<td>33</td>
<td>31</td>
<td>Recovery of oral doses did not differ between cases and controls.</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>11 female controls</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rogers et al., 1997</td>
<td>2 males, 2 females</td>
<td>No</td>
<td>$^{2}\text{H}_{2}$-folic acid (220 µg)</td>
<td>$^{13}\text{C}_{5}$-folic acid (220 µg)</td>
<td>24</td>
<td>1.8</td>
<td>1.7</td>
<td>Similar absorption and in vivo behaviour of oral doses of $^{2}\text{H}<em>{2}$-folic acid and $^{13}\text{C}</em>{5}$-folic acid.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wei et al., 1996</td>
<td>7 males</td>
<td>10000 µg of folic acid for $^{2}\text{H}_{4}$-folic acid</td>
<td>$^{2}\text{H}_{2}$-folic acid$_6$</td>
<td>48</td>
<td></td>
<td>$^{12}$</td>
<td>$^{9}$</td>
<td>Recovery of oral doses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study (Gregory et al., 1990)</td>
<td>Participants</td>
<td>Dose</td>
<td></td>
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<tr>
<td></td>
<td>11 males</td>
<td>2000 µg of folic acid for 7 days</td>
<td>2H4-folic acid (300 µg)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>UER</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IV: Intravenous; 1</td>
<td>UER greater than 1 is theoretically impossible and therefore indicates oral and IV dose is handled differently in the body. Therefore, UER cannot indicate the absolute folate absorption;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UER =0.96 when both 2H2-folic acid and 13C5-folic acid are given orally;</td>
<td>Differences between treatments were significantly different (p&lt;0.05);</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean value of 6 test foods (water 1, orange juice, tomato, lima beans, citrate pH 4.1, water 2);</td>
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</tr>
</tbody>
</table>
Table 8 Acute studies using urine response following administration of single stable isotope-labelled folates to determine folate bioavailability

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Presaturation</th>
<th>Oral dose (µg)</th>
<th>Sampling duration (hr)</th>
<th>Recovery of oral dose (%)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davis et al., 1995 (241)</td>
<td>10 cases (with NTD-affected pregnancy)</td>
<td>2x1000 µg of folic acid for 30 days</td>
<td>$^2$H$_4$-folic acid (400µg)</td>
<td>24</td>
<td>9</td>
<td>No differences in urinary excretion between cases and controls.</td>
</tr>
<tr>
<td></td>
<td>10 controls</td>
<td></td>
<td>$^2$H$_4$-folic acid (400µg)</td>
<td></td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>
2.5 The intestinal biosynthesis of folate

2.5.1 Overview of bacterial synthesis of folate in colon

The human colon has been reported to contain to $10^{13}$ microorganisms per gram of intestinal content. While more than 50 phyla have been identified, the most prevalent phyla of colonic bacteria include Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (243). Based on sequencing of 16S rRNA, it has been estimated there are more than 1000 microbial species in the human gastrointestinal tract (244), predominantly anaerobic bacteria. It is believed that the fecal microbiota of human adults is generally stable over time with inter-individual variations in the composition (245, 246). The microbiota composition can be influenced by several factors, such as diet (247), age (248), host genetics, luminal pH, intestinal transit time and geographical locations (249, 250). It has been found that Bacteroides is the most dominate genus, followed by Bifidobacteria (248).

The human gut microbiota is known to synthesize and secrete into the lumen several nutrients, such as amino acids (251), vitamin K and certain B-vitamins, including folate (252). The pathway through which bacteria synthesize folate is well-characterized. It involves conversion of guanosine triphosphate (GTP) to 6-hydromethyl-7,8-dihydropterin then to chorismate and PABA in sequence. Dihydropterin pyrophosphate and PABA then go through a condensation reaction to form folate. Many bacteria present in the colon have the enzymatic machinery to produce folate while other bacteria have an obligate requirement for preformed folate. For example, the concentration of folate in yogurt is affected depending on the starter cultures used. A combination of Streptococcus (S) thermophilus and Lactobacillus (L) bulgaricus (253) may increase the folate content of yogurt; whereas the use of lacotbacilli alone (without other folate producers in the starter) may reduce folate level in fermented products (254) as generally
lactobacilli (except for *L. plantarum*) have an obligate requirement for preformed folate. It has been shown that *L. plantarum* is able to secrete folate when cultured in a medium that is folate-free but in the presence of PABA (255, 256). This finding is in an agreement with genome sequencing analysis showing the de novo PABA synthesis pathway is present in *L. plantarum* but generally not in other strains of lactobacilli (257). Bifidobacteria are generally major folate producers. All strains of *Bifidobacterium (B. bifidum* and *B. infantis* have been identified as high folate-producing species whereas *B. breve, B. longum* and *B. adolescentis* are low folate-producing species (258). Furthermore, the amount of folate being secreted extracellularly appears to be strain-specific (30). In a folate-free medium, it has been shown only 17 out of 76 strains of Bifidobacteria have the ability to produce folate with the highest extracellular folate levels being produced by four strains of *B. adolescentis* and two strains of *B. pseudocatenulatum* (30). Subsequent in vivo studies confirmed that folate status of rats was improved (259) and the folate content in feces of humans increased (260) following the administration of high folate-producing strains of Bifidobacteria.

Based on experimental evidence, other bacteria present in human colon appear to synthesize folate including *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides vulgatus, Clostridium difficile* and *Escherichia coli* (32). In a recently published study of the potential metabolic capabilities of the human gut microbiota, Magnusdottir et al. reported that nearly all Bacteroidetes, Fusobacteria and Proteobacteria appear to harbor the genetic material required for de novo PABA synthesis and hence are expected to produce folate (32). In contrast, most of Firmicutes are unable to synthesize folate mostly due to the lack of the enzymatic machinery for PABA synthesis. The authors estimated that complete folate synthesis is expected in 43% of the 256 human gut microbiota tested.
In vitro studies suggest that folates produced by *S. thermophilus*, a common starter used for industrial dairy preparations, contains three glutamates in their polyglutamyl tail and hence would potentially be more bioavailable than naturally occurring food folates where the glutamate chain length is typically longer (256). Kim et al. (33) reported the folate content in fecal samples from human infants was $93 \pm 93$ nmol/d (Adequate Intake level for an infant (0-6 months) = 65 µg, 150 nmol and for an infant (7-12 months) =80 µg, 180 nmol), 66% of which were short-chain folates, indicating folates found in the colon are likely bioavailable.

2.5.2 Regulation of bacterial synthesis of folate in colon: fibre

2.5.2.1 Evidence showing fibre intake has an impact on folate status

Previous work in our laboratory suggests that the folate status of rats can be influenced by changes in colonic bacterial load and microbial community composition (261, 262). Semchuk et al. fed rats diets containing human milk solids (a source of prebiotic oligosaccharides) with or without a sulfa drug. Sulfa drugs added to the diet prevent colonic bacteria from utilizing PABA to synthesize folate. Inclusion of a sulfa drug to a diet containing human milk reduced folate concentrations in blood by 50% compared to the diet without the drug (261). In another study, rats were fed with diets containing human milk, cow milk or goat milk solids; unlike human milk cow and goad milk contain low concentrations of oligosaccharides. The highest Bifidobacteria count in the cecum of rats in this experiment was observed in the group receiving the human milk solids diet, which also resulted in the highest plasma folate concentrations (262). It has been further shown that folate status of rats (36, 37) and humans (33, 38, 263) are associated with fibre or prebiotics intake in the diet. Studies have shown that when fibre intake is increased through the addition of xylan, wheat bran or beans to diet in rats by random assignment, a higher folate content in the liver and fecal samples is observed. These data suggest higher colonic
bacterial folate synthesis in response to fibre and the synthesized folate is bioavailable for rats (36). Thoma et al. reported rats receiving a diet containing citrus pectin or oligofructose had higher folate concentrations in plasma, erythrocyte and colonic tissue than rats receiving diet supplemented with cellulose (unfermentable source of fibre) (37). Kim et al. showed that the feces of exclusively human milk-fed infants contained approximately 2-fold greater folate content compared to feces of infants fed with infant formula (33). The differences in folate content in feces was thought partially due to the change of colonic microbiota as a result of the greater amount of oligosaccharides found in human milk compared to infant formula (33). In an observational study (n=224), Houghton et al. (38) found that dietary fibre intake was positively associated with folate status of adolescent females, even after controlling for dietary folate intake. The authors estimated every gram increase in nonstarch polysaccharide intake was associated with a 1.8% increase in serum folate concentration, likely through promotion of growth of the bacterial folate producers in the colon (38). In a randomized clinical trial, Wolever et al. reported that patients with type 2 diabetes receiving miglitol had higher serum folate concentrations compared to patients treated with metformin. Miglitol improves glycemic control through competitively inhibiting absorption of carbohydrate and hence, increases the amount of fermentable substrate entering the colon. In contrast, metformin exerts its hypoglycemic effects through improving insulin sensitivity and reducing hepatic glucose output. These authors also proposed that the increase in the availability of fermentable substrate in the colon following miglitol treatment promoted the growth of bacteria favorable for folate production, leading to the observed higher serum folate concentrations in their subjects (263). Taken together, the inclusion of fermentable substrates, such as fibre or prebiotics, likely effects the net production of bacterially synthesized folate production in the colon.
2.5.2.2 Fibre intake regulates the growth of folate producing colonic bacteria

Fermentable substrates, such as dietary fibre that escape intestinal digestion and enter colon intact, are the main substrates available for colonic bacteria growth. Water soluble fibres can be completely fermented whereas water insoluble fibres are more resistant to fermentation. These substrates are thought to have an impact on colonic environment and microbiota via fermentation activities (264). Fermentable dietary fibre provides the requisite substrate necessary to increase the total bacteria load in the colon (265, 266). Further, both epidemiological and short-term intervention studies suggest that total habitual intake of dietary fibre or feeding fibre/prebiotics on a short-term basis may lead to change in the composition of human gut microbiota (247).

Plant-based diets, high in fruit/legume fibre, are associated with predominance of Bacteroidetes, especially *Prevotella spp* whereas Western diet, typically low in fibre is associated with decrease in the beneficial Firmicutes (267-270).

As described in section 2.5.2.1, folate is only produced by specific strains of bacteria, of which *Bifidobacteria* spp have been extensively studied. Short-term intervention studies in both animal models (271) and humans (adults and infants) (272-275) have demonstrated an increased concentration of *Bifidobacteria* following administration of short-chain oligofructose (271), oligosaccharides (272), lactulose (273), very long chain inulin (274) and inulin extracted from Jerusalem artichoke or chicory (275). Using 16S rRNA sequencing, the impact of dietary fibre on specific strains of *Bifidobacteria* spp. has been investigated. It has been shown that humans (n=12) randomly assigned to inulin (5g, twice daily) for 21 days have a relative increase in the abundance of *Bifidobacteria* spp. in feces compared to controls (no supplement) (276). Furthermore, more than a 4-fold increase in the relative abundance of *B. adolescentis* and a 2.7
fold increase in \textit{B. bifidum} and \textit{B. longum} were observed following inulin treatment. In a placebo-controlled, double blind clinical trial, obese women (n=30) were randomly assigned to receive 16 g of/d of inulin-type fructans or placebo for three months (277). Increases in \textit{B. adolescentis}, \textit{B. pseudocatenulatum} and \textit{B. longum} were observed in feces of subjects receiving the inulin-type fructans (277). Among healthy volunteers (n=59) provided with galactooligosaccharide at 7g/d for 7 days, increase in the ratio of \textit{Bifidobacterium} spp. to total bacteria was observed (278). In the same study, the growth rate of \textit{B. bifidum}, \textit{B. infantis}, \textit{B. longum} and \textit{B. animalis (lactis)} in feces in batch cultures with added galactooligosaccharide was greater than in feces with added sugar (controls) (278). Likewise, Davis et al. fed 18 subjects the prebiotic galactooligosaccharide at four increasing dosages (0, 2.5, 5, and 10 gram/day) for 21 days (279). The consumption of >5g/d of galactooligosaccharide led to a 5 to 10 fold increase in the relative abundance of \textit{Bifidobacterium} spp in feces and a 3 to 4 fold increase in \textit{B. adolescentis}, \textit{B. longum} and \textit{B. catenulatum}.

In addition to manipulating the level of ingestion, there has been a growing interest in exploring whether specific type of dietary fibre such as prebiotics and resistant starch can selectively promote the growth of specific bacteria. For example, in an \textit{in vitro} model examining fermentation of different sources of insoluble fibre, the most abundant type of bacteria recovered from wheat bran were clostridial cluster XIVa, while the most abundant bacteria recovered from high amylose starch were \textit{B. adolescentis}. (280). Ivarsson et al. conducted an animal study in which pigs were fed diets containing one of four fibres: pectin from chicory forage; pectin from sugar beet pulp; arabinoxylan from wheat bran; and arabinoxylan from grass meal (281). After 14 days of feeding, the chicory forage diet resulted in a higher relative abundance of \textit{Prevotella} spp. in feces compared to pigs fed other diets. In a more recent study, it was found that feeding a
diet containing 10% pea fibre for 30 days increased *Lactobacillus* counts in the ileum of weanling piglets compared to those fed a control (no fibre) diet or a diet rich in soy bean fibre (10%). In the same study, Bifidobacterium counts in the colon were higher in those fed a wheat bran rich diet compared with those on control and the soy bean fibre diet (282). Taken together, if the intention is to promote the growth of a specific gut bacterium, both specific fibre type or combination of fibres and dose need to be considered.
3. Rationale, objectives and hypotheses

3.1 Rationale

Like many other nutrients, whole body folate homeostasis in the human body is maintained through a balance of input and output of folate. The overarching goal of the thesis is to investigate various issues related to the “input side” of folate homeostasis. Folate is available from different sources including naturally occurring folate in foods, and synthetic folic acid that can be obtained from fortified food and supplements (6). Folate produced by bacteria residing in the colon comprises a significant depot of naturally occurring folate and may also contribute to whole body folate homeostasis and to folate requirements of the colonocyte specifically. The focus of my thesis is on the evaluation and manipulation of several of folate sources including folic acid used as a fortificant in foods and colonic bacterial synthesis.

The mandatory folic acid fortification program is regarded as a very successful public health initiative that has led to an approximate 50% reduction in NTD rates in Canada (4). It is currently mandatory to fortify white wheat flour at the level of 150 µg per 100 g of flour. Although in the Canada’s Food Guide, all Canadians are encouraged to consume more whole grains due to their contribution of magnesium, fibre and the well-known health benefits related to decreased risk of some chronic diseases (25-28, 283-289), folic acid fortification of whole grains is not under the current mandate. Hence, the current folic fortification policy in Canada is in conflict with the portfolio of evidence suggesting increased consumption of whole grains to reduce the risk of chronic diseases. In fact, the justification for recommending only 50% of consumed grain products to be whole grains, versus more, was that because only white wheat flour is fortified with folic acid (290). The impact of extending folic acid fortification to include whole wheat flour in Canada is unknown. Therefore, for Study I, we conducted a modelling
exercise to evaluate the impact of adding folic acid to whole wheat flour on the folate intake distribution of the Canadian population at the national level in order to ensure that this would not expose Canadians to excessive levels of folic acid intake.

Another important factor that has an impact on the “input side” of folate nutrition is the contribution of folate synthesized by colonic microbiota. While it has been established that pre-formed folate can be absorbed across the human colon (34, 35), very little is known about the factors affecting folate biosynthesis of gut microbiota and factors influencing colonic folate absorption in human. To date, there has been evidence generated from well-controlled animal studies showing that dietary fibre or fermentable substrates in the diet have an impact on the folate status of the host, which is likely due, at least in part, to changes in bacterial folate biosynthesis in the colon through the promotion of growth of folate producers (36, 37, 261, 262).

In an observational study of adolescent females consuming lactoovovegetarian, semivegetarian or omnivorous diets, the authors reported that every gram increase in nonstarch polysaccharides intake was associated with a 1.8% increase in serum folate concentrations (38). Also, Wolever et al. reported that patients with type 2 diabetes receiving miglitol appear to have higher serum folate concentrations compared to patients treated with metformin. It was proposed that the increase in the availability of fermentable substrate in colon followed by miglitol treatment promoted the growth of bacteria favorable for folate production leading to the observed higher serum folate concentrations (263). However, there is no direct evidence demonstrating whether colonic folate contents could be manipulated through modifying the fibre level in diet of humans. Therefore, Study II aimed to investigate how manipulating the provision of fibre in the diet may impact the quantity of bacterially-synthesized folate produced in the colon in human subjects.
We previously confirmed that pre-formed isotopically-labelled test doses of folate ($^{13}$C$_5$-5-formyltetrahydrofolate) can be absorbed across human colon after direct infusion to the colon during colonoscopy (34) or after targeted delivery to the colon by specially-designed enteric coated caplets (35). The logical next step would be to investigate whether newly-synthesized folate produced by gut microbiota can be absorbed across the human colon. Biosynthesis of folate from precursor (e.g. PABA) by gut microbiota would likely take some time to reach steady-state in the body. Therefore, investigation of the absorption of bacterially-synthesized folate will likely require administration of multiple doses of an isotopic-labelled precursor and will involve collection of serial biomedical samples, e.g. plasma or urine. Since multiple sample collection over several days will likely be required, it would be useful to implement a less invasive sampling approach than blood sampling. The use of urine may be a suitable alternative to blood sample collection to study folate kinetics in this context. Furthermore, folate bioavailability from different foods has been measured using the appearance of labelled folate in both plasma and urine following administration of a test dose of labelled folate (201). However, colonic folate absorption has only been determined using plasma analysis in human (34, 35). Therefore, Study III aimed to investigate the feasibility of using a non-invasive means of assessing the bacterial contribution of folate produced in the colon by measuring stable isotopes of folate in urine.

The approach of this thesis can be summarized using Figure 4 where Study I investigated the impact of a hypothetical fortification scenario while Study II and III evaluated how the production of folate of colonic origin could be manipulated and how to measure colonic folate absorption using a less invasive approach. Collectively, Study I, II and III provided information
to better understand the physiology and applications of the input side of the equation of folate nutrition.

Figure 4 Thesis approach. This thesis includes three studies investigating the contribution coming from fortification and colonic bacterially-synthesized folate to understand the “input side” of folate nutrition.

3.2 Objectives

Study I: By modelling data from the 2004 Canadian Community Health Survey 2.2 (n=35,107), I assess the effect of extending the mandatory folic acid fortification program in Canada to whole wheat flour on the change in the prevalence of folate inadequacy and the percentage of the population with folic acid intakes above the UL.

Study II: To investigate whether colonic folate content in humans can be manipulated by switching the diet of a sample of African Americans (n=20) normally consuming a Western diet (high fat, low fibre) to a diet traditionally consumed by rural South Africans (low fat, high fibre)
for two weeks and similarly switching the diet of a sample of rural South Africans (n=20) to a Western diet for two weeks.

Study III: To determine if folate absorption across human colon in adult human subjects (n=7) can be assessed by measuring urinary enrichment using LC/MS/MS in response to oral and IV administration of a test dose of $^{13}$C$_5$-5-formyltetrahydrofolate.

3.3 Hypotheses

Study I: Extending folic acid to whole wheat flour in Canada will improve the prevalence of folate inadequacy and will not increase the proportion of the population with intakes of synthetic folic acid above the UL.

Study II: In a sample of African Americans consuming a Western diet (high fat, low fibre) and switched to a traditional rural South African diet (low fat, high fibre) for two weeks, subjects will show an increase in the folate content of colonic evacuant. In contrast, in a sample of rural South Africans switched to a Western diet, subjects will show a decrease in the folate content of colonic evacuant.

Study III: The appearance of labelled folate in urine can be detected after oral and IV administration of a test dose of $^{13}$C$_5$-5-formyltetrahydrofolate and will be a suitable biomarker for future investigation of measuring the availability of folate biosynthesized in the colon.
4. Thesis study I: Modelling demonstrates that folic acid fortification of whole wheat flour would reduce the prevalence of folate inadequacy in Canadian whole wheat consumers

Chapter 4 has been published.


4.1 Abstract

Background: Mandatory folic acid fortification of white wheat flour and selected other grain products reduced the prevalence of neural tube defects in Canada. Fortification of whole wheat flour is not permitted.

Objective: The objective of this study was to model the impact of adding folic acid to whole wheat flour on the folate intake distribution of Canadians.

Methods: Twenty-four hour dietary recall and supplement intake data (n=35,107) collected in the 2004 Canadian Community Health Survey 2.2 were used to calculate the prevalence of folate inadequacy (POFI) and proportion of folic acid intakes above the UL. In model 1, folic acid was added to whole wheat flour-containing foods in amounts comparable to that mandatory for white wheat flour-containing foods. In model 2, a 50% overage of folic acid fortification was considered. Model 3 and 4 included assessment of folate intake distributions in adult whole
wheat consumers with or without a fortification overage. Software for Intake Distribution Estimation was used to estimate usual folate intakes.

**Results:** Mean folate intakes increased ~ 5% in all sex and age groups when whole wheat foods were fortified (Models 1 and 2 (p<0.0001)). Folic acid fortification of whole wheat flour-containing foods did not change the POFI or percentage of intakes above the UL in the general population, whether supplement users or non-users. Among whole wheat consumers, POFI was reduced by 10 percentage points after fortification of whole wheat flour-containing foods (95% CIs did not overlap). The percentage of whole wheat consumers with intakes above the UL did not change.

**Conclusion:** While folic acid fortification of whole wheat flour-containing foods is unlikely to change the POFI or proportion of folic acid intakes above the UL in the general Canadian population, this fortification strategy may reduce the POFI in adult whole wheat consumers.

**Keywords:** folate; folic acid; fortification; whole wheat flour; prevalence of folate inadequacy; Tolerable Upper Intake Level; folate intake distribution
4.2 Introduction

Folate is a generic term for a family of compounds that facilitate transfer of one-carbon units in the body. In this role, folate is required for DNA and RNA synthesis and repair, amino acid metabolism and methylation-mediated epigenetic programming (1, 291). To reduce the incidence of NTDs associated with suboptimal maternal intakes, mandatory folic acid fortification of the food supply was implemented in North America in 1998. In Canada, it is mandatory to fortify white wheat flour and “enriched” cornmeal with 150 µg of folic acid per 100 g, and “enriched” pasta with 200 to 270 µg of folic acid per 100 g (24). In the years following mandatory fortification, the prevalence of NTDs in Canada decreased by approximately 50% (4). While mandatory fortification of white flour and selected grain products was a public health success, whole wheat flour is not fortified in Canada.

Epidemiological evidence drawn from several large, prospective cohort studies suggests that intake of whole grains, including whole wheat, is protective against coronary artery disease (25-28). In addition, recent population-based cohort studies demonstrate inverse relationships between whole grain intake and the incidence of type 2 diabetes and some cancers (283-286). Further, consumption of whole grains was found to be inversely associated with all cause mortality (27, 287). Therefore, all Canadians are encouraged to consume at least half of their grain products as whole grain products (29).

Unlike unfortified white wheat flour, whole wheat flour does contain a natural source of folate. However, given public health policy to encourage the consumption of whole grain products, are whole wheat flour consumers at a disadvantage with respect to folic acid and should folic acid fortification of whole wheat flour in Canada be considered? Before contemplating a change in the fortification policy, it is important to understand how the fortification of whole wheat flour
would impact the folate intake of Canadians. In addition to prevention of NTDs, adequate folate intakes have been associated with a number of other positive health outcomes including a reduction of other birth defects (e.g. cleft lip with or without cleft palate), colorectal cancer, stroke and neuropsychiatric disorders (1, 292). However, high intake of folic acid ingested as supplements can result in masking of vitamin B\textsubscript{12} deficiency (1). In addition, there are reports in the literature, albeit inconsistent, that find an association between supraphysiologic intakes of folic acid with a number of other negative health consequences including progression of neoplastic cells to cancer, and increased risk of asthma, respiratory infection, wheezing and central adiposity in the offspring of mothers consuming supplemental folic acid during pregnancy (5, 9, 15-17, 293, 294).

The overarching goal of this study was to model the impact of extending folic acid fortification in Canada to whole wheat flour on folate intake distribution of Canadians using data from the CCHS 2.2 (295). Specific objectives included estimating the change in POFI and the percentage of individuals with folic acid intakes above the UL, defined as the highest nutrient intake thought to pose no adverse health risk (1). Importantly, this modelling exercise considered folic acid fortification of whole wheat flour at levels currently applied to white wheat flour, as well as with a 50\% fortification overage of white flour which we reported in 2009 based on direct analyses of foods in the Canadian marketplace (296).

4.3 Materials and methods

Data source

The CCHS 2.2, a nationally representative, cross-sectional survey, conducted in 2004, was used for the present analyses (295). General health and dietary intake data were collected by Statistics
Canada and under the authority of the Statistics Act of Canada from 35,107 respondents. Children who were younger than 1 y, and women who were pregnant or lactating were excluded from our analysis. Respondents included in the analysis were stratified by the DRI age and sex groups defined by the Institute of Medicine (1). Dietary intake data were collected using the Automated Multi-Pass 24 hr Recall Method by an in-person interview (297). A second 24 hr recall was collected from a subsample of 10,786 respondents 3-10 days later by telephone interview. Supplement use data (frequency and dose) were collected by participant recall. Respondents were classified as supplement users if they consumed a vitamin and/or mineral preparation containing folic acid in the past 30 days on at least one occasion.

**Model 1: dietary folate intakes with whole wheat flour-containing foods fortified at the mandatory level of comparable foods made with white wheat flour**

Food items listed in Health Canada’s Nutrition Survey System (298) made of whole wheat flour were identified using the food description that contained the term “whole wheat”. The nutrition composition of foods in the Health Canada Nutrition Survey System was based on the Canadian Nutrient File version 2001b (299), which was primarily derived from the USDA Nutrient Database SR 13 and amended to reflect Canadian foods where significant differences exist (300). In Model 1, the total folic acid content of whole wheat flour-containing foods was set to be equivalent to the current mandatory amount added to fortified white wheat flour (i.e. 150 µg folic acid/100 g of white flour). For example, if a white flour-containing food was listed as containing x µg of folic acid per gram and its 100% whole wheat flour containing counterpart contained 0 µg of folic acid per gram, we added x µg of folic acid in our modelling exercise to the 100% whole wheat product. If the whole wheat product contained some white wheat flour containing y
µg of folic acid, the difference in folic acid compared to the white flour only product was added to the modelled whole wheat product \((x-y)\) on a gram basis. When an exactly comparable white wheat flour food could not be found, a group of similar white wheat flour-containing foods was identified and the average folic acid content from these foods was used to model the folic acid content of the whole wheat food.

To model the folate intake distribution in response to the hypothetical fortification of whole wheat flour-containing foods, the Health Canada’s Nutrition Survey System codes were used to identify whole wheat flour-containing food items that were consumed by respondents from the food record of each individual. The adjusted folic acid content of whole wheat flour-containing foods was used to compute the predicted usual folate intake.

**Model 2: dietary folate intakes with whole wheat folic acid fortification adjusted for a 50% anticipated overage**

In 2009, we reported that the total folate content (naturally occurring plus synthetic folic acid) in fortified white flour-containing foods in Canada were approximately 50% higher on average than mandatory levels and values included in the 2007 version of the Canadian Nutrient File (296, 299). In order to estimate the folate intake distribution of Canadians that better reflects the actual folic acid content in the food supply, Model 2 included a 50% folic acid fortification overage in both white and whole wheat flours.

**Models 3 and 4: folate intakes of adult whole wheat consumers with and without inclusion of a fortification overage**
Respondents who reported consuming food products containing the term “whole wheat” in their 24-hour recall at least once were determined to be “whole wheat consumers”. Model 3 estimated the folate intake distribution in adult whole wheat consumers if folic acid was to be added to whole wheat foods at the current mandatory level for white wheat flour whereas Model 4 included the 50% folic acid fortification overage. Due to the small sample size, we did not conduct these analyses in children.

**Accounting for differences in bioavailability of synthetic folic acid and naturally occurring forms of folate**

As suggested by the Institute of Medicine for determining total folate intake, we accounted for differences in the bioavailability of naturally occurring food folate and synthetic folic acid by multiplying the folic acid consumed as part of food by 1.7 to arrive at µg DFE (1). This assumes folic acid consumed with food is 1.7 times more available than natural folates from food. Synthetic folic acid consumed on an empty stomach is nearly 100% bioavailable (301). We assumed that synthetic folic acid consumed as a supplement was ingested on an empty stomach and therefore multiplied this source of folic acid by 2 to arrive at µg DFE. As such, total daily folate intakes in DFEs were calculated using the following equation:

\[ \text{DFE} = \mu g \text{ food folate} + (\mu g \text{ folic acid from foods} \times 1.7) + (\mu g \text{ folic acid from supplements} \times 2) \]  

*(1)*

**Statistical analysis**

SAS for Windows (version 9.3; SAS Institute Inc, Cary, NC) was used to set up the data files, adjust the folic acid content of foods, estimate the folate intake distributions and perform all
statistical analyses. The POFI was determined by the using the Estimated Average Requirement (EAR) cut-point method as described in detail previously (302). Briefly the EAR cut-point method allows for determination of the percentage of individuals who have “usual” folate intakes (adjusted for bioavailability) below the EAR for each DRI sex and age group. Supplement users and non-users were analyzed separately as suggested by Garriguet (303). Folic acid intake from supplements was added to dietary folate intake before respondents’ usual intake was estimated. The SIDE (Software for Intake Distribution Estimation) program (version 1.0, Department of Statistics and Center for Agricultural and Rural Development, Iowa State University, Iowa) (304) was used to simulate respondents’ usual (long-term) intake.

The proportion of the population with folic acid intakes above the UL was also determined by SIDE. The UL for folic acid (1000 µg/day) was set by the IOM to reflect the quantity of synthetic folic acid associated with masking of vitamin B_{12} deficiency (1). Therefore, the percentage of the population with intake levels above the UL was determined by using the intake of folic acid only and not naturally occurring folates.

For determination of both POFI and proportion of intakes above the UL, a bootstrap balanced technique was performed to estimate variance of the analyses given the complex and multistage nature of the sampling for the CCHS 2.2. Prevalence estimates of <5% were not reported since they are at the tails of usual intake distribution generated by SIDE and are considered less precise. Differences in POFI and the proportion above the UL with and without folic acid being added to whole wheat flour were assessed by whether or not the 95% confidence intervals overlapped and using unpaired t-tests in a secondary analysis. Paired t-test was used to compare the change in mean folate intakes with and without folic acid fortification of whole wheat flour.
4.4 Results

Estimated folate intakes of Canadians

There were small (<5%) but significantly higher mean total folate intakes in all DRI sex and age groups in the population as a whole with folic acid fortification of whole wheat flour-containing foods regardless of supplement use (Table 9 and 10, p<0.0001). For example, among women 19 to 30 years of age that did not consume a folic acid-containing supplement, mean folate intakes increased from 419±15 to 441±15µg DFE/d after modelling folic acid fortification of whole wheat flour at the mandatory level for white wheat flour (Model 1, p<0.0001).

Prevalence of folate inadequacy and proportion of the population with folic acid intakes above the UL

Inclusion of folic acid in whole wheat flour-containing foods did not lead to a statistically significant decrease in the POFI for any DRI sex and age group, regardless of supplement use or whether white wheat flour and currently fortified products contained the mandatory folic acid level (Model 1) or a 50% folic acid fortification overage (Model 2) (Table 9 to 11). Again, differences were judged by whether or not the 95% confidence intervals overlapped in the POFI before and after modelling folic acid fortification. Most findings did not change using unpaired t-tests (less conservative) in a post-hoc secondary analysis, except for female non-supplement users > 71 years (Model 1) and adult female non-supplement users and supplement users (food source only) (Model 2) where a reduction in the POFI was observed with folic acid addition to whole wheat-containing foods (unpaired t-test, p<0.05).
Table 9 Total folate intake and prevalence of folate inadequacy (POFI) of Canadian children and adolescents with and without modelled folic acid fortification of whole wheat flour at the current mandatory level for white wheat flour (Model 1)

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample size</td>
<td>EAR (µg DFEs)</td>
</tr>
<tr>
<td>1-3 y</td>
<td>120</td>
<td>1537</td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1537</td>
<td>263 ± 7</td>
</tr>
<tr>
<td>Supplement users</td>
<td>615</td>
<td>296 ± 9</td>
</tr>
<tr>
<td>Foods only</td>
<td>296 ± 9</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td>515 ± 10</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>4-8 y</td>
<td>160</td>
<td>2052</td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1287</td>
<td>463 ± 10</td>
</tr>
<tr>
<td>Supplement users</td>
<td>1287</td>
<td>359 ± 8</td>
</tr>
<tr>
<td>Foods only</td>
<td>359 ± 8</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td>567 ± 9</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>Male, 9-13 y</td>
<td>250</td>
<td>1674</td>
</tr>
<tr>
<td>Sample size</td>
<td>Without whole wheat fortification</td>
<td>With whole wheat fortification</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>(µg DFEs)</td>
<td>(µg DFEs/d)</td>
</tr>
<tr>
<td>Supplement users</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foods only</td>
<td>475</td>
<td>431 ± 20</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td>700 ± 20</td>
</tr>
<tr>
<td>Female, 9-13 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1608</td>
<td>391 ± 9</td>
</tr>
<tr>
<td>Supplement users</td>
<td>434</td>
<td>416 ± 20</td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>683 ± 30</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, 14-18 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>2094</td>
<td>544 ± 10</td>
</tr>
<tr>
<td>Supplement users</td>
<td>302</td>
<td>534 ± 30</td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>1240 ± 60</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, 14-18 y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[^1]: EAR: Estimated Average Requirement; Total folate intake = EAR ± standard error
[^2]: POFI: Probability of Folate Intake; % of EAR
<table>
<thead>
<tr>
<th>Sample size</th>
<th>EAR (μg DFEs)</th>
<th>Total folate intake (μg DFEs/d)</th>
<th>POFI (%)</th>
<th>Total folate intake (μg DFEs/d)</th>
<th>POFI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-supplement users</td>
<td>1995</td>
<td>417 ± 9</td>
<td>28 (23, 34)</td>
<td>430 ± 9*</td>
<td>25 (20, 31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Without whole wheat fortification</td>
<td>With whole wheat fortification</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>350</td>
<td>449 ± 20</td>
<td>23 (12, 34)</td>
<td>462 ± 20*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foods only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1150 ± 50</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>1160 ± 50*</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foods + Supplements</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 All values are mean ± SEs, obtained from each individual’s first 24 hr dietary recall data; DFE, dietary folate equivalents.
2 All values are expressed as probability (lower limit, upper limit of 95% confidence interval). No differences were found in the POFI with or without fortification of whole wheat flour (95% confidence intervals overlap).
*Different from without whole wheat fortification, P<0.05 (Student’s paired t test, P< 0.0001)
Table 10 Total folate intake and prevalence of folate inadequacy (POFI) of Canadian adults with and without modelled folic acid fortification of whole wheat flour at the current mandatory level for white wheat flour (Model 1)

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(µg DFEs)</td>
<td>Total folate intake</td>
</tr>
<tr>
<td></td>
<td>(µg DFEs/d)</td>
<td>(%)</td>
</tr>
<tr>
<td>Male 19-30 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1537</td>
<td>562 ± 20</td>
</tr>
<tr>
<td>Supplement users</td>
<td>324</td>
<td>596 ± 30</td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>1460 ± 40</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>2178</td>
<td>517 ± 10</td>
</tr>
<tr>
<td>Supplement users</td>
<td>570</td>
<td>491 ± 20</td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>1460 ± 60</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without whole wheat fortification</td>
<td>With whole wheat fortification</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>Sample size</td>
<td>EAR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(µg DFEs)</td>
</tr>
<tr>
<td>51-70y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>2051</td>
<td>436 ± 9</td>
</tr>
<tr>
<td>Supplement users</td>
<td>672</td>
<td>497 ± 20</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1650 ± 50</td>
</tr>
<tr>
<td>&gt;71 y</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1212</td>
<td>370 ± 10</td>
</tr>
<tr>
<td>Supplement users</td>
<td>385</td>
<td>444 ± 20</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1660 ± 70</td>
</tr>
<tr>
<td>Female</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>19-30 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-supplement users</td>
<td>1452</td>
<td>419 ± 20</td>
</tr>
<tr>
<td>Supplement users</td>
<td>463</td>
<td>383 ± 20</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1300 ± 50</td>
</tr>
<tr>
<td>Age Group</td>
<td>Non-supplement users</td>
<td>Supplement users</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------</td>
<td>------------------</td>
</tr>
<tr>
<td></td>
<td>Sample size</td>
<td>EAR (µg DFEs)</td>
</tr>
<tr>
<td>31-50 y</td>
<td>1995</td>
<td>411 ± 10</td>
</tr>
<tr>
<td></td>
<td>855</td>
<td>391 ± 10</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1520 ± 50</td>
</tr>
<tr>
<td></td>
<td>Foods + Supplements</td>
<td>320</td>
</tr>
<tr>
<td>51-70 y</td>
<td>2278</td>
<td>373 ± 9</td>
</tr>
<tr>
<td></td>
<td>1126</td>
<td>373 ± 10</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1560 ± 50</td>
</tr>
<tr>
<td></td>
<td>Foods + Supplements</td>
<td>320</td>
</tr>
<tr>
<td>&gt; 71 y</td>
<td>1890</td>
<td>314 ± 8</td>
</tr>
<tr>
<td></td>
<td>879</td>
<td>333 ± 10</td>
</tr>
<tr>
<td></td>
<td>Foods only</td>
<td>1540 ± 60</td>
</tr>
<tr>
<td></td>
<td>Foods + Supplements</td>
<td>320</td>
</tr>
</tbody>
</table>
All values are mean ± SEs, obtained from each individual’s first 24 hr dietary recall; DFE, dietary folate equivalents.

All values are expressed as probability (lower limit, upper limit of 95 % confidence interval). No differences were found in the POFI with or without fortification of whole wheat flour (95 % confidence intervals overlap).

*Different from without whole wheat fortification, P<0.05. (Student’s paired t test, P< 0.0001)
Table 11 Prevalence of folate inadequacy (%) and proportion of adult Canadians with intakes above the UL (%) with and without folic acid being added to whole wheat flour adjusted for a 50% anticipated overages (Model 2)\(^1\)

<table>
<thead>
<tr>
<th>Sample size</th>
<th>EAR (µg DFEs)</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
<th>UL (µg)</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 to &gt; 71 y</td>
<td>320</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Non-supplement users</td>
<td>7014</td>
<td>4 (2, 5)</td>
<td>2 (1, 4)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Supplement users</td>
<td>1951</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>4 (2, 6)</td>
<td>3 (1, 5)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>16 (13, 19)</td>
<td>17 (14, 20)</td>
<td></td>
</tr>
<tr>
<td>Female Non-supplement users</td>
<td>7615</td>
<td>14 (12, 17)</td>
<td>10 (8, 13)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Supplement users</td>
<td>3323</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td>18 (13, 23)</td>
<td>12 (8, 16)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>14 (12, 16)</td>
<td>15 (13, 17)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) All values are expressed as probability (lower limit, upper limit of 95% confidence interval). No differences were found in the POFI and in the percentage of participants above the UL with or without fortification of whole wheat flour (95% confidence intervals overlap).
In addition, inclusion of folic acid in whole wheat flour-containing foods at levels currently mandatory for white wheat flour (data not shown) or modelled with a 50% overage did not affect the percentage of Canadians with intakes above the UL from dietary sources alone (Table 11, adults only). In all instances, these values were zero or approached zero in most DRI and sex age groups. When supplemental folic acid intake was included in the analysis, <5 to 15% of supplement users exceeded the UL, but this did not increase significantly (<5 to 17%) when whole wheat flour fortification was modelled with the current mandatory level of folic acid or a 50% fortification overage (Table 11 and 12).

Whole wheat consumers

Thirty-six percent of participants 19 years of age and older who did not use a supplement (i.e. n=5211 out of 14629, unweighted sample) reported consuming at least one whole wheat flour-containing food and were designated a “whole wheat consumer” (Table 13). Among supplement users, 44% (n=2319 out of 5274, unweighted sample) were determined to be whole wheat consumers.

Among adult whole wheat consumers that did not consume a folic acid supplement, the POFI was reduced from 21% to 10% when whole wheat was fortified at the mandatory level (Model 3) or from 10% to 3% with a 50% fortification overage (Model 4, 95% CIs do not overlap). Among supplement users, when considering dietary sources only, the POFI was reduced from 26% to 13% in Model 3 or from 12% to 4% in Model 4 with a fortification overage included (95% CIs do not overlap) (Table 13). Regardless of whether folic acid was added to whole wheat flour or not in the modelling exercise, the POFI was virtually nonexistent among supplement users when supplemental folic acid intake was included in the analysis.
No whole wheat consumers exceeded the UL for folic acid from food alone, regardless of the level of folic acid fortification modelled in whole wheat foods (Table 13). Thirteen to 18% of whole wheat consumers that consumed a folic acid-containing supplement had intakes above the UL. There was no significant increase in the proportion of whole wheat consumers with intakes above the UL with whole wheat flour fortification (Table 13).
<table>
<thead>
<tr>
<th>Sample Size</th>
<th>UL (µg)</th>
<th>Without Whole Wheat Fortification (%)</th>
<th>With Whole Wheat Fortification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male and Female 1-3 y</td>
<td>615</td>
<td>300</td>
<td>7 (4, 11)</td>
</tr>
<tr>
<td>4-8 y</td>
<td>1287</td>
<td>400</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td>Male 9-13 y</td>
<td>475</td>
<td>600</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td>14-18 y</td>
<td>302</td>
<td>800</td>
<td>14 (7, 21)</td>
</tr>
<tr>
<td>19-30 y</td>
<td>324</td>
<td>1000</td>
<td>5 (2, 9)</td>
</tr>
<tr>
<td>31-50 y</td>
<td>570</td>
<td>1000</td>
<td>11 (7, 15)</td>
</tr>
<tr>
<td>51-70 y</td>
<td>672</td>
<td>1000</td>
<td>11 (8, 14)</td>
</tr>
<tr>
<td>&gt; 71 y</td>
<td>385</td>
<td>1000</td>
<td>13 (8, 18)</td>
</tr>
<tr>
<td>Female 9-13 y</td>
<td>434</td>
<td>600</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
<tr>
<td>14-18 y</td>
<td>350</td>
<td>800</td>
<td>13 (8, 18)</td>
</tr>
<tr>
<td>Sample size</td>
<td>UL (µg)</td>
<td>Without whole wheat fortification (%)</td>
<td>With whole wheat fortification (%)</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>---------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>19-30 y</td>
<td>463</td>
<td>9 (6, 13)</td>
<td>10 (6, 14)</td>
</tr>
<tr>
<td>31-50 y</td>
<td>855</td>
<td>14 (10, 18)</td>
<td>14 (10, 18)</td>
</tr>
<tr>
<td>51-70 y</td>
<td>1126</td>
<td>11 (8, 14)</td>
<td>11 (8, 14)</td>
</tr>
<tr>
<td>&gt; 71 y</td>
<td>879</td>
<td>12 (9, 15)</td>
<td>12 (9, 16)</td>
</tr>
</tbody>
</table>

1 No Canadians (supplement users or non-users) had folic acid intakes > UL from diet alone.
2 All values are expressed as probability (lower limit, upper limit of 95% confidence interval). No significant differences were found in the percentage of participants above the UL with or without the fortification of whole wheat flour (95% confidence intervals overlap). Folic acid intake comes from both dietary and supplemental sources.
Table 13 Whole wheat consumers sub-analysis
Prevalence of folate inadequacy (%) and proportion of adult Canadians whole wheat consumers with intakes above the UL (%)

Prevalence of folate inadequacy (Folic acid intakes above the UL)

<table>
<thead>
<tr>
<th>Sample size</th>
<th>EAR (µg DFEs)</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
<th>UL (µg)</th>
<th>Without whole wheat fortification</th>
<th>With whole wheat fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-supplement users</td>
<td>5211</td>
<td>320</td>
<td>21 (18, 25)</td>
<td>10 (7, 13)</td>
<td>10 (7, 12)*</td>
<td>3 (1, 4)*</td>
</tr>
<tr>
<td>Supplement users</td>
<td>2319</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foods only</td>
<td></td>
<td></td>
<td>26 (21, 31)</td>
<td>12 (7, 17)</td>
<td>13 (9, 17)*</td>
<td>4 (2, 6)*</td>
</tr>
<tr>
<td>Foods + Supplements</td>
<td></td>
<td></td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
<td>&lt;5 (&lt;5, &lt;5)</td>
</tr>
</tbody>
</table>

Footnote: All values are expressed as probability (lower limit, upper limit of 95% confidence interval).

* The prevalence of folate inadequacy (POFI) with fortification is significantly different from the POFI without the fortification (95% confidence intervals do not overlap).
4.5 Discussion

The results of this modelling exercise suggest that extending folic acid fortification to include whole wheat flour-containing foods, not currently permitted in Canada, would result in a statistically significant but incrementally small (<5%) increase in mean total folate intakes of Canadians as a whole. Further, our preplanned statistical analyses (overlap of 95% confidence intervals) indicate this increase would not change the POFI of Canadians in the general population. However, fortifying whole wheat flour with folic acid would result in 10 percentage point decrease in the POFI among adult whole wheat consumers. While the prevalence of blood folate concentrations in the deficient range (<305 nmol/L) is negligible among healthy Canadians, our results suggest that fortification of whole wheat flour may ensure that whole wheat consuming-women of reproductive age have red blood cell folate concentrations maximally productive against a NTD (900 to 1000 nmol/L) (44, 45, 145). This finding is important as it can be anticipated as food consumption patterns among Canadians move away from white wheat to whole wheat four, women of reproductive age will be less exposed to folic acid fortified foods as currently mandated. We and others previously reported about 20% of Canadian females of reproductive age have blood folate concentrations below that which is maximally protective against an NTD (145, 305). Importantly adding folic acid to whole wheat flour, even with a 50% folic acid fortification overage, would not likely increase the percentage of individuals with intakes above the UL, regardless of whether they were from the general population or were specifically whole wheat consumers. This should provide reassurance that folic acid fortification of whole wheat flour, would not put Canadians at risk of the negative consequences of very high levels of folic acid including masking of vitamin B₁₂ deficiency. The lack of impact on the population as a whole reflects the relatively low proportion of the
population who consumed whole wheat flour-containing foods when the CCHS 2.2 data were collected and the relatively low amount they consume

Fortification of the food supply is an effective strategy in addressing micronutrient deficiencies and provides the opportunity to impact the entire population (306). Such has been the case in Canada where mandatory folic acid fortification of white wheat flour, “enriched” cornmeal and “enriched” pasta has virtually eliminated classic folate deficiency among healthy Canadians as assessed using red blood cell folate concentrations and was very effective in reducing folate-responsive NTDs (1, 4, 24, 145). The strategy, however, is in conflict with contemporary dietary guidance to increase consumption of whole grain foods. For example, currently Health Canada in its 2007 Food Guide recommends that Canadians consume a diet rich in whole grains by choosing at least half of their daily grain servings from whole grain sources (29). While the etiology is no doubt multifactorial, and the contribution of increased whole grain consumption is unknown, it is interesting to note that since its nadir in 2006, the prevalence of NTDs in Canada has steadily increased from 4.4 to 5.7 cases per 10,000 total births in 2010 (123). An important first step in considering any policy change related to the fortification of whole wheat flour with folic acid consists of modelling the impact on folic acid intake to ensure that the important gains made in improving the folate status of reproductive-age women are preserved whilst not unduly exposing the non-target population to unsafe levels of folic acid.

The age groups with the highest prevalence of dietary folate inadequacy in CCHS 2.2 were Canadians over the age of 71. For example, among females > 71 years of age in the CCHS 2.2, approximately 58 and 47% of non-supplement and supplement users, respectively, reported dietary intakes of folate below the EAR. After modelling folic acid fortification of whole wheat
flour, the POFI among non-supplement and supplement-using females >71 years of age was 46 and 36%, respectively. This reduction, while possibly meaningful, did not reach statistical significance as determined by overlapping 95% confidence intervals. Further, it needs to be acknowledged that biomarker data from the Canadian Health Measures Survey suggest a very low prevalence of folate deficiency in this age group (145).

A concern for the elderly population is that intakes of synthetic folic acid may mask vitamin B<sub>12</sub> deficiency, which, if undetected could lead to or exacerbate irreversible neurological damage (1). MacFarlane et al. estimated that approximately 4.5% (95% CI: 2.9, 6.1) of Canadians 60-79 years are vitamin B<sub>12</sub> deficient as assessed by a serum vitamin B<sub>12</sub> level <148 pmol/L (152). Further, approximately 17.1% (14.8, 19.3) have a serum vitamin B<sub>12</sub> concentration in the “marginal” range of 148 to 220 pmol/L. It should be noted that Canadian regulatory guidance recommends that multivitamin supplements containing >199 µg folic acid should also contain vitamin B<sub>12</sub> (307). This recommendation would reduce the risk of vitamin B<sub>12</sub> deficiency and therefore the risk of masking vitamin B<sub>12</sub> deficiency secondary to supplemental folic acid intake (307). In the present analysis, no adult, including the elderly, had intakes of synthetic folic acid from diet alone above the UL whether or not they were whole wheat consumers or whether a 50% fortification overage was applied to the model. Overall, our analysis confirmed that folic acid supplement use, and not folic acid fortification of the food supply, even if extended to whole wheat flour, is a risk factor for folic acid intakes above the UL (61, 111, 112).

There are several limitations to our analysis. First, data from the CCHS 2.2 were collected in 2004 and may not accurately reflect current dietary intakes. For example, it is possible that the consumption of whole wheat flour-containing foods has increased in Canada since 2004. While
38% of adults in our sample consumed at least one whole wheat flour-containing food in their 24-hour recall, there has been significant growth in new product launches and product labels bearing whole grain claims (308). Further, the recent Tracking Nutrition Trends 2013 survey reported that over 70% of adult Canadian respondents indicated in an on-line survey that an important factor influencing their food choices was whether a food is a source of whole grain (309). This suggests that Canadians may be replacing white flour containing food products with products that contain whole wheat flour, which could lower folic acid intake. As such, if our analysis had been performed using more recent dietary data, and if Canadians are indeed consuming more whole wheat flour, we would anticipate that folic acid fortification of whole wheat flour may have a greater impact on the folate intake distribution in the Canadian population. Specifically, a significantly lower POFI among Canadians may be observed with whole wheat flour fortification, and likely a larger reduction in the POFI among whole wheat flour food consumers. Second, the folic acid content in rice recorded in Health Canada’s Nutrition Survey System used in the present analysis did not accurately reflect the actual folic acid content of rice in Canada. Except for heavily processed rice, most rice and rice products in Canada are not fortified with folic acid. However, most rice in Health Canada’s Nutrition Survey System includes folic acid as a fortificant. Based on our calculations, this resulted in an underestimate of the POFI by about 4% and an overestimate of the percentage of the population above the UL by about 3% (data not shown). However, since rice was not the targeted food vehicle for our modelling exercise, the extra folic acid content in rice was embedded in the analysis of both pre and post fortification of whole wheat flour. Third, while the CCHS 2.2 is generalizable to >98% of the Canadian population from all 10 provinces, it is not representative of those living in Northern Canada, specifically Nunavut, Yukon and the Northwest Territories.
Further, caution should be exercised in applying these results to other countries that have a different food supply and food consumption patterns (310).

In conclusion, based on CCHS 2.2 data of 2004, we would anticipate fortification of whole wheat flour-containing foods would increase total folate intake in the Canadian population and would improve POFI in adult whole wheat consumers without risk of increasing the proportion of the population with intakes above the UL. However, adding folic acid to whole wheat flour containing foods would likely not significantly change the POFI or the prevalence of folic acid intake above the UL in the general Canadian population based on dietary patterns reflected in the CCHS 2.2 of 2004. In the absence of more current national dietary data in Canada, a future modelling exercise using local datasets to gauge the impact of broader whole wheat flour consumption on the POFI and intakes above the UL is warranted.

4.6 Acknowledgements

The authors would like to acknowledge the assistance from Yaseer Shakur who assisted in setting up the analysis. We also want to thank Carmina Ng at Statistics Canada for data releasing and other technical support.

Statement of author contributions to manuscript:

Y.M.C., D.L.O. and A.J.M. conceptualized and designed the study. YMC conducted the analyses and wrote the first draft of the manuscript. All authors contributed to data interpretations and manuscript editing. All authors have read and approved the final content of the manuscript.
5. Thesis study II: Increasing the consumption of fibre-rich foods in African Americans increases colonic folate contents

Yen-Ming Chan, Deborah L. O’Connor, Stephen J. O’Keefe

A brief communication in preparation for submission to a journal.

5.1 Abstract

We investigated whether feeding a diet traditionally consumed by rural South Africans (high fibre low fat) to African Americans (n=20) and feeding rural South Africans (n=20) a Western diet for two weeks affects colonic folate synthesis. The high fibre low fat diet consumed by African Americans produced a 53% increase in colonic folate concentration compared with the baseline values (p=0.0003). No change was observed in rural South Africans consuming a Western diet. A high fibre low fat diet might result in greater bacterial synthesis of folate.
5.3 Introduction

Folate, a water-soluble B-vitamin, mediates the transfer of one-carbon moieties involved in nucleotide biosynthesis and biological methylation reactions (1). As such, folate plays an important role in human health and disease. Suboptimal intakes of folate are associated with a number of undesirable health consequences, including anemia, cognitive impairment, cancer and neural tube birth defects (1, 8, 311). In Canada, mandatory folic acid fortification of the food supply in 1998 led to an approximate 50% reduction in neural tube defects (4). An emerging body of evidence, however, suggests that high intakes of synthetic folic acid may be associated with adverse health effects including masking of vitamin B$_{12}$ deficiency (1), accelerated tumor progression, altered DNA methylation and in utero epigenetic programming, and aberrant embryonic developments in mice (312, 313) and humans (314). A better understanding of the “input side” of folate nutrition will help set dietary and vitamin supplement recommendations that strike the right balance between the known benefits and possible risks.

Unlike humans, many species of colonic bacteria can synthesize folate (31). We and others have shown that the quantity of folate produced in the colon frequently exceeds dietary intakes, and that folate can be absorbed across the colon in both humans and animal models (33-35, 189). In carefully controlled animal studies, it has been demonstrated that bacterial biosynthesis of folate in the colon can be altered by the quantity and type of fibre in the diet or the inclusion of human milk solids (source of the oligosaccharides, a prebiotic) (36, 37, 261, 262). These alterations have also been shown to impact the folate status of the host. However, there is a paucity of data on what impact, if any, dietary manipulation has on net bacterial biosynthesis of folate in the colon of humans. In a cross-sectional study, we found that for each 1 gram increase in nonstarch polysaccharide in the diet, a 1.8% increase in serum folate concentration was observed, even
after controlling for dietary and supplemental folic acid intake (P<0.001) (38). To the best of our knowledge, however, there has been no direct experimental evidence showing whether folate colonic contents can be manipulated by modifying the diet in humans. Since folate produced by bacteria residing in colon comprises a significant depot of naturally occurring folate that may help meet one’s folate requirement, it would be important to investigate how the production of this source could potentially be regulated.

The aim of this study was to investigate whether colonic folate content in humans (African Americans) can be manipulated by switching a typical Western diet (high fat, low fibre) for two weeks with one traditionally consumed by rural South Africans (high fibre, low fat) (267, 315).

5.4 Methods and materials

Subject and diet

This present sub-study was conducted within a larger feeding trial (316) that examined whether differences in fat and fibre intake as a result of a dietary pattern switch between African Americans and rural South Africans yielded reciprocal responses in mucosal biomarkers and changes in the microbiome associated with the risk of colon cancer. Key outcomes for the parent study included mucosal epithelial proliferation, mucosal inflammation, and microbiota composition (316). Details of the feeding trial have been extensively published elsewhere and are summarized briefly again here (316). A group of age and sex-matched African Americans (n=20) from Pittsburgh, PA USA and rural South Africans (n=20) from a rural area in Kwazulu-Natal (Empangeni) in South Africa were recruited through local advertisements and provided with compensation for participations. Individuals were eligible if they were healthy, 40-65 years of age and had a body mass index (BMI) between 18-35 kg/m². Subjects were excluded if they
had gastrointestinal diseases; had invasive cancers in the past 5 years; recently underwent gastrointestinal surgery or had a chronic gastrointestinal conditions affecting gastrointestinal function; had a renal, hepatic or bleeding disorders; took antibiotics or other medications, prebiotics and probiotics that could affect bacterial biosynthesis or folate metabolism 12 week prior to the enrollment (316). The study consisted of two phases made up of a home environment phase (day 0 - day 14) and a dietary intervention phase (day 15 - day 29). All subjects started with the two-week home environment phase where they consumed their usual home diet. Three-day dietary recalls were collected on a subset of subjects (n=12 in rural South Africans and n=10 in African Americans). On day 0 of the home environment phase, subjects drank 2L of a bowel wash-out solution over 30 minutes prepared in the O’Keefe lab [polyethylene glycol (60g/l, molecular weight 3350) plus electrolytes] over 30 minutes to a) sample the total colonic contents and b) to prepare the colon for colonoscopy. The evacuants expelled over the subsequent 3 hr were collected, weighed, homogenized and aliquoted. The colonoscopy was conducted to collect colonic mucosal biopsies for epithelial proliferation outcomes reported previously (316). The intervention diets were designed using dietary data previously collected on samples of African Americans and rural South Africans by our group (317, 318). During the dietary intervention phase (day 15- day 29), rural South Africans were provided with a typical Western diet consisting of 30-35 kcal/kg ideal body weight, and a % total energy macronutrient distribution of 27% protein, 52% fat, 21% carbohydrate, and 12g/d fibre). Typical foods provided included hotdogs, fries, steak and hamburgers. During the intervention, African Americans were provided with a typical high fibre low fat, rural South African diet (30-35 kcal/kg ideal body weight, 16% protein, 18% fat, 63% carbohydrate, and 55 g/d fibre). The intervention diet, on a 3-day rotation menu consisted of a “meilie meal” (samp and beans)-based
diet supplemented with corn kernels and sugar beans. The diet also consisted of white potatoes, bread, cabbage, mango, banana, vegetable oil and small portions of animal-based protein (e.g. eggs and chicken). All meals during the intervention were prepared and consumed by African Americans at the University of Pittsburgh Clinical Translational Research Center and by rural South Africans at a local rural lodging facility in South Africa. Subjects were encouraged to consume all the foods that were provided. In rare instances where foods could not be finished, leftovers were weighed. Colonic evacuants were collected again on day 29 prior to the final colonoscopy and completion of the study. The colonic evacuants were homogenized, aliquoted and kept frozen at -80°C until further analysis.

Two weeks of dietary intervention was chosen with consideration and evidence that colonic bacterial metabolism and related epithelial proliferation, key outcomes of the parent study, can be modified by diet within the 2-week window in humans (319-321).

**Analytical analysis of colonic evacuants**

Folate in evacuants was extracted using the tri-enzyme digestion method (322). Thereafter, folate concentrations were measured by the microbial assay (323). Certified pig liver (13.3 mg folate/kg, Pig Liver, BCR 487, IRMM, Geel, Belgium) was used to assess the accuracy and reproducibility of the assay. We found the average folate concentrations of the pig liver reference standard was 12.4 ± 0.9 mg/kg with an inter-assay CV of 7.5% (n=4).

**Statistical analysis**

Difference between African American and rural South Africans for baseline variables were assessed using the Mann-Witney test. The differences in dietary composition between groups
and changes in dietary composition before and after intervention within group were analyzed using PROC MIXED (SAS 9.4, SAS Institute, Cary NC). The main effects of diet on the total and concentration of folate of colonic evacuants were assessed using PROC MIXED (SAS 9.4, SAS Institute, Cary NC). A p-value < 0.05 was considered statistically significant.

The sample size for the parent study was calculated to be able to detect the differences in epithelial proliferation (Ki 67), production of butyrate, breath hydrogen and methane between the two groups at an alpha=0.05 and 80% power, using values from previous studies (317, 318) and unpublished pilot data from our group.

5.5 Results

Subject characteristics

There was no difference in the mean age of African Americans or rural South Africans (Table 14). While African Americans were both heavier and taller than the rural Africans (p<0.5), there was no difference in BMI between the two groups. The intervention diet was well-tolerated and the body weight change of each subject at the end of the intervention remained within 2 kg of their home day 0 baseline weight (316).
Table 14 Baseline characteristics of the study population<sup>1</sup>

<table>
<thead>
<tr>
<th></th>
<th>Rural South Africans (n=20)</th>
<th>African Americans (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>M=10/F=10</td>
<td>M=11/F=9</td>
</tr>
<tr>
<td>Age (y)</td>
<td>54.8 ± 1.0</td>
<td>55.6 ± 0.8</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>73.1 ± 3.3</td>
<td>87.1 ± 4.0*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.5 ± 2.2</td>
<td>172.5 ± 2.4*</td>
</tr>
<tr>
<td>Body mass index (Kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>27.7 ± 1.5</td>
<td>29.4 ± 0.8</td>
</tr>
</tbody>
</table>

<sup>1</sup>: Average ± SE; * p<0.05

**Dietary intakes**

During the home environment phase, mean (± SD) dietary intakes of rural South Africans were estimated to contain lower fat (45 ± 27 vs 98 ± 41 g/d) and soluble fibre (3 ± 3 vs 6 ± 3 g/d) but higher amounts of carbohydrate (391± 69 vs 292 ± 100 g/d), insoluble fibre (26 ± 14 vs 9 ± 3 g/d) and total dietary fibre (28 ± 14 vs 15 ± 6 g/d), compared to African Americans (p<0.05) (Table 15). During the intervention phase, African Americans consumed lower amounts of protein (87 ± 16 vs 160 ± 31 g/d), fat (42 ± 7 vs 137 ± 27 g/d) but higher amounts of carbohydrate (345 ± 73 vs 120 ± 24 g/d), soluble fibre (4 ± 1 vs 2 ± 0 g/d), insoluble fibre (40 ± 5 vs 7 ± 1 g/d) and total dietary fibre (43 ± 5 vs 8 ± 2 g/d), compared to rural South Africans consuming a Western diet (p<0.0001) (Table 16).
Table 15 Usual home (baseline) diet consumed by rural South African and African Americans\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Rural South Africans (n=12)</th>
<th>African Americans (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>2403 ± 483</td>
<td>2454 ± 863</td>
</tr>
<tr>
<td>Total protein (g/d)</td>
<td>71 ± 31</td>
<td>92 ± 30</td>
</tr>
<tr>
<td>% Kcal from protein</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>45 ± 27</td>
<td>98 ± 41(^*)</td>
</tr>
<tr>
<td>% Kcal from fat</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>Total carbohydrate (g/d)</td>
<td>391 ± 69</td>
<td>292 ± 100(^*)</td>
</tr>
<tr>
<td>% Kcal from carbohydrate</td>
<td>66</td>
<td>48</td>
</tr>
<tr>
<td>Total dietary fibre (g/d)</td>
<td>28 ± 14</td>
<td>15 ± 6(^*)</td>
</tr>
<tr>
<td>Insoluble fibre (g/d)</td>
<td>26 ± 14</td>
<td>9 ± 3(^*)</td>
</tr>
<tr>
<td>Soluble fibre (g/d)</td>
<td>3 ± 3</td>
<td>6 ± 3(^*)</td>
</tr>
<tr>
<td>Resistant starch (g/d) #</td>
<td>38</td>
<td>1</td>
</tr>
<tr>
<td>Folate (µg/d)</td>
<td>368 ± 354</td>
<td>504 ± 296</td>
</tr>
<tr>
<td>Iron (mg/d)</td>
<td>11 ± 8</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>293 ± 266</td>
<td>820 ± 368(^*)</td>
</tr>
</tbody>
</table>

\(^*\) p<0.05; \(^1\): Average ± SD; \(^#\) Estimated values (324)
<table>
<thead>
<tr>
<th></th>
<th>Rural South Africans (n=20)</th>
<th>African Americans (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>2375 ± 474</td>
<td>2216 ± 406</td>
</tr>
<tr>
<td>Total protein (g/d)</td>
<td>160 ± 31</td>
<td>87 ± 16*</td>
</tr>
<tr>
<td>% Kcal from protein</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>Total fat (g/d)</td>
<td>137 ± 27</td>
<td>42 ± 7*</td>
</tr>
<tr>
<td>% Kcal from fat</td>
<td>52</td>
<td>18</td>
</tr>
<tr>
<td>Saturated fatty acids (g/d)</td>
<td>53 ± 10</td>
<td>8 ± 2*</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (g/d)</td>
<td>14 ± 3</td>
<td>7 ± 2*</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (g/d)</td>
<td>50 ± 10</td>
<td>18 ± 3*</td>
</tr>
<tr>
<td>Total carbohydrate (g/d)</td>
<td>120 ± 24</td>
<td>345 ± 73*</td>
</tr>
<tr>
<td>% Kcal from carbohydrate</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td>Total dietary fibre (g/d)</td>
<td>8 ± 2</td>
<td>43 ± 5*</td>
</tr>
<tr>
<td>Insoluble fibre (g/d)</td>
<td>7 ± 1</td>
<td>40 ± 5*</td>
</tr>
<tr>
<td>Soluble fibre (g/d)</td>
<td>2 ± 0</td>
<td>4 ± 1*</td>
</tr>
<tr>
<td>Resistant starch (g/d) #</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Folate (µg/d)</td>
<td>424 ± 85</td>
<td>772 ± 134*</td>
</tr>
<tr>
<td>Iron (mg/d)</td>
<td>21 ± 4</td>
<td>17 ± 4**</td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>541 ± 108</td>
<td>592 ± 188</td>
</tr>
<tr>
<td>Vitamin A (RAE, µg/d)</td>
<td>6411 ± 1279</td>
<td>829 ± 174*</td>
</tr>
<tr>
<td>Vitamin C (mg/d)</td>
<td>31 ± 6</td>
<td>116 ± 38*</td>
</tr>
</tbody>
</table>

* p<0.0001; ** p<0.001; †: Average ± SD; # Estimated values (324)
Colonic folate contents in evacuants

There was no difference in the mean folate contents in colonic evacuants of rural South Africans and African Americans at baseline; whether expressed as concentration or as total content (concentration x evacuant volume) (Figure 5 and 6). The Western diet intervention did not affect the folate concentration or total folate contents in colonic evacuants of the rural South African subjects. However, the high fibre low fat increased both the folate concentration (1.5 ± 1.3 vs 2.3 ± 1.3 µg/mL; p=0.0003) and total folate contents (2197 ± 2483 vs 3107 ± 1811 µg/d; p=0.0037) from baseline in African Americans. In addition, the African Americans group had higher folate concentration (2.3 ± 1.3 vs 1.3 ± 1.2 µg/mL; p=0.0045) and total folate contents (3107 ± 1811 vs 2157 ± 1956 µg; p=0.04) compared to rural South Africans post-intervention.
Figure 5 Colonic folate concentrations (µg/ml) before and after a two week feeding intervention in a sample of rural South Africans (n=20) and African Americans (n=20).

The baseline diet for rural South Africans was high in fibre and low in fat whereas the baseline diet for African Americans was low in fibre and high in fat. The intervention diet for rural South Africans was low in fibre and high in fat whereas the intervention diet for African Americans was high in fibre and low in fat.
Figure 6 Total colonic folate content (µg) before and after a two week feeding intervention in a sample of rural South Africans (n=20) and African Americans (n=20).

The baseline diet for rural South Africans was high in fibre and low in fat whereas the baseline diet for African Americans was low in fibre and high in fat. The intervention diet for rural South Africans was low in fibre and high in fat whereas the intervention diet for African Americans was high in fibre and low in fat.
5.6 Discussion

Our results indicate that a two-week switch to a higher fibre, low fat diet (43 g/d fibre, 18% total energy as fat) leads to higher colonic folate contents in a sample of African Americans who usually consumed a Western diet (15 g/d fibre, 36% of total energy as fat). To the best of our knowledge, this study provides the first direct experimental evidence that colonic folate contents can be manipulated by dietary modification in humans.

We speculate that the change in colonic folate contents are due to, at least in part, increased bacterial biosynthesis of folate in the colon. It has been previously established that the capacity of the colonic microbiota to generate folate is tremendous (33). The adult human gastrointestinal tract consists of $10^{13}$ to $10^{14}$ microorganisms, including bacteria that can synthesize folate by condensation of PABA with dihydropterin pyrophosphate. These bacterial may then secrete synthesized folate into the intestinal lumen. Other bacteria in the community have an obligate requirement for pre-formed folate. Magnusdottir et al. estimated based on an analysis of the metabolic capabilities of bacteria found in the colon that approximately 43% of bacterial species are able to synthesize folate (32). The main fuel source for colonic bacteria are fermentable substrates, such as dietary fibre and resistant starch that escape digestion in the small intestine and enter the colon intact (264, 265). As the quantity of dietary fibre or prebiotics consumed increases, so does total bacteria load and short chain fatty acid production in colon (264, 265, 267). All available evidence, primarily from animal models, suggests as total bacterial load increases so does net folate production in the colon (262, 325). Furthermore, both epidemiological (267-270) and short-term intervention (271-275) evidence suggest that the quantity and type of fibre consumed may also impact microbial diversity and composition. Specifically, diets high in animal products but low in fermentable carbohydrate increases the
abundance of Bacteroides and lowers Firmicutes. We and others have proposed that enhancing the concentration of Bifidobacteria species (Actinobacteria phylum) increases the production of folate in the colon (38, 261, 262) as several strains of this bacteria, such as B. adolescentis and B. pseudocatenulatum, are potent folate synthesizers (30, 32, 258).

The higher colonic folate contents we found in African American following a high fibre, low fat diet illustrate the principal of how a source of folate generated in the colonic lumen ensure the folate requirements of the colonocyte are met in the event of suboptimal dietary intakes of folate. Meta-analyses of randomized clinical trials has consistently shown that suboptimal dietary intakes of folate are associated with an increased risk of colon cancer (1, 56). While we acknowledge that the proposed link between bacterial biosynthesis of folate the risk of colon cancer in populations with low dietary folate intake is speculative, there are several lines of evidence suggesting this area of research merits future investigation. In the previous investigation, we found while RBC folate was much lower in a group of rural South Africans, colonic folate contents were similar to that of African Americans (39). It suggests that much of folate of colonic origin might remain and be used in mucosa or within the splanchnic bed for normal metabolism and cellular repair. We proposed in this previous publication that bacterial biosynthesis in the colon might be the reason why low folate status as measured by blood measures does not lead to higher risk of colon cancer in rural South Africans (39). Data from our current parent study indeed demonstrated increased saccharolytic fermentation and short chain fatty acid production in our sample of African Americans switched to the high fibre low fat intervention diet illustrating increased capability of the colonic microbiome to increase bacterial folate biosynthesis (316). The increased saccharolytic fermentation in African Americans corresponded to a shift from high occurrence of Bacteroides towards higher relative abundance
of Firmicutes (316). Our results suggest dietary switch between the two groups led to reciprocal changes in the composition of gut microbiota (316). While the change in gut microbiota related to folate production was not specifically analysed in this present study, there is ample evidence suggesting that soluble fibre (276, 279) and type 1 resistant starch (Hi-maize whole grain corn flour provided to African Americans) (326) is Bifidogenic. Therefore, we speculate that the gut microbiota composition in response to the high fibre low fat diet consumed by African Americans led to a favorable net effect on folate production in the colon.

Unlike the change in total colonic folate content and mean colonic folate concentration related to the dietary switch to the high fibre low fat diet in the African Americans, we observed no such change in colonic folate contents of rural South Africans switched to the low fibre, high fat diet. This finding is consistent with our previous observations where the folate contents in colonic evacuants in a sample of African Americans and rural South Africans did not differ (39). There could be a number of explanations of why we did not see differences in colonic contents in rural South Africans and African Americans at baseline or between rural South Africans at baseline and rural South Africans at the end of the two-week intervention. The net difference in total fibre content between the African Americans and rural South Africans at baseline (13 g/d) and change in fibre contents between baseline and intervention in the rural South Africans (20 g/d) was smaller than that of the African Americans switched to the high fibre low fat diet (28 g/d) and that of African Americans compared to rural South Africans during the intervention (35g/d). Therefore, the difference in fibre intake between the two groups at baseline or among rural South Africans at baseline and after the feeding intervention might be too small to generate a detectable difference in colonic folate content. Alternatively, a 2-week intervention period may have been too short for rural South Africans whose fibre intake was decreased by 20g to show an effect on
colonic folate content. It has been suggested the adaption of colonic fermentation may take 9-12 months to develop in response to change in fibre intake in humans (327). Of note, in this current intervention study, while no differences in colonic folate contents was noted, we reported rural South Africans experienced a decrease in saccharolytic fermentation and butyrogenesis in the colon following the switch to the low fibre high fat diet (316).

There are a number of limitations of this study. While recent evidence confirms that changes in dietary fibre composition can alter gut microbiome within a day, it is unknown whether the changes in colonic folate production among African Americans consuming the high fibre, low fat diet would be sustained for the long-term (328, 329). Understanding the long-term effect of dietary manipulation on the microbiome is particularly important because of evidence suggesting the gut microbiota may take up to one year to reach equilibrium in response to changes in fibre or other treatments (327, 330). A second acknowledged limitation of this study was that the baseline and intervention diets differed in their folate content. While it is generally believed that most dietary folate is absorbed in the small intestine, we cannot discount that some of the differences in colonic folate contents were due to differences in dietary folate intake. However, although dietary folate intake was higher in African Americans in the intervention phase, the difference in colonic contents greatly exceeds the difference in dietary folate by 2.5 fold.

In summary, our study provides the first direct evidence in humans that colonic folate contents can be enhanced by increased fibre consumption in a sample of healthy adults normally consuming a Western diet characterized by high fat and low fibre content. The change in colonic content may be in part due to the regulation of folate production by the microbiota in the colon.
5.7 Acknowledgement

The authors would like to acknowledge the technical support provided by Dr. Susanne Aufreiter for colonic folate contents measurements.
6. Thesis study III: Folate is absorbed across the human colon: evidence by assessment of urinary excretions in response to oral administration of $^{13}$C$_5$-5-formyltetrahydrofolate

Yen-Ming Chan, Susanne Aufreiter, Hayley Craig-Barnes, Michael Leadley, Alanna Lakoff, Bairbre Connolly, Paul B Pencharz, Deborah L. O’Connor

6.1 Abstract

**Background:** We previously established folate is absorbed across the human colon using plasma folate kinetics. Measurement of labelled folate in urine would be less invasive and likely critical to facilitate longer-term studies under steady-state conditions.

**Objective:** The objective of the study was to evaluate folate bioavailability in the colon using urinary folate excretions following oral administration of $^{13}$C$_5$-5-formyltetrahydrofolate delivered quantitatively to the colon.

**Method:** Healthy adults were ingested a 400 µg of $^{13}$C$_5$-5-formyltetrahydrofolate containing pH-sensitive enteric caplet for quantitative delivery to the colon. Twenty-four-hour urine was obtained the day prior to this test dose and three subsequent days thereafter. After a washout period (>3 weeks), subjects received the same test compound (100 µg) via IV injection. Twenty-four-hour urine collections were carried out the day before the IV test dose and two days after.

**Results:** Changes in urinary total $^{13}$C$_5$-5-methyltetradrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate excretions in response to both caplet and IV test doses were observed (p<0.0001). Changes in
the urinary recovery of labelled folate dose was also significant over time in both caplet and IV arms (p<0.0001). The mean UER (% oral $^{13}$C isotope dose excreted/% IV $^{13}$C isotope dose excreted) was 0.06 ± 0.02. The molar ratios (e.g. labelled versus unlabelled) of $^{13}$C$_5$-5-methyltetrahydrofolate in urine and plasma were correlated (r=0.7, p<0.0001). However, the level of enrichment in urine as well as the UER was low.

**Conclusions:** Labelled folate was detectable in the urine following oral and IV doses of $^{13}$C$_5$-5-formyltetrahydrofolate confirming that folate is absorbed in the colon. Our current results were in agreement with our previous investigation where we showed colonic folate absorption using plasma enrichment. Thus, the appearance of labelled folate in urine might be a suitable biomarker for future investigation of measuring the availability of folate biosynthesized in the colon. At the current level of analytical sensitivity, the use of urine enrichment may be useful to serve as a confirmatory measure for colonic folate bioavailability. In future studies, % urinary enrichment with $^{13}$C$_5$-5-methyltetrahydrofolate may be improved by establishing a pre-study $\geq$2-week protocol whereby subjects consume a daily folate supplement to saturate tissue stores.
6.2 Introduction

Folate is a generic term for a family of compounds that are required for growth and cell division. An increase in the folate intake of women, as a result of folic acid fortification of the food supply in Canada, has led to a 50% reduction in birth defects (e.g. neural tube defects) (4). However, it has been proposed that higher intakes of folate in the synthetic form (i.e. folic acid) added to foods or provided in vitamin supplements may be associated with a number of adverse health outcomes including masking of vitamin B\textsubscript{12} deficiency and progression of neoplastic cells to cancer and increased risk of asthma, respiratory infection, wheezing and central adiposity in offspring of mothers consuming supplemental folic acid (5, 9, 15-17, 293, 294). Admittedly, this literature is very conflicting and has been polarizing. Recently, there has been growing interest in investigating the possibility of bacterially-synthesized folate serving as a complementary source of folate for humans. A more comprehensive understanding of all sources of folate contributing to the “input side” of folate metabolism will help to establish dietary and supplemental folic acid recommendations that achieve the right balance of health benefits and risks.

Many bacteria commonly residing in the colon can synthesize folate through a process that involves condensation of PABA with dihydropterin pyrophosphate. It has been shown that the amount of folate in the mammalian colon can exceed dietary requirements, and is in a form that can be absorbed (at least across the small intestine) (33, 39). Aufreiter et al. conducted the first study to provide direct evidence that folate can be absorbed across the intact colon of humans (34). During colonoscopy, a physiological dose (648 nmol) of naturally occurring folate (\textsuperscript{13}C\textsubscript{5}-5-formyltetrahydrofolate) was infused into the cecum of adults. Study results showed that \textsuperscript{13}C\textsubscript{5}-5-
methyltetrahydrofolate appeared in plasma after cecal infusion at a rate of 0.6 ± 0.2 nmol/h compared to 7 ± 1.2 nmol/h after IV injection of $^{13}$C$_5$-5-formyltetrahydrofolate.

In the study of Aufreiter et al., much of the colonic bacteria of the humans was removed in preparation for colonoscopy (34). To investigate colonic absorption of folate with an intact microbiota, a method to deliver test material to the colon was developed and validated by our research team. Specifically, barium sulphate caplets coated with a unique pH dependent acrylic copolymer were developed to non-invasively and quantitatively deliver stable isotopes of folate past the ileocecal junction of adult humans (190). These newly designed pH-dependent release caplets were utilized in a recently completed experiment to deliver an 855 nmol oral dose of $^{13}$C$_5$-5-formyltetrahydrofolate to the colon of human volunteers (35). Their gut microbiota was kept intact and disintegration of the caplets was monitored by fluoroscopy. The apparent absorption rate of folate absorption was determined to be 46% using the mathematical model described by Wright et al. (191). This finding suggests that a significant amount of folate can be absorbed across the human colon in the presence of an undisturbed microbiota.

The folate bioavailability from different foods has previously been determined by the measurement of labelled folate in both plasma and urine. Colonic folate absorption in humans using stable isotopes, to the best of our knowledge has only been evaluated using folate plasma enrichment (34, 35). In addition, while it has been confirmed pre-formed folate can be absorbed in the colon, it still needs to be determined whether folate synthesized by colonic bacteria can be absorbed. To carry out this work, multiple doses of labelled- PABA (a precursor for folate biosynthesis) likely will need to be administered and some time will need to elapse for bacteria to take-up PABA, convert it into folate, secrete it into the colonic lumen and be absorbed.
Multiple blood sampling over many days may be considered demanding, impractical and costly. A medical facility is required to conduct such a procedure. In contrast, collection of 24-hour urine samples to measure isotope enrichment may be a more practical alternative in evaluating the bioavailability of bacterially-synthesized folate from the colon. Lakoff et al. reported the colonic folate absorption using determination of the appearance of labelled folate in plasma following oral administration of $^{13}$C-5-formyltetrahydrofolate (35). We aimed to assess colonic folate absorption using the urine samples collected in the same study to confirm that folate is absorbed across the colon and to assess the feasibility of measuring urinary enrichment with folate in future research.

6.3 Subject and methods

Study population

The study protocol and procedures have been previously described (35). Briefly, healthy adult individuals (n=9), aged 18 to 65 year were recruited. After initial screening using a telephone questionnaire, potential subjects were invited to attend an on-site screening session at the Physiological Research Unit at The Hospital for Sick Children. Blood samples were collected from the potential subjects for determination of folate status, vitamin B$_{12}$, and pyridoxal-5-phosphate concentrations as well as a complete blood count, electrolyte levels and MTHFR C677T genotype. Potential subjects were excluded if they met one or more conditions from the following list: homozygous of the T allele for MTHFR C677T genotype, abnormal blood results including abnormal folate status and other blood chemistries, with chronic diseases (eg, inflammatory bowel or celiac disease), recent gastrointestinal surgery or recent use of medications (eg, dilantin, metformin, antacids, laxatives, or antibiotics) known to affect folate
metabolism. In addition, individuals who regularly consumed more than one alcoholic drink/day or were currently smoking were also excluded. Eligible subjects were asked to refrain from vitamins and mineral supplement consumption for ≥ 2 weeks before the first day of the study and to avoid alcohol consumption for 24 hr before the labelled folate dose was given. Written informed consent was obtained by all subjects. The study protocol was approved by the human research ethics board at The Hospital for Sick Children. The Clinical Trial Application for this study was approved by the Therapeutic Products Directorate, Health Canada.

**Test compound and dose formulations**

Detailed information on the composition of both caplet and IV test doses have previously been reported (35). Briefly, the isotopically labelled test compound, $^{13}\text{C}_5$-$\text{C}_5$-formyltetrahydrofolate was formulated as a calcium salt (Merck Eprova AG, Schaffhausen, Switzerland). The test compound was then incorporated into barium caplets for oral delivery past the ileocecal junction. Caplets were manufactured by The Toronto Institute of Pharmaceutical Technology. Each caplet contained 855 nmol $^{13}\text{C}_5$-$\text{C}_5$-formyltetrahydrofolate (400 µg), 64% (wt:wt) barium sulfate, 7% (wt:wt) polyvinyl pyrillidone K90, 13% (wt:wt) microcrystalline cellulose and 4% (wt:wt) of sodium starch glycolate (JRS Pharma). The coating of the caplet contained two pH-sensitive methyl acrylic copolymers: Eudragit L100 (threshold at pH 6.0) and Eudragit S100 (threshold at pH 7.0) (Evonik Industries AG) in a 3:1 ratio. The choice of using $^{13}\text{C}_5$-$\text{C}_5$-formyltetrahydrofolate rather than other reduced forms of folate was because 5-formyltetrahydrofolate appears to be much more stable as compared to other reduced folates.

For the IV test dose, physiologic saline (pH 7.0) was used to dissolve $^{13}\text{C}_5$-$\text{C}_5$-formyltetrahydrofolate to prepare a test dose of 214 nmol (100 µg) $^{13}\text{C}_5$-$\text{C}_5$-
formyltetrahydrofolate. The IV test dose was prepared and stored in the Department of
Pharmacy at The Hospital for Sick Children.

**Study protocol**

The study protocol has been previously reported elsewhere (35) and is illustrated in Figures 7
and 8. In brief, on study day 1, 24 hr prior to the caplet dose ingestion, baseline blood (5ml) and
24 hr urine samples were collected. On day 2, after an overnight fast, at around 6 am, subjects
consumed the caplet containing 855 nmol (400 µg) of $^{13}$C$_5$-5-formyltetrahydrofolate immediately
followed by a standardized breakfast that was confirmed to provide low folate and residue (190).
To monitor the anatomical location and the disintegration pattern of each caplet, *in vivo*
fluoroscopic images of the abdominal area commenced approximately 2-hour post-dose and
hourly thereafter until approximately 6 pm or earlier if the caplet was determined to be
completely disintegrated. Fluoroscopic imaging was conducted by experienced certified
technicians in the Image Guided Therapy Department at The Hospital for Sick Children.
Subjects were instructed to continue collecting 24-hour urine samples on days day 2 through day
4. On day 2, hourly blood samples (5mL) was collected via an indwelling catheter once the
caplet had exited the stomach as determined by fluoroscopic imaging and also at 24 hr (day 3)
and 48 hr (day 4) after the caplet ingestion. Subjects had ad libitum access to meals and snacks
consisting of foods low in folate content once the caplet had passed through the pyloric
sphincter.

After a wash-out period of $\geq$ 3 weeks, subjects underwent a 3-day test period where on day 1, a
baseline blood sample was collected followed by IV administration of $^{13}$C$_5$-5-
formyltetrahydrofolate (214 nmol, 100 µg) after an overnight fast. After 15 minutes, a blood
sample (5mL) was collected and subsequent samples were collected at 30-minute intervals for 4 hr. During blood collection, low folate and low fibre snacks were provided ad libitum. On days 1-3, 24 hr urine samples were collected. A dietary record of food intake during day 1-4 of the caplet phase and day 1-3 of the IV arm was collected. Dietary intake and composition was analysed (Food Processor SQL version 10.2.0; Esha Research).

Figure 7 Study protocol of the caplet phase.

A caplet containing 0.4mg of $^{13}$C$_5$-5-formyltetrahydrofolate was given in the morning of day 2. Twenty four-hour urine sample was collected from day 1 through day 4.
A dose of 0.1 mg of $^{13}$C$_5$-5-formyltetrahydrofolate was given in the morning of day 2 through IV injection. Twenty four-hour urine sample was collected from day 1 through day 3.

Blood and urine sample analyses of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate

Blood samples for analysis of plasma and RBC folate were collected in EDTA tubes, transported on ice and processed as described previously (34). To preserve the folate in urine samples and prevent bacterial growth, 2.5 mmol of sodium ascorbate was added to each opaque bottle used to collect urine. Urine was stored at 4 °C until the end of the 24-hour collection period and thereafter, samples were pooled and stored at -80 °C. Folates in blood were extracted and the ratio of $^{13}$C labelled to unlabelled folates was analysed with LC/MS/MS as previously described (35). Urinary folates were extracted from 4 mL samples and were spiked with $^{13}$C-folic acid internal standard. The sample then was mixed with 3 mL of 1% ammonium formate buffer.
(pH=2.9, 0.5% ascorbic acid) and equilibrated at 4°C for 20 minutes. Each sample was centrifuged at 4°C at 5000xg for 20 minutes and filtered through a Supor ® membrane filter (0.2µm). Before loading the urine samples for clean-up, phenyl cartridges (1mL) were conditioned with 2 mL each of acetonitrile, methanol, and the 1 % ammonium formate buffer (pH 2.9, no ascorbic acid). Thereafter, samples were loaded on the phenyl columns, allowed to equilibrate for 10 minutes and were then washed sequentially with 3mL of 0.05 % ammonium formate buffer (pH=3.4, 0.5% ascorbic acid). Folates were eluted from the columns using an elution buffer of 0.5mL; 49% water, 40% methanol, 10% acetonitrile, 1% concentrated acetic acid and 0.5% ascorbic acid. The urine enrichments of \(^{13}\text{C}_5\)-5-formyltetradrofolate and \(^{13}\text{C}_5\)-5-methyltetradrofolate were analysed using LC/MS/MS, an Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) connected to a Sciex Q-Trap 5500 mass spectrometer (SCIEX, Framingham, MA, USA). Immediately prior to analysis, extracted samples were mixed 1:1 with 1% acetic acid in deionized H₂O. Samples (20 µL) were separated chromatographically on a Phenomenex Kinetex PFP column (1.7 µm, 100Å, 30 x 2.1 mm) (Phenomenex, Torrance, CA) running a gradient mobile phase over 6 minutes at a flow rate of 150 µL/min, consisting of: MPA - H₂O + 1% Acetic acid and MPB – 4:1 Methanol: Acetonitrile + 1% Acetic acid. The mobile phase gradient was: t=0 min: 5%B; t=1.5 min: 5%B; t=3.0 min: 70%B; t=3.2 min: 5%B; t=6.0 min: 5%B.

Quantification of \(^{13}\text{C}_5\)-5-formyltetrahydrofolate (479.2/299.2 m/z) and \(^{13}\text{C}_5\)-5-methyltetrahydrofolate (465.2/313.2 m/z) was carried out by comparing sample results to a standard curve generated using known quantities of \(^{13}\text{C}_5\)-5-formyltetrahydrofolate and \(^{13}\text{C}_5\)-5-methyltetrahydrofolate that were added to pooled urine. The internal standard used was \(^{13}\text{C}_5\)-5-
folic acid (447.2/295.2 m/z). Unlabelled 5-methyltetrahydrofolate (460.2/313.2 m/z) concentrations were extrapolated using the $^{13}$C$_5$-5-methyltetrahydrofolate curve.

**Determination of urinary excretion of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate**

The amount of daily $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate excreted into urine was calculated by multiplying the concentration of $^{13}$C$_5$-5-methyltetrahydrofolate or $^{13}$C$_5$-5-formyltetrahydrofolate by the total volume of urine excreted of the day. Recovery of isotopically labelled folates (% dose recovered) was calculated by dividing the quantity (ng) of either $^{13}$C$_5$-5-methyltetrahydrofolate or $^{13}$C$_5$-5-formyltetrahydrofolate excreted in urine by the administered dose of $^{13}$C$_5$-5-formyltetrahydrofolate multiplied by 100. The UER was expressed as % oral $^{12}$C isotope dose excreted/% IV $^{13}$C isotope dose excreted.

**Statistical methods**

SAS for Windows (Version 9.2; SAS Institute Inc, Cary, NC) was used to generate descriptive statistics (e.g., mean, SE). Changes in the unlabelled and labelled folate concentration in urine, and the percent recovery of the labelled test dose of folate in urine over time was analyzed by repeated measures analysis of variance (ANOVA) (PROC MIXED) with sample as the main effect and quadratic sample or cubic sample as necessary. Correlations between plasma and urine parameters were conducted using Pearson’s correlation.
6.4 Results

Subject characteristics

Subject characteristics of the study population have previously been reported (35). In brief, nine subjects (3 female and 6 male subjects, 22-26 y) successfully completed both caplet and IV arms of the study. All subjects had normal folate, vitamin B\textsubscript{12} and pyridoxal-5-phosphate status. No subject included in the study was homozygous for the T allele of MTHFR C677T. Dietary folate intakes 14 hr after caplet ingestion and 4 hr after IV injection were 47.9 ± 8 and 12 ± 1.2 µg DFE, respectively.

Caplet transit and disintegration

As previously described (35), our fluoroscopic imaging analysis revealed that no disintegration was observed before the ileocecal junction for all caplets. Complete caplet disintegration in the colon was observed in 6 of 9 subjects although 8 subjects showed some initial caplet disintegration. However, plasma uptake of \textsuperscript{13}C\textsubscript{5}-methyltetrahydrofolate after caplet ingestion was not observed in 2 out of 9 subjects (n=1 with complete caplet disintegration; n=1 without caplet disintegration) and as such, these two subjects were removed from subsequent plasma and urine analyses.

Urinary total folate excretion

In the caplet arm, mean total urinary folate excretions were 8718 ± 757 (mean ± SE), 10589 ± 779, 8454 ± 1009 and 12214 ± 1143 ng for the pre-dose, 24, 48 and 72 hr post-dose 24-hour urine collections, respectively. Day to day differences in the total amount of folate excreted was statistically significant (p=0.04). In the IV arm, mean total urinary folate excretions were 10000
± 2566 (mean ± SE), 19521 ± 3102 and 17028 ± 6228 ng for pre-dose, 24 and 48 hr post-dose 24-hour urine collections, respectively. As in the caplet arm of the study, man differences in daily excretion were statistically significant.

**Urinary folate excretion of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate**

Among 7 subjects that showed plasma uptake of $^{13}$C$_5$-5-methyltetrahydrofolate post caplet ingestion, we did not detect either $^{13}$C$_5$-5-methyltetrahydrofolate nor $^{13}$C$_5$-5-formyltetrahydrofolate in the urine samples of 2 subjects. Urinary total $^{13}$C$_5$-5-methyltetrahydrofolate excretions for pre-dose, 24 and 48 and 72 hr post caplet ingestion were 0, 12.2 ± 7.1, 3.2 ± 1.1 and 3.0 ± 1.4 ng, respectively (Figure 9). The change in total amount of urine $^{13}$C$_5$-5-methyltetrahydrofolate over time was significant (p=0.0012). No $^{13}$C$_5$-5-formyltetrahydrofolate was detected in urine pre or post of caplet ingestion. There was also a significant change (p<0.0001) in urinary total $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate excretions in response to the IV injection of $^{13}$C$_5$-5-formyltetrahydrofolate (0, 106.0 ± 16.0 and 27.7 ± 8.6 ng for pre-dose, 24, 48 and 72 hr, respectively) (Figure 10). The mean urinary recovery of $^{13}$C$_5$-5-methyltetrahydrofolate was found to be 0.007 ± 0.003 % of the caplet dose over the 72 hr post-dose period (Figure 11 and Table 17). Over the 48 hr post-IV period, the mean urinary recovery of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate combined was 0.13 ± 0.02 % of the IV dose (Figure 12 and Table 17). The change in the urinary recovery of labelled folate dose was significant (p<0.0001) over time in both caplet (Figure 11) and IV (Figure 12) arms. The UER ranged from 0 to 0.16 with an average of 0.06 ± 0.02.
Table 17 Urinary folate excretion profile of study participants (n=7)

<table>
<thead>
<tr>
<th>Urinary excretion of $^{13}$C$_5$-5-methyltetrahydrofolate over 0-72 hr post caplet dose (ng)</th>
<th>Value$^1$</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary excretion of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate over 0-48 hr post IV dose (ng)</td>
<td>133.7 ± 20.0</td>
<td>(50-218)</td>
</tr>
<tr>
<td>% recovery of caplet dose over 72 hr</td>
<td>0.007 ± 0.003</td>
<td>(0-0.021)</td>
</tr>
<tr>
<td>% recovery of IV dose over 48hr</td>
<td>0.13 ± 0.02</td>
<td>(0.04-0.22)</td>
</tr>
<tr>
<td>Urinary excretion ratio (UER)$^2$</td>
<td>0.06 ± 0.02</td>
<td>(0-0.16)</td>
</tr>
</tbody>
</table>

$^1$Mean ± SE; $^2$ UER: % oral $^{13}$C isotope dose excreted/% IV $^{13}$C isotope dose excreted; IV: intravenous
Figure 9 Mean ± SE urinary excretion of $^{13}$C$\textsubscript{5}$-methyltetrahydrofolate in 24-hour urine collected prior to (pre-dose) and 24, 48 and 72 hr post administration of a caplet containing $^{13}$C$\textsubscript{5}$-5-formyltetrahydrofolate.

The change in mean urine $^{13}$C-5-methyltetrahydrofolate per 24 hr was significant ($p<0.0012$) over time. No $^{13}$C$\textsubscript{5}$-5-formyltetrahydrofolate was detected in urine in the caplet arm.
Figure 10 Mean ± SE urinary excretion of $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5-formyltetrahydrofolate in 24-hour urine collected prior to (pre-dose) and 24 and 48 hr post intravenous injection of $^{13}$C$_5$-5-formyltetrahydrofolate.

The change in mean urine $^{13}$C$_5$-5-methyltetrahydrofolate and $^{13}$C$_5$-5formyltetrahydrofolate per 24 hr was significant (p<0.0001) over time.
Figure 11 Mean ± SE percentage of the oral dose of $^{13}$C5-formyltetrahydrofolate (400µg) recovered in urine over pre-dose, 24, 48 and 72 hr post-dose periods in the caplet arm.

The change in mean percentage of the recovered dose was significant (p=0.0058) over time.
Figure 12 Mean ± SE percentage of the intravenous (IV) dose of $^{13}$C$_5$-5-formyltetrahydrofolate (100µg) recovered in urine over pre-dose, 24 and 48hr post-dose period in the IV arm.

The change in mean percentage of the recovered dose was significant (p<0.0001) over time.
6.5 Discussion

Our study was the first investigation using determination of labelled folate in urine to provide direct evidence that folate, delivered to the colon by enteric-coated caplets, is absorbed across human colon. This observation is in agreement with our previous work that used the same study population and measurement of labelled folate in plasma (35). The molar ratios (M+5 to M+0) for $^{13}$C$_5$-5-methyltetrahydrofolate in plasma previously reported was significantly correlated with the urinary $^{13}$C$_5$-5-methyltetrahydrofolate enrichment values obtained using our current analysis ($r=0.7$, $p<0.0001$). The colonic folate absorption observed in humans is also in agreement with earlier animal work where labelled folate was delivered to the cecum and colon and subsequent colonic folate absorption was confirmed by subsequent appearance of labelled folate in plasma, liver and kidneys (187, 189).

The mechanisms through which bacterially-produced folate is absorbed across the colon are not well understood. It has been, however, postulated that the mechanism may be similar to that of the small intestine. In the small intestine, polyglutamylated folate, naturally abundant in food, needs to be hydrolysed to the monoglутamyl form which can be then absorbed. Hydrolysis occurs in the brush border of the small intestines of humans and pigs by GCPII (155, 156). Subsequent to hydrolysis, monoglutamylated folate is absorbed across the apical side of the enterocyte. Folate absorption, at physiological concentrations, is thought to be facilitated primarily by PCFT (pH optimum 5.8) (156).

In the colon, folates are likely found in both mono- as well as polyglutamylated forms; our previous research suggests ~50% of the folates in the stools of infants and piglets are monoglutamylated (33). Folates secreted by bacteria into lumen of colon are monoglutamylated,
which is the form that is readily absorbed in the small intestine (256, 331). In addition, the lysis of prokaryotes and eukaryotic cells in the colon yield a source of polyglutamylated folate which likely needs to be hydrolyzed if it is to be absorbed. Both GCPII transcripts and GCPII activity have been reported in the colon, though at a low level (157, 332). Bacteria themselves contain low levels of folate conjugase (333). In the present study, we examined the uptake of pre-formed monoglutamylated folate (e.g. $^{13}$C$_5$-5-formyltetrahydrofolate). At present, it is unclear whether the polyglutamylated fraction of folate in the colon can be absorbed. The PCFT, responsible for absorption of monoglutamylated folate, is expressed in the colon (156); though it is uncertain whether the pH at the absorptive surface of the colon is sufficiently low to allow for appreciable PCFT activity (165).

In order to directly assess whether bacterially synthesized folates are absorbed across the colon, $^{13}$C$_6$-PABA, a precursor of bacterially synthesized folate, could be provided to human subjects in our specially designed enteric coated caplets and the appearance of $^{13}$C$_6$-folate could be assessed in the plasma or urine. Given biosynthesis of folate will likely take some time, blood sampling would not be feasible and hence our interest in assessing the suitability of measuring the uptake of $^{13}$C$_6$-folate in urine.

The urinary recovery of labelled folate was found to be 0 to 0.02 % during the 72 hr post dose period in the caplet arm as a result of colonic absorption. In previous investigations where folate bioavailability targeting absorption of dietary and supplemental folate in the small intestine was evaluated with single stable isotope dose administration, urinary recovery of labelled folate was found to range from 0.1 to 2 % over 36 to 48 hr post-dose (203, 204, 239). Colonic folate absorption reported herein yielded much lower urinary recovery compared to small intestinal
absorption of folate. Complete urinary recovery of isotopic folate is not expected because of fecal excretion, catabolism, uptake by other folate-requiring bacteria in the colon, and *in vivo* retention by the host (including the splanchnic bed and renal reabsorption).

Our current protocol did not include a saturation period where subjects are typically provided with unlabelled folic acid (≥1 mg folic acid/d) for ≥ 1 week prior to the administration of labelled folate to saturate tissue stores. In studies of dietary and supplemental folate absorption across the small intestine, when a single dose of labelled folate follows a pre-dose saturation period, urinary recovery of labelled folate ranged from 10-60% of the oral dose and 21-25% of the IV dose (209, 212, 215, 242). Further, the subject-to-subject variation in urinary excretion of the labelled folate was lower due to reduced hepatic and tissue retention of absorbed folate from the test dose. Therefore, the use of a pre-saturation protocol is thought to be quantitatively useful and improves the precision of the estimate of folate bioavailability—at least from the small intestine (204).

However, such an approach can also be questioned because it involves non-physiological intakes of folate with different *in vivo* kinetics compared to a non-saturation protocol (203, 208, 232). Specifically, high doses of folate result in high enterohepatic recirculation of folate leading to high biliary folate. It remains unclear whether this high biliary folate would have an impact on absorption of a labelled test dose of folate (204). Furthermore, in vitro and animal evidence suggests over-supplementation of folate may down regulate folate uptake in the small intestine (172, 173).

To calculate the absolute absorption of an oral dose in a context of a stable isotope protocol involving both oral and IV administration of a test dose, one can evaluate the UER. The use of UER to determine absolute absorption however needs to fulfill the assumption that handling of
oral and IV isotopes by the body are similar, in terms of the *in vivo* kinetics, metabolic disposition and excretion (203, 239). The mean UER (0.06 ± 0.02) reported in the current study appears to greatly depart from 1 indicating that oral isotope of $^{13}$C$_5$-5-formyltetrahydrofolate, absorbed in the colon has a very different kinetics pattern than that of the IV isotope dose. Previous investigations administering both oral and IV doses of labelled folic acid consistently reported a UER greater than 1, indicating urinary excretion of labelled folate derived from the oral labelled folic acid dose being larger compared to the IV labelled folic acid dose (204, 239). Since the IV dose is considered to be 100% bioavailable, this is theoretically impossible. To correct this, Finglas suggested the use of reduced labelled folate as test dose in dual-labelled stable isotope protocol (239) which was adopted by the current study. To the best of our knowledge, the current investigation is the first study that reported UER followed by IV administration of a labelled dose of reduced folate. Our results showed colonic folate bioavailability led to a much lower UER compared with previous studies that examined bioavailability of dietary and supplemental folate across the small intestine.

There are a number of limitations to this study. Firstly, we acknowledge the final sample size (n=7) was quite small and hence the results obtained from a larger sample size may be required before the findings can be applied to the general population. Secondly, labelled folate catabolites were not measured in this study. Buttner et al., showed that after a test dose of labelled folate was given, the amount of labelled folate catabolites recovered in the urine was 3 times higher than the amount of labelled folate that was recovered (334). Since the recovery of labelled urinary folate was low in our study, analysis of labelled folate catabolites in the urine would have greatly enhanced the sensitivity of the model.
In summary, labelled folate is detectable in urine as evident by an increase in urinary excretion of $^{13}$C$_5$-5-methyltetrahydrofolate as well as $^{13}$C$_5$-5-formyltetrahydrofolate following administration of an enteric coated caplet containing $^{13}$C$_5$-5-formyltetrahydrofolate directly delivered to the colon. Our current results are in agreement with our previous investigation where we demonstrated colonic folate absorption using plasma enrichment. Thus, the appearance of labelled folate in urine might be a suitable biomarker for future investigation of measuring the availability of folate biosynthesized in the colon. The enrichment of labelled folate in urine was however low and with high inter-subject variation at the current level of analytical sensitivity and the current single dose stable isotope administration protocol. The sensitivity of this model may be improved by pre-saturating the tissues of subjects by administration of unlabelled folic acid.
7. Thesis overall discussion, conclusion and future directions

7.1 Discussion and conclusion

Folate is available from different sources including naturally occurring folate in foods, and synthetic folic acid that can be obtained from fortified food products and supplements. Folate produced by bacteria residing in the colon comprises a significant depot of naturally occurring folate. The aim of this thesis was to investigate the contribution and manipulation of the “input side” of folate nutrition focusing on folic acid fortification and bacterial biosynthesis of folate to folate uptake.

Firstly, in this thesis, I examined how extending folic acid fortification to whole wheat flour in Canada might affect the prevalence of folate inadequacy in the population and the percent of individuals with intakes of synthetic folic acid above the UL. Our modelling exercise predicted fortifying whole wheat would be unlikely to have an impact on either the prevalence of folate inadequacy or on the percentage of individuals with folic acid intakes above the UL in the general population. However, fortifying whole wheat would benefit whole wheat consumers by reducing the prevalence of folate inadequacy while not putting whole wheat consumers at additional risks.

Secondly, I examined how manipulating the provision of fibre in the diet may impact the quantity of bacterially synthesized folate produced in the colon. My results showed that increasing the consumption of dietary fibre increased colonic folate content in a sample of African Americans. The change in colonic folate content is likely due to, at least in part, the change in folate production in the colon in response to the increase in dietary fibre consumption.
The final aim in my thesis was to investigate the feasibility of using a non-invasive means of assessing the bacterial contribution of folate produced in the colon through the measurement of stable isotopes of folate in urine. My results showed that labelled folate was detectable in the urine post both oral and IV doses of $^{13}$C$_5$-5-formyltetrahydrofolate. The molar ratios (labelled vs unlabelled) of $^{13}$C$_5$-5-methyltetrahydrofolate in urine and plasma were correlated. It was therefore feasible to measure colonic folate absorption by measuring urinary excretion of labelled folate. Measurement of urinary enrichment could be a suitable biomarker in addition to plasma kinetics, when investigating colonic bacterially-synthesized folate absorption in future studies.

While the mandatory folic acid fortification of white wheat flour has led to a 50% reduction in the prevalence of NTDs in Canada, fortifying whole wheat flour is not currently permitted (4, 24). In Canada’s Food Guide, due to the contribution of magnesium, fibre and well-known health benefits of whole grains (25, 26, 28, 265, 283-289), all Canadians are encouraged to consume at least 50% of grain products as whole (29). The rationale for this recommendation at 50% versus higher was that only white wheat flour is fortified with folic acid (290). It is clear that the current folic acid fortification policy in Canada is in conflict with the portfolio of evidence favoring an increased consumption of whole grains. In this thesis, a modelling exercise was conducted to evaluate the impact of adding folic acid to whole wheat flour on folate intake distribution in the population. We found that in the general population, regardless of supplement use, folic acid fortification of whole wheat flour-containing foods would not change the prevalence of folate inadequacy or percentage of intakes above the UL. Among adult whole wheat consumers, prevalence of folate inadequacy would be reduced by 10 percentage points after folic acid fortification of whole wheat flour-containing foods was modelled. The percentage
of whole wheat flour consumers with intakes above the UL would not change. We concluded that the prevalence of folate inadequacy or proportion of folic acid intakes above the UL in the general Canadian population is unlikely to be affected by fortifying whole wheat flour-containing foods with folic acid; however, the prevalence of folate inadequacy in adult whole wheat consumers may be reduced.

Fortification is an important contributor to the “input side” of folate nutrition. My thesis demonstrates an example of how the factor of fortification could be manipulated and how the folate intake distribution could be affected at a national level. In considering a fortification strategy, it is important to consider who would be the targeted population. Folic acid fortification of the food supply was made mandatory in Canada to reduce the prevalence of folate-dependent NTDs. Thus, the target population was women of reproductive age. In the case of Study I, whole wheat consumers would be the target population that might benefit from an extension of the current mandatory folic acid fortification policy to whole wheat flour. I chose to define whole wheat consumers as individuals who reported to have consumed a whole wheat food item at least once (regardless of the amount) in their 24 hr food recalls within the CCHS 2.2 (2004) dataset. This approach allowed me to capture almost all individuals who consumed whole wheat containing food items in the current cohort. The additional folic acid that whole wheat consumers would consume under the theoretical fortification of whole wheat flour was calculated using data on the actual amount of whole wheat product consumed by the individual. Canada’s Food Guide recommends the consumption of at least 3-4 servings of whole grains for adults daily. However, 3-4 servings of whole grains was not used as a cut-off to select whole wheat consumers because the resulting subgroup would only represent a small group of the population. Whole grain consumption has been found to be low in the US (335-337). Results
from the USDA’s Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996 showed that 70% of American adults consumed some whole grain and only 7% of the US population met their whole grains requirement, i.e., three servings or more of the whole grains per day (335). The CSFII survey also showed that American adults consumed an average of one serving of whole grain per day (335). More recently, using data from the NHANES 1999 to 2004 showed that the percentages of subgroups that consumed more than three servings of whole grains per day are as follows: children/adolescents, adults aged 19 to 50 yr and adults aged > 51 yr were 1.5 - 4.3, 4.8 and 6.6%, respectively (336, 337). While equivalent Canadian data is not available, my present analysis showed that only 38% of Canadian adults were whole wheat consumers (who reported to have consumed some whole wheat). Among non-supplement users, 36% were whole wheat consumers whereas among supplement users, 44% were whole wheat consumers. Since whole wheat consumption rate was already low in the current cohort, if a higher cut-off of serving size of whole grain consumption was used, the population of whole wheat consumers would be even smaller and the modelled prevalence of folate inadequacy as well as percentage of intake level above the UL would not have represented the population. My analysis is also in accordance with previous investigations where it has been consistently shown that folic acid fortification of foods does not lead to the intake level above the UL. In contrast, folic acid supplement use is the driving force behind excessive intakes (61, 111, 112). Health Canada has been considering allowing the addition of folic acid to the food supply, using whole wheat flour as the selected food vehicle. My analysis confirms that whole wheat consumers would benefit from fortification of whole wheat flour with folic acid while the entire Canadian population would not be exposed to additional risk of receiving too much folic acid. Therefore, fortifying whole wheat flour with folic acid would be an acceptable public health initiative. In addition, if
whole wheat flour was permitted to be fortified with folic acid, the dietary recommendation of at least 50% of grain consumption as whole grains may be revised to 100%. Since whole grain intake is currently low in North America, a public health message on promoting whole grain consumption might be useful and might help achieve the recommended fibre intake in the population.

In fact, fibre intake may have an impact on colonic bacterially-synthesized folate, which is another important factor contributing to the “input side” of folate nutrition. Since the amount of folate found in the colon well exceeds daily folate requirements of adults, and a significant fraction of colonic folate appear to be in the readily absorbable monoglutamyl form, folate produced by gut microbiota in the colon may provide a complementary source of naturally occurring folate (33, 39). Therefore, it is important to understand the bioavailability of bacterially synthesized folate from the colon and investigate ways to manipulate the folate production of colonic origin. In Study II, we investigated whether manipulating the level of dietary fibre in diet would have an impact on colonic folate contents.

In Study II, colonic fluid samples from a group of African Americans (who normally consume a low fibre Western diet), and a group of rural South Africans (who normally consume a high fibre diet) were studied at baseline and after a 2-week reciprocal dietary switch (dietary intervention). During the intervention, rural South African participants were provided with a typical African American diet (protein 27%, fat 52%, carbohydrate 20% and fibre 8g/d). In addition, during the intervention, African American participants were provided with a typical rural South African diet (protein 16%, fat 18%, carbohydrate 63% and fibre 43g/d). My results showed that compared to their baseline diet, African Americans received 28g more fibre during the invention which was
associated with a 53% increase in colonic folate concentrations relative to their usual baseline diet. During the intervention, African Americans also received 35 g more dietary fibre compared to rural South Africans. Colonic evacuants obtained post-intervention demonstrated this increase in fibre intake was associated with 76% higher colonic folate concentrations in African Americans compared to rural South Africans. At baseline, when the differences in fibre intake was smaller (13g), colonic folate content appeared to be similar between rural South Africans and African Americans, consistent with our previous findings (39). The anticipated decrease in colonic folate content in rural South Africans after the intervention where fibre intake was reduced was not observed. It should be noted that the fibre intake was only lowered by 20g compared to the baseline in rural South Africans. Our study provides the first direct evidence that colonic folate contents can be manipulated by modifying fibre intake in the diet, likely through the regulation of folate production capacity of colonic bacteria. Our findings further suggest the amount of fibre that is required to achieve a net increase in colonic folate content might be an important point to consider when planning a diet that aims to promote colonic bacterial folate production. According to the IOM, the Adequate Intake for fibre is 38g/day and 25g/day for male and female, respectively (338). In Study II, African Americans during the intervention received a diet containing ~45g of fibre/day resulting in favorable change in colonic folate contents. To ensure host’s utilization of folate of colonic origin, we needed to further investigate if bacterially synthesized folate can be absorbed in the colon.

It has been previously confirmed that pre-formed folate can be absorbed across the human colon (34, 35). Our group, using data on the colonic folate absorption rate (calculated using pre-formed folate), together with the total folate content of the colon estimated 322-396 µg of folate could be being potentially absorbed daily across the colon (35). Of note, the RDA of folate for
adults is 400 µg DFE (1). However, it has yet to be demonstrated whether folate produced by the colonic bacteria can be absorbed. To confirm this, our group conducted a pilot study where subjects were provided with a single dose of labelled PABA, the precursor of folate biosynthesis, using a caplet that is designed to disintegrate in the colon. Our preliminary analysis did not detect the appearance of newly-synthesized folate in plasma up to 72 hr post-dose. We hypothesized that multiple dosing of the precursor as well as serial sample collections over an extended period of time would likely be required to achieve steady-state and obtain a detectable response in the site where measurement is carried out. Therefore, a less invasive sampling approach would be required. In this context, measurement of labelled folate in urine might be a feasible alternative to the choice of plasma where taking multiple blood draws is known to be associated with both some risks and some practical limitations.

In Study III, we used the urine samples collected from a previous study where we demonstrated folate is absorbed across the human colon using plasma kinetics (35). Our analyses show that changes in total urinary $^{13}\text{C}$-5-methyltetrahydrofolate and $^{13}\text{C}$-5-formyltetrahydrofolate in response to both caplet and IV test doses were observed. The UER (% oral $^{13}\text{C}$ isotope dose excreted/% IV $^{13}\text{C}$ isotope dose excreted) ranged from 0 to 0.16 with an average of 0.06 ± 0.02. Therefore, we confirmed that labelled folate is detectable in urine as a result of colonic absorption and the current analyses is in agreement with our previous work showing colonic folate absorption using plasma (34, 35). The molar ratios of $^{13}\text{C}$-5-methyltetrahydrofolate in urine and plasma were correlated suggesting the appearance of labelled folate in urine and plasma followed a similar trend. However, labelled folate enrichment in urine as well as the UER is low and had a high subject-to-subject variation. Therefore, at the current level of analytical sensitivity and isotope administration regimen, the use of urine enrichment may serve
as a confirmatory measure for colonic folate bioavailability that would be better interpreted in conjunction with folate kinetics in plasma. To develop a less invasive protocol is always important in the area of stable isotope research because a less invasive protocol would allow kinetics work to be extended to more vulnerable groups, such as pregnant women and the pediatric population (339). A more practical approach, i.e. urine sample collection, could be more commonly employed, as it costs much less and would not require specialized medical facilities. In the context of studying colonic folate absorption, urine sampling has the potential to fulfill the aforementioned general advantages and more importantly, provides a useful tool to study steady-state folate kinetics that would otherwise require multiple blood samples. Study II and III collectively provide evidence for the contribution of bacterially-synthesized folate to the “input side” of folate nutrition. Study III provides an important tool to study the contribution of folate production in the colon. If folate synthesized by gut microbiota is bioavailable, folate of colonic bacterial origin could serve as a complementary source of natural occurring folate. It would be useful to evaluate how colonic bacterial origin could potentially be a useful folate source contributing to one’s folate requirement. Results obtained from Study II support the idea that production of colonic folate could be manipulated by dietary approaches and further elucidate one of the factors affecting the “input side” of folate nutrition.

In summary, the major findings and significance of the thesis can be illustrated using Figure 13.
Figure 13 Overall summary and significance of the thesis.

My thesis provides a framework to demonstrate how the “input side” of folate nutrition has an impact on the development of dietary recommendations that achieve folate requirements but are associated with a low risk. Extending folic acid fortification to include whole wheat flour would be a useful public health strategy to deliver folic acid while promoting whole wheat/grains consumption but would not expose the entire population at additional risk. A total of 45g of fibre/day may help optimize the colonic folate contents which may serve as a complementary pool that could be used to achieve one’s daily folate requirement through colonic absorption. Folate colonic absorption is still not well understood and requires further characterization. The use of urine sample provides a useful tool for on-going investigations on bacterially-synthesized folate bioavailability which will further refine dietary recommendations related to folate nutrition.
7.2 Future directions

In Study I, we showed extending folic acid fortification to whole wheat flour would not likely change the prevalence of folate inadequacy or the percentage of intake levels above the UL in the general Canadian population. However, whole wheat consumers would benefit from fortification of whole wheat flour. Therefore, based on the analysis of Study 1 which addressed the risks and benefits of adding folic acid to whole wheat flour, my study results support the fortification of whole wheat flour in addition to the current fortification strategy. It should be noted however, that this modelling exercise was conducted using the CCHS 2.2 of which the dietary data was collected in 2004. Since the new Canada Food Guide promoting whole grain consumption was released in 2005, we anticipate that current whole grain consumption has increased in Canada. In the U.S, the whole grain consumption has increased according to the analysis of NHANES 2009-2010 compared to NHANES 1999-2004 (336, 337, 340) and this trend is believed to be the same in Canada. Therefore, it would be prudent to repeat our modelling exercise using a database reflecting more current dietary intakes of Canadians. In fact, a repeat of the nutrition module of CCHS is currently underway from data collection in 2015. We anticipate the impact of extending folic acid fortification to whole wheat on the folate intake distribution of whole wheat consumers will be greater in modelling data from the new CCHS if whole grain consumption has increased as most believe it has. We also believe it would be useful to better understand socio-demographic, nutritional status and health characteristics of whole wheat consumers. A better understanding of the targeted population is important when implementing or extending a fortification policy. In particular, while folate deficiency is virtually non-existent in Canada, about 20% of females of childbearing age have a folate status under the cut-off that is thought to be protective against NTDs (145). Using the NHANES 2003-2006 dataset, our group
previously found women who smoked, did not consume a folic acid containing supplement, non-Hispanic black women, and women of “other” races (non-Hispanic, non-black, non-white) were more likely to have sub-optimal RBC folate status (data not published). In Canada, a report using the Canadian Health Measure Survey has stated that being younger, and having a lower income and lower education level appear to be the risk factors of having sub-optimal folate status (<906 nmol/L) among non-supplement users (305). Therefore, it would be useful to further clarify whether fortifying whole wheat flour with folic acid could reach that fraction of the population that is at risk for having sub-optimal folate status. Lastly, the current modelling exercise only focuses on fortification of whole wheat flour as whole wheat flour-containing foods represent 70% of whole grain consumption (data not reported). It would be useful to evaluate the impact on folate intake if folic acid fortification was to be extended to other non-white flours, including oats, barleys and ryes.

One of my thesis goals was to establish an experiment model that is practical for future investigations on the absorption of bacterially-synthesized folate of the colon origin. The use of urine samples might be a useful alternative to plasma samples which would become impractical when multiple oral doses of labelled precursor might be needed for folate biosynthesis in the colon. In that case, collection of biospecimens for an extended period of time would be required to capture the metabolic processes. In fact, it has been suggested that administration of multiple oral labelled test doses would lead to a more stable response in the measurement site and is a more sensitive model to study folate kinetics (208). In our current analysis, while urinary excretion of labelled folate was detectable, enrichment was low and showed with high subject-to-subject variation. In previous studies when folate enrichment in urine was analyzed to determine the bioavailability of dietary or supplemental folate across the small intestine, a pre-saturation
period where subjects consume ≥1000 µg/day of folic acid prior to experimentation to saturate tissues was employed. The common criticism for this approach is that this experimental design does not reflect normal physiological conditions. However, when we tried to quantify colonic folate absorption, we observed that the % recovery of oral dose and % recovery of IV dose as well as the UER in our study is 10 to 100 times lower than the values reported in the literature when folate absorption in the small intestine was measured and when subjects also did not undergo a pre-saturation period (203, 204, 239). Therefore, pre-loading folate to standardize liver and tissue stores may be even more beneficial for the purpose of colonic folate absorption measurement. Therefore, the next step could be to develop a protocol that involves inclusion of a pre-saturation period followed by multiple doses of labelled precursors, delivered to the colon for production of colonic folate which could be measured using the appearance of labelled folate in both plasma and urine collected over a period of time.

We showed colonic folate contents could be manipulated by the change in fibre level in the diet. Nevertheless, it remains unknown how the shift in gut microbiota composition is related to folate contents in the colon in response to dietary changes. Gut microbiota composition has been analyzed and the analysis of genomes of the gut microbiota is also available for this study population (270, 316). Since the capacity of folate production appears to be strain specific, the next step would perhaps be to analyze the change in gut microbiota composition and correlate the colonic folate content with the gut microbiota composition. In addition, the intervention adopted in Study II was only 14 days. Recent evidence does confirm changes in dietary composition in regards to carbohydrate and fibre can detectably alter gut microbiome within a day (328, 329). However, it should be noted that an earlier controlled-feeding study suggested the increase in Bifidobacteria counts in response to fibre intake returned to baseline after 15 weeks of feeding.
Therefore, long-term effect of increase carbohydrate/fibre intake on the regulation of colonic folate production needs to be further studied.

In addition, information on the folate status of the study participants pre and post dietary intervention were not available for Study II. Therefore, whether the change in colonic folate contents contributes to changes in folate status could not be determined in Study II. Plasma and RBC folate concentration in response to long term dietary intervention has been used as a measure to determine folate bioavailability. In future studies measuring blood concentrations of folate would assist in understanding the actual impact of changes in the colonic folate content on the host. In a future study, ideally a randomized control trial, subjects should be randomized to different levels of fibre and plasma and RBC folate concentrations could be determined to estimate the bioavailability of folate produced by gut microbiota. Differences in colonic gut microbiota composition and colonic folate content in response to fibre would be assessed. This approach is similar to previous research by our group using a rat model where feeding human milk solids (containing oligosaccharides) was found to increase Bifidobacteria counts in the colon and cecum, total cecal folate as well as to be associated with increased folate status in rats (262). Also, the proposed study might become an alternative to the stable isotope protocol to determine the bioavailability of colonic bacterially-synthesized folate as plasma and RBC folate concentrations responding to fibre intake would indicate whether folate produced by gut microbiota is absorbed and hence is bioavailable (34, 35).
8. References


12. Stevens VL, McCullough ML, Sun J, Jacobs EJ, Campbell PT, Gapstur SM. High levels of folate from supplements and fortification are not associated with increased risk of colorectal cancer. Gastroenterology 2011;141(1):98-105, e1.


60. Bailey RL, Mills JL, Yetley EA, Gahche JJ, Pfeiffer CM, Dwyer JT, Dodd KW, Sembros CT, Betz JM, Picciano MF. Unmetabolized serum folic acid and its relation to folic acid


82. Hibbard BM. The role of folic acid in pregnancy; with particular reference to anaemia, abruption and abortion The Journal of obstetrics and gynaecology of the British Commonwealth 1964;71:529-42.
83. Hibbard BM, Hibbard ED, Jeffcoate TN. Folic acid and reproduction. Acta obstetricia et 
gynecologica Scandinavica 1965;44(3):375-400.

84. Mulinare J, Cordero JF, Erickson JD, Berry RJ. Periconceptional use of multivitamins 

85. Bower C, Stanley FJ. Dietary folate as a risk factor for neural-tube defects: evidence from 
a case-control study in Western Australia. The Medical journal of Australia 

86. Milunsky A, Jick H, Jick SS, Bruell CL, MacLaughlin DS, Rothman KJ, Willett W. 
Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of 

87. Werler MM, Shapiro S, Mitchell AA. Periconceptional folic acid exposure and risk of 

88. Shaw GM, Schaffer D, Velie EM, Morland K, Harris JA. Periconceptional vitamin use, 
dietary folate, and the occurrence of neural tube defects. Epidemiology (Cambridge, 


90. Recommendations for the use of folic acid to reduce the number of cases of spina bifida 
and other neural tube defects. MMWR Recommendations and reports : Morbidity and 
mortality weekly report Recommendations and reports / Centers for Disease Control 

91. Society of Obstetrics and Gynaecologists of Canada Genetics Committee. 
Recommendations on the use of folic acid for the prevention of neural tube defects: 

92. Henshaw SK. Unintended pregnancy in the United States. Family planning perspectives 

93. Food and Drug Administration. Food standards: amendment of standards of identity for 
enriched grain products to require addition of folic acid. Federal Register 1996;61:8781- 
97.

94. Allen L, de Benoist B, Dary O, Hurrell R. Guidelines on food fortification with 
micronutrients. The World Health Organization, Food and Agriculture Organization of the 
United Nations; 2006. Available from: 

95. Food and Nutrition Board. Dietary Reference Intakes: Guiding Principles for Nutrition 


98. Canadian Public Health Association. 


175. Jing M, Tactacan GB, Rodriguez-Lecompte JC, Kroeker A, House JD. Proton-coupled folate transporter (PCFT): molecular cloning, tissue expression patterns and the effects of


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251. Metges CC. Contribution of microbial amino acids to amino acid homeostasis of the host. The Journal of nutrition 2000;130(7):1857S-64S.


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