Total Folate and Synthetic Folic Acid Content in the Food Supply and Its Influence on Absorption Across the Colon

by

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Abstract

To reduce the risk of neural tube defect-affected pregnancies in Canada, folic acid fortification of foods is mandated, and women planning a pregnancy are advised to consume a folic acid-containing supplement. This research aims to determine the amount of folic acid in fortified foods and understand the impact of folic acid on folate absorption in the colon. In study one, analysis of fortified foods by microbial analysis and mass spectroscopy showed 65% higher folate values than in the Canadian Nutrient File. In study two, an on-going randomized clinical trial, we seek to investigate the influence of folic acid on folate absorption in the colon. Data herein verify the feasibility of the protocol and that the feeding and supplement intervention produced two distinct groups in terms of blood folate status. This work will facilitate an improved understanding of available sources of folate to inform future folate supplementation and fortification recommendations.

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TABLE OF CONTENTS

List of Tables

List of Figures

List of Appendices

List of Abbreviations

BMI	Body Mass Index
CCHS	Canadian Community Health Survey
DFE	Dietary Folate Equivalents
DHF	Dihydrofolic
DHFR	Dihydrofolate reductase
DV	Percent Daily Value
EAR	Estimated Average Requirement
FDA	Food and Drug Administration
LC/MS-MS	Liquid Chromatography Mass Spectrometry
NHANES	National Health and Nutrition Examination Survey
NTD	Neural Tube Defect
RBC	Red Blood Cell
RDA	Recommended Daily Allowance
THF	Tetrahydrofolic
USDA	United States Department of Agriculture

1.0 Introduction

The vitamin folate is a generic descriptor for a family of Vitamin B9 compounds, including folic acid, which all share a pteroylglutamic acid core.[1] Folate acts as a co-factor for one carbon metabolism and is primarily responsible for DNA and RNA biosynthesis and repair, as well as DNA methylation to convert homocysteine to methionine necessary for protein synthesis. Methionine can also be converted to S-adenosylmethionine (SAM) which is involved in numerous methylation reactions in the body. It cannot be synthesized by mammals so it must come from diet and dietary supplements or generated from bacterial species present in the colon.[1,2] Folate is naturally found in dark green leafy vegetables, legumes, citrus fruit juices. As of 1998 Health Canada opted for mandatory fortification of all enriched flour and cereal grain products with folic acid. Canadian guidelines have stated that all white wheat flour be fortified with 140-150 micrograms/100 g of folic acid and pastas with 200 micrograms/100 g folic acid. A study done by De Wals and colleagues demonstrated an up to 50% reduction in neural tube defects upon the implementation of this guideline.[3]

Folate requirements are highest during the anabolic stage of the lifecycle including pregnancy, lactation, early fetal and post-natal life.[4] The Nutrition Canada Survey conducted in 1970 indicated the risk of folate deficiency across all age groups, however a higher risk of folate deficiency was seen in pregnant women, females 20-39 and males over 65.

More recently, in the national representative Canadian Health Measures Survey using data from 5604 Canadians aged 6-79 years old, reported a dramatic shift in the folate over the last 50 years. Specifically classic folate deficiency as assessed by a determined a folate level of 305 nmol/L or less, was shown to be virtually non-existent.[5] Further, a significant proportion of participants had blood folate values which were very high (e.g. non-physiological).

The totality of evidence in the research literature is that there is a U-shaped relationship between the intake of folate and risks. In order to establish an optimal dietary recommendation, we need to understand the input side of folate nutrition. It is imperative to determine the current folate intake of Canadians, which includes the amount of folate present in the foods we consume. A study by Shakur et al. from our research group 10 years ago assessed the folate content in Canadian fortified foods post mandatory fortification. Ninety-five of the top consumed Canadian fortified foods across 7 different categories were analyzed.[6] Total folate concentrations were calculated using the tri-enzyme digestion method followed by a microbiological assay. The analyzed folate content of the foods was found to be on average approximately 50% higher than what was mandated and were not reflective of the values listed in the Canadian Nutrient File or on food labels.[6] To the best of our knowledge there have been no direct reports of the actual folic acid concentration of foods since these analyses 10 years ago.

Traditionally, dietary and supplemental sources of folate were assumed to be the only contributors to the input-side of folate nutrition, however it has recently been demonstrated that folate absorption and metabolism in the colon, specifically microbial folate biosynthesis may play a role in meeting the folate requirements in humans. The current Dietary Reference Intakes for folate do not consider the potential contribution of microbially synthesized folate.[17]Despite the presence of high concentrations of folate produced by the intestinal microbiota, the current recommendations are based solely upon oral intake of both folate and folic acid fortified foods and supplements.[8] The size of the colonic folate depot has been studied and found to exceed RDA's in quantity thereby suggesting it is a source of the vitamin that may influence whole body folate status. Studies previously done on colonocytes in vitro, and by our group and others with rats, and humans suggest folate absorption across the colon.[9–16] The rationale of this study was to

further understand the factors contributing to the input side of folate nutrition and the fundamental processes involved in folate absorption and transport in the colon of humans.

The first objective of this study was to analyze the top consumed Canadian fortified foods for their total folate and folic acid content and compare it against reported Canadian Nutrient File and label values. 89 Canadian fortified foods were analyzed for their total folate and synthetic folic acid content. These analyzed values were compared to values in the Canadian Nutrient File and Folate values listed on food labels. The findings reported in chapter three of this thesis allow us to consider all input sources of folate when determining estimated requirements of folate in the diet, and can help establish new guidelines for optimal intake.

The second objective of this study was to assess how folic acid supplementation influences colonic folate absorption and metabolism in healthy adults. This will be accomplished by an on-going clinical trial that aims to assess the expression of two major folate transporters hypothesized to be responsible for folate absorption in the colon of healthy adults. All participants received an adult multivitamin and were then randomized to receive a supplement containing either 0 or 400 μg folic acid over the course of 16 weeks. Within this study, the primary objective is to evaluate the impact of 0 and 400 μg supplemental folic acid on total folate concentrations within the colonic mucosa by measuring folate levels. The secondary objectives include evaluating the impact of 0 and 400 μg supplemental folic acid on the regulation of PCFT and RFC in the ileum and colon by assessing the expression of mRNA transcripts and proteins, and determining the degree to which GCPII and GGH are present in the lumen of the ileum and colon by quantifying enzyme activity and expression of mRNA transcripts and proteins. This work will lead to the beginning of a deeper understanding of colonic folate absorption and metabolism, resulting in more appropriate dietary and supplemental

folate recommendations.

For the purpose of this thesis, we sought to focus on the feasibility of the protocol proposed, and whether the feeding and supplement interventions were successfully able to produce two distinct groups in regards to blood folate status. The findings reported in chapter four of this thesis were obtained from eight healthy adults. Baseline and 8-week RBC and plasma folate values are provided, as well as folate intake using the ASA24 dietary assessments to gather information on the current folate status of participants within the study.

2.0 Review of the Literature

2.1 Folate Structure and Function

The origins of folate date back to 1930 when a cure for "tropical macrocytic anemia" particularly in pregnant women was sought after. During her trip to India, Lucy Wills discovered marmite, a form of yeast extract to be an effective treatment for the disease as an anti-anemic factor.[17] Shortly thereafter Mitchell and colleagues isolated the active component of this yeast extract from spinach which is now known as folate, one of the water-soluble vitamins part of the B complex.[18]

The term folate is a generic descriptor for a family of vitamin B_9 compounds, including synthetic folic acid that share a pteroylglutamic acid core. Due to the variation in the number of glutamate residues, carbon substitutions, and reduction state of the pteridine ring, there are different forms of the coenzyme.[19] Pteroylglutamic acid, commonly referred to as folic acid, is the most stable and oxidized form of the vitamin, and used in food fortification along with vitamin supplements. The pteroylglutamic acid core structure is composed of para-amino benzoic acid (PABA) which is connected to a pteridine ring by way of a methylene bridge forming pteroic acid (Figure 1). Further in the core structure the amino group of L-glutamic acid is joined to the carboxyl group of PABA via a peptide bond. Folic acid was first synthesized into its oxidized form in 1945 where it was determined to have a molecular weight of 441.4 g/mol.[20]

The primary biological role of folate is the transfer of one-carbon units.[1] Folate acts as a co-factor in nucleotide synthesis, therefore it is necessary for both DNA and RNA biosynthesis and repair. It also aids in the re-methylation of homocysteine to produce methionine, an amino acid essential in protein synthesis. Methionine can further be converted to S-adenosylmethionine

(SAM) which is involved in numerous methylation reactions in the body, including epigenetic programming early in life and biosynthesis of several hormones and neurotransmitters including epinephrine.[21,22]

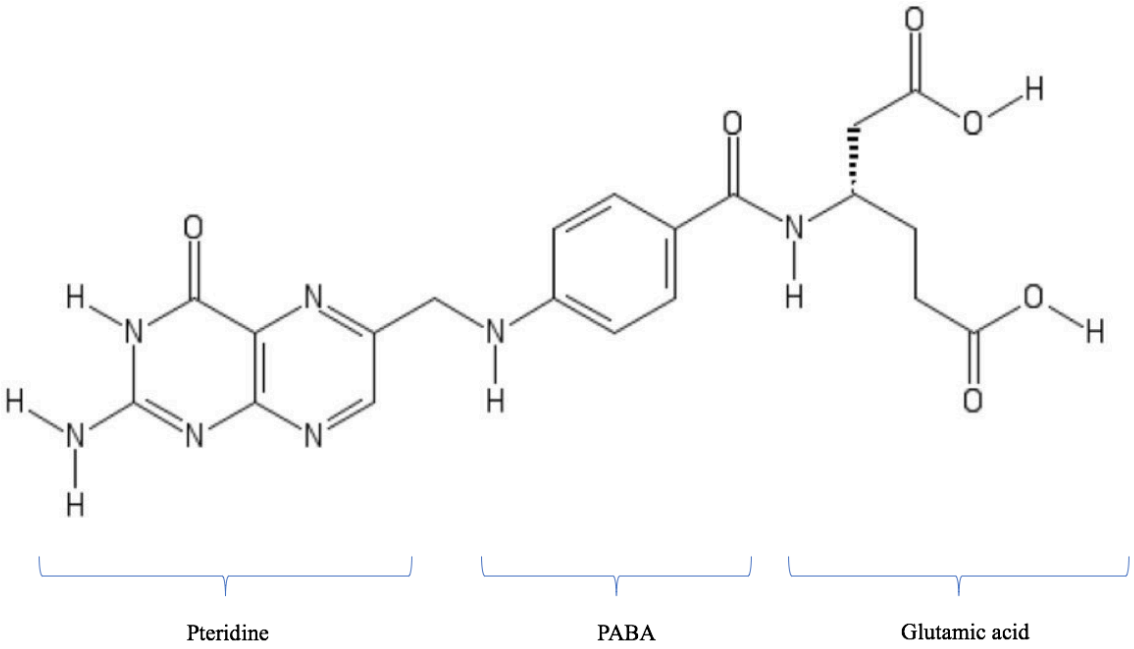


Figure 2.1.1 The structure of folate

2.2 Dietary sources of Folate

Mammals lack the expression of dihydropteroate synthase, an enzyme required to couple PABA to the pteridine ring and are therefore unable to synthesize folate de novo.[23] An exception to this is folate synthesized via the intestinal microbiota which can be absorbed across the colon and integrated into tissue.[24] Due to this, mammals must obtain the majority of their folate from dietary and supplemental sources.

2.2.1 Natural sources

In most naturally occurring foods, folate is usually found in its reduced polyglutamate form.[1] Natural folates tend to be easily oxidized in heat, light, pressure or under acidic conditions due to their unstable nature. This instability can cause loss of folate activity in foods over the course of days and weeks, as well as loss of folate content during handling and processing.[25] Losses of folate totaling almost 50-75% can occur during food harvesting, processing, preparation and storage causing preparation methods to be major determinants of folate bioavailability. Some methods of processing may lead to the loss of folate via diffusion into processing water. For example, there have been high losses of folate reported in frozen citrus juices compared to their unprocessed counterparts.[26] In general, it is understood that the bioavailability of natural folate is approximately 50% that of supplemental folic acid consumed on an empty stomach.[1] This may be a result of several factors including incomplete release from cellular food matrices and degradation of natural forms while in the gastrointestinal tract.[27] The major forms of folate in food include 5-methyltetrahydrofolate (5-MTHF) and 10-formyltetrahydrofolate (10-formylTHF). Natural sources of folate include dark green leafy vegetables such as spinach and broccoli, asparagus, citrus fruit juices such as orange juice, nuts, legumes and can vary by food item.[28] High amounts of folate are found in liver, organ meats, and yeast. Table 2.2.1 displays the folate content of fresh cut vegetable products obtained using microbiological assay, in comparison to their USDA reference standard.

Table 2.2.1 Folate content in fresh-cut vegetable packed products

Food Product	Folate content (ug/100g)	Reference Data- USDA (ug/100g)
Spinach	141±21	194
Broccoli	70±12	63
Pineapple	11±1	18
Coconut	10±4	26
Carrot	19±4	19
Potato	12±6	30
Iceberg Lettuce	35±10	29
Corn on the cob	15±4	42

Adapted from Fajardo et al. (2015) [29]

2.2.2 Synthetic sources

Unlike natural folate, folic acid in the fully oxidized monoglutamate form and is thus highly stable. Folic acid has a higher bioavailability than natural folate, approaching almost 100% when consumed as a supplement on an empty stomach, and 85% when consumed with food; of note studies regarding availability of folic acid preceded high intakes since mandatory folic acid fortification of the food supply.[27] This higher bioavailability may be due to the fact that folic acid does not have to break away from cellular food matrices, it comes in a monoglutamated form and is less prone to degradation whilst in the gastrointestinal tract. In Canada and the US, synthetic folic acid is added as a fortificant to white wheat flour and as such it may be found in a number of foods containing white wheat flour including breads, pastas, cereals, oatmeal, granola bars, rolls and crackers. Further additional folic acid can be added to food products labelled enriched

such as enriched pasta. Over-fortification of foods is often used as a means to ensure the folate content in the product remains above that claimed on the nutrition label at the end of its shelf-life; however, it isn't uncommon for fortification overages to be almost 2-fold as much as needed.[27,30] Folic acid is commonly used alone or as a multivitamin supplement among North Americans, with up to 40% of the population reporting consuming a folic acid supplement.[31] The Percent Daily Value (DV) is used on food labels in Canada and was developed to aid consumers in determining if a serving of food is high or low in a certain nutrient. As of 2016, the current DV for folate used on food labels in Canada is transition to 400 μg per serving, compared to the previous 220 μg. Food labels are required to list the folate content of a product if it has been fortified with the nutrient. Foods containing nutrients that are 20% or more of the DV are considered to be sources high in those nutrients.[32]

Table 2.2.2 Selected food sources of folate and folic Acid

Food	DFE (ug/serving)	Percent DV*
Breakfast cereals (fortified)	100	25
Rice (white, cooked, ½ cup)	54	14
Spaghetti (enriched, cooked, ½ cup)	45	11
Spinach (raw, 1 cup)	58	15
Broccoli (cooked, ½ cup)	52	13
Bread (white, one slice)	32	8
Orange juice (3/4 cup)	35	9
Banana (one whole)	24	6
Lettuce (1 cup)	64	16
Avocado (raw, ½ cup)	59	15
Kidney beans (1/2 cup)	46	12
Milk (1 cup)	12	3
Eggs (one whole, hard-boiled)	22	6
Beef liver (3 oz)	215	54
Fish (cooked, 3 oz)	12	3
Chicken breast (3 oz)	3	1

Adapted from U.S. Department of Agriculture Agricultural Research Service (2019) [33]

2.2.3 Recommendations

The recommended dietary allowance (RDA) of folate for men and women in North American is 400 micrograms/day of dietary folate equivalents (DFE).[1] This recommendation is set slightly higher for pregnant and lactating women to 600 micrograms/day of and 500 micrograms/day of DFE respectively, to account for the increased demands during these physiological states. The Recommended Daily Allowance (RDA) for children is 150 micrograms/day of DFE for those aged 1-3 years old, 200 micrograms/day of DFE for those aged 4-8 years old, 300 micrograms/day of DFE for those 9-13 years old, and 400 micrograms/day of DFE for those aged 14 and above (Figure 2.2.3).[1] These recommendations are based upon the amount of folate required to maintain a normal blood cell folate concentration. In order to protect against the adverse effects of high folic acid, the upper limit set for safe intake is 1000 micrograms/day of folic acid for adults. Health Canada recommends that low risk women capable of becoming pregnant supplement their diets with 400 micrograms of folic acid daily in addition to folate from natural sources.[1,34] Dietary folate equivalents were established to take into account the differences in bioavailability of the different forms of natural and synthetic folate.

Table 2.2.3 Recommended daily allowance for folate instituted by the Institute of Medicine

Cohort (Age/Demographic)	RDA (μg DFE/ day)
1-3	150
4-8	200
9-13	300
14+	400
Pregnant	600

Adapted from Institute of Medicine (1998) [1]

2.3 Folic Acid Fortification and Status

2.3.1 History of Association between Folic Acid and Neural Tube Defects

NTD's are characterized as a group of severe birth defects occurring as a result of partial or complete failure of the neural tube to close within 28 days of conception.[35] NTD's represent the second most common congenital abnormality in the world and are often associated with fetal and infant mortality, morbidity, disability, and economic costs.[35,36] Approximately 260, 100 births were affected by NTD worldwide in 2015.[37] First reported associations between NTD and folate deficiency as a risk factor were seen by Hibbard and colleagues in 1965.[38] Following this, Smithells and colleagues attempted to further study this relationship and through conducting several control trials found that the recurrence rate of NTD was reduced from 5.9% to 0.5% with a periconceptional supplementation of a multivitamin containing 0.36 mg of folic acid.[39–43]Despite these results, these trials were not considered substantial evidence due to the non-randomization of the groups and mode of delivery of folic acid via multivitamin supplement.

The first study to report the use of folic acid during the periconceptual period as a tool to prevent recurrence of NTD's was by Laurence et al.[44] In 1981, this group of researchers conducted a double-blind randomized control trial that looked at the effect of 2 mg of folic acid per day in the periconceptional period on preventing NTD recurrence and found no recurrences to occur amongst women that received supplementation ($p<0.04$). Soon after in 1991, a group of researchers in the UK sought to understand folate supplementation from a prevention aspect. In a randomized double-blind trial, 1817 women who had previous history of a NTD-affected pregnancy were recruited from 33 centers in 7 countries across the world.[45] These women were randomly allocated into one of four groups (folic acid supplementation, other vitamins, both, and neither). A 72% reduction in NTD reoccurrence risk was observed in the folic acid

supplementation group that received 4 mg/day during the periconceptional period. Subsequently, another large randomized control trial in Hungarian women found 0.8 mg folic acid supplementation obtained through a multivitamin demonstrated a 93% reduction in the incidence of NTD's.[46]

Other case-control studies conducted provided similar evidence regarding a reduced risk of prevalence in NTD's in women consuming a folic acid supplement of up to 0.8 mg/day in addition to natural dietary sources of folate.[42,47–50] These results led to the development of a folic acid recommendation of 0.4 mg/day for women planning to become pregnant during the periconceptional period and first trimester of pregnancy.[51]

The results of the Canadian maternity experiences survey conducted in 2008 suggested that of 6421 women surveyed, 57.7% reported consuming a folic acid supplement during their periconceptional period, and 89.7% reported consuming folic acid during the first trimester of pregnancy.[52] With this trend of high folic acid consumption, a study conducted by Masih and colleagues in 2015 studied a cohort of 368 pregnant women who consumed a supplemental intake of 1000 mg/d of folic acid before conception and during early pregnancy.[53] They found a high prevalence of prenatal supplement use amongst these women resulting in intakes of folic acid above the RDA from supplements alone.

2.3.2. Rationale for Fortification

As of 1998 Health Canada opted for the fortification of all enriched flour and cereal grain products with folic acid. Canadian guidelines have stated that all white wheat flour be supplemented with 140-150 μg/100 g of folic acid and pastas with 200 μg/100 g folic acid.[54] Due

to the body of knowledge surrounding folate as essential during the periconceptual period, this mandatory folic acid supplementation program was implemented with the intent of providing a protective effect against the development of neural tube defects (NTD's). Developers of this initiative set out to increase folic acid consumption by an additional 0.1 to 0.2 mg per day to obtain beneficial effects.[55] A Canadian population-based study conducted by De Wals and colleagues found a significant reduction in the prevalence of NTD's post fortification totaling almost 50%.[3] This reduction was the greatest in those geographical areas where the baseline levels were high. Several other studies found a significant increase in dietary intakes and blood levels of folic acid during the full-fortification period.[5,56,57]Due to the success of the folic acid fortification intervention in North America, other countries that have adopted the use of a mandatory folic acid supplementation program include South Africa, Chile, and Costa Rica.[58] Table 2.3.2 summarizes the history of the implementation of mandatory folic acid fortification in countries across the world.

It was after this study that it was realized the NTD burden was preventable through consumption of mandatory folic acid fortified food products before conception.[59] After mandatory fortification was opted in 1998, a number of studies were conducted to observe the effects of this supplementation program. In 2002, a study found women in Ontario the prevalence of NTD's affected pregnancies among 336, 963 women who underwent maternal serum screening over a period of 77 months declined from 1 to 13 per 1000 pregnancies prior to fortification to 0 to 58 per 1000 pregnancies post ($p<0.0001$).[60] As mentioned previously, a study conducted by De Wals and colleagues found the prevalence of NTD's decreased from 1.58 per 1000 births prior to fortification, to 0.86 per 1000 births post fortification resulting in a 46% reduction.[3] A higher reduction rate of 53% was observed was observed for cases of spina bifida compared to

anencephaly and encephalocele.

Table 2.3.2 History of mandatory folic acid fortification in countries across the world

Country	**Fortification mandated**	**Year mandated**
United States	140 ug/ 100 g	1998
Canada	150 ug/ 100 g	1998
Costa Rica	180 ug/ 100 g	1998
Chile	220 ug/ 100 g	2000
Guatemala	40 ug/ 100 g	2001
Argentina	220 ug/ 100 g	2002
South Africa	150 ug/ 100 g	2003
Honduras	180 ug/ 100 g	2007
Fiji	200 ug/ 100 g	2009
China	200 ug/ 100 g	2012
Kenya	150 ug/ 100 g	2012
Australia	250 ug/ 100 g	2014
Afghanistan	100 ug/ 100 g	2014
Zimbabwe	200 ug/ 100 g	2016
Brazil	180 ug/ 100 g	2017
Ethiopia	200 ug/ 100 g	2017
India	10 ug/ 100 g	2018

Adapted from Crider et al. (2011)[61]

2.3.3 The Canadian Nutrient File

The Canadian Nutrient File (CNF) is an online database created by Health Canada that reports the energy, macronutrient and micronutrient composition in up to 5690 unbranded foods.[62] Values are based on USDA values of food and mathematically modified to reflect amounts in the Canadian food supply. The most recent 2015 version of the database provides estimates on total folate, naturally occurring folate, and synthetic folic acid, and DFE found in founds. In conjunction with dietary records, the CNF has been used as a tool to understand the folate intake of Canadians. The CNF consists of unbranded foods and does not provide product specific information. It combines different brands of similar products to produce an average amount of nutrient in a food, and not the exact nutrient composition in a specific food. The methods used to analyze the folate content in these foods remain variable. The nutrient database continues to be updated with values obtained via laboratory methods, however the database remains incomplete at this point. Although the nutrient values for foods such as those in the "breads" category have been updated as such, foods that fall under the category of "pastas" are either based on USDA values of food and mathematically modified to reflect amounts in the Canadian food supply, or calculated from other data.[63]

A lack of incorporation of possible overages of nutrients added to fortified foods is also problematic. Overages of folic acid are routinely added to fortified foods to ensure that it does not degrade to less than mandated levels throughout the products' shelf life.[6] Unincorporated overages, along with the outdated laboratory methods used to create the nutrient database, suggest that folic acid intake may have likely been grossly underestimated in most prior studies.[6]

2.3.4 Folate content in the Food Supply

To date, there have only been a few studies that have examined the folate content in the food supply since mandatory folic acid fortification in North America in 1998. A paper by Rader et al. analyzed approximately 150 top-selling fortified grain products in the United States, and found that folate levels in these foods were significantly higher than stated on the label, about double the levels mandated at the time.[64] In contrast, a 2004 study by Tamura and colleagues measured the total folate content of 92 white and whole-wheat breads. They found no differences between the mandated and actual folate levels in white breads containing folic acid fortified white wheat.[65] Tamura et al. concluded that their study showed that the levels of fortification in the United States declined since the initial post-fortification high, and that monitoring of folic acid fortification may be necessary. Canadian and American fortification regulations are similar but differ in important ways including mandatory folic acid fortification of rice in the United States but not Canada. A study done by Shakur and colleagues 10 years ago (2009), reported on the total folate content of 95 of the most frequently consumed folic acid fortified foods in the Canadian food supply, and found significantly higher folate values than what was stated on the label.[6] They reported the actual amount of folate in fortified foods is approximately 50% higher than what was mandated.

Table 2.3.4 Summary of folate content analyzed in the food supply

Reference	Mandated level at the time	Number of foods analyzed	Type of food analyzed	Food folate content
Rader et al. 2000	140 ug/ 100 g	150	Grain products	Significantly higher than label value
Tamura et al. 2004	140 ug/100 g	92	White and Whole-wheat breads	No difference between mandated and actual levels
Shakur et al. 2009	150 ug/100 g	95	Breads, Rolls, Buns, Cereals, Packaged Desserts, Pastas, Cookies, and Crackers	Double mandated levels

2.3.5 Folate Status of Canadians Post-Fortification

In the years' post-fortification, alongside the decline in NTD's, an increase in the folate status of Canadians was also observed. In 2010, a group of researchers sought to determine the RBC folate concentrations of inpatients in three major downtown Toronto Hospitals.[66] They collected the RBC folate concentrations of 2,563 inpatients as part of their recommended inpatient testing and found that only 4 (0.16%) of RBC folate levels were classified as deficient (<254 nmol/L).[66] Using data from the 2007-2009 Canadian Health Measures Survey, Colapinto and colleagues aimed to assess the folate status of a nationally representative sample of Canadians, including a

subset of women of childbearing age.[5] They measured RBC folate in a cross-sectional sample of 5248 Canadians aged 6 to 79. Their results indicated approximately 16% of the sample fell under the high RBC folate threshold of 1450 nmol/L. They also found, virtually 100% of Canadians met or exceeded the RBC folate concentration cut-off level of deficiency (<305 nmol/L), and 40% of the general population had high RBC folate concentrations (>1360 nmol/L) (Figure 2.3.5).[5] 22% of women of childbearing age had RBC folate concentrations below that established for maximal neural tube defect-risk reduction (< 906 nmol/L). Moreover, those who consumed a folic acid supplement were more likely than those who did not, to have high RBC folate levels.[67]

The normal range for RBC folate in children is >362 nmol/L, and adults is 317-1422 nmol/L.[68] According to the 2017 Statistics Canada report, mean RBC folate levels for those aged 6-9 and 20-79 were 1,352 nmol/L and 1,407 nmol/L, respectively.[69] In 2015, Masih et al. found high maternal RBC folate concentrations of 2417 nmol/L and 2793 nmol/L in a cohort of Canadian pregnant women during early pregnancy and upon delivery, respectively.[53] A recently published study in 2018 examined a cohort of Toronto women for their blood folate concentrations. The researchers found that among 235 women of child bearing age, approximately 7% had total blood folate concentrations of less than the 906 nmol/L, the minimum value required for protective effects against NTD's.[70,71]Research continues to suggest folate deficient is nearly nonexistent, and Canadians are approaching higher RBC folate levels.

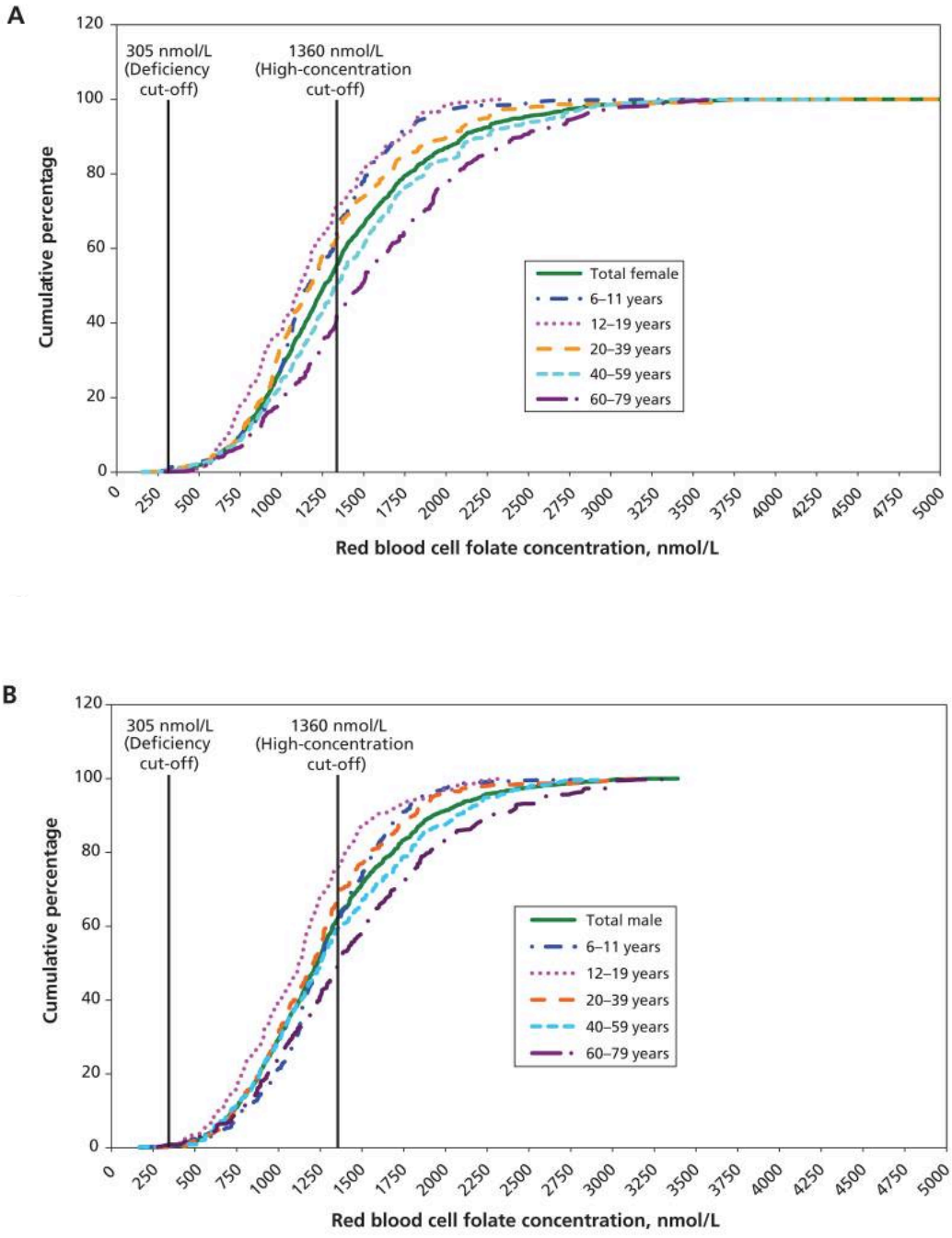


Figure 2.3.5 Cumulative percentile distributions of red blood cell folate concentrations by age group among female (A) and male (B) participants in the Canadian Health Measures Survey, 2007-2009. Deficient (305 nmol/L) and high (1360 nmol/L) folate concentrations are indicated by vertical lines. Adapted from Colapinto et al. (2015) with permission.[5]

2.4 Folate Metabolism

Folic acid cannot be synthesized in the body because mammals are unable to join the pteridine ring to PABA.[72] When consumed, dietary folic acid must be converted into the biologically active compound which requires the action of dihydrofolate reductase (DHFR). DHFR converts folic acid either partially into the intermediate dihydrofolic acid (DHF) reduced at C7 and C8 of the pyrazine ring, or completely into tetrahydrofolic acid (THF) reduced at C5, C6, C7, C8 of the ring. The basic role of folate within the cell is in one-carbon metabolism by accepting and transfer of one carbon groups such as methyl, methylene, hydroxymethyl, methyenyl, formyl, and formimino substitutions.[72] These single carbon groups are attached to N5 of pteridine ring, N10 of PABA or both nitrogen of the THF.

THF occurs mainly as 5methylTHF, 5,10,methylTHF and 10formylTHF derivatives in the body.[72] The main circulating form of folate is 5-methylTHF. Cellular folate one carbon metabolism occurs in the cytoplasm and mitochondria. In the mitochondria one-carbon metabolism is required for the synthesis of formate, formylate methionyl-tRNA and glycine.[72] The primary role of folate is to participate in the metabolism of nucleic acids and to support DNA and RNA biosynthesis and repair. Polyglutamated THF is a commonly used substrate in nucleotide synthesis and the methylation cycle.[72] The essential amino acids, methionine and cysteine, are synthesized in the methylation cycle from homocysteine. The enzyme methionine synthase transfers methyl from 5-methyl-THF on homocysteine to form methionine and THF. This regenerated methionine can then later be converted to S-Adenosyl Methionine.[72] Summarized in Figure 2.4.1 are the common one-carbon metabolism pathways. Folate is able to cross the cell membrane once it is in its monoglutamated form. Once in the cell, the glutamate tail is elongated via folylpolyglutamate synthetase which is necessary for retaining the structure

of folate within cells; the affinity of folate to key enzymes is also affected by the length of the polyglutamic tail.[73] Folate is metabolically active when fully reduced and polyglutamated to THF acid.[74] The length of these polyglutamate chains may differ between cell types and organelles.[75] The methylenetetrahydrofolate reductase (MTHFR) gene is responsible for the conversion of 5,10-methylenetetrahydrofolate to its active form 5-methyltetrahydrofolate.[76] This enzymatic reaction is part of a multistep process to produce methionine from homocysteine which is involved in the formation of S-adenosylmethionine, DNA synthesis and numerous other methylation reactions in the body.[76] Individuals with a 677C→T polymorphism in this gene that typically process the re-methylation of homocysteine to methionine less efficiently. High concentrations of folate are thought to stabilize MTHFR.

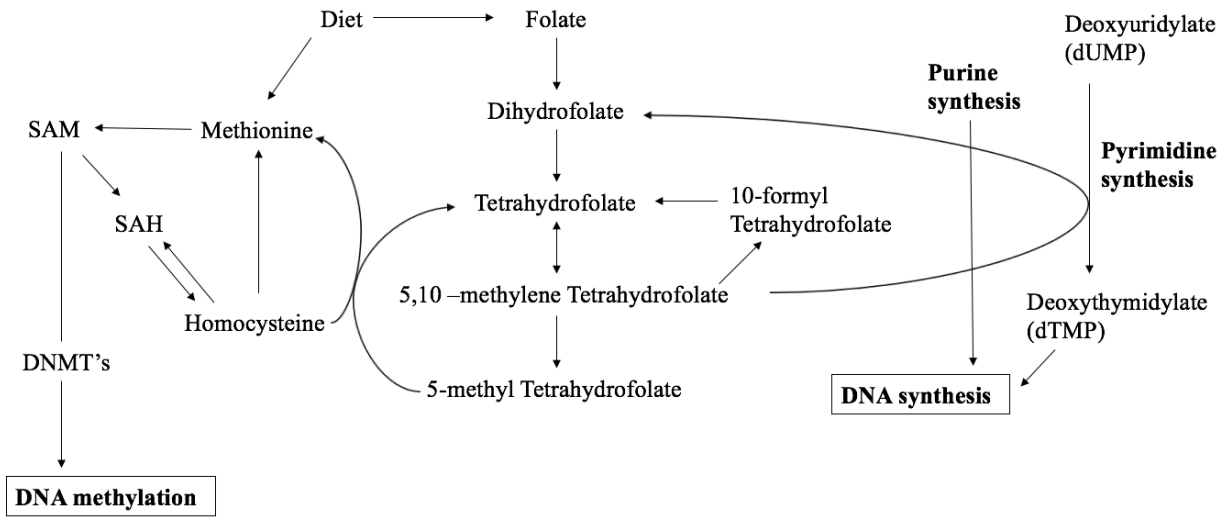


Figure 2.4 Folate-dependent one-carbon metabolism pathway of folate. Abbreviations: S-adenosylhomocysteine, SAH; S-adenosylmethionine, SAM; Thymidine Monophosphate, TMP; Uridine Monophosphate, UMP; DNA methyl transferase, (DNMT). Adapated from: Lamprecht and Lipkin, Nature Reviews (2003)[77]

2.4.1 Folate Elimination

Folate elimination refers to the irreversible loss of the nutrient via processes of metabolism or excretion.[78] Daily folate excretion in humans is an estimated 0.3-0.8% of the folate body pool.[72] It is excreted in both active and inactive forms in the urine and bile. Within the kidney folate that remains intact may be reabsorbed by either FRα and PCFT in the kidney and subsequently moved into circulation to be redistributed and taken up into tissues by RFC and organic anion transporters.[79–82]Folate can also be broken down in the tissues in an oxidative process converting ferritin to PABA, a urinary catabolite.[83,84] In addition, folate may be secreted into bile; most of which is believed to be reabsorbed via enterohepatic circulation. Large quantities of folate are excreted in feces as a result of gastrointestinal secretions, folate produced via bacterial biosynthesis and lyses colonocytes.[85]

2.5 Folate Absorption across the small intestine

Dietary folate is absorbed across the proximal small intestine and subsequently enters systemic circulation via the hepatic portal vein.[7] Once taken up by the liver, these folates can either be transported to the periphery by re-entering the systemic circulation through the hepatic vein, or be stored as a polyglutamate folate. Some monoglutamylated folates absorbed by the liver can be secreted into the bile and sent to the enterohepatic circulation for reabsorption which occurs through the small intestine.

As previously mentioned, mammals are incapable of de novo folate biosynthesis and must consume foods rich in folate to obtain this vitamin. Most dietary folates are in the polyglutamylated form. In order for the absorption of folate to occur it must be in its bioavailable monoglutamylated form.[86] The deconjugation of polyglutamylated folate to monoglutamylated

folate is primarily facilitated by the enzyme glutamate carboxypeptidase 2, GCPII (EC number 3.4.17.21) which is concentrated in the apical brush border of the jejunum of the small intestine in humans.[18,73,83] This enzyme operates at an optimum pH of 6-7. GCPII mRNA has been previously identified in the colonic tissue of humans, however at lower concentrations as compared to the small intestine.[87] Another enzyme called γ-Glutamyl hydrolase (operates at an optimum pH of 4.5-6) located intracellularly and within lysosomes, is believed to be involved in the deconjugation of folate but to a lesser extent.[7,88] Folates that exist intracellularly have been observed to comprise of up to ten polyglutamates, while folates in the serum are in their monoglutamated form.[83,89,90]

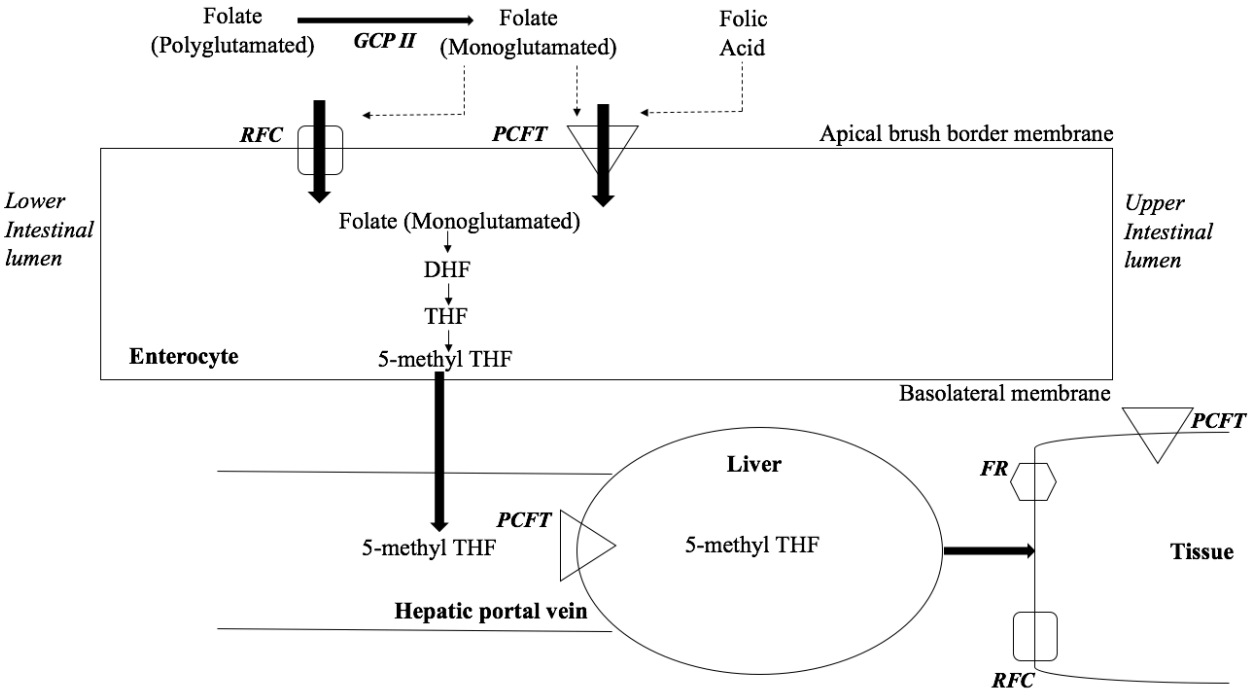


Figure 2.5 Absorption of folate across the small intestine. Abbreviations: Tetrahydrofolate (THF). Enzymes: (1) Glutamate Carboxypeptidase II (GCPII); Folate Transporters: Protein-Coupled Folate Transporter (PCFT), pH optimum of 5.5; Reduced Folate Carrier (RFC), pH optimum of 7.4; Folate Receptor (FR). Adapted from Zhao et al. (2009) [80]

2.5.1 Receptors, Hydrolases and Transporters

The uptake of folate by the intestine occurs primarily through three mechanisms. At intraluminal concentrations of 10 μmol/L and above, deconjugated folates are transported through enterocytes without modification by a non-saturable ion-mediated passive diffusion process.[91,92] The transport of reduced folate compared to folic acid occurs more quickly by this route.[93] At physiological pH folate exhibits lipophobic properties, therefore this method of absorption is thought to play a minimal role in the intestine.[80] At intraluminal concentrations of less than 10-20 μmol/L, the absorption of monglutmylated folates occurs via active transport by a saturable pH and energy dependent process (pH optimum 5-6.[94,95] The mRNA expression of two folate carrier proteins, reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT) was observed in the intestine.[96] RFC (encoded by the *SLC19A1* gene) is a facilitative carrier found in the apical brush-border membrane of the small intestine that participates in carrier-mediated absoption.[97] The enzyme operates at a pH optimum of 7.4, a relatively neutral pH, and is believed to be the primary carrier of folate in systemic circulation into cells. RFC has a high affinity for folates in their reduced form (Kt=2-7 μM), however a low affinity for synthetic folic acid (Ki=100-200 μM). RFC expression is greatest in the duodenum causing more transporters to be localized on the upper half of the intestinal villus.[96,98] Similar to RFC, PCFT (encoded by *SLC46A1* gene), consists of 12 transmembrane domains. The enzyme operates at a pH optimum of 5.8 making it relatively acidic. PCFT has been found along the small intestine, kidney, brain, retina, liver, and placenta.[99] It has a high affinity for folate in its reduced form (1-3 μM), as well as a high affinity for synthetic folic acid.[97] The expression of PCFT is lower in areas such as the ileum, cecum, and rectum, moderately in the jejunum, and most concentrated in the duodenum. Table 2.5.4 summarizes the characteristics of these two transporters. FR α and β are membrane-bound folate

receptors which serve as the primary method of plasma folate (5-MTHF) transport into the cytoplasm through the process of endocytosis.[80] FR α is primarily located in the kidney, uteri epithelial cells, retina, placenta, and choroid plexus, while FR β is found mainly in the spleen, placenta, thymus, and $CD34^+$ monocytes. Both FR α and β are not thought to be involved in the intestinal transport of folate, however are expressed in tumours.[80,82,100] They have a higher affinity for synthetic folic acid compared to RFC and PCFT (K_d 1-10 nM), while RFC and PCDT have a higher affinity for folate in its reduced form.[80,91,101]

Table 2.5.1 Review of intestinal folate transporters, reduced folate carrier (RFC) and the proton coupled folate-carrier (PCFT)

Characteristics	**RFC**	**PCFT**
Localization	**Kidney, liver, small intestine, colon, choroid plexus**	**Kidney, liver, brain, retina, placenta, small intestine, colon**
Expression	**Small intestine (apical basolateral membrane) and colon (villus tip)**	**Small intestine (apical basolateral membrane) > colon**
Binding affinity for reduced folates	**High receptor binding affinity (2-7 μM)**	**High receptor binding affinity (4 μM)**
Binding affinity for folic acid	**Very low competitive inhibitor binding affinity (150-200 μM)**	**Low receptor binding affinity (1 μM)**
Optimal pH	**Neutral, 7.4**	**Acidic, 5.5**

Adapted from: Zhao et al. (2009)[80]

2.6 Folate Production, Absorption across the colon and Bioavailability

2.6.1 Physiology of the colon

The large intestine differs from the small intestine in that it consists of a more viscous luminal environment that allows for a slower transit time, substrate availability and consists of different ranges of intra-luminal pH which affect the growth of gut bacteria. The large intestine, also referred to as the colon, is approximately 1.5 m long and makes up the longest segment of the gastrointestinal tract. It begins at the ileocecal junction and terminates at the anus.[102] Relative to the small intestine, the large intestine has a smaller internal surface area.[103] The small intestine comprises of villi which are covered with several microvilli that contribute to its larger surface area, while the colon does not have villi. The different sections of the colon are as follows: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anal canal. The pH varies across different sections of the colon. The main pH of the lumen of the ileum is approximately 7.5, which drops immediately to 5 in the cecum area and slowly rises along the length of the colon. The pH of the mid colon region is around 6.6-6.8 and the distal colon is 7.04-7.14.[104,105] Similar to the small intestine the pH of the mucosal surface is more acidic than the pH of the colon contents.

2.6.2 Intestinal biosynthesis of folate

In 1941 Mitchell and colleagues were the first to suggest the possibility of microbial synthesis of folate in mammals.[18] Studies conducted in the late 1940's found the amount of folate synthesized in the intestinal microbiota was 3-5 times that of the recommended dietary intake.[106,107] In 2004, Kim and colleagues analyzed infant fecal samples for folate to find 52.5 ± 30.1% were in their monoglutamated form which is readily absorbed across the small intestine.[8] The total folate content was found to be 63% of what the adequate intake is for infants aged less than 5 months.

A study by O'Keefe and colleagues sought to examine the influence of the diet on colonic mucosa health by looking at microbiota and cancer risk. After the ingestion of a standard 2L polyethylene glycol solution collected over 3 hours, the adult colonic evacuants of a population of South Africans and Caucasians exceeded the RDA of 400 μg/day for adults by 699 ± 131 and 860 ± 129 μg, respectively.[108] Thus, both these studies were vital in demonstrating there is a large pool of folate that exists within the large intestine which contributes to exceeding or approaching recommended dietary intakes.

Many colonic bacterial species are responsible for the production of folate using a series of enzyme catalyzed reactions.[109,110] This process consists of 7 steps to allow the conversion of 6-hydroxymethyl-H_2-pterin pyrophosphate (GTP) to THF. The mechanism of bacterial folate synthesis can be explained in Figure 2.6.2.

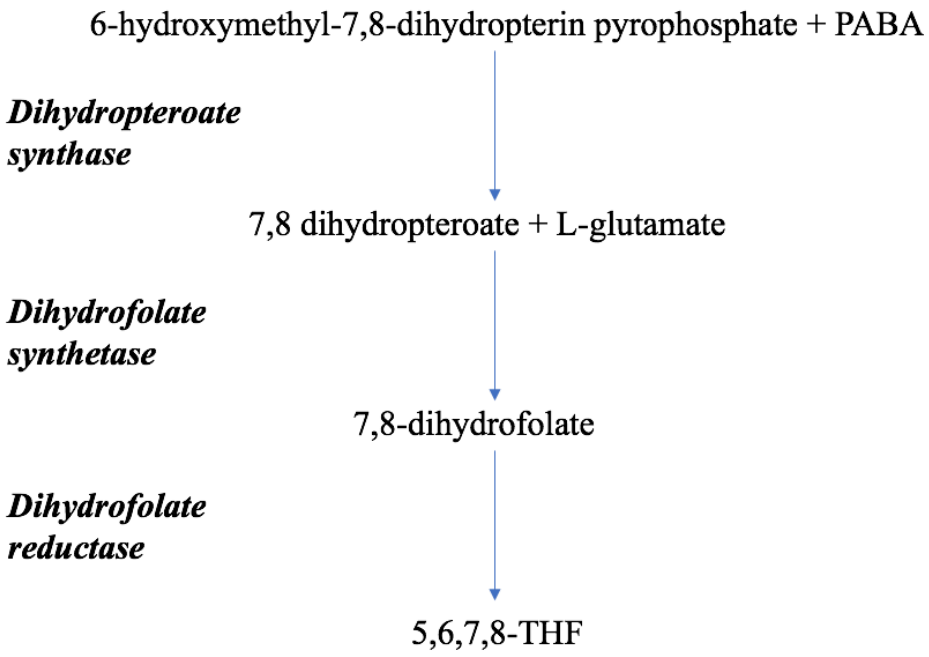


Figure 2.6.2 Final steps in the bacterial biosynthesis pathway of tetrahydrofolic acid. Adapted

from Bermingham & Derrick, (2002)[110]

Amongst the population of colonic bacteria there are folate consumers and folate synthesizers. Bifidobacteria and Streptococcus thermophilus have been identified as bacterial species with the ability to synthesize folate de novo and Lactbacillus as a bacterial species that reduces folate levels.[111] A study conducted by Lin and Kim found the form of folate produced in the body, either 5-methylTHF or 5-formylTHF, during metabolism can vary according to bacterial strain. An example of this is the bacterial species L. casei was found to synthesize mono and diglutamated forms of 10-formylTHF.[112,113] The folate consumers may delay the rate of folate biosynthesis by folate producers and thus there has been difficulty accurately estimating the colonic rate of folate biosynthesis. The region of the colon in which the most folate is produced is still unknown. Ahmed and colleagues sought to characterize bacterial communities within the colon and found no significant differences in bacterial count between the ascending, transverse and descending sections of the colon.[114] Additional research is required to quantify which specific regions of the colon contribute to greater folate production.

2.6.3. Folate absorption across the colon

Although folate absorption across the small intestine has been confirmed, absorption of folate across the large intestine remains of interest. In a study conducted by Rong and colleagues the absorption of folate across the colon was demonstrated in rats after the administration of ^{3}H-labelled PABA injections. Post injection it was observed that folate was incorporated into the liver therefore confirming intestinally synthesized folate is bioavailable to the rat through the colon.[9] Another study conducted by Thomas and colleagues in 2003 further verified this by cecal injection of rats with ^{3}H-labelled PABA and observing the recovery of ^{3}H label in rat livers.[115] In 2005, Asrar and O'Connor used a cecal injection of ^{3}H –labelled folic acid and ^{3}H-labelled

PABA to quantify the absorption of folate across the colon of piglets and found more than 20% of the dose provided was located as folates in the liver, kidney, urine and feces.[10] Similarly, studies conducted by Aufreiter et al. and Lakoff et al. looked at the absorption of folate through the colon in humans via cecal infusion and caplet ingestion respectively. The study conducted by Aufreiter et al. 684 nmol of [^{13}C]- [6S]-glutamyl-5-formylTHF was infused into the cecum of adults and blood samples were collected.[116] Absorption across the colon was confirmed by the appearance of the metabolite in the plasma at a mean appearance rate of 0.6+0.2 nmol/h. In the study by Lakoff et al. an enteric coated caplet containing 5-formylTHF was ingested by adults.[117] Caplet disintegration in the colon was monitored via fluoroscopic imaging and the change in plasma folate levels was found to be significant after caplet ingestion.[117] The rate of appearance of the folate metabolite in the plasma was 0.33±0.09 nmol/h and due to the significant time spent in the colon, the mean absorption across the colon was 46%. Results of both these studies suggest colonic folate absorption is possible. Although there is evidence for in vitro human colonic folate absorption, there is evidence lacking for in vivo data.

2.6.4 Folate Bioavailability

The term "bioavailability" is used to describe the overall efficacy of nutrient utilization. This encompasses how much of the nutrient is utilized by the body after being absorbed via the gastrointestinal tract from food sources, clinically, or produced by gut microbiota. Factors affecting bioavailability include the form of the nutrient, amount, and food preparation/handling methods. The efficacy of utilization is dependent on factors such as nutrient transport, metabolism, catabolism, excretion and retention.[91,92] Other factors affecting bioavailability include aspects varying in individuals such as dietary components that may be present in their enterohepatic circulation, dose-dependence, and the actions of the intestinal microbiota which

may either enhance or hinder the availability of a nutrient.[92] Due to the difference in bioavailability of the different forms of folate, dietary folate equivalents (DFE's) are used to account for this. Folic acid consumed with food has a bioavailability of 85%, while food folate stands less than that at 50% bioavailability.[1] Therefore folic acid consumed with food is 1.7 times more bioavailable than food folate. Dietary folate equivalents can be calculated to determine the Estimated Average Requirement (EAR) using the following formula: μg of DFEs provided = μg of food folate + (1.7 × μg of folic acid). This means 1 DFE is defined as 1 microgram of natural sources of folate, 0.5 micrograms of a folic acid supplement consumed on an empty stomach, or 0.6 micrograms of folic acid from foods that have been fortified or supplements consumed alongside food.[1]

2.7 Folate Health

A growing body of evidence suggests that Folate is an essential vitamin for mammals due to its critical role in cellular metabolism. It has been discovered to have a preventative effect in disease onset and progression.[118] Several epidemiological studies have noted an association between folate deficiency and anemia, NTD development risk, a number of cancers, atherosclerosis, and neuropsychiatric disorders.[119–122] Due to the observed associations, many studies evaluating the beneficial effects of folate for health were conducted.

Following the implementation of folic acid fortification in the US grain supply, there was a small but significant decline of 6% in the prevalence of orofacial clefts observed.[123] Research conducted in elderly populations have found an association between adequate folate consumption and the prevention of stroke.[124] Folic acid supplementation may also serve protective effects against low infant birth weight, placental abruption, and preeclampsia.[125,126]

The beneficial effects of folate supplementation for women and their offspring are well known, however there is additional research that suggests paternal periconceptional supplementation of folate and folate gene polymorphisms are of interest. One study found healthy men that consumed more than 700 micrograms of folate per day had lower frequencies of sperm aneuploidy.[127]

Although folate is known to contribute to a number of health benefits, other research suggests folic acid supplementation may be associated with potentially harmful effects such as cancer promotion, decreased natural killer cell cytotoxicity activity, resistance to anti-inflammatory drugs, anti-epileptic treatments, and anti-folate based chemotherapy.[128–130] A study conducted by Mason and colleagues looked at the relationship between colorectal cancer and folic acid supplementation in both Canada and the US and found an increase in incidence in the post fortification period compared to the downwards trend observe pre-fortification.[131] As more research is conducted and more evidence comes to light, it is possible the relationship between folate and disease prevention may be more complicated than previously thought as folic acid may have a tumor promoting effect.

2.7.1 Consequences of suboptimal folate status

As mentioned previously, the folate status of Canadians today is higher than it has been in the past few decades. Folate requirements are generally higher during anabolic stages of the life-cycle including pregnancy, lactation and early fetal and post-natal life.[132] There are several well-documented adverse health outcomes associated with chronically inadequate folate consumption in humans such as spina bifida and anencephaly and neuroblastoma in offspring of folate deficient mothers, colorectal and cervical cancer, anemia during pregnancy, and low infant birth weight and premature birth.[133–136] Folate deficiencies have also been observed in many

neuropsychiatric and neurodegenerative disorders such as depression and dementia.[137] However, folate status appears to have a particularly great impact on the risk of carcinogenesis in numerous human tissues, most notably those of the colorectal system.[134] Since folate is involved in the process of DNA methylation, deficiencies of the vitamin are likely harmful due to an interruption of this pathway. The health outcomes due to folate inadequacy causes it to be an area of growing research.

Neural Tube Defects

Folate has been deemed essential for normal fetus brain, skull, and spine development, especially during the first few weeks of pregnancy since this period of time is critical for the closing of the neural tubes. A regular consumption of folic acid supplements by women pregnant, capable of becoming pregnant, or planning a pregnancy is crucial to reduce the risk of NTD's.[1] A Cochrane systematic review by De-Regil L and colleagues found that first and second time occurrence of NTD's was prevented by the use of folic acid supplementation.[138] There was also no evidence no evidence of preventive or negative effects of folic acid supplementation on other birth defects such as cleft palate or cleft lip. Several countries across the world, including Canada, US, Costa Rica, Chile, and South Africa showed a reduction in NTD affected pregnancies post fortification (Table 2.7.1.a).[39] In most countries, there is a reduction of 10% to 80% in total NTD prevalence associated with the implementation of folic acid food fortification.

Table 2.7.1.a Impact of folic acid fortification of flour on neural tube defects

Location	Pre fortification	Post fortification	NTD prevalence (per 10,000 births) Pre fortification	NTD prevalence (per 10,000 births) Post fortification	% Reduction	Reference
Canada, Ontario	1994-1997	1998-2000	11.3	5.8	48	Ray et al. 2002
Canada, Quebec	1992-1997	1998-2000	19.8	13.0	34	De Wals et al. 2003
Canada, Nova Scotia	1991-1997	1998-2000	2.6	1.2	55	Persad et al. 2002
Canada, Newfoundland	1991-1997	1998-2001	4.4	1.0	78	Liu et al. 2004
Canada	1993-1997	2000-2002	16.9	8.6	49	De Wals et al. 2007
USA	1995-1996	1998-1999	3.8	3.1	18	Honein et al. 2001
USA	1995-1996	1998-1999	0.8	0.6	26	Williams et al. 2002
USA, Maine	1993-1996	1998-2000	1.2	1.1	13	Palomaki et al. 2003
USA, Rhode Island	1991-1996	1998-2000	3.8	3.3	13	Lambert-Messerlian et al. 2004
USA, Arkansas	1993-1995	1999-2000	10.9	13.0	34	Simmons et al. 2004
USA, California	1989-1996	1998-2003	8.5	7.2	15.5	Chen et al. 2008
USA, South Carolina	1996-1997	2008-2009	13.4	9.7	27.6	Collins et al. 2011

Brazil	2000-2004	2005-2006	17.2	5.1	29.2	Pacheco et al. 2009
Brazil	1998-2001	2005-2007	31.4	24.3	22.6	Lopez-Camelo et al. 2010
Chile	1999-2000	2001-2002	17.0	10.1	40.5	Hertrampf et al. 2004
Chile	1998-2001	2001-2002	19.8	10.1	48.9	Lopez-Camelo et al. 2005
Costa Rica	1996-1998	1999-2000	9.7	6.3	35.0	Chen et al. 2004
South Africa	2003-2004	2004-2005	14.1	9.8	30.5	Sayed et al. 2008
Jordan	2000-2001	2005-2006	18.5	9.5	48.6	Amarin et al. 2010
Argentina	1998-2001	2005-2007	24.5	12.3	49.7	Lopez-Camelo et al. 2010
Iran	2006-2007	2007-2008	31.6	21.9	31.0	Abdollahi et al. 2011

Adapted from Imbard et al. (2013) and Castillo-L et al. (2013)[39,139]

Stroke and CVD

In recent years, there has been a well-established link between vascular disease and one-carbon folate metabolism along with the homocysteine to methionine conversion. It is suspected that a lower folate intake can lead to higher homocysteine levels which have been positively correlated to higher incidences of stroke.[140] Thus, high levels of plasma homocysteine are often an indicator of folate deficiency. A study conducted by Kilmer McCully in 1969 demonstrated homocysteine

may exert harmful effects on the vascular wall via atherosclerosis and therefore is a risk factor for developing cardiovascular disease.[141] A longitudinal study by Weng et al. followed 1772 adults over an average of 10.6 years. They looked at the association between folate and ischemic stroke and found that folic acid therapy significantly reduced the risk of first stroke in individuals with a baseline folate deficiency who had never smoked and in hypertensive smokers with normal folate levels who did not have a cardiovascular disease.[142] A prospective cohort study demonstrated a 25% reduction in serum homocysteine lowered the risk of stroke by 24% and the risk of coronary heart disease by 18% while another study found low plasma folate levels/folate intake was associated with increased stroke risk.[143,144] In a study by Qin et al. folic acid supplementation was seen to reduce the risk of first stroke associated with an elevated total cholesterol in hypertensive adults without a history of major cardiovascular diseases by 31%.[145] A meta-analysis of randomized control trials demonstrated a reduction in stroke risk by 10% and in overall cardiovascular disease by 4% upon folic acid supplementation.[146] Results from these studies collectively suggest folic acid deficiency is associated with a higher risk of stroke and cardiovascular disease.

Cancer

Since folate is required for vital cellular metabolic processes such as DNA and RNA biosynthesis and repair, it is critical in cancer prevention.[147] Insufficient blood folate concentrations and the misincorporation of uracil into DNA are inversely related to one another causing the disruption of two pathways in cancer: abnormal methylation reactions and increased breaking of the chromosomes.[147] Inadequate levels of folate have been associated with various conditions such as breast, cervix, pancreas, lung, stomach, and colon cancer, neuroblastoma, and leukemia.[120,134,147,148] Despite the number of health benefits that occur as a result of folic acid

fortification, specifically the reduction in NTD's, controversy remains regarding the supplementation of folic acid being detrimental in some respect and conducive of other diseases. Table 2.7.1.b is a summary of a meta analyses looking at folate intake and associated cancer risk by cancer type. Folate intake has been linked to several types of cancers; colon cancer in particular. Therefore, further information regarding this association is required to establish a conclusive link. Multiple studies have indicated folic acid supplementation has been recognized as a preventative tool against the increased risk of colorectal cancer that may be caused by a diet low in folate.[149–151] A study by French et al. demonstrated a reduction in the incidence of neuroblastoma among infants post folic acid fortification of 60%.[135] It has also been shown that inadequate folate status of the mother during pregnancy can result in low infant birthweight, premature birth, anemia during pregnancy and long-term adverse health outcomes.[136] The implementation of mandatory fortification has allowed for strides in the health of Canadians. Recent data from the 2007-2009 Canada Health Measures Survey suggests that folate deficiency is virtually non-existent in Canada post fortification.[5,152]

Table 2.7.1.b Review of meta-analyses investigating folate intake and cancer risk in humans

Cancer Type	Reference	Folate Measurement	Number of Cases	Results
Breast	Chen et al. 2014	Dietary folate intake	16 prospective	Not significant
	Zhang et al. 2014	Folate intake	14 prospective observational	Not significant
	Liu et al. 2014	Dietary folate	15 prospective cohort	Not significant
	Tio et al. 2014	Dietary folate	36 studies	Not significant (upon adjustment)
Bladder	He H et al. 2014	Dietary folate	13 studies	Significant (Decreased risk of bladder cancer)
Head and Neck	Fan C et al. 2017	Folate intake	9 case-control studies	Significant (decreased risk of head and neck cancer in highest folate intake compared to lowest)
Colorectal Cancer	Moazzen et al. 2018	Supplemental folate intake	13 RCT and cohort studies	Not significant
Esophageal	Zhao et al. 2017	Dietary folate intake	14 case-control 1 cohort dietary folate intake	Significant (Decreased risk with highest folate intake)
	Tio et al. 2014	Dietary folate	9 case-control	Significant (Dietary folate associated with decreased risk of esophageal cancer)
Gastric	Tio et al. 2014	Dietary folate intake	16 studies	Not significant
Lung	Zhang et al. 2014	Dietary folate intake	9 cohort studies	Not significant in general population, Significant (Low intake reduced lung cancer risk in women, High intake reduced

				risk in men)
Pancreatic	Tio et al. 2014	Dietary folate	8 studies	Significant (Dietary folate associated with decreased risk of pancreatic cancer)
	Lin et al. 2013	Dietary folate intake	13 studies	Significant (Decreased pancreatic cancer risk with higher dietary folate intake)
Prostate	Wang et al. 2014	Dietary folate intake	10 studies	Not significant
	Tio et al. 2014	Dietary folate	11 studies	Not significant (marginally decreased risk)
Ovarian	Li et al. 2013	Dietary folate	8 studies	Significant (High dietary folate associated with reduced risk of ovarian cancer upon adjustment)

S = statistically significant ($P < 0.05$)
NS = not statistically significant ($P > 0.05$)
Adapted from Pieroth et al. (2018) and Moazzen et al. (2018)[153,154]

2.7.2 Supra-physiological Folate Status

There is a clear u-shaped relationship between folic acid intake and adverse health consequences. Although the benefits of folic acid supplementation during the periconceptional period for the reduction of NTD's are well known, the effects of a supraphysiological folate status has emerged as a concern. Recommendations by the Society of Obstetricians and Gynecologists of Canada

(SOGC) state pregnant women at a low risk for NTD's consume a folic acid supplement of 400 to 1000 μg/day, and that those at a high risk of NTD's consume a folic acid supplement of 5000 μg/day.[155] However, until very recently in Canada, prenatal vitamins contained at a minimum the tolerable upper intake level of 1000 μg/day. A study conducted in London, Ontario demonstrated a mean daily intake of 2148 μg/day DFE in pregnant women consuming a folic acid supplement along with dietary folate.[156] Similarly, in an American study the median folic acid intake was 1129 μg/day, above the daily recommended amount.[157] Tam et al. found that the consumption of a high dose of folic acid (5000 μg/day) during the first 12 weeks of supplementation almost doubles the median plasma concentration of unmetabolized folic acid.[158]

There are a number of studies that suggest a link between elevated blood values of folate during asthma but other studies have not found and association. For example, one study found that children of mother's that had a high total folate intake level from diet and supplementation at 22 weeks gestation had a higher 23% risk of asthma at age 7 compared to those children who were born to mothers' with a low total folate intake.[159] Brown et al. conducted a systematic review of 10 large prospective cohort studies to assess whether exposure to folate via folic acid supplementation in amounts greater than the recommended dose influenced chronic health outcomes such as asthma. They found a majority of the studies did not suggest an association between maternal folate exposure and the development of childhood asthma and allergy.[160] There continue to be gaps in the research and the authors agree the findings are not sufficient to make a firm conclusion regarding this association.

Effects of a high folate status include the masking of vitamin B_{12} deficiency, changes in immune function, an increased risk of prostate cancer, and changes in methylation epigenetic

programming.[161–164] High levels of folic acid also interfere with the activity of antifolate drugs such as pyrimethamin, sulfasize, methotrexate and can cause a decrease in natural killer cell cytotoxicity, epigenetic instability, and the selection of disease alleles such as the MTHFR gene.[130,165] A study by Morris and colleagues found elderly individuals with a low vitamin B12 status, a high serum folate of >59 nmol/L was associated with cognitive impairment and anemia.[166] A high folic acid dose (>1000 μg/day) was also found to be associated with poor birth outcomes such as reduced birth height and weight.[167]

There have been some chemoprevention trials conducted in which a tumor-promoting effect of folic acid supplementation has been observed by individuals.[168] The idea is that a surplus of folate in tissues may also introduce a higher risk of carcinogenesis because the high folate levels allow existing neoplastic lesions to grow more quickly.[169] A review of several meta-analyses conducted over the period of 2013 and 2018 by Pieroth et al. suggest a folate deficiency indicated by lower serum levels, may lead to an increase in the risk of several cancers, including head and neck, esophagus, oral cavity and pharynx, pancreatic, bladder, and cervix. They also found a low folate intake in combination with a high alcohol intake may increase the risk of breast cancer.[153]

There has been a dual modulatory effect observed on folate and development of colon cancer specifically. Table 2.7.2 summarizes the effects of high vs. low levels of folic acid and colorectal cancer risk.

Table 2.7.2 Dual modulatory role of folate in colorectal cancer development

	Folate Deficiency	**Folate Supplementation**	**Supraphysiological folate**
Normal Tissue Cancer Risk	Increased	Decreased	Increased
Neoplasm Cancer Risk	Decreased	Increased	Increased

Adapted from: Kim et al. (2007)[170]

2.8 Folate Analysis in Foods

Since mandatory fortification 20 years ago, few studies have investigated the folate content in foods. Among those that have investigated, analyzed amounts exceed those mandated as part of national food fortification regulations, indicated in food composition tables or found on label claims.[6,171,172] During the early post fortification period there were concerns with inaccurate estimates of folate intake. The traditional methods often underestimated for actual folate content in foods due to the partial release of folate from the food matrix, as well as partial hydrolysis of the polyglutamated folate.[1] The buffer solution used previously to homogenize samples demonstrated incomplete recovery of folate compared to a more effective extraction buffer such as the one used by Tamura and colleagues.[65,173] The tri-enzyme approach was seen to yield a 2-fold greater concentration of folate in foods analyzed in the 1997 study conducted by Tamura et al. Soon after, Pfeiffer and colleagues verified the efficacy of the tri-enzyme digestion method to analyze grain products along with unfortified foods.[174]

Due to the nature of folate as susceptible to oxidative damage, exposure to heat and light are

often limited in these methods, and an antioxidant such as ascorbate is added. Folate bioavailability can be measured using three common methods which include microbiological assay or LC-MS/MS. The Microbiological assay is able to measure total folate in blood samples using Lactobacillus rhamnosus (ATCC 7469).[175] In order to achieve deconjugation of polyglutamate forms and complete extraction of folate from food matrices, this method often requires the samples to be pretreated using enzymes such as protease.[173,174,176–178] Another common method used for measuring folate bioavailability includes combining the different techniques of liquid chromatography (LC) with tandem mass spectrometry (MS/MS). This process allows is more sensitive and allows the production of distinct daughter fragmentation, a shorter processing time, along with lower losses of folate to oxidative breakdown.[179–181]High pressure liquid chromatography can often be used in combination with microbiological assay to determine total folate.[179] Table 2.8.1 compares the folate obtained in foods via two different methods.

In a study conducted by Shakur et al. in 2009, approximately 10 years post-fortification, a total of 87 white wheat food products (bread, pasta, rolls, crackers) were analyzed via tri-enzyme digestion method.[6] Due to the susceptibility of folate to oxidative damage, exposure to heat and light were limited and the addition of ascorbate served as an antioxidant. Foods were homogenized using the Wilson-Horne buffer (50mM CHES-HEPES buffer with 2% ascorbic acid and 0.2 M 2-mercapto- ethanol (pH 7.85)) and stored in a -80°C freezer until analysis.[65] Once ready for analysis, the aliquots were thawed and treated with protease to free folate from food matrices and binding proteins and proceed to undergo a microbiological assay and assess total folate concentration. They found that the folate content of the analyzed food products was 151% ± 63 of the CNF value.[6]

Table 2.8.1 Comparison of total folate (PteGlu ug/100 g) found in different foods analyzed via liquid chromatography–tandem mass spectrometry and microbiological assay

Food	**Ptero Glut**	**LC-MS/MS**
Chickpeas	254*	393
Pistachios	93*	112
Spaghetti	20	17
Mung beans	238*	433
Vegetable Mix	311	336
Wheat germs	277*	392

*Indicates significant difference from LC-MS/MS value ($p<0.05$)

Adapted from Ringling & Rychlik (2017)[182]

Chapter 3

3.0 STUDY 1: Folate and Synthetic Folic Acid Content in Canadian Fortified Foods 20 Years Post Mandatory Fortification

3.1 Abstract:

Objectives: In 1998, Health Canada mandated folic acid fortification of white flour and enriched grain products to reduce the prevalence of neural tube defects (NTDs). In 2009, we reported that the analyzed folate content of 95 of the mostly commonly purchased folic acid fortified foods in Canada was on average 151±16% of that reported in the Canadian Nutrient File (CNF). The aim of this study was to assess whether 20 years after mandatory fortification, the CNF values for folate and folic acid accurately reflect amounts determined by direct assessment.

Methods: Using the 2007 ACNielsen Company data, in 2009 and 2019 we attempted to acquire 10 to 15 of the most commonly purchased folic acid-fortified foods from each of the following categories: “breads”, “rolls and buns”, “cookies”, “crackers”, “cooked pastas”, “prepackaged desserts”, and “ready-to-eat cereals”. Total folate concentrations in foods were determined by tri-enzyme digestion method and microbial assay. Synthetic folic acid concentrations were determined using stable-isotope liquid chromatography-mass spectrometry. Analyzed values were compared to the product label values and 2015 CNF values which were calculated using unbranded foods.

Results: Our data show with the exception of “cooked pastas”, the total folate content of foods (n=89) was significantly higher than the CNF values across all categories, ($p<0.05$) and on average, 167% ± 54 of the CNF values. The synthetic folic acid content in foods determined

using LCMS/MS was significantly higher than CNF values for all categories except “cooked pastas” ($p<0.05$), and on average 188% ± 94 of the reported CNF folic acid values. Compared to 2009 values, the mean analyzed total folate values were significantly higher across the “breads”, “rolls and buns”, “cookies” and “crackers” food categories ($p<0.05$). Similarly, directly analyzed folate content was higher than indicated on the Nutrition Facts label across all analyzed categories ($p<0.05$). Folic acid values obtained using LCMS/MS were significantly lower than values obtained using microbiological assay across the “rolls and buns” and “crackers” categories ($p<0.05$).

Conclusion: These data suggest, 20 years after mandatory fortification of the food supply, product label values and CNF values which include unbranded foods do not accurately represent the amounts of total folate and synthetic folic acid in foods. Hence dietary estimates established using the CNF many significantly underestimate actual intakes due to continued overages in folic acid fortification.

3.2 Introduction:

Folate, a water-soluble B vitamin, plays a major role in one-carbon metabolism and the proper closure of the neural tube during the early weeks of pregnancy. Due to the well-established link between folate intake and the reduced prevalence of NTDs it is recommended that women capable of becoming pregnant consume a 400 μg folic acid-containing multivitamin supplement.[155] Folic acid is a synthetic form of folate. National Canadian data in 2008 suggest that only 57.7% of women took supplementary folic acid prior to conception.[52] Due to concerns over the number of unplanned pregnancies and lack of adherence to the folic acid supplement recommendation, in 1998, Health Canada mandated the fortification of all white wheat and enriched grain products. The minimum mandated level of folic acid fortification for white wheat flour and pasta was fortified with 150 μg/100 μg and up to 270 μg /100 μg of folic acid, respectively.[54]

Upon the implementation of mandatory fortification, a study by De Wals and colleagues found the incidence of NTD's lowered by approximately 50% in Canada.[3] However, others reported an increase in folate intake and blood folate indices.[183,184] Despite these successes, a growing body of literature suggests that there is a U-shaped relationship with folate intake.[170] In addition to NTDs, Suboptimal intakes of folate have been linked to colorectal and cervical cancer, neuroblastoma, anemia during pregnancy, and low infant birth weight and premature birth.[133–136] There have been additional associations made regarding low folate intake and congenital defects such as cleft lip, vascular disease, and neuropsychiatric disorders.[137] In contrast, supplemental folic acid intake in addition to folic acid food fortification has produced very high levels of folic acid in sub-populations of Canadian and has been associated with adverse outcomes in some

studies but not others. For example, supraphysiological folate status has been linked to the masking of a vitamin B-12 deficiency, carcinogenesis, specifically prostate and colorectal cancer, changes in immune function, and changes in methylation epigenetic programming.[161–164]

Studies from the United States conducted in the earlier period post mandatory fortification (1998-2000) suggested the folate amounts analyzed in foods may be twice that of what was mandated at the time in the Unites States.[64] In contrast, results from a subsequent 2004 study analyzed 92 white and whole-wheat breads and found no difference in actual vs mandated folate levels. These findings shed light on the possible decline in levels of fortification in the United States since the initial post-fortification high, and suggested monitoring of folic acid fortification may be necessary.[65]

In Canada, Shakur et al. conducted the first direct assessment of folate in the Canadian food supply post mandatory fortification. They compared directly analyzed total folate values for 95 fortified foods to product label values and the values listed in Canada's main reference nutrient database, the Canadian Nutrient File (CNF). The Canadian nutrient file is a database for Canadian nutrient values of foods, which includes unbranded foods. In 2009, Shakur et al. found the folate content of the foods was approximately 50% higher than those of the values reported in the 2007b CNF, and folate values were significantly higher than what was stated on the label.[6,185] Neither the study of Shakur et al nor the earlier publications from the United States reported values specifically for folic acid added to foods as a fortificant; rather total folate concentrations of foods were measured and overages could reflect higher concentrations of naturally occurring folates.

The aim of this study, then, was to assess if after 20 years of mandatory fortification, the values listed in the CNF and food label values for folate accurately reflect the amounts determined by direct assessment, and whether changes have been made to address the overage of fortification in the food supply 10 years post these findings. Further, using a liquid chromatography-mass spectrometry (LCMS/MS) approach, we aimed to specifically quantitate the synthetic folic acid content of foods in comparison to total folate obtained via microbiological assay.

3.3 Methods:

Selection of foods

The products to be analyzed were chosen from a comprehensive list of the most commonly purchased foods across households in Canada. Data from the 2001 Food Expenditure Survey (FOODEX) was used to identify the number of households that reported the purchase of a given category of food, accounting only for categories where foods were fortified with folic acid. The FOODEX is representative of 98% Canadians and contains household level data collected in 2001 from more than 10,000 dwellings. The data collected by trained interviewers included detailed information on food expenditures for each household over the duration of two weeks. The 2007 ACNeilsen company data set was further used to identify 10 to 15 of the most commonly purchased folic acid fortified foods from the following categories: “breads”, “rolls and buns”, “cookies”, “crackers”, “cooked pastas”, “prepackaged desserts”, and “ready-to-eat cereals”. Using this list, the top 15 fortified food brands from each food category purchased by more than 20% of households, and the top 10 brands of fortified foods from each food category purchased by 15-20% of households were analyzed. A total of 92 products were purchased from

grocery stores in Toronto and Montreal (depending on availability) between June 2018 and February 2019. All foods were analyzed prior to their respective expiration dates.

Folate and Folic acid content based on CNF and label values

CNF values were obtained from the most recent available version of the database (2015) found on the Health Canada website.[62] The CNF consists of unbranded foods and reports values of naturally occurring folate, synthetic folic acid, and total folate. The values reported are primarily based on the USDA Nutrient Database for Standard Reference (Release 27, 2014) and modified mathematically to reflect Canadian guidelines for mandatory folic acid fortification.[63]

The "breads", "rolls and buns", "cooked pasta" and "ready-to-eat cereals" food categories had folate listed in their Nutrition Facts Table on the product labels. Most often folate was expressed as % Daily Value (%DV). In Canada, the Daily Value for folate was previously set to 220 μg following the Recommended Nutrient Intakes for Canadian adult males 18 years of age and older in 1983, however has recently been increased to 400 μg as of 2019.[186] The folate content of all foods were calculated using the former DV and serving size, excluding pastas which were calculated from the latter DV.

Laboratory analysis

Foods were analyzed as purchased, except for pasta which was prepared according to package instructions. Samples were homogenized (1g/10 mL) in 50 mmol/L CHES-HEPES buffer with 0.2 mol/L 2-mercaptoethanol and 2% ascorbic acid (pH 7.8). Homogenized samples were stored at -80°C until analysis.[187] Using the tri-enzyme digestion method, 500 μL aliquots of the thawed

homogenate were treated with protease, conjugase and amylase enzymes to allow for the release of folate from the food matrices and to remove the polyglutamate chain from naturally occurring folates.[178,187,188] Once converted into their microbiologically assayable forms, the total folate concentration of the supernatant was determined using *Lactobacillus rhamnosus* (ATCC No. 7469; American Type Tissue Culture Collection, Manassas, VA) with folic acid used to generate a standard curve.[189]

Duplicates of three different dilutions of the enzyme treated homogenates were transferred to a 96-well plate, incubated at 37°C and turbidity assessed using a microplate reader (590 nm). The accuracy and reproducibility of these assays were assessed daily using standard reference materials: (1) Fortified Breakfast Cereal, SRM 3233 (Gaithersburg, MD) with a certified value of 15.1 ± 1.2 mg folate/kg. Our analysis of the breakfast cereal reference material yielded a folate concentration of 14.5 ± 2.1 mg/kg, with an overall inter-assay CV of 7.4% (n=29). The R-squared value of the standard curve for all assays conducted was on average 0.9981.

Synthetic folic acid in the enzyme treated homogenates were determined by stable isotope dilution tandem mass spectrometry (LC/MS-MS) in the Analytical Facility for Bioactive Molecules facility at The Hospital for Sick Children after solid phase extraction (SPE), following the methods of Pfeiffer et al. in our laboratory.[190]

Briefly, standard curves were prepared from spectrometrically verified stock solutions of folic acid (Merck Eprova, Switzerland), and aliquots were stored at -80°C until needed; a solution of $^{13}C_5$ folic acid was used as internal standard (Merck Eprova, Switzerland). Digested samples and

standards spiked with $^{13}C_5$ folic acid in 1 % ammonium formate, 0.5% ascorbic acid buffer (pH 3.2) were loaded onto phenyl cartridges (1mL) (Phenomenex, Torrance. CA) which had previously been conditioned with 2 mL each of methanol, acetonitrile and 1 % ammonium formate buffer (pH 3.2). Samples were allowed to equilibrate for 1 minute. The loaded cartridges were then washed sequentially with 3mL of 0.05 % ammonium formate buffer (pH=3.4, 0.25% ascorbic acid). Folic acid was eluted from the columns using an elution buffer (1 mL of 49% water, 40% methanol, 10% acetonitrile, 1% concentrated acetic acid and 0.5% ascorbic acid). Eluted solutions were analyzed using an AB Sciex QTRAP 5500 triple quadrupole MS system (Agilent 1290 UHPLC system, (Agilent Technologies, Santa Clara, CA, USA). Samples were separated chromatographically on a Kinetex PFP (50 x 3.0 mm, 2.6 μm particle size) column (Phenomenex, Torrance CA) running a gradient mobile phase (A=water + 1% acetic acid, B= 4:1 methanol:acetonitrile +1% acetic acid), starting at 95% A and holding for 1.5 minutes then changing to 30% A by 3 minutes and holding for 1 minute and then back to starting conditions for a total of 8 minutes.

Mass spectrometry parameters were optimized by infusion of standards using 2 μg/ml solution at a flow rate of 10 μL/min, curtain gas at 30 psi, ion source voltage at 4300 V, temperature at 500 ˚C, ion source gasses (1 & 2) at 50 psi, collisionally; activated dissociation gas (CAD) at medium and MRM settings in Positive Mode. Folic acid and $^{13}C_5$ folic acid were monitored at the transitions m/z 442.4 → m/z 295.1 and m/z 447.4 → 495.1, respectively.

The inter-assay accuracy and precision were determined with the use of Fortified Breakfast Cereal, SRM 3233. The mean concentration we obtained was 15.9 ± 1.3 ng/mL with an inter-assay CV of 7.8% (n=18).

Statistical Analyses

Results are presented as means and standard deviations. All statistical analyses were performed using R software (version 1.1456 © 2009-2018, RStudio, Inc.). Paired t-tests were conducted comparing analyzed folate results in each food category to the previously analyzed 2009 values, CNF, and label values. A P-value of <0.05 was considered significant.

3.4 Results:

Of the 92 foods analyzed, three brands of pasta identified using the ACNeilsen data were classified as whole wheat pasta. Although all three brands did contain amounts of folic acid, they were not subject to mandatory fortification and not enriched with folic acid therefore these three products were excluded from all analyses. The results from a total of 89 fortified food products across seven food categories are presented. The folate content of the analyzed foods, as determined by microbial assay, as a percentage of amounts reported in the CNF was, on average, 65% higher. The mean analyzed total folate values were 167% ± 54 over the reported CNF total folate values. The mean analyzed total folate values were higher than the values reported in the CNF for all food categories except "cooked pastas" ($p<0.05$) (Table 3.4.1). In comparison to the previously obtained 2009 values using microbiological assay, the analyzed mean 2019 values were significantly higher across the "breads", "rolls and buns", "cookies", and "crackers" food categories ($p<0.05$) (Table 3.4.1).[6] "Cookies", on average contained the highest amount of folate relative to the CNF values (196% ± 63) (Table 3.4.1).

The amount of folate present in the foods was reported on the product labels of only the "breads", "rolls and buns", "cooked pasta" and "ready-to-eat cereals" categories. Table 3.4.2 displays a comparison of the mean analyzed total folate values to the label folate values. The

analyzed values were on average 87% higher than the label values across all categories ($p<0.05$) except for "cooked pasta" which was 69% lower than the label values.

Comparison of the folate values determined by microbial assay (total folate) and LCMS/MS (synthetic folic acid) is presented in Table 3.4.3. The folic acid values obtained using LCMS/MS were, on average 12% lower than values obtained using microbiological assay across the "rolls and buns" and "crackers" categories ($p<0.05$) (Table 3.4.3).

Synthetic folic acid values obtained via LCMS/MS were compared to food folic acid values listed in the CNF (Table 3.4.4). The synthetic folic acid content of the analyzed foods as a percentage of amounts reported by the CNF was, on average, 77% higher. The reported CNF folic acid values were significantly lower across all food categories except "cooked pasta" ($p<0.05$) (Table 3.4.4). "Cookies", on average contained the highest amount of folic acid relative to the CNF values (251% ± 89) (Table 3.4.4). The mean analyzed folic acid values were 188% ± 94 over the reported CNF folic acid values.

Table 3.4.1: Comparison of the analyzed food folate content to values reported in the Canadian Nutrient File (CNF) and 2009 reported values

Food Category[1]	n	Analyzed Total Folate Value[2] (μg/100 g)		Mean CNF Value Total Folate[2] (μg/100 g)		% of CNF Value Total Folate[3]	
		2009	2019	2009	2019	2009	2019
Breads	14	131 (28)	163 (41)[5]	107 (16)	99 (18)[4]	127 (29)	168 (38)
Rolls and Buns	14	126 (22)	178 (39)[5]	106 (8)	107 (8)[4]	116 (19)	167 (39)
Cookies	15	94 (29)	117 (28)[5]	59 (14)	64 (18)[4]	167 (56)	196 (63)
Crackers	10	116 (32)	195 (51)[5]	80 (11)	85 (47)[4]	188 (57)	195 (80)
Cooked Pastas	7	102 (45)	93 (23)	47 (14)	82 (11)	172 (72)	116 (35)
Prepackaged Desserts	14	79 (31)	72 (26)	77 (0)	45 (14)[4]	133 (59)	161 (45)
Ready-to-eat Cereals	15	146 (36)	152 (34)	105 (36)	107 (30)[4]	137 (104)	153 (55)

[1] Foods were analyzed as purchased with the exception of pasta, which was cooked according to package directions

[2] Mean ± Standard Deviation

[3] Analyzed values as a % of the CNF Folate Value

[4] Significantly different from 2019 analyzed values (Student's paired t-test; $P < 0.05$)

[5] Significantly different from 2009 analyzed values (Student's paired t-test; $P < 0.05$)

Table 3.4.2: Comparison of the analyzed food folate content to values reported on the food labels

Food Category[1]	n	Analyzed Values Total Folate[2] (μg/100 g)	Mean Label Values Total Folate[2,3] (μg/ 100g)	% of Label Value[4]
Breads	9	145 (31)	99 (35)[5]	162 (57)
Rolls and Buns	7	179 (44)	96 (29)[5]	178 (50)
Cooked Pastas	7	93 (23)	299 (87)[5]	33 (9)
Ready-to-eat Cereals	15	152 (34)	59 (5)[5]	257 (58)

[1] Foods were analyzed as purchased with the exception of pasta, which was cooked according to package directions

[2] Mean ± Standard Deviation

[3] Calculated from the % Daily Value listed on the Nutrition Facts Label.

[4] Analyzed values as a % of the Label Values

[5] Significantly different from analyzed values (Student's paired t-test; $P < 0.05$)

Table 3.4.3: Comparison of food folate determined using microbiological assay and LCMS/MS

Food Category[1]	n	Microbiological Assay Total Folate[2] (μg/100g)	LCMS/MS Folic Acid[2] (μg/ 100g)
Breads	14	163 (41)	149 (42)
Rolls and Buns	14	178 (39)	147 (38)[3]
Cookies	15	117 (28)	112 (38)
Crackers	10	195 (51)	157 (66)[3]
Cooked Pastas	7	93 (23)	79 (35)
Prepackaged Desserts	14	72 (26)	70 (31)
Ready-to-eat Cereals	15	152 (34)	142 (52)

[1] Foods were analyzed as purchased with the exception of pasta, which was cooked according to package directions

[2] Mean ± Standard Deviation

[3] Significantly different from analyzed values obtained via microbiological assay (Student's paired t-test; $P < 0.05$)

Table 3.4.4: Comparison of the analyzed food synthetic folic acid content to values reported in the Canadian Nutrient File (CNF)

Food Category[1]	n	Analyzed LCMS/MS Folic Acid[2] (µg/ 100g)	CNF Folic Acid[2] (µg/ 100g)	% of CNF Value Folic Acid[3]
Breads	14	149 (42)	85 (25)[4]	178 (60)
Rolls and Buns	14	147 (38)	80 (13)[4]	186 (50)
Cookies	15	112 (38)	46 (20)[4]	251 (89)
Crackers	10	157 (66)	65 (49)[4]	146 (161)
Cooked Pastas	7	79 (35)	75 (11)	108 (55)
Prepackaged Desserts	14	70 (31)	30 (11)[4]	236 (84)
Ready-to-eat Cereals	15	142 (52)	100 (35)[4]	155 (72)

[1] Foods were analyzed as purchased with the exception of pasta, which was cooked according to package directions

[2] Mean ± Standard Deviation

[3] Analyzed values as a % of the CNF Folic Acid Value

[4] Significantly different from analyzed values (Student's paired t-test; $P < 0.05$)

3.5 Discussion:

Results of this study indicate that twenty years after mandatory folic acid fortification of the food supply in Canada, a significant discrepancy exists between the analyzed total folate levels and those reported in the CNF and on product labels. The analyzed total folate content, determined by microbial assay, and folic acid values, assessment by LCMS/MS were on average 67% and 88% higher than those reported in the CNF, respectively. These data are consistent with the findings from our laboratory by Shakur et. al in 2009 who reported a 50% overage of analyzed total folate values compared to CNF values.[6] We acknowledge part of the discrepancy between CNF values and those determined by direct assessment reflects, in part, the CNF database is unbranded. For example, whereas in our direct analysis we analyzed the top 15 folic acid fortified breads as determined by point-of-purchase data, there is a single total folate and folic acid value for white bread. Further the CNF is modified to reflect different food fortification regulations in Canada.[63] It is comprised of data derived from the USDA Nutrient Database for Standard Reference; hence it is quite likely the actual folate content of foods manufactured and consumed in the U.S. differ from that in Canada.[33] Moreover, some foods available in the United States have different formulations than those in Canada, therefore may also contribute to the difference in folate content. The Canadian government calls for a mandatory minimum level of folic acid fortification in flour. However, unlike the regulations in the United States, there was no upper limit to these fortification levels which led to concerns of over-fortification.[54] Alongside this, there was speculation that this applied to other mandated fortified micronutrients (iron, thiamin, riboflavin, etc.), suggesting the food supply may also contain overages of these, resulting in intakes above the tolerable upper level. In April of 2012, the Government of Canada implemented a food program to assess the nutritional compliance of enriched flour in imported

and manufactured foods.[191] They assigned an acceptable tolerance level of 120 μg/100 g to 260 μg/100 g for folic acid in enriched foods and defined values 175% above the regulatory level as "unsatisfactory". Our findings illustrate the analyzed values in the categories of "Rolls and buns" and "Ready-to-eat cereals" were unsatisfactory as they were more than 175% over the product label values.

Another concern when looking at actual folate levels and the CNF is the inadequacy of analysis methods for folate used to create the nutrient databases. Over the past few years the Government of Canada has made efforts to improve the consistency of methods used to report nutrients in the CNF. Foods continue to be updated with values from the tri-enzyme digestion method or LCMS/MS on an on-going basis, however the database remains incomplete at this point.[62] The nutrient values of some foods such as those that fall under the category of "breads" and "ready-to-eat cereals" have been updated via analysis in a Canadian laboratory, whereas foods in categories such as "pastas" are either modeled after USDA data with no changes, modified statistically to reflect Canadian food, or calculated from the data other than the USDA which could cause there to be an underestimation of the amount of folate in the food supply.[63]Moreover, the CNF consists of unbranded foods and therefore does not provide product specific information to Canadian consumers. It combines different brands of similar products to produce an average amount of nutrient in a food, and not the exact nutrient composition in a specific food. Measures should be taken to ensure the CNF is up to date and accurately reflects what is present in the food supply.

Mean label values of folate in products were found to be lower than the analyzed total folate

values, which is consistent with the literature.[6] This may be due to unincorporated overages. Higher amounts of folic acid are often incorporated into products to ensure they do not degrade to levels less than those mandatory throughout the products' shelf life. The magnitude of the overages of the analyzed foods compared to the label claims, is similar to the effect size observed of the overages compared to the CNF values. This likely reflects that most manufacturers use CNF values to construct what is listed on the product label. All mean label values were significantly lower than analyzed values except for "cooked pastas" which was significantly higher. The label value for "cooked pastas" was on average 299 μg/100 g (Table 3.4.2). To obtain an accurate representation of the folic acid consumed in the Canadian diet, we analyzed pastas according to package instructions and prepared it as it would be consumed. However, the value reported on the product label speaks to the folate that would be present in raw and uncooked pasta. When cooked, the pasta water is discarded causing some of the folate to be lost. Therefore, the amounts present in cooked pasta are much less than those reported on the label. We observed a loss of approximately 74% of folate upon the cooking of the pastas analyzed, which is similar to the 70% loss observed with values of raw to cooked pasta reported in the CNF, as well as previous findings.[25,62] This may be a future point of improvement for pasta food product labels. In addition, although not represented in statistical analyses above, the whole-wheat pastas did contain trace amounts of both folate and folic acid. Similar to the overages seen in previous studies, these overages continue to exist in the food supply today.

LCMS/MS is an established method used for measurement of many different forms of folate, including the synthetic form, folic acid. When comparing values obtained using the two different methods (microbiological assay vs. LCMS/MS) we used to analyze foods, similar results were

obtained across all categories, except for “Rolls and buns” and “Crackers” ($p<0.05$). The “Rolls and buns” and “Crackers” categories had higher amounts of sodium in them as compared to the breads. This observed difference may be due to the sensitivity of LCMS/MS in detecting sodium, or possibly to sample components which may introduce matrix effects.[192] For example, the presence of small hydrophilic molecules such as sodium can cause ion suppression and therefore may have caused some folic acid to go undetected. To our knowledge, this study is the first to determine the synthetic folic acid content in the Canadian food supply since mandatory fortification in 1998.

There are a few limitations in the work presented in this thesis. The national food consumption data (FOODEX) used to identify folic acid containing food categories and ACNeilsen company data to identify brands, were those from 2001 and 2007 respectively, therefore may not be representative of the top categories and fortified foods consumed in Canada as of 2019. Thus, any new fortified foods now on the market that are being consumed frequently were not included, and any shifts that may have occurred in the dietary patterns of the Canadian population is likely not reflected. The samplings methods used in this study may also be a limitation. Multiple samples of the food products from different stores were not purchased or used in the analysis to account for batch effects. The entire portion of the product purchased was not homogenized in the sample analyzed, and may therefore not be representative of the whole food. Moreover, compared to the study by Shakur et al. which analyzed 92 fortified foods, 89 foods on the Canadian market were analyzed due to some products that are now unavailable or discontinued.

The strengths of this study include the use of two different methods (Microbiological assay and LCMS/MS) to determine the total folate and synthetic folic acid content in the foods, respectively. These two methods performed in line with one another, allowed for a greater understanding of the relationship between the different forms of folate consumed and their expected contributions in the diet. Moreover, the use of a suitable fortified breakfast cereal control material as a standard alongside the foods analyzed allowed for a more accurate comparison of the folate form of interest.

The mandatory folic acid fortification of the food supply has been a public health success and has resulted in an approximately 50% reduction in NTDs in Canada.[3] According to the 2017 Perinatal health indicators for Canada report, in 2014 the prevalence of congenital anomalies increased to 430.5 per 10,000 total births across Canada (excluding Quebec).[193] Similarly, there was increase in the prevalence of NTD's in 2014 to 5.7 cases per 10,000 total births, in comparison to the reported 4.4 cases per 10,000 total births in 2013. This recent report speculating a possible increase in the prevalence of NTD's has left researchers questioning where this problem stems from. With the rise in folic acid in the food supply and lack of folate deficiency in Canada, this may not be the likely cause of this observed increase. Overages still appear to exist as they did in the first 10 years post-fortification. It is possible there may be other contributing factors to the prevalence of NTD's that may have previously been overlooked.

Despite this clear discrepancy in results, it is still up for debate whether folate overages are really a problem. In 2011, Colapinto et al. analyzed the red blood cell (RBC) folate concentrations of the general Canadian population and displayed folate deficiency in Canada is virtually non-

existent.[5] Their findings indicated almost 100% of Canadians met or exceeded the established cut-off for RBC folate deficiency (<305 nmol/L), and that 40% of the population had high RBC folate concentrations (>1360 nmol/L). The incidence for NTD's is low, and approximately 88% of women remain protected meeting the cut off (>906 nmol/L) optimal for minimizing risk of NTD's.[5,67]

However, data collected in 2017 from Statistics Canada suggests males and females aged 20 to 79 have RBC folate concentrations of 1407 nmol/L which not only classifies as high, but is also just under the clinically defined upper limit of the normal range (1422 nmol/L).[68,69] Moreover, a growing body of literature suggests adverse outcomes of a supraphysiological folate status. A high folic acid intake has been associated with a masking of Vitamin B12 deficiency in adults, changes in immune function, and introduces a higher risk of carcinogenesis leading to an increased risk of prostate cancer and changes in methylation epigenetic programming.[161–164] The belief is that existing neoplastic lesions may grow more quickly in the presence of high folate levels.[118–120,169] This research has been inconclusive and further suggests there due to the variability there may be a genetic influence in adverse outcomes associated with increased folate intake.[130,194]

Upon these findings, concerns of mass medication have also risen. Should flour consumed by the entire population be fortified for the benefit of a few? Is it necessary to supplement the diet of all adults with fortified folic acid in flour, instead of focusing solely on women of child-bearing age and pregnant women? Countries such as Ireland in the UK, have delayed imposing mandatory folic acid fortification due to the potential role folic acid may play in increasing the risk of

colorectal cancer.[131,195] Assessments of voluntary folic acid food fortification, RBC and serum folate levels within population sub-groups, and up to date information on the prevalence of pregnancies affected by NTD's were all used to estimate folic acid requirements.[196] Upon this research, it was decided folic acid fortification was not required in the country at the time and thus postponed. Therefore, continuing to monitor the actual amount of folate in the food supply to help inform total folate intake is necessary to influence public policy and guidelines regarding folic acid consumption.

The 2007 Canada Food Guide recommended 50% of grain products consumed be whole grain, leaving 50% of nutrition to come from white wheat flour. For this reason, the use of food modelling for micronutrients has been called into question. Some microorganisms in the colon produce folate.[110] This further suggests, dietary intakes alone should not be used to estimate usual intakes with regards to micronutrients.

The results of this study can help inform the answers to questions regarding public health. For example, should we alter the level of fortification of the food supply? Should we alter the amount of folic acid in supplements? Which populations are at an increased risk of high folate intakes? These overages, in addition to the various input sources of folate nutrition mentioned previously may be a cause for concern. The use of folic acid supplementation in addition to the diet should consider the nutritional condition and genetic background of the individual. For example, those that have a nutritional deficiency due to anemia or a genetic mutation such as the MTHFR C677T polymorphism should avoid consuming additional folic acid from supplements. Moving forward, we recommend the practice of folic acid fortification and supplementation

adopted by a country be cohort specific, for example recommendations specific to pregnant women or those at risk of folate deficiency as opposed to the entire adult population. Countries that have already adopted this practice should be wary and continue to monitor RBC folate levels along with folate dietary intake to determine a safe level of fortification for their population.[197]

A significant difference and persistent inconsistency observed between expected and actual values upon mandatory folic acid fortification is a cause for change in dietary recommendations and updating public policy. Although the benefits of folate are well-known, it is necessary that all input sources of folate nutrition be taken into account when estimating the current folate status of the Canadian population. The 2015 Canadian Health Measures Survey found approximately 79% of people surveyed consumed folic acid through a supplement.[198] Based on our findings, it is likely that the overages of folic acid in our food supply, in combination with supplemental folic acid consumed in multivitamins, along with natural dietary sources of folate, and the folate produced by the colonic microbiota, our general population is approaching an overall higher folate intake that goes well beyond our requirements.

We recommend reconsidering the use of folate on product labels, along with the use of unbranded foods to comprise the CNF database. These numbers can often be misleading and call into question the use of dietary data to estimate folate intake of the population. Furthermore, it may be beneficial to define an upper limit to fortification guidelines in Canada to combat the over fortification of foods to meet product shelf-life. Improving quality control and the testing of the nutrients present in the foods may also be a noteworthy point in industry.

In conclusion, our findings indicate actual folate and folic acid levels continue to be higher than mandated levels 20 years' post fortification. This varies by category. Further monitoring of nutrient content in fortified foods is required to establish a balance between the risks and benefits of folate when informing guidelines.

Chapter 4

4.0 STUDY 2: Factors Affecting Colonic Folate Absorption and Metabolism in Humans

4.1 Abstract:

It is generally believed that dietary sources of folate are primarily absorbed in the small intestine. However, recent evidence from our team and others suggests that the colon may play a more significant role in the absorption of dietary folate than previously understood. The aim of this study is to assess how folic acid supplementation influences colonic folate absorption and metabolism in healthy adults. This will be accomplished by randomizing 24 participants to receive a multivitamin supplement containing either 0 or 400 μg folic acid. In order to ensure that two distinct groups are produced at the end of the 16-week intervention in terms of folate status and exposure to folic acid, bread and pasta containing low levels of synthetic folic acid will be provided and subjects will be instructed on how to avoid folic acid fortified foods. The expression of two major folate transporters (PCFT and RFC) hypothesized to be responsible for folate absorption in the colon of healthy adults will be assessed as well as the expression of certain folate hydrolases believed to be responsible for converting naturally occurring folate to its bioavailable form. As part of my MSc program, I took the lead in setting up and launching this research project. The project is on-going. Herein, I provide the study protocol and blood folate results from the first 8 participants (4 in each group) to verify the feasibility of the research protocol and that the feeding and supplement intervention successfully produced two distinct groups in terms of folate status. This work will lead to a deeper understanding of colonic folate absorption and metabolism, resulting in more appropriate dietary and supplemental folate recommendations.

4.2 Introduction:

The term folate is a generic descriptor for a family of compounds, including synthetic folic acid, that share a pteroylglutamic acid core and whose primary biological role is the transfer of one-carbon units.[1] Folate is known for its function as a co-factor in nucleotide synthesis – necessary for DNA and RNA biosynthesis and repair – and for the re-methylation of homocysteine to produce the amino acid methionine.

Mammals cannot synthesize folate, hence an exogenous source is required.[1] In 1998, Canada introduced a mandatory folic acid supplementation program that required all white wheat flour products to be fortified with 150 μg folic acid per 100 g of flour in order to reduce the risk of folate dependent neural tube defects during pregnancy.[54] Presently, Canadians consume the majority of their dietary folate from fruits, orange juice and vegetables – which contain natural sources of folate – but also from breads, rolls and pasta which contain folic acid added as a fortificant.[53,199–201] In the past it was assumed that dietary or supplemental sources of folate were the only contributors to the input-side of folate homeostasis. However our research program has changed this understanding, demonstrating that folate absorption and metabolism in the colon, specifically microbial folate biosynthesis, likely plays a role in meeting the folate requirements of mammals, including humans.[8,202,203]

4.2.1 Absorption of folate across the small intestine

Most naturally occurring dietary folates possess a negatively charged polyglutamate side chain. In order for absorption to occur, the side chain must be removed, yielding a monoglutamylated folate. The enzymatic deconjugation of polyglutamylated folate to bioavailable

monoglutamylated folate occurs at a pH optimum of roughly 6-7.[86] In humans and pigs, it is thought that this reaction is primarily facilitated by the enzyme GCPII (EC number 3.4.17.21; pH optimum 6.5) known to be primarily located in the brush border of the jejunum, a section of the small intestine.[7] γ-Glutamyl hydrolase is also found in human tissues that store folate, including hepatocytes, and is crucial for folate export from cells.[7,88] γ-Glutamyl hydrolases has also been identified in human intestinal tissue, however the enzyme is located intracellularly and within lysosomes, as opposed to being part of the intestinal brush border.[7,88] Importantly, it remains unclear to what degree GCPII and γ-glutamyl hydrolases are present in the human colon. Consequently, the extent to which both enzymes are involved in the deconjugation of folate in the human colon also remains to be determined.

Following deconjugation, it is generally believed that monoglutamylated folates are predominantly absorbed in the small intestine by one of two transporters, PCFT or RFC. RFC (encoded by the gene *SLC19A1*, pH optimum 7.4) is a facilitative carrier found in most cells in the body, including the apical brush-border membrane of the small intestine.[97]

4.2.2 Folate absorption across the colon

Much less work has been done to date regarding the mechanisms of folate absorption in the colon. Recent research from our group has demonstrated that monoglutamylated forms of folate are absorbed across the colon in humans, and it is estimated that >50% of colonic folate is monoglutamylated.[8,117] It is known that both RFC and PCFT are expressed in the cecum and colon of animals, albeit at much lower concentrations than in the proximal small intestine.[204–206]

Sources of monoglutamylated folate in the colonic lumen include folate synthesized by bacteria and secreted in the lumen, 5-methylfolate lost during enterohepatic circulation, and synthetic sources of folic acid consumed from supplements and fortified foods. In the past it was generally believed that most, if not all, synthetic folic acid was absorbed in the small intestine. However, to the best of our knowledge this has not been assessed.[1] Monoglutamylated folate could be a relevant source of colonic folate given the dramatic increase in consumption of synthetic folic acid from supplements and folic acid fortification of the food supply.[5]

It is less clear if colonic polyglutamylated folates that reside in the colon as a result of bacterial cell death, sloughing of cells from colonic tissue or not absorbed in the small intestine can be converted to the absorbable monoglutamylated form. Indeed GCPII transcripts are present in the colon, however they are found at a much lower concentrations than in the small intestine and it is known that bacteria themselves contain folate conjugase, usually at low levels.[87,207] This opens up the possibility that a portion of polyglutamylated folates may be deconjugated and subsequently absorbed in the colon; however this remains to be confirmed.

There are a number of limitations surrounding the available data regarding colonic folate absorption in humans. Although it is known that both RFC and PCFT are expressed in human colonocytes, it is not known to what degree these specific transporters are present in the colon of healthy humans. To date, the majority of studies conducted identifying the presence of RFC and PCFT in colonocytes have been performed using cell lines or tissue harvested from organ donors.[11,97,208] To our knowledge, no study has investigated the mRNA and protein concentrations

of both RFC and PCFT present in human colonic tissue retrieved as biopsies. It also remains to be determined if folate status and availability of folic acid influences RFC and PCFT expression at the level of the colon. Ashokkumar et al. noted a reduction in [^{3}H]-folic acid uptake by Caco-2 cells in media designed to mimic folate over-supplementation.[209] This reduction was associated with a decline in RFC protein and in mRNA levels of RFC and PCFT.

Secondly, it remains unclear to what degree GCPII and γ-glutamyl hydrolase are present in colonic mucosa. Due to the fact that a proportion of colonic folates are polyglutamylated, it is of interest to determine if GCPII and γ-glutamyl hydrolase may be responsible for the necessary deconjugation required to yield an absorbable form of folate.

The overall aim of this research study is to develop a more thorough understanding of the fundamental processes involved in folate absorption in the colon of humans. To accomplish this, three objectives are proposed:

1) Evaluate the impact of 0 and 400 μg supplemental folic acid on the regulation of proton coupled folate transporter (PCFT) and reduced folate carrier (RFC) in the ileum and colon by assessing the expression of mRNA transcripts and proteins.
2) Determine the degree to which two intestinal folate hydrolases, glutamate carboxypeptidase II (GCPII) and γ-glutamyl hydrolase, are present in the lumen of the ileum and colon by quantifying enzyme activity and expression of mRNA transcripts and proteins.
3) Evaluate the impact of 0 and 400 μg supplemental folic acid on total folate concentrations within the colonic mucosa by measuring folate levels.

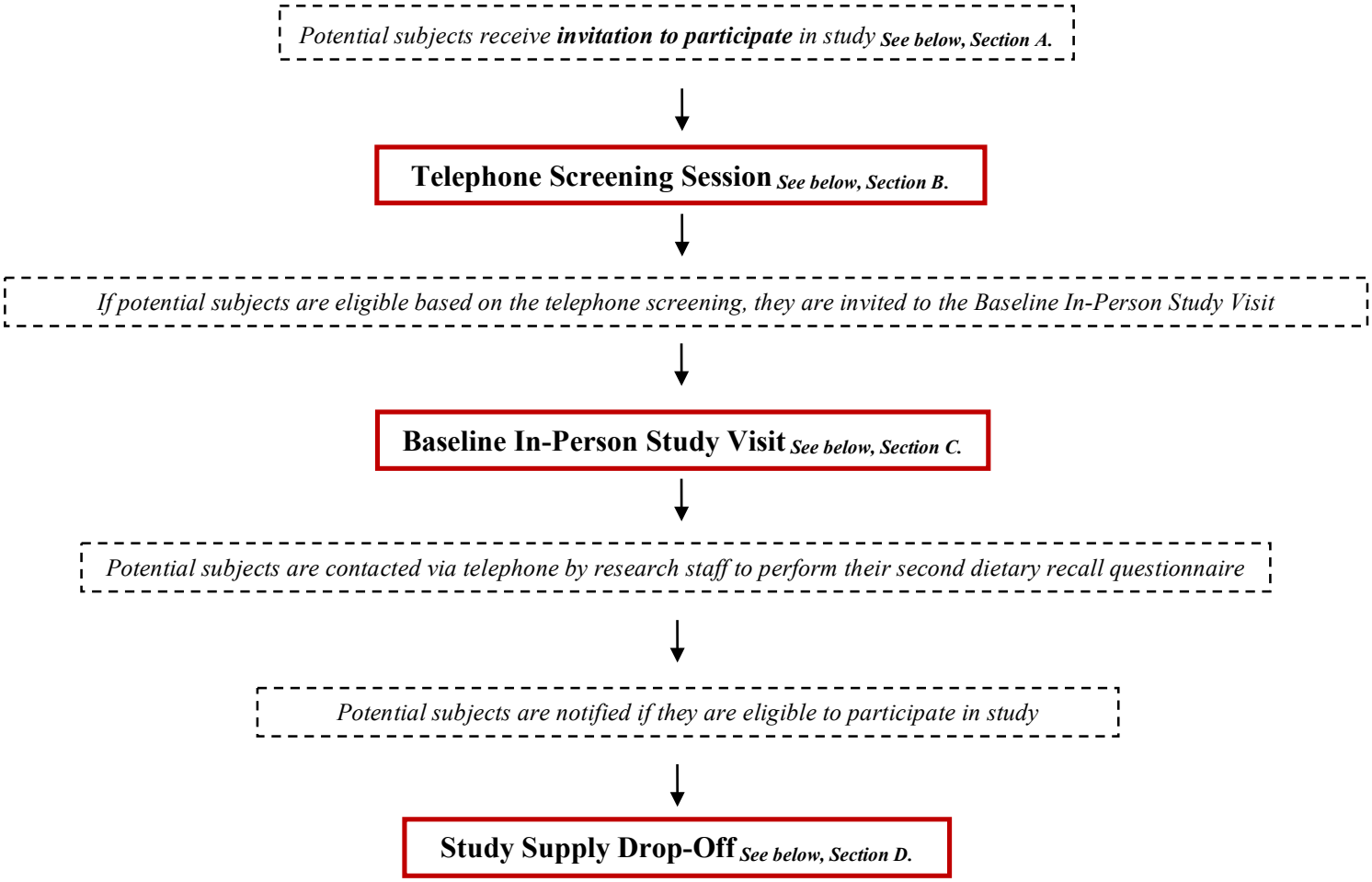
The current research study is on-going. I took the lead in setting up and launching this research project. The purpose of this data chapter is to describe our preliminary findings regarding feasibility of the study protocol and determine whether we could successfully produce two distinct groups with regard to folate status with the proposed feeding and supplement intervention.

4.3 Subjects and Methods:

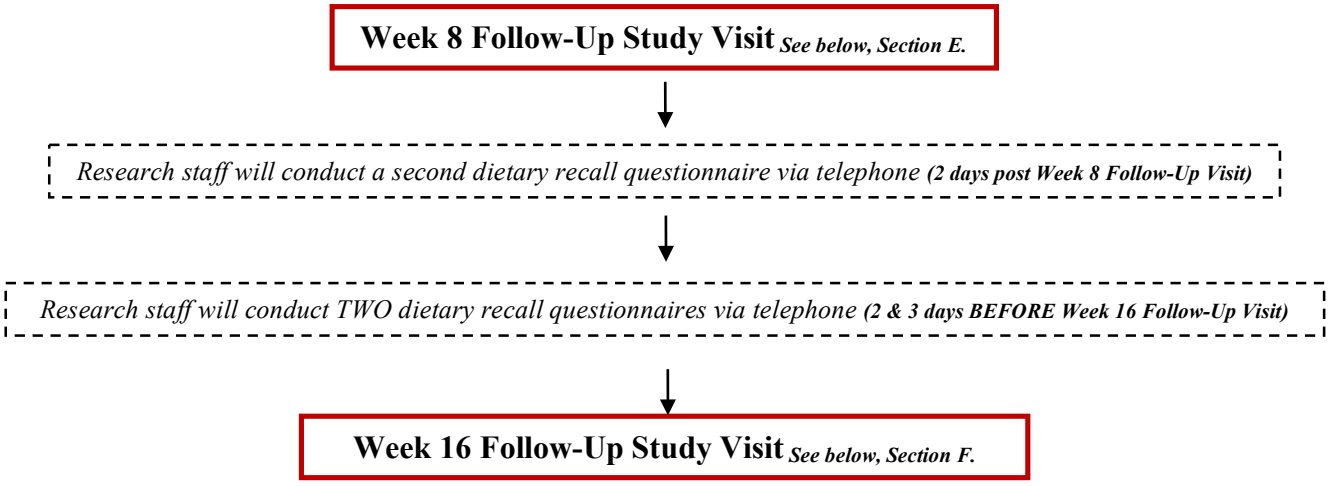
To address the study objectives, a 16-week longitudinal open-labelled randomized clinical study is being conducted. Recruitment and screening require three separate steps comprised of an initial invitation to participate, followed by a Telephone Screening Session and finally a Baseline In-Person Study Visit as detailed in Figure 4.3.1. Healthy adults were recruited from colonoscopy waiting lists in the Division of Gastroenterology at St. Michael's Hospital. If the patient expressed interest in the study and agreed to be contacted, a member of the research study personnel contacted them to provide further information regarding the study, extended an invitation to participate and complete a Telephone Screening Session.

Figure 4.3.1 – Depiction of timeline for subject recruitment and participation.

A) <u>Recruitment, Screening and Supply Drop-Off</u>



B) <u>Follow-Up Study Visits</u>



Telephone Screening Session and Inclusion Exclusion Criteria

If patients express interest in participating in the study, they were screened over the telephone to determine eligibility. Telephone screening was conducted by research staff from St. Michael's Hospital using the telephone-screening questionnaire (**Appendix A**) that includes questions relating to the inclusion and exclusion criteria outlined below. Documents to be utilized to explain study information, including an information letter and study consent form (**Appendices B & C**), were sent to potential subjects via e-mail for review upon request.

Inclusion criteria

Patients scheduled to have a screening colonoscopy were considered eligible to participate if they met all of the following criteria:

1) Males ≥18 years old and ≤75 years old;

2) Females who are pre-menopausal that have had a hysterectomy or tubal ligation, post-menopausal (at least 1 year) and ≤75 years old;

3) Describe themselves as generally healthy.

4) Recommended to have a colonoscopy examination by their Doctor

Exclusion criteria

Potential subjects were considered ineligible if they met one or more of the following criteria:

1) They have a history of gastrointestinal disease (such as Crohn's disease, ulcerative colitis, celiac disease) and/ or gastrointestinal cancers;

2) They have had a previous colon resection;

3) They are regularly using medications that may affect gastrointestinal pH or folate metabolism (e.g. proton pump inhibitors, phenytoin, sulfasalazine, phenobarbital, primidone, or have used oral antibiotics within the last 2 weeks);
4) On a regular basis they consume >2 alcoholic drinks/day for women or >3/day for men;
5) They are currently smokers;
6) They are folic acid supplement users or have used folic acid supplements or multivitamins containing folic acid in the last 4 months;
7) They have a bleeding disorder (such as hemophilia)
8) They are unlikely able to discontinue anti-coagulant therapy prior to colonoscopy

Baseline In-Person Study Visit

Participants who are deemed as potentially eligible based on the initial Telephone Screening Session were invited for a Baseline In-Person Study Visit at the Division of Gastroenterology at St. Michael's Hospital. The purpose of the Baseline In-Person Study Visit is to further explain the study, obtain written informed consent, collect a blood sample and conduct a 24-hour dietary recall. This visit begins with a detailed explanation of the study delivered by research staff or graduate students, followed by a period where patients may ask any questions they may have. Written informed consent to participate in the study is obtained from each patient (see **Appendix C** for study consent form).

At the Baseline In-Person Study Visit, a 24-hour dietary recall using the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool, Canadian version (2016) was conducted by a trained research assistant. Participants were also informed that a second dietary

recall would be performed over the phone sometime within the following week, and an appointment was scheduled. Together, these two 24 hour recalls were used to approximate typical daily nutrient intakes. The ASA24 is a web-based program based on the Automated Multiple-Pass Method 24-hour recall method developed by the United States Department of Agriculture.[210,211] Anthropometric measurements (height, weight, waist, hip, Body Mass Index) were also collected according to standardized procedures.[212]

Upon completion of the 24-hour dietary recalls, research nurses from Division of Gastroenterology draw a venous blood sample. These samples are then analyzed to determine plasma and red blood cell (RBC) folate concentrations, complete blood count (CBC) and to genotype the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C→T variant.

Attendees of the Baseline In-Person Study Visit were invited to participate in the intervention phase of the study except in following situations:

1) Their 24-hour dietary recall indicate extreme dietary irregularities or under-reporting (e.g. they consume <1000 kcal/day);
2) They have baseline RBC folate levels <306 nmol/L or >2150 nmol/L;
3) They have a hemoglobin level of <100 g/L or demonstrate another clinical relevant abnormal result from the CBC;
4) They are homozygous for the C- to- T677 substitution (i.e. TT genotype) in the MTHFR gene. *N.B.* This single nucleotide polymorphism (SNP) is believed to influence MTHFR activity and blood folate values in individuals with low folate intakes.
5) They have a serum vitamin B12 level of ≤148 pmol/L

Intervention

Once eligibility to participate in the intervention was confirmed, subjects were randomized to either consume a 400 µg folic acid supplement (Folic Acid, Life Brand) or not, using a computer-generated randomization schedule stratified by gender. All participants, regardless of treatment arm, were instructed to consume an adult multivitamin containing no folic acid (Adult Multivitamins, Life Brand). All participants were instructed to follow a low folic acid-fortified diet for the duration of the 16-week intervention. The 16-week intervention allows for a complete turnover of RBC's. In an attempt to limit dietary intake of folic acid from fortified sources, participants were provided with bread and pasta containing no or low levels of added synthetic folic acid as determined in our laboratory by direct assessment. Participants were also instructed to limit their intake of products such as cookies, cakes, doughnuts, biscuits and rolls as these products have been found to be among the primary sources of fortified folic acid among Canadians.[53,199] Subjects either opted to pick study supplies (e.g. supplements, bread, pasta) up from St. Michael's Hospital or requested the supplies be delivered to their home. Participants were provided with sufficient supplies to last until the 8-Week Follow-up Study Visit.

8-Week Follow-Up Study Visit

Participants were asked to return to St. Michael's Hospital at 8 weeks (± 3 days) to complete another 24-hour dietary recall, collect additional study supplies and provide a blood sample. Participants were advised that a second 24-hour dietary recall would be performed over the phone two days later, and a time-slot was scheduled. Like the Baseline In-Person Study Visit, both 24-hour recalls were administered by a trained study team member and performed using ASA24.

At this study visit, participants were provided with a second 8-week supply of low folic acid bread and pasta and study supplements to last until the end of the intervention. Lastly, participants were asked to provide a 6 mL venous blood sample, collected by research nurses, for assessment of plasma and red blood cell folate concentration, to confirm response to the intervention.

16-Week Follow-up Study Visit

The 16-Week Follow-up Study Visit was scheduled at the end of the 16-week intervention (± 3 days). Prior to this visit, two 24 hour dietary recalls were performed on 2 and 3 days prior to the colonoscopy. At the 16-week study visit, a colonoscopy was performed. Participants were asked to adhere to the colonoscopy preparatory instructions as outlined by the Division of Gastroenterology at St. Michael's Hospital (see **Appendix D** for preparatory instructions). At this visit, research nurses collected a final 6 mL venous blood sample from participants along with a final measurement of the participant's weight.

The colonoscopy was carried out in the usual manner. Tissue biopsies were collected during the standard colonoscopy. Three small samples of 15 mg (total of 45 mg) were collected from each of the: 1) terminal ileum; 2) cecum; 3) ascending colon; 4) descending colon. Tissue biopsies were cleaned immediately with a cold phosphate-buffered saline solution. Samples were then placed in Eppendorf Safe-Lock tubes and snap frozen in dry ice. Tissue samples were transported to Peter Gilgan Centre for Research and Learning (PGCRL) at The Hospital for Sick Children and placed in the -80ºC freezer until further analyses.

Laboratory Methods

Assessment of screening and outcome measures from blood and tissue samples was performed at various laboratories in downtown Toronto. Complete blood counts and serum vitamin B12 analyses are being performed at the Diagnostic Laboratories at St. Michael's Hospital. Plasma and RBC folate levels are being analyzed in Dr. O'Connor's laboratory located in the Peter Gilgan Centre for Research and Learning (PGCRL) at The Hospital for Sick Children. The blood samples collected for plasma and red blood cell folate analyses were collected into EDTA-treated tubes, shielded in aluminum foil, and processed and frozen within 2 hours of blood draw. 100 μL aliquots of whole blood samples for each participant were diluted 10-fold with ascorbic acid and deionized water (1%wt:vol). In order for pteroylpolyglutamate hydrolase (EC 3.4.19.9) to convert the polyglutamated folates to their assayable form, samples were further incubated at 37 °C for 30 min. The remaining whole blood was spun in a centrifuge (1500 X g for 20 min at 4 °C). Plasma obtained upon centrifugation was collected and stored with the addition of sodium ascorbate (1% wt:vol) to prevent any oxidation of the folate. All samples were stored at -80 °C until analyzed. Total folate content was determined via the standard microbiological assay method using the *Lactobacillus rhamnosus* (ATCC No. 7469; American Type Tissue Culture Collection, Manassas, VA) test organism and 5-methyltetrahydrofolate to generate the standard curve.[189] Plasma and whole blood samples were transferred into a 96-well plate at three different dilutions. Duplicate plates were produced and analyzed. The reproducibility and accuracy of the assays were assessed using a frozen human plasma folate standard reference material with a certified value (30.6 nmol/L Frozen Human Plasma, SRM 1950, National Institute of Standards and Technology, Gaithersburg, MD). Our analysis of the human plasma control standard yielded a total plasma folate content of 29.3 + 2.7 nmol/L, with a CV of 9.1%. To determine RBC folate,

plasma folate was subtracted from whole blood folate and a correction for hematocrit was performed.

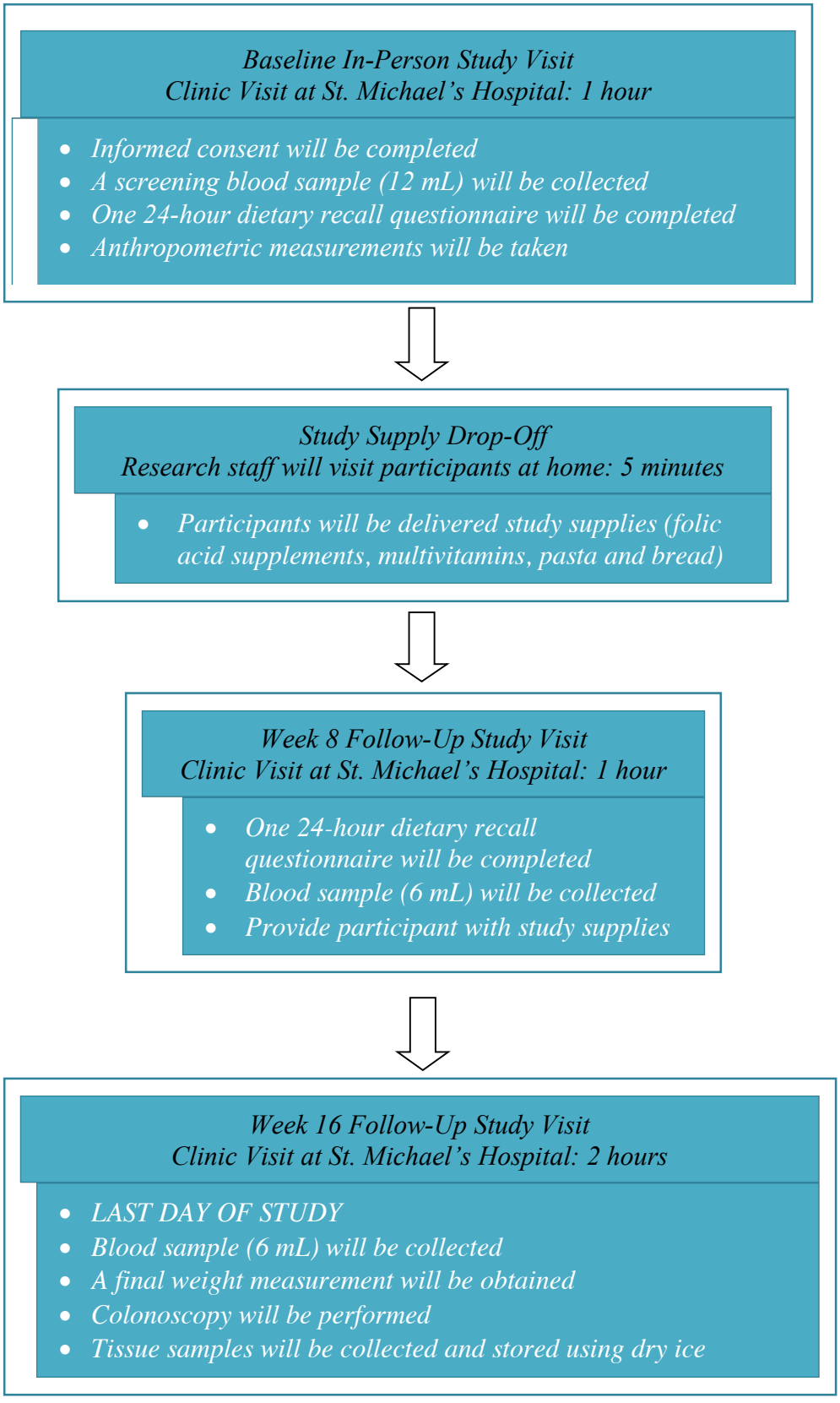
1) The presence of the MTHFR C677T variant was determined at the The Centre for Applies Genomics TCAG Facilities. The Hospital for Sick Children. Genotyping was performed using TaqMan™ , a method involving PCR of the region surrounding the SNP, followed by hybridization of fluorescent probes that are complementary to the two versions of the SNP.[213]

2) Quantification of mRNA transcripts and proteins of PCFT, RFC, GCPII and γ-glutamyl hydrolases will be completed in Dr. Bendayan's laboratory at the Leslie Dan Pharmacy Building at the University of Toronto. Transcripts and proteins will be determined according to methods described previously.[206,214,215] Semi-quantitative real-time PCR (qRT-PCR) will be performed using the ViiA-7 Real Time PCR System (Applied Biosystems). RPL32 will be used as the housekeeping gene. Primers sequences will be designed and verified for specificity by using the Basic Local Alignment Search Tool (BLAST). For Western Blot analysis, protein samples will be extracted using RIPA lysis and extraction buffer. Blots will be quantified using Quantity One software (Bio Rad).

3) GCPII and γ-glutamyl hydrolase activity will be assessed by research staff in Dr. Bendayan's laboratory at the Leslie Dan Pharmacy Building at the University of Toronto. Enzyme activity will be determined according to the method created by Krumdieck and Baugh with modification.[216] Analyses will be performed at a pH of 6.5 in order to inhibit intracellular folate hydrolase.

4) Colonic mucosa folate content will be determined by microbial assay by research staff in Dr. O'Connor's laboratory at the Peter Gilgan Centre for Research and Learning (PGCRL) at The Hospital for Sick Children.[8]

Figure 4.3.2 Study visit diagram



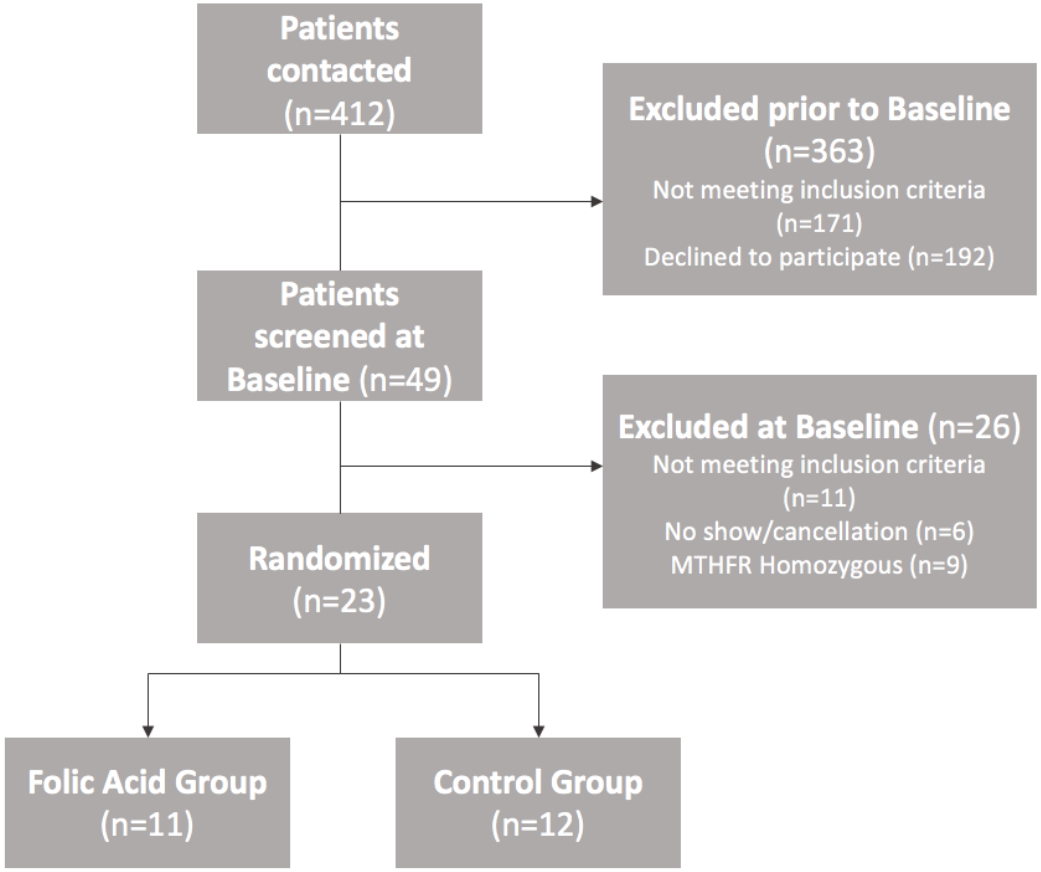
Sample Size and Statistical Analyses

While the primary outcome measures of this proposed study is the expression of folate transporter mRNA transcripts and proteins in colonocytes, there is insufficient data in the literature relating to these outcomes on which to base a sample size calculation. Therefore, a calculation was performed based on available RBC folate concentration data and previously observed standard deviations. Previous studies were considered that examined supplementation with 400 μg of folic acid per day in healthy adults over a period of 12 weeks – a design similar to this proposed study.[217–219] A sample size of 12 in each group (N=24) would provide 80% power to detect a 1.5 SD difference in RBC folate levels between study groups assuming an α=0.05 and attrition of 4 subjects per group.[125] It was anticipated that a total of 48 patients will be screened via telephone and 35 patients will the screened in-person before a final sample size of 24 is reached.

Descriptive statistics were generated for all variables of interest using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC). Frequency and percent will be calculated for categorical variables whereas mean, standard deviation, median and interquartile range (IQR) will be determined for continuous variables. In terms of comparative statistics, continuous data measured at a single visit will be analyzed using t-tests and categorical variables by Chi-square or Fisher's Exact tests as appropriate. Outcome variables assessed at multiple times will be assessed using repeated measures linear regression statistics. The Mann Whitney U test/Wilcoxon RankSum Test was performed for two sample mean comparisons of continuous variables at each time point. A p value of <0.05 indicated significance.

4.4 Results

Figure 4.4.1 Progress of participants through each stage of the study as of November 15, 2019



Screening and enrollment into the study occurred between January 2019 and December 2019. Figure 4.4.1 illustrates the progress of participants through the study as of November 2019. A total of 412 patients were contacted via telephone to partake in the study. Of these patients, 363 were excluded after the Telephone Screening Session as they either did not meet the inclusion criteria, or after learning about the study declined. 49 patients were further screened during the Baseline In-Person Study Visit, after which 26 were excluded due to later discovery of not meeting the inclusion criteria, no showing up for the visit, or genotyping results revealing the presence of the MTHFR C677T homozygous mutation. As of November 2019, a total of 23

participants are enrolled within the study. 18 participants have completed the 8-week study visit, and 15 participants have completed the 16-week colonoscopy. We examined the dietary folate intake and blood folate values for the first 4 participants that completed the intervention in each group.

Of the first 8 participants that completed the intervention, they were on average 63 years of age with an average BMI of 26. The subject baseline characteristics are summarized in Table 4.4.2. Subject characteristics, including age, BMI, dietary folate intake, and RBC and Plasma folate concentration, were not significantly different between the two groups at baseline (Table 4.4.3).

Table 4.4.2 Folic acid and control group at baseline in-person study visit

Characteristic	**Folic Acid Group (n=4)**	**Control Group (n=4)**
Gender		
Male	1	2
Female	3	2
Age	61 (6)	66 (4)
Height (cm)	162 (5)	165 (11)
Weight (kg)	68 (10)	70 (5)
BMI	26 (4)	26 (3)
RBC Folate (nmol/L)	1086 (187)	1112 (183)
Plasma Folate (nmol/L)	23 (10)	35 (15)
Hb (g/L)	138 (10)	145 (6)
Serum Vitamin B12 (pmol/L)	305 (145)	289 (116)

Mean ± SD of participant characteristics at Baseline In-Person study visit by treatment group. No statistically significant differences were found between treatment groups.

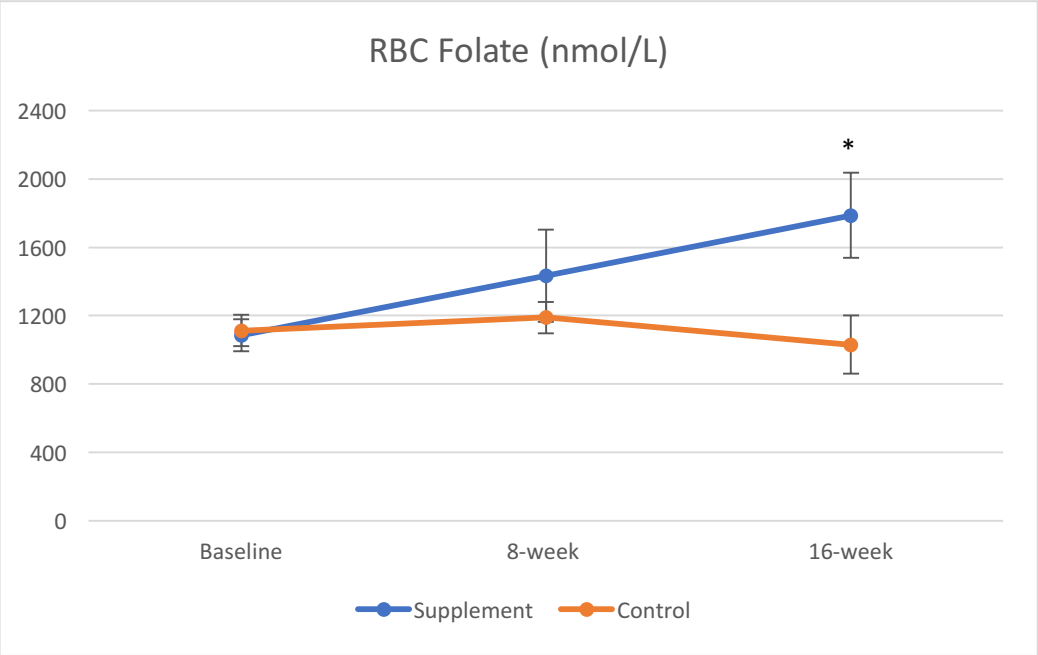
Table 4.4.3 Daily dietary and supplemental intakes of participants (n=8) across the intervention period

	Baseline	8-week	16-week
Total Caloric Intake (kcal)			
Supplement	2576 (1198)	1911 (384)	1734 (585)
Control	2049 (602)	1838 (883)	2182 (979)
Protein (g)			
Supplement	73 (22)	74 (23)	63 (18)
Control	87 (30)	92 (53)	90 (52)
Carbohydrate (g)			
Supplement	316 (176)	248 (65)	198 (77)
Control	198 (74)	205 (123)	188 (61)
Fat (g)			
Supplement	119 (58)	68 (15)	69 (32)
Control	89 (29)	68 (39)	92 (46)
Folate (DFE)			
Supplement	493 (277)	1160 (464) *	1110 (611) *
Control	379 (120)	383 (210)	393 (248)

Mean ± SD values are reported above
* Significantly different from control group ($p<0.05$)

Participants completed a total of 6 dietary recalls were during the course of the study. The values reported in Table 4.4.4 are the averages of two dietary recalls conducted within one week of the study visit. Values for total caloric intake, protein, carbohydrate, fat and folate were collected in both groups at three time points. The folate dietary intake (DFE) is reflective of total folate dietary intake, including the use of folic acid supplements. Mean total caloric intake, protein, carbohydrate, and fat were not significantly different between groups at each respective time point. The average dietary folate intakes prior to intervention did not differ significantly between

groups. Folate (DFE) intake in the supplement group was significantly higher than the control at 8-weeks and 16-weeks of the intervention ($p<0.05$).



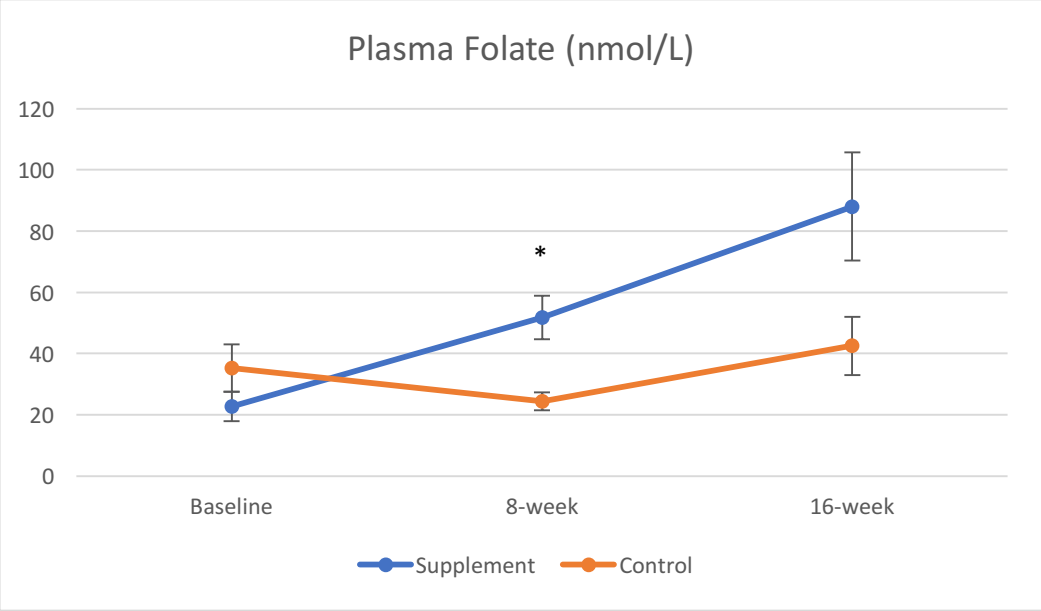


Figure 4.4.4 Mean ± SEM red blood cell and plasma folate concentrations of subjects during the 16-week intervention period.

Due to small sample size of 4 participants in each group, repeated regression linear statistics were not performed to determine a trend in outcome variables across time. Using pairwise comparisons, we found no statistically significant differences in RBC or Plasma folate levels between groups at Baseline. RBC folate levels were significantly higher in the supplement group compared to the control group at 16-weeks ($p<0.05$). Plasma folate levels were significantly higher in the supplement group compared to the control group at 8-weeks ($p<0.05$). A proportional increase is seen with both RBC and Plasma folate and supplement use.

Percent compliance for the folic acid supplement group taking both multivitamins and folic acid supplements at the 8-week visit mark was 85% and 101%, respectively. Percent compliance for the control group taking only multivitamins at this time was 89%. Similarly, at 16-weeks, the percent compliance for the folic acid supplement group taking multivitamins and folic acid supplements was 79% and 93%, respectively.

4.5 Discussion:

Results from this preliminary analysis suggest participants were very successful at adhering to the dietary and supplement intervention of the present study. None of the participants who commenced the intervention as of January 2019 have dropped out of the study. Upon analysis, our findings indicate two distinct groups of RBC folate levels have formed in the 16-week period intervention. While we acknowledge that we did not see a statistically significant difference in plasma folate concentration at 16 weeks, we attribute this to the small sample size. (Figure 4.4.5.).

Our blood folate findings reflect excellent adherence of participants to consuming the supplements as randomized. Among the four subjects randomized to receive the folic acid supplement, pill counts at the 8-week and 16-week visits indicate they consumed on average 97% of the folic acid supplements provided. This numbers speaks to the success in the adherence of participants to the feeding and supplement interventions. Further, the 24-hour recall data show two distinct groups were produced in terms of folate intake, but not other nutrients.

The Recommended Dietary Allowance of Folate for adults is set to 400 µg per day.[1] 1 μg of folic acid consumed as a supplement is equivalent to 2.0 µg DFE. This is reflected in the dietary folate intake values of the supplement group at 8-weeks and 16-weeks, which average to 1160 and 1110 DFE, respectively. Folate intake levels from the folic acid supplement consumed daily in the treatment group are expected to be 800 µg of DFE. This accounts for approximately 200 µg of DFE in the supplement group diet that is coming from other sources of folate input nutrition. The mean ± SD dietary folate intake of the control group at the 16-week mark was 393 (248) DFE. This value was similar to that found in Canadian men and women of the same age of 445 (139) and 378 (103) DFE, respectively.[220] The dietary folate intake of our supplement group at 16-weeks, 1110 (611) DFE, was also comparable to this cohort of Canadian men and women of 1654 (767) and 1589 (1014) DFE, respectively.[220]

According to the 2017 statistics Canada report, the range of RBC folate levels for adults aged 20 to 79 was 1,407 nmol/L 95% CI [1,258, 1,556].[69] This value is similar to that observed in individuals of the same age range in our folic acid supplement group at the end of the intervention. The mean RBC folate of the participants in the folic acid supplement group at 16-

weeks was above the upper limit of this range by approximately 27% at 1787± 496 nmol/L. Despite all participants adhering to a low synthetic folic acid diet for the duration of the intervention, these higher levels suggest there are other sources of folate contributing to the high folate status of Canadians. This could include folate from natural sources, fortified foods, and folate produced from colonic microbiota. Further monitoring of these potential sources is required to gain a deeper understanding of the contributing factors of folate status and inform guidelines for dietary and supplemental folate intake.

The RBC folate values of the 400 µg folic acid supplement group at 16-weeks were 1.7x higher compared to that of the control group. Similarly, the plasma folate values of the 400 µg folic acid supplement group at 16-weeks were 2.1x higher compared to that of the control group. This is consistent with the effect size observed in previous studies examining a 400 µg supplementation of folic acid per day in healthy adults over a 12-week.[217–219]

RBC folate levels of both groups at baseline were within normal range (317-1422 nmol/L) prior to the start of the intervention (Table 4.4.2).[68] These participants were not consuming multivitamins containing folic acid or folic acid supplements for at least 4 months prior to their baseline visit. Upon the completion of intervention, the supplement group displayed an average RBC folate status of above the normal range at 1787± 496 nmol/L (Figure 4.4.4). Similar to the data obtained from the 2017 Statistics Canada report, Canadians and participants in this study are shown to have adequate folate status prior to folic acid supplementation, questioning the necessity of folic acid supplementation for the diets of the general population, excluding pregnant women.[69]

Limitations of this study thus far include the small sample size of participants analyzed. A total of 8 participants from the expected 24, with 4 participants randomized in each group were studied and included in statistical analysis. All dietary recalls were self-reported and thus may not be an accurate indicator of what was consumed by participants. Difficulties with recruitment included participants unwilling to change their diets for the duration of the 16-week intervention period. Although study criteria call for the inclusion of individuals aged 18-75, participants enrolled were generally elderly individuals all over the age of 50, who had been recommended to have a colonoscopy by their physician. Therefore, these findings may not be easily generalizable to younger adults.

Strengths of this study thus far include assigning all participants to a uniform diet of consuming low synthetic folic acid products allowing any effects of folic acid on the blood observed to be an isolated finding. Two 24 hour dietary recalls were collected within one week of each and averaged out to more accurately reflect the participant's dietary intake. Furthermore, the randomized design of the study allowed for direct comparison between treatment groups, and minimization of allocation and selection bias. The scope of the study was large including a variety of patients. Population was vast age group and demographics. Moreover, objectives 2 and 3 of this study will include study live human tissue colonic biopsies for folate transporters and enzymes.

Bowen et al. considered expansion, adaptability, integration, and practicality as factors when discussing the feasibility of a study.[221] Our results show this intervention could be easily expanded to include a different population, or those in a different setting. The high adherence to both the feeding and supplement intervention display the intervention is adaptable to participant

schedules and easily integrated to fit their diets. The intervention proved to be practical as participants were provided with study supplies regularly at study visits or to their homes, and maintained regular communication with a member of research staff.

Our preliminary results display we were able to produce distinct groups with the intervention proposed. Consistent with previous literature, as expected, red blood cell folate levels increased to display a shift in dietary and supplemental intake levels by 16-weeks of intervention.[222] The RBC and plasma folate levels, along with the dietary folate intake values of the control group remained consistent across the duration of the intervention. We anticipate the trend of differences observed between supplemental and control groups will continue for all 24 participants once enrolled in the study. The results obtained infer this protocol is feasible and will continue to produce reliable and valid results. The proposed study will further our understanding of the fundamental processes of folate metabolism and absorption in the colon. A more complete understanding of colonic folate absorption will help inform future revision of folate intake recommendations. Results from this study may help to establish the optimal intakes of dietary folate, and meeting the requirements of humans, without the potential health risks related to over-exposure to synthetic folic acid.

This study was funded by the Natural Sciences and Engineering Research Council of Canada. The ClinicalTrials.gov registration number for this study is: NCT03421483.

5.0 Discussion, Conclusions, and Future Directions

5.1 Discussion and Conclusions

Folate, also referred to as Vitamin B9 is necessary for DNA and RNA biosynthesis and repair, and one-carbon folate metabolism.[1] Folate cannot be synthesized in humans so it must be obtained from dietary sources, however recent evidence suggests that some folate may be produced via colonic microbial biosynthesis.[1,2] A growing body of literature indicates both a deficiency and surplus of folate can be harmful. Folate deficiency is associated with several negative health outcomes, some of which are neuropsychiatric and neurodegenerative diseases.[137] Folate deficiency also possesses the ability to suppress tumor growth and cell proliferation of neoplastic loci.[118] Thus, folate intake was thought to have a protective effect against the risk for colorectal cancer.[223] However, recent evidence suggests folate may play a dual modulatory role in carcinogenesis.[119] Although there is a well-established link between folate intake and the reduction in the prevalence of NTD's, there is some concern that an excessive amount of folate, specifically as supplemental folic acid, may lead to the masking of a vitamin B12 deficiency, changes in immune function such as reduced natural killer cell cytotoxicity, an increased risk of prostate cancer, and changes in methylation and epigenetic programming.[130,161–164,224] Several drugs (anti-malaria, anti-cancer, antibiotics) are also known to have anti-folate properties.[225–227]

Like many other nutrients, there appears to be a U-shaped relationship between folate intake and health risks. Studies conducted in the United States during the period immediately post mandatory folic acid fortification of the food supply suggest significantly more folic acid was added to folic acid-fortified foods than mandated (called folic fortification overage).[64,65] To establish optimal dietary recommendations for folate, the input side of folate nutrition must be considered. Thus, determining the current folate intake of Canadians, which includes the amount

of folate present in the foods, naturally occurring and as a fortificant, is necessary. Furthermore, previous research from our team and that of others suggest bacterial folate synthesis in the colon may also to the total body folate status.[8,116] This thesis aimed to further understand the factors contributing to the input side of folate nutrition and the fundamental processes involved in folate absorption and transport in the colon of humans.

In study one of this thesis (Chapter 3), I sought to conduct a direct assessment of the top consumed Canadian fortified foods for their total folate and folic acid content. These values were then compared against the values listed in the CNF and product labels. A total of 89 of the most commonly consumed folic acid fortified foods were analyzed for their total folate and synthetic folic acid content. Study one has allowed for a greater understanding of the amount of folate and folic acid present in the Canadian food supply in comparison to the mandated levels. In 2012, the Government of Canada implemented a food program to assess the nutritional compliance of enriched flour in imported and manufactured foods.[191] An acceptable tolerance level of 120 μg/100 g to 260 μg/100 g was assigned for folic acid in enriched foods to serve as a guide. The findings indicate there continues to be an overage of folic acid in foods, however these levels are not above the acceptable tolerance level, and that values stated in the CNF and product labels continue to be inaccurate and significantly lower than directly assessed values across certain food categories.

Nutrients obtained via supplemental intake have been previously overlooked when evaluating dietary inadequacy mainly due to methodological concerns.[228,229] However, not incorporating the nutrient contribution from supplemental sources can lead to incorrect estimates of nutrient inadequacy and intakes above the UL. With the increasing supplement use amongst the Canadian

population, the risk for over supplementation of micronutrients is also higher. Data from the 2015 Canadian Community Health Survey indicates approximately 79% of the total respondents (7,820,400) aged 19 and over consumed a folic acid supplement.[198] Continued research brings to light that the greater issue may be the dose of 400 μg in folic acid supplements being consumed on top of fortified foods in the food supply that is contributing to the higher folate status of the population. Given these findings, moving forward, future dietary intakes of micronutrients should account for nutrient contributions from both dietary and supplemental sources.

The use of folate on product labels has further been called into question as the values reported are often misleading to consumers. Our data shows, some foods such as pasta report the raw value of folate instead of the cooked value. Although the cooked value is more indicative of the nutrient amount present in the food when consumed, the general population is unaware of this which can lead to making misinformed purchases. Similar to this, using unbranded foods in the CNF database doesn't reflect the variability of foods on the market, or their respective folate content. We recommend information regarding nutrient content be relayed as accurately as possible to allow for Canadians to make informed purchases.

The second study described in this thesis (Chapter 4) is an open-label randomized clinical trial that aims to assess the influence of supplemental folic acid on colonic absorption and metabolism; specifically the impact on expression of folate transporters in the colon and the folate content in colonic mucosa. My role in this project was writing the study protocol, launching the study and assessing the feasibility of the protocol in terms of subject burden and and whether the feeding and supplement intervention recommendations produced two distinct groups of folate status at the 16-week mark. All participants were asked to consume a diet low in

synthetic folic acid fortified foods, and replace their breads and pastas with whole wheat or gluten-free products. Each participant was provided with an adult multivitamin free of synthetic folic acid, and either a 0 μg or 400 μg supplement of folic acid depending on their assigned treatment group.

The preliminary results from the first eight participants that completed the intervention were presented. One μg of folic acid received as a supplement is equivalent to 2 μg of DFE. The DFE for an individual consuming a 400 μg supplement of folic acid on this diet is expected to be approximately 800 μg DFE. The high dietary intake folate values in the group consuming a daily 400 μg supplement of folic acid, were above the expected amounts of 800 μg, at approximately 1110 DFE (Table 4.4.4.). Similarly, the RBC folate levels in the supplement group were higher than the clinically defined upper limit of 1422 nmol/L, at 1787± 496 nmol/L (Figure 4.4.5).[68] This suggests there are multiple sources of input folate nutrition contributing to the high folate status in Canadians, one of which may be the overages in the food supply. Trace amounts of folate and folic acid were found in whole wheat products analyzed, although excluded from statistical analyses. This suggests, that we are still receiving dietary folate from unfortified sources along with fortified sources.

These findings suggest that folate overages continue to persist in the food supply. These food overages in addition to the consumption of folic acid supplements, and folate produced by the colonic microbiota contribute to the content of monoglutamylated folate in our bodies, and thus our increasing folate status as a population. Therefore, this information is informative when understanding how to modify current folate guidelines.

5.2 Future Directions

The results obtained from this thesis provide an insight into further research to be conducted and the various practical applications of these findings. Given the overages of folic acid in the food supply, and these overages being common consistently across certain food categories (breads, rolls and buns, and ready-to-eat cereals), further monitoring of folic acid fortification is required.

The data from study two suggest two distinctive groups are being formed at 16-weeks thus suggesting the protocol is feasible. Once complete, results from the study will help inform the influence of supplemental folic acid on the expression and activity of various folate transporters and enzymes in different regions of the colon. Localization and regulation (upregulation vs downregulation) of folate uptake. High supplemental folate levels are suspected to promote carcinogenesis and enhance the growth of pre-existing neoplastic lesions.[118,223] This information can be used to further investigate the risk of colonic cancer associated with folic acid consumption, and the dual modulatory effects at play.

Recent data suggests supplement users are at an increased risk for overconsumption of folic acid and elevated red blood cell folate levels.[220] As a result, it is imperative the general population stays informed on the risks associated from overconsumption of supplements. The findings of this thesis suggest the risks and benefits of food fortification be carefully assessed, specifically when above mandated levels (due to overages), and that FA supplement use also be monitored in the general population. It is necessary for the different sources of folic intake to be examined to formulate guidelines reflective of the actual folate status, and needs of the Canadian population.

Further studies using different amounts of folic acid supplementation will allow for the understanding of the effects of different doses of folic acid on colonic folate levels, transporters

and enzymes. Using nationally representative data from the Canadian Health Survey Measure, this data can be further used to model dietary folate intakes across different cohorts/subgroups in the Canadian population, such as in pregnant and lactating women. It can also be used to study other potential factors contributing to individual and population folate status.

We recommend nutrient intake guidelines be established for certain subgroups, and that vitamin/mineral supplement use be reevaluated as it may not be necessary for the Canadian population. Folic acid deficiency is virtually non-existent in Canada.[5] Given this trend, it is likely that other micronutrients present in fortified foods or consumed via supplements/multivitamins are also present at higher levels in the general population, thereby eliminating the need for such supplements, and calling for a shift in fortification guidelines. This may include lowering the current mandated levels, or setting an upper mandated limit to fortification of nutrients. Moreover, incorporating quality control methods to verify nutrient amounts in foods may be useful to addressing the issues in over fortification.

Study one was the second of its kind conducted in Canada that analyzed the food supply for total folate. In addition to assessment of total folate content assessed by microbiological assay, using a sophisticated LCMS/MS procedure we were also to specifically measure synthetic folic acid content. Study two displayed the feasibility of the protocol and the success anticipated with the feeding and supplement interventions proposed. Once complete, the results of study two will help inform guidelines that influence folate intake in the Canadian population. These guidelines should be reflective of the effects of fortification, supplement use, and folate absorbed through the colon. Further research is required on the input sources of folate nutrition, and the increasing folate status of the Canadian population in order to strike a balance between risks and benefits of fortification and supplement use, and effectively manage health outcomes.

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7.0 Appendices

Appendix A

TELEPHONE SCREENING QUESTIONNAIRE

Should patients express interest in participating in the study, research staff will contact each potential participant over the phone. The purpose of the Telephone Screening Session is to determine eligibility based on pre-established inclusion and exclusion criteria.

Before proceeding with the questionnaire, the following information will be conveyed:

1. The research staff member conducting the questionnaire will identify herself/himself.

2. The staff member will state that they are calling from St. Michael's Hospital or The Hospital for Sick Children regarding a research study that the patient expressed interest in.

3. The staff member will state the name of the research study:
   - *Factors Affecting Colonic Folate Absorption and Metabolism in Humans*

4. The staff member will identify the principal investigators:
   - Dr. Deborah O'Connor, Professor at The University of Toronto and Senior Scientist at SickKids
   - Dr. Young-In Kim, Professor at The University of Toronto and Gastroenterologist at St. Mike's

5. The staff member will indicate the purpose of the research study and briefly describe the study procedures:
   - **Example script**: *Folate is an important B-vitamin used by your body that helps to produce and maintain new cells. Folate is especially important during pregnancy for the prevention of birth defects and later in life to ensure a healthy colon. We want to study how synthetic folate (folic acid) supplementation affects the absorption of folate across the colon. The study will take 16 weeks (4 months) to complete and would require you to make three visits to the Gastroenterology Clinic at St. Mike's. At each visit we will take your height, weight, hip circumference, waist circumference, and collect a blood sample. During the final visit your colonoscopy will be performed in the usual manner. In addition, small samples of tissue will be collected for our research. During the 16 week period we will provide you with supplements with and without folic acid and bread and pasta that contains low levels of folic acid.*

6. The staff member will offer to send electronic copies of the consent form via e-mail that describe the study in detail.

7. The staff member will explain to the potential subjects that she or he would like to ask some questions regarding their health, medication and diet history to determine if the patient is eligible to participate in the study. The staff member will indicate that the questionnaire will take approximately 5-10 minutes to complete, and then they will ask if the subject is willing to answer some questions.

8. The staff member will indicate that **only** study team members will have access to the answers provided by the participants.

9. The staff member will state that the study is funded by Natural Sciences and Engineering Research Council of Canada.

10. Lastly, the staff member will ask if the patient has any questions before proceeding.

INCLUSION CRITERIA

1. Describes themselves as generally healthy? ☐ Yes ☐ No

2. If the patient is female:

- Are you age 75 or under? ☐ Yes ☐ No
- Are you pre-menopausal and have had a hysterectomy or tubal ligation? ☐ Yes ☐ No
- Are you post-menopausal (for at least 1 year)? ☐ Yes ☐ No

3. If the patient is male:

- Are you between the ages of 18-75? ☐ Yes ☐ No

4. Have you been recommended to have a colonoscopy by your Doctor? ☐ Yes ☐ No

EXCLUSION CRITERIA

1. Do you have a history of gastrointestinal disease and/or gastrointestinal cancer? ☐ Yes ☐ No
   - *(e.g. Crohn's disease, ulcerative colitis, celiac disease)*

2. Have you previously had a colon resection? ☐ Yes ☐ No

3. Do you regularly consume more than:

   Women – more than 2 alcoholic drinks per day? ☐ Yes ☐ No

   Men – more than 3 alcoholic drinks per day? ☐ Yes ☐ No

4. Do you currently smoke cigarettes? ☐ Yes ☐ No

5. Do you regularly take any of the following medications: ☐ Yes ☐ No
   - Anticonvulsants (e.g. Phenytoin, Phenobarbital, Primidone)
   - Proton pump inhibitors (e.g. Nexium)
   - Oral antibiotics within the past 2 weeks (e.g. sulfonamides)

6. Do you take folic acid or multivitamin supplements

   containing folic acid currently? ☐ Yes ☐ No

   - In the past 4 months? ☐ Yes ☐ No

7. Do you have a bleeding disorder (such as hemophilia)? ☐ Yes ☐ No
8. Are you able to discontinue anti-coagulant therapy

   prior to colonoscopy? ☐ Yes ☐ No

Patient is: ☐ Eligible to continue ☐ Ineligible to continue

If the potential subjects are **not eligible** based on this questionnaire, study team member will inform the subjects and thank them for their time.

If the potential subjects are **eligible** based on this questionnaire, the study team member will offer to schedule them for a further on-site screening visit (Baseline In-Person Study Visit).

Scheduling of Baseline In-Person Study Visit

Preferred date & time #1: _____ _____
Date Time

Signature: _____ Date: _____

Appendix B

How to Limit the Intake of Folic Acid Fortified Foods

As of 1996, Health Canada opted for fortification of white wheat flour and grain products labelled enriched with folic acid—a synthetic form of the B vitamin folate. This Health Canada initiative was done to improve the folate intakes of pregnant women. Adequate intakes of folate around the time of conception reduces the risk neural tube defects, a type of birth defect. In the healthy Canadian population, folate deficiency is now rare.

For this study, we are asking you to reduce your intake of synthetic folic acid—the form of folate used in fortified foods. This can be done by reading food labels and reducing your intake of foods containing white wheat flour.

Some examples of foods that contain white wheat flour are:

- Pasta (white)
- Bread (white)
- Cake
- Cookies
- Doughnuts
- Pies
- Crackers

Words to look for in the ingredient list of food labels to know whether a food contains synthetic folic acid include:

- "enriched unbleached flour"
- "folic acid"
- "enriched wheat flour"

Nutrition Facts

Serving Size: 1 Slice (34g)
Servings Per Container: 20

Calories 90
Calories from Fat 10

Amount/serving	%DV*	Amount/serving	%DV*
Total Fat 1g	1%	**Total Carbohydrate** 17g	6%
Saturated Fat 0g	0%	Dietary Fiber Less than 1g	3%
Trans Fat 0g		Sugars 2g	
Cholesterol 0mg	0%	**Protein** 3g	
Sodium 180mg	7%		

Vitamin A 0% • Vitamin C 0% • Calcium 4% • Iron 6%
Thiamine 10% • Riboflavin 6% • Niacin 6% • Folic Acid 8%

*Percent Daily Values (DV) are based upon a 2,000 calorie diet. Your Daily Values may be higher or lower depending upon your calorie needs.

Nutrients	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Saturated Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

INGREDIENTS: ENRICHED UNBLEACHED FLOUR (WHEAT FLOUR, MALTED BARLEY FLOUR, NIACIN, REDUCED IRON, THIAMINE MONONITRATE, RIBOFLAVIN, FOLIC ACID), WATER, HIGH FRUCTOSE CORN SYRUP, POTATO FLAKES, CONTAINS LESS THAN 2% OF EACH OF THE FOLLOWING: YEAST, SOYBEAN OIL, SALT, WHEAT GLUTEN, DOUGH CONDITIONERS (MONOGLYCERIDES, SODIUM STEAROYL LACTYLATE, ASCORBIC ACID, CALCIUM SULFATE, ENZYMES), VINEGAR, MONOCALCIUM PHOSPHATE, CALCIUM PROPIONATE (PRESERVATIVE). **CONTAINS: WHEAT**
THIS PRODUCT IS MANUFACTURED ON EQUIPMENT THAT PROCESSES PRODUCTS CONTAINING SESAME

PAN-O-GOLD "VILLAGE HEARTH" BAKERS, ST. CLOUD, MN 56302 • FARGO, ND 58108 • MINNEAPOLIS, MN 55447 • SUN PRAIRIE, WI 53590 www.panogold.com VH CTGWI

INGREDIENTS:ENRICHED WHEAT FLOUR, WATER, SUGAR, YEAST, SOYBEAN AND/OR CANOLA OIL, SALT, SOY FLOUR, CALCIUM PROPIONATE, VEGETABLE MONOGLYCERIDES, SODIUM STEAROYL-2-LACTYLATE. MAY CONTAIN WHEAT GLUTEN, SORBIC ACID.

May contain: sesame seeds

INGRÉDIENTS : FARINE DE BLÉ ENRICHIE, EAU, SUCRE, LEVURE, HUILE DE SOYA ET/OU CANOLA, SEL, FARINE DE SOYA, PROPIONATE DE CALCIUM, MONOGLYCÉRIDES VÉGÉTAUX, STÉAROYL-2-LACTYLATE DE SODIUM. PEUT CONTENIR GLUTEN DE BLÉ, ACIDE SORBIQUE.

Peut contenir : graines de sésame

Appendix C

St. Michael's
Inspired Care.
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UNIVERSITY OF TORONTO

SickKids
THE HOSPITAL FOR SICK CHILDREN

LETTER OF INFORMATION AND CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE OF PROJECT: Factors Affecting Colonic Folate Absorption and Metabolism in Humans

Investigators:

The University of Toronto and The Hospital for Sick Children

Deborah L. O'Connor, PhD	Overall Principal Investigator	(416) 813-7844

St. Michael's Hospital

Young-In Kim, MD	Site Principal Investigator	(416) 864-5848
Sub-Investigators:		
G. Kandel, MD	Division of Gastroenterology	(416) 864-3093
N. Marcon, MD	Division of Gastroenterology	(416) 864-3092

After regular work hours, dial (416) 864-5431 and ask to speak to the gastroenterology fellow on call.

Research Personnel

Maria Cirocco, RN	Gastrointestinal Research, St. Michael's Hospital	(416) 864-6060 ext. 2965
Nancy Basset, RN	Gastrointestinal Research, St. Michael's Hospital	(416) 864-6060 ext. 2964
Heather White, CRA	Gastrointestinal Research, St. Michael's Hospital	(416) 864-6060 ext. 2671
Aneta Plaga, RA	Translational Medicine, SickKids	(416) 813-5894
Siya Khanna, BSc	Translational Medicine, SickKids	(416) 813-5894

Conflicts of Interest: **None of the research staff have any conflicts of interest.**

Sponsorship: **The sponsor of this research is Dr. Deborah L. O'Connor and the University of Toronto. The study is funded by the Natural Sciences and Engineering Research Council of Canada (NSERC).**

INTRODUCTION

We understand that your gastroenterologist has recommended that you have a colonoscopy. The colonoscopy will be explained later in this document. We are inviting you to consider participating in a research study in addition to that examination. Here we describe the objectives of the study and inform you of what you would need to do if you decide to participate.

Before agreeing to participate in this research study, it is important that you read and understand this research consent form. This form provides all the information we think you will need to know in order to decide whether you wish to participate in the study. If you have any questions after you read through this form, ask your study doctor or study personnel. You should not sign this form until you are sure you understand everything on this form. It is important that you are as accurate as possible with your study doctor with respect to your health history and any medication you may be taking in order to prevent any unnecessary harm to you should you decide to participate in this study.

PURPOSE OF THE RESEARCH

Folate, sometimes called folic acid, is a vitamin needed for growth, development and tissue repair. Pasta and white wheat flour are fortified with synthetic folic acid in the Canadian food supply. Adequate blood folate levels have been shown to reduce the incidence of some birth defects (spina bifida) and certain cancers, including colon cancer. Until recently, it was generally believed that folate was absorbed through the small intestine. However recent results from our research team and others have shown that the colon (i.e. large intestine) may play a more significant role than we previously assumed.

The purpose of this study is to examine how consuming a folic acid supplement affects the absorption of folate across the colon, and if enzymes that convert some naturally occurring forms of folate exist in the colon. Specifically, we are interested in assessing two enzymes and two transporters responsible for folate absorption in the colon.

This is a randomized study that involves the use of over the counter multivitamin supplements and folic acid supplements. Randomization is a process where the study group to which you are assigned is selected by chance, like the flipping of a coin. This means you have a 50/50 probability of receiving either the treatment (multivitamin and folic acid supplement) or the control (multivitamin supplement). The study will be open-labelled. This means that both you and your study doctor will know the study group to which you have been assigned, and thus if you are taking a multivitamin supplement with folic acid or not.

A total of 24 people will participate in this study for the duration of 16 weeks (~4 months). All participants will be from St. Michael's Hospital. Half of the study participants (12 people) will be provided with a daily multivitamin supplement containing folic acid and the other half will receive a multivitamin supplement containing no folic acid.

At the end of the 16-week study, participants will come in to St. Michael's Hospital for their clinically scheduled **colonoscopy**. This procedure will be described later in this document. During the colonoscopy, your gastroenterologist will take **small tissue samples,** called biopsies, from four sections of the small and large intestines. Participants will not be able to feel this happening. These samples will be frozen and our research team will later analyze them for the enzymes and transporters

that we believe are involved in folate absorption as well as the amount of folate the tissues contain. The samples will be stored in the laboratory until all analysis has been completed and manuscripts have been published. Once complete they will be destroyed.

The Research Ethics Board of St. Michael's Hospital and The Hospital for Sick Children have reviewed this study.

DESCRIPTION OF THE RESEARCH:

You have been scheduled for a colonoscopy, which is an examination of the inside of your colon using an endoscope. You are being invited to consider participating in this study because you are a healthy adult and your gastroenterologist has recommended that you have a colonoscopy exam. If you choose not to participate in the research study you will still receive the same standard and level of care at St. Michael's Hospital , including your scheduled colonoscopy, and follow-up by your physician.

This study will take place in the Gastroenterology Clinic at St. Michael's Hospital. During the study, we will need your participation for a total of 16 weeks. Your colonoscopy will be performed at the end of the 16 weeks. The blood and tissue samples collected during this study will be analyzed at St. Michael's Hospital and at the Peter Gilgan Centre for Research and Learning (PGCRL) at The Hospital for Sick Children. While you can **refer to page 10** of this document for a depiction of the study timeline, here is a description of what the study would entail:

A. Baseline In-Person Study Visit (Time Required: 1 hour)

At your first visit to the Gastroenterology Clinic at St. Michael's Hospital, a research staff member will explain the study to you in detail and will answer any questions that you may have. He or she will also review this consent form with you.

The research staff member will then guide you through a 24-hour dietary recall. This is a questionnaire that asks about the food and drinks you have consumed in the past 24-hours. It will take approximately 30 minutes to complete. The research staff member will also schedule a time, convenient to you, to call you over the telephone within the following week to complete a second 24-hour dietary recall. Height, weight, waist circumference, and hip circumference, will also be collected at this visit according to standardized procedures.

Lastly, a nurse will take a blood sample from your arm, a total of 12 mL (about 3 teaspoons) of blood. This blood will be analyzed to make sure you do not have anemia and to gauge your current state of nutrition. Research staff will also analyze your blood sample to determine if you have a genetic polymorphism (mutation) in an enzyme called methylene tetrahydrofolate reductase (short form is MTHFR). A mutation in the MTHFR enzyme will change how your body processes folate. Results from this baseline visit will be used to determine whether or not you are eligible to participate in this study.

The MTHFR gene is the only gene that will be examined. No additional genetic tests will be performed on any blood samples collected during the study. When the results are available, the registered nurse that collected your blood will phone you. All information will remain confidential

and private. Your results will remain in a secured location and will only be used for the purpose of determining your eligibility to participate in this study. After the Baseline In-Person Study Visit you will be invited to participate in the intervention phase of the study except in the following situations:

6) Your 24-hour dietary recall indicate extreme dietary irregularities or under-reporting
7) Your baseline RBC folate levels are above or below the clinical cutoff
8) Your hemoglobin level is below 100 g/L or your bloodwork demonstrates a clinically relevant abnormal result;
9) You are homozygous for the C- to- T677 substitution
10) You have a serum vitamin B12 level of ≤148 pmol/L

B. Study Supply Drop-Off (Time Required: 5 minutes)

If you are eligible to continue on in the study, we will provide you with the study supplies that you will need. You may opt to pick up your study supplies from St. Michael's Hospital or they can be delivered to your home by research staff members or courier. As previously mentioned, you will be provided with a daily supplement with or without folic acid.

Study supplies will include your study supplements, but also bread, pasta. The bread and pasta we provide will be either gluten free or 100% whole wheat depending on your preference. These contain low levels of synthetic folic acid and therefore will differ from most white wheat products found in grocery stores. They are however commercially available in Canada. You will be asked to replace your usual bread and pasta products with the ones we provide you for the duration of the 16-week study. You will also be given a list of certain products (such as cookies, cakes and other baked goods that contain white wheat flour) that you should limit your intake of while you are in the study. You will be provided with sufficient supplies to last until the 8-Week Follow-up Study Visit (see below).

C. 8-Week Follow-Up Study Visit (Time Required: 1 hour)

After 8 weeks (halfway through the study) you will return to St. Michael's Hospital. During this visit, you will complete another 24-dietary recall with a research staff member and a nurse will draw a 6 mL (1.5 teaspoons) blood sample to determine your folate status. You will also be provided with another 8-week supply of study materials (bread, pasta and study supplements). Any unused products or packages of food can be returned to study staff at this visit. A research staff member will also schedule a time to call you over the telephone or via Skype two days later to complete a second 24-hour dietary recall.

D. 16-Week Follow-Up Study Visit (Time Required: 2 hours)

A research staff member will contact you a week before your 16-Week Follow-Up Study Visit to schedule your last two 24-hour dietary recalls. These recalls will be conducted via telephone or Skype and will take place at approximately two and three days before your 16-Week Follow-Up Study Visit.

At your 16-Week Follow-Up Study Visit, your colonoscopy will be performed in the usual manner. You will be asked to follow the preparatory procedures for colonoscopy outlined by your Gastroenterologist at St. Michael's Hospital. These instructions will have been provided to you via mail or e-mail by your doctor's office. The study nurse will also review these instructions with you.

When you come for your colonoscopy, the study nurse will draw a final 6 mL (1.5 teaspoons) blood sample. A final measurement of your weight will also be obtained during this visit.

A colonoscopy is the insertion of a flexible tube-shaped scope a bit larger than the size of a finger into your rectum. The scope has a lens and a light which allows the doctor to see a video image of your colon on a screen. The scope also has a working channel, which is a hollow tube incorporated in the scope, which allows the doctor to introduce instruments, such as catheters to specific areas of the colon. The scope is gently pushed inside your colon so the physician can examine the inside of the entire length of your colon. While your doctor is completing the colonoscopy, he or she will also remove three small tissue samples each from four areas of your small and large intestine. This is the portion of the procedure that is for research and not part of your standard clinical care.

The colonoscopy procedure is not painful but may be uncomfortable while the scope is passed through the colon due to bloating. Intravenous sedation will be provided if you find the procedure uncomfortable. If you receive sedation during the colonoscopy procedure, you will require someone to drive you home at the end of this study visit.

Blood samples and tissue samples that are collected over the course of the study will be used for research purposes only in the context of this study. Samples will be stored in a secure research freezer until a research staff member analyzes them. Samples will be destroyed in accordance with safety procedure at St. Michael's Hospital and The Hospital for Sick Children after the analysis and final report are complete. Samples will not be linked to you in any way; a numeric identifier will be used instead of your name.

POTENTIAL HARMS, DISCOMFORTS OR INCONVENIENCES

Colonoscopy/Endoscopy:

The risks of colonoscopy will have been discussed with you already as part of your clinical care. As a reminder, some of the more common risks are described. If you agree to be in this study, you will be exposed to risks associated with the standard colonoscopy, which include a risk of over sedation, of possible perforation (creating a small hole) in the colon wall, and possible minor bleeding from biopsy or infection. These conditions occur in less than 1% of patients and may resolve spontaneously; however, if they are very severe you may require antibiotics or an operation to correct the problem. Additionally, you may experience some abdominal discomfort and bloating as described earlier.

Risks of Biopsies:

Biopsies are routinely obtained during colonoscopy and may cause pain, bleeding, swelling, and/or infection at the site of the biopsies. These risks are considered small and are difficult to quantify. However, you will be monitored after your colonoscopy as per routine practice.

Blood draws:

There may be a small amount of bleeding when blood is taken from a vein. There may also be slight discomfort and bruising or redness at the blood draw site that will usually disappear in a few days.

Study visits:
You may be inconvenienced by having to travel to St Michael's Hospital on three occasions. Study obligations also require you to take time away from your regular schedule.

REPRODUCTIVE RISKS

There are no reproductive risks to participants associated with this research study.

POTENTIAL BENEFITS

There are no direct benefits to volunteers from participation in this study. However, your participation is appreciated as the results will be used to better understand colonic folate absorption and will help inform future revision of folate intake recommendations.

A study team member will discuss the results of the research with participants on request, once the study is completed.

ALTERNATIVES TO PARTICIPATION

If you choose not to participate in this study, you will receive a standard screening colonoscopy as scheduled by your doctor.

PROTECTING YOUR HEALTH INFORMATION

In addition to information regarding your blood tests, tissue samples and dietary questionnaires, personal information such as your age, height, weight will be collected to strengthen our research study. Any records that identify you will remain confidential and not publicly available. Results of this study will not be commercialized. If the results of the study are published, you will remain completely anonymous.

The results of the tests described will be used for research purposes only in the context of this study. Results of your colonoscopy will be communicated to you by your doctor in the usual manner.

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law makes us do this. For example, the law could make us give information about you in the following circumstances:

- If a child has been abused
- If you have an illness that could spread to others
- If you or someone else talks about suicide (killing themselves), or
- If the court orders us to give them the study papers

In the event of clinically relevant incidental findings, you will be notified. The Research Ethics Board of St Michael's Hospital, employees of the granting agency funding the study (NSERC), or the regulator of the study may see your health records to check on the study without violating your confidentiality. For example, if necessary, they may look at your records as they pertain to this study, in order to assure that the study is conducted properly and that certain federal government rules about research are being followed.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. Following completion of the research study, the data will be kept as long as required by the St. Michael's Hospital "Records Retention and Destruction" policy. The data will then be destroyed according to these policies.

STUDY REGISTRATION AND STUDY RESULTS

The results of this study may be presented at a scientific conference or published in a scientific journal. If you are interested in obtaining the results of the study, you can contact the study doctor. We expect that the results of the study will be available in 2 years.
You will never be personally identified in any publication, report, or presentation that may come from this study.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This website will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.
The registration number for this study is NCT03421483.
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COMMUNICATION WITH YOUR FAMILY DOCTOR

As the study is not being conducted by your family doctor, we would like to notify him/her of your participation in the study, if you agree. With your agreement this could also include any other medical practitioners you see for treatment but who are not involved in the research. In this case, the study doctor will send a letter to your physician(s) that outlines the details of the study. It is important to know that your personal information (e.g., your name and date of birth) will be on this letter. There is the risk of the unintentional release of information should the letter be intercepted. The decision of notifying your treating physician(s) is entirely up to you.

POTENTIAL COSTS OF PARTICIPATION AND REIMBURSEMENT

You will be compensated for your time spent at St. Michael's Hospital and reimbursed for travel costs of $35.00 for the Baseline In-Person Study Visit and $35.00 for Week 8 Follow-Up Study Visit. For the Week 16 Follow-Up Study Visit you will receive $50.00.

COMPENSATION FOR INJURY

If you suffer an injury from the study procedure(s) or from taking the study drug or participating in this study, medical care will be provided to you in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form waive your legal rights nor release the study doctors, sponsor or involved institutions from their legal and professional responsibilities.

PARTICIPATION AND WITHDRAWAL

Your participation in this research study is purely voluntary. If you choose not to participate, you and your family will continue to have access to customary care at St. Michael's Hospital. If you decide to participate in this study you can change your mind without giving a reason, and you may withdraw from the study at any time without any effect on the care you and your family will receive at St. Michael's Hospital. You should inform your study doctor or study coordinator of your decision and about any problems that you experience during the study. If you choose to stop your participation in this study, for example prior to the final analysis, all your information including any samples collected will be withdrawn from the study and destroyed if possible.

Your participation may also be stopped without your consent, for example, if your study doctor feels that it is not in your best interest to continue or if you do not follow study directions. The investigators may terminate their involvement in the study at any time. The study sponsor and St. Michael's Hospital Research Ethics Board, have the right to terminate the study at any time.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

NEW FINDINGS OR INFORMATION

We may learn new things during the study that you may need to know. We can also learn about things that might make you want to stop participating in the study. If so, you will be notified about any new information in a timely manner. You may also be asked to sign a new consent form discussing these new findings if you decide to continue in the research study.

RESEARCH ETHICS BOARD CONTACT

If you have any questions regarding your rights as a research participant, you may contact the Chair, Research Ethics Board, St. Michael's Hospital, at (416) 864-6060 ext. 2557, during business hours.

The Research Ethics Board is also required to do periodic review of ongoing research studies. As part of this review, someone may contact you from the Research Ethics Board to discuss your experience in the research study.

STUDY CONTACT

If during the course of this study you have any questions or concerns regarding your study participation, of a health problem that may or may not be related to the study, or if you experience a research-related injury, please contact one of the study doctors or study nurses (listed on the front page of this consent form), during business hours.

In case of an emergency, please go to the nearest emergency department and let them know that you are in a study, and the principal investigator's name. If you are worried about some adverse effect that you are experiencing and want to talk to the study doctor, outside of regular business hours, you can contact him through the gastroenterology fellow on call.

TIMELINE FOR SUBJECT PARTICIPATION

C) Screening and Supply Drop-Off

Baseline In-Person Study Visit
Clinic Visit at St. Michael's Hospital: 1 hour

- Informed consent will be completed
- A screening blood sample will be collected
- A 24-hour dietary recall will be completed
- Anthropometric measurements will be taken

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You will be contacted via telephone by research staff to perform a second dietary recall questionnaire within the following week

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You will be notified via telephone if you are eligible to participate in our study

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Study Supply Drop-Off
Research staff will visit you at home: 5 minutes

- Study supplies will be delivered to you (supplements, bread and pasta)

D) Follow-Up Study Visits

Week 8 Follow-Up Study Visit
Clinic Visit at St. Michael's Hospital: 1 hour

- A blood sample will be collected
- A 24-hour dietary recall will be completed
- Study supplies provided

↓

Research staff will conduct a second dietary recall questionnaire via telephone ***(within the following week post Week 8 Follow-Up Visit)***

↓

Research staff will conduct TWO dietary recall questionnaires via telephone ***(2 & 3 days BEFORE Week 16 Follow-Up Visit)***

↓

Week 16 Follow-Up Study Visit
Clinic Visit at St. Michael's Hospital: 2 hours

- A blood sample will be collected
- A final weight measurement will be obtained
- Your standard colonoscopy will be performed and small tissue samples will be collected

TITLE OF PROJECT: Factors Affecting Colonic Folate Absorption and Metabolism in Humans

CONSENT:

By signing this consent form, I acknowledge that:

- The research study has been explained to me, and my questions have been answered to my satisfaction.
- I have been informed of the alternatives to participation in this study.
- I know that I have the right not to participate and the right to withdraw without affecting the quality of medical care at St. Michael's Hospital for me and for other members of my family.
- The potential harms and benefits (if any) of participating in this research study have been explained to me.
- I have been told that I have not waived my legal rights nor released the investigator, sponsor, or involved institutions from their legal and professional responsibilities.
- I know that I may ask now, or in the future, any questions I have about the study.
- I have been told that records relating to me and my care will be kept confidential and that no personal information will be disclosed without my permission unless required by law.
- I have been given sufficient time to read the above information.
- I will be given a copy of the signed and dated consent form.

Making your choice

I agree that the study doctor may inform my family doctor or treating physician(s) (as specified) about my participation in this research study (please select one and initial):

YES_____ Initials _____ ; NO ____ Initials _____

If YES: Name of Family Doctor and/or Treating Physician(s):

_____

I hereby consent to participate in this study. I will be given a signed copy of this consent form.

_____	_____	_____
Name of Participant (print)	Signature of Participant	Date

I confirm that I have fully explained the nature of this study to the above-named participant and believe that he/she understands it.

_____	_____	_____
Name and Position of Person* Obtaining Consent (print)	Signature of Person Obtaining Consent	Date

* By signing I confirm that I have fully explained the nature of this study to the participant and believe that he/she has understood it.

Appendix D

St. Michael's
Inspired Care. Inspiring Science

Young-In Kim, MD, FRCPC
Professor of Medicine
Division of Gastroenterology
St. Michael's Hospital
16CC-038, 30 Bond Street
Toronto, ON, M5B 1W8
Phone: 416-864-5848
Fax: 416-864-5994

Appointment Date:
Procedure Time:
Arrival Time:

Colonoscopy Instructions – MOVI-PREP

Your physician has arranged for you to have a procedure called a colonoscopy. This is a procedure where the colon is evaluated with a fiber-optic camera under sedation medications. In order to have a good look at the colon, you have to take medications the night before to clean the colon.

IMPORTANT NOTE:

Confirmations: Please confirm by calling the office at 416-864- 5848.
If your procedure is not confirmed at least **1 WEEK** before your appointment,
IT WILL BE CANCELLED.

1. Book the day off work the day of your procedure.

2. The procedure involves sedation medications which wear off quickly, so ensure that you are not driving or making significant decisions until the day after your procedure. **A responsible adult must drive you home after the procedure**. If you plan on taking a cab/uber, please have a famiy or friend accompany you home. You will be at the hospital for roughly 2 and a half to 3 hours in total.

3. You MUST bring your health card with you and the hospital card (if you have one).

4. A few days before your procedure, go to the pharmacy and purchase **MOVIPREP** which is a colon cleaning solution usually located behind the counter. It is a powder that eventually will make 2 litres of solution that must be completed entirely for the procedure to be optimal.

5. The day before the procedure, you should have nothing solid to eat. **ONLY have clear fluids** including; apple juice, white grape/cranberry juice, coconut water, ginger-ale, 7UP, Sprite, soup broth, black coffee or tea (no cream or milk), Jell-O, popsicles, Gatorades (NO Red, blue or purple) etc. is also allowed. Anything that you can see through, you can have, if you cannot see through it, DO NOT drink it. No dairy products and no alcohol all day. NO NUTS AND SEEDS 5 DAYS PRIOR.

DATE OF COLONOSCOPY:

If you are on iron medications, stop 4 days prior to your test.

Please inform the doctor if you are on anti-coagulants (blood thinners), at least five days before the procedure.

ONE WEEK BEFORE – PLEASE AVOID RAW CEREALS, FRUITS (with small seeds, and thick skin ie, berries, kiwi, grapes,) AND NUTS.

WHAT TO EXPECT:

The bowel cleansing effect is improved by the amount of clear fluids that you drink. Cramps and diarrhea are expected so stay close to a toilet. Drink large quantities of clear fluids to avoid dehydration.

If you have problems tolerating the bowel prep, notify the doctor's office for further instructions.

Purchase boxes of **PICO-SALAX(Purg-odan)** one with two packets and a second box with one packet (**DO NOT read the pamphlet that comes with the box.)** from your pharmacy.

START CLEAR FLUIDS FIRST THING IN THE MORNING THE DAY BEFORE YOUR PROCEDURE, CONTINUE UNTIL THE DAY OF PROCEDURE.

Gatorade is recommended along with juices, black tea/coffee, broth (beef/chicken), jello popsicles, Kool-Aid, soft drinks, plain sugar candies and water.

NO MILK or ORANGE JUICE OR RED COLOURED LIQUIDS.

At 3:00pm the day before the procedure, drink one packet of PICO-SALAX mixed with cold water in a mug. Continue to drink fluids until the next sachet,

At 7pm, the day before procedure, drink second packet of PICO-SALAX mixed with cold water in a mug. Continue to drink fluids until the next sachet,

If your colonoscopy is in the morning or you are from out of town then at 10:00 pm the day before the procedure, drink a third packet of PICO-SALAX mixed with cold water in a mug.Continue to drink fluids until bedtime.

If your colonoscopy is in the afternoon, then at 6:00 am the day of the procedure, drink a third packet of PICO-SALAX mixed with cold water in a mug.

Continue to drink as much clear fluids as you can until the time of your procedure.

Arrange to have someone drive you home after the procedure as you will be receiving intravenous sedation prior to the procedure.

Report to the hospital 1 hour before assigned time for registration. ST MICHAEL'S HOSPITAL, ENDOSCOPY 16TH FLOOR, CARDINAL CARTER WING. TAKE THE NORTH ELEVATORS, PLEASE USE THE QUEEN STREET ENTRACE, AND FOLLOW THE BLUE LINE TO NORTH ELEVATORS.

THIS APPOINTMENT MUST BE CONFIRMED AT LEAST 72 HOURS BEFORE. IF THE APPOINTMENT IS NOT CONFIRMED THERE IS A CHANCE OF IT BEING CANCELLED.

DR KANDEL – 416 864 3093

After a clear fluid diet all morning:

*** If your procedure is *BEFORE* 12noon, follow the instructions below:**

a) At 5:00 PM take the first dose: Mix one Sachet A and one Sachet B together with 1 litre of cold water (you can add ice cubes to make your preparation cold). Drink all the solution, and follow by at least 2-3 litres of any clear fluids.

b) At 9:00 P.M. take the second dose: Mix one Sachet A and one Sachet B together with 1 litre of cold water (you can add ice cubes to make your preparation colder). Drink all the solution, and follow by at least 2-3 Litres of any clear fluids. You can continue to drink clear fluids up to midnight. After midnight no more fluids.

*** If your procedure is *AFTER* 12noon, follow these instructions below:**

a) At 6:00 PM the day before the procedure, take the first dose: Mix one Sachet A and one Sachet B together with 1 litre of cold water (you can add ice cubes to make your preparation colder). Drink all the solution, and follow by at least 2-3 litres of any clear fluids.

b) At 5:00 AM the morning of your scheduled procedure, take the second dose: Mix one Sachet A and one Sachet B together with 1 litre of cold water (you can add ice cubes to make your preparation colder). Drink all the solution, and follow by at least 1-2 litres of any clear fluids. No More fluids after 8:00 A.M.

6. After the first or second litre is complete, you will start to get diarrhea-like bowel movements. It will continue for about two hours after you ***finish drinking the prep***.

7. St. Michael's Hospital is located at the corner of Queen and Victoria Streets. Enter through the Queen Street entrance and follow the blue line to the Cardinal Carter Wing North elevators to the 16th floor Endoscopy Unit and register at the desk at the following date and time (which is approximately 1 hour before your procedure is scheduled):

8. Please bring a list of all medications that you are taking to the Endoscopy unit. If you do not have a heart condition and are on aspirin (ASA) please stop taking it 5 days before the procedure. Also stop taking any medication that contains Iron 5 days prior.

9. If you are on **coumadin (Warfarin), dabigatran (Pradax), clopidogrel (Plavix), rivaroxaban (Xerelto), ticagrelor (Brilinta), any injectional blood thinner (such as heparin, enoxaparin, dalterparin) or ticlopidine (Ticlid)** then please contact our office at least TWO WEEK before the procedure at 416-864-5848. We may schedule you to see Dr. Kim in the clinic before your procedure.

**Cancellations must be done at least 5 days prior to the procedure (unless you have been given an emergency appointment, in which case cancellations are not permitted). If you do not show for an appointment or cancel without sufficient notice, the time is wasted and patients on our urgent waiting list suffer. The waiting list for elective endoscopic procedures at St. Michael's Hospital can exceed 18 months. If you miss your appointment without good reason, you will not be rescheduled.