Dietary Intake and Status of Folate, Vitamin B_{12} and Vitamin B_6 in Pregnant Women in Alberta

by

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Abstract

A multivitamin supplement containing folic acid is recommended during pregnancy. However, few women are counselled by a dietitian during pregnancy. The purpose of this study was to determine folate, vitamin B_{12} and vitamin B_6 status and to estimate the contribution of food and supplements to the intake of folate, vitamin B_{12} and vitamin B_6 in pregnant women. The B-vitamins intakes were estimated in women (N=599) in the Alberta Pregnancy and Outcomes and Nutrition cohort during pregnancy and at 3-months postpartum using multiple 24-hour recalls and supplement intake questionnaires. Red blood cell folate (RBCF) and plasma folate, holotranscobalamin and pyridoxal 5-phosphate were measured. A quarter of the women had sub-optimal folate status in the first trimester of pregnancy and over half the women had abnormally high folate status suggesting that supplementation during pregnancy is not appropriate in a cohort of women considered to be healthy and a low risk for nutritional deficiencies. The prevalence of vitamin B_{12} and vitamin B_6 deficiency was very low in the cohort. The percentage of women with intakes of the B-vitamins below the EAR was negligible during pregnancy but increased during 3-months postpartum. The risk of inadequacy of folate, vitamin B_{12} and vitamin B_6 from food alone was 25, 19, and 33 times higher respectively compare to total intake (food + supplement). During pregnancy and postpartum a high proportion of the women (59% to 85%) had folic acid intakes that exceeded the upper level. Even in a group of healthy women with low risk pregnancies and high socio-economic status, the use of supplemental folic acid, vitamin B_{12} and vitamin B_6 is required to ensure women meet dietary perinatal

recommendations. Guidance is needed in recommending the appropriate supplemental dose of folic acid.

Preface

This thesis is an original work by Faiqa Fayyaz. The research project Alberta Pregnancy Outcomes and Nutrition (APrON), of which this thesis is a part, received research ethics approval from the Health Research Ethics Boards at the University of Alberta (Pro 00002954) and the University of Calgary (E22101).

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Faiqa Fayyaz

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LIST OF ABBREVIATIONS

AI	Adequate Intake
APrON	Alberta Pregnancy Outcomes and Nutrition
BMI	Body Mass Index
CI	Confidence Interval
CNF	Canadian Nutrient File
CoA	Coenzyme A
CRC	Colorectal Cancer
DFE	Dietary Folate Equivalents
DHFR	Dihydrofolate Reductase
DMR	Differentially Methylated Regions
DNA	Deoxyribonucleic Acid
dTMP	Thymidine Monophosphate
EAR	Estimated Average Requirement
FFQ	Food Frequency Questionnaires
FR	Folate Receptor
HCP1	Heme Carrier Protein 1
HoloHC	Holohaptocorrin
HoloTC	Holotranscobalamin
HR	Hazard Ratio
hRFC	Reduced Folate Carrier 1
IGF2	Insulin-like Growth Factor 2
LOD	Limit of Detection

MCV	Mean Corpuscular Volume
MMA	Methylmalonic Acid
MS	Methionine Synthase
MTHFR	5-Methyltetrahydrofolate Reductase
NHANES	National Health and Nutrition Examination Survey
NHP	Natural Health Products
NK	Natural Killer Cell
NTD	Neural Tube Defects
OR	Odds Ratio
PABA	Para-Aminobenzoic Acid
PCFT	Proton Coupled Folate Transporter
PL	Pyridoxal
PLP	Pyridoxal 5-Phosphate
PM	Pyridoxamine
PN	Pyridoxine
RBCF	Red blood cell folate
RDA	Recommended Dietary Allowance
RR	Relative Risk
RTE	Ready To Eat
SD	Standard Deviation
SGA	Small-for-Gestational-Age
SIQ	Supplement Intake Questionnaire
SPSS	Statistical Package for the Social Sciences

THF	Tetrahydrofolate
UL	Tolerable Upper Intake Level
UMFA	Unmetabolized Folic Acid
USDA	United States Department of Agriculture

CHAPTER 1: Introduction and Literature Review

1 Introduction

1.1 Folate

Folate is water soluble B-vitamin which is present as structurally related derivatives. It consists of an aromatic pteridine ring joined to para-aminobenzoic acid (PABA) which is attached to glutamate moiety through a peptide bond. Folates found in nature contain a polyglutamate tail is comprised of up to eleven glutamate moieties. Each folate vitamer can differ in oxidation state of pteridine ring and N-5 or N-10 substitution (Figure 1.1). Folic acid is a stable, oxidized and synthetic form of folate, which is present in fortified foods or pharmacological supplement. Folate in the form of reduced tetrahydrofolate (THF) act as coenzyme to help the transfer of one-carbon units in a variety of reactions involved in amino acids and nucleotides metabolism. They also mediate the conversion of serine and glycine, and play a role in histidine catabolism.

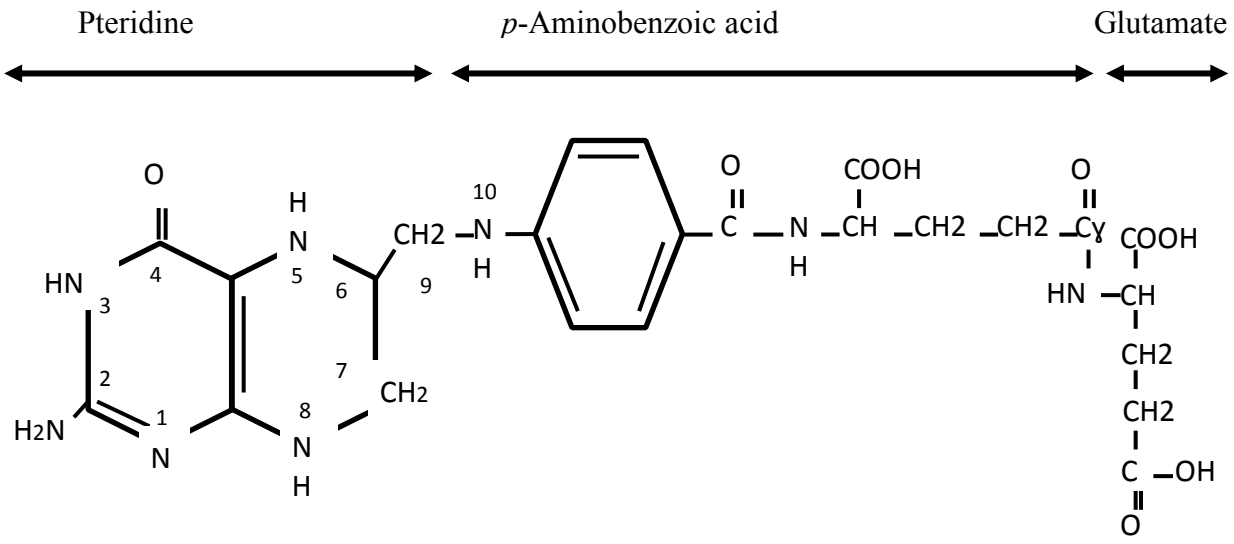


Figure 1.1: Structure of folate (THF).

1.1.1 Dietary Sources

1.1.1.1 Naturally occurring food folate

Human cannot synthesize folate and thus continuous supply from varied dietary sources is required to carry on some vital body functions. According to Canada's Food Guide, excellent sources of folate are beans, vegetables including spinach, okra, asparagus, broccoli and salads.

1.1.1.2 Folic acid in fortified food products and supplements

In addition to naturally occurring folate, it is also available in enriched grains, pasta and cornmeal and in supplements in the form of folic acid. The mandatory folic acid fortification was introduced in Canada for all types of white flour and enriched pasta and cornmeal to provide additional ~100 µg (150 µg/100g of flour) of folic acid per day to the women of child bearing age to decrease the risk of Neural Tube Defects (NTD).

1.2 Vitamin B_{12}

The vitamin B_{12} refers to a group of cobalt-containing vitamers known as cobalamins which consist of a cobalt atom surrounded by a heme-like planar corrin ring which in turn is coupled to phosphoribo-5,6-dimethylbenzimidazolyl group (Figure 1.2). Vitamin B_{12} as a cofactor is of key importance for two metabolic reactions; (1) vitamin B_{12} in the coenzymatic form of methylcobalamin in the methionine synthase reaction which converts homocysteine to methionine and (2) as adenosylcobalamin in the L-methylmalonyl CoA mutase reaction to convert L-methylmalonyl CoA (MMA) to succinyl CoA.

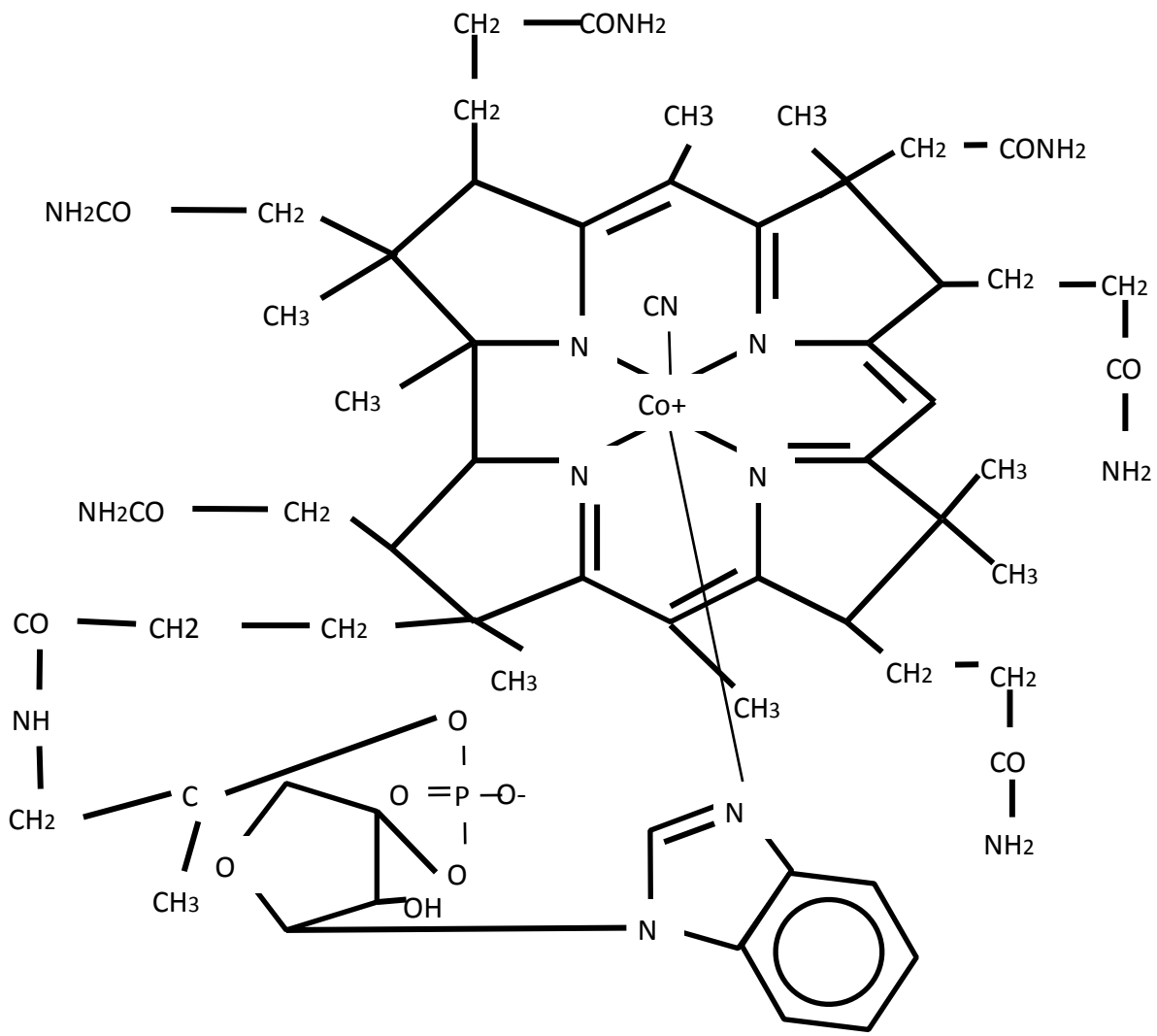


Figure 1.2: Structure of vitamin B_{12} (cyanocobalamin).

1.2.1 Dietary Sources

1.2.1.1 Naturally occurring vitamin B_{12}

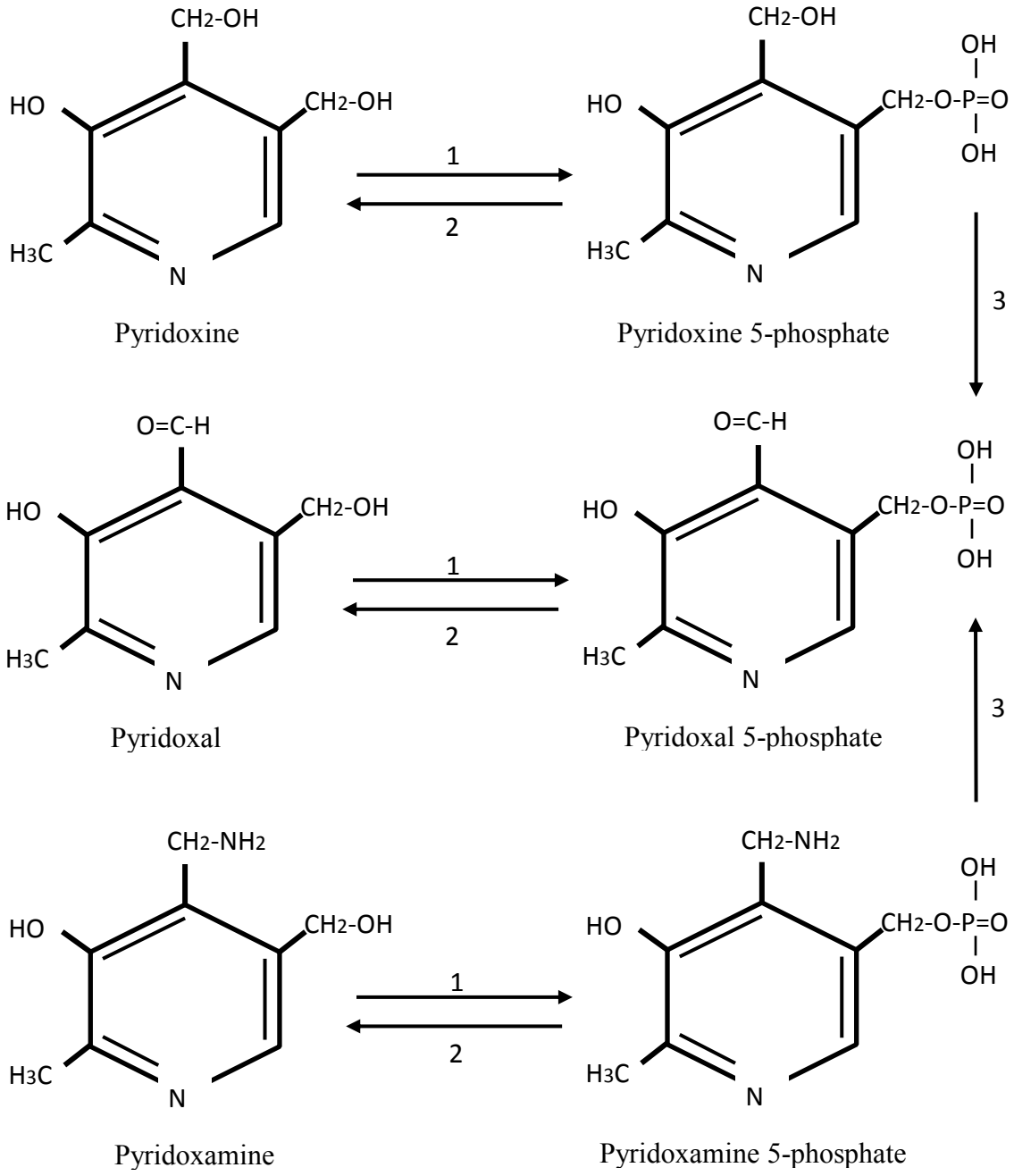
According to Canada's Food Guide, richest sources of vitamin B_{12} in the Canadian diet are the foods that come from animal's sources including meat, chicken, fish and shellfish, eggs, and milk and milk products.

1.2.1.2 Vitamin B_{12} from supplements

Vitamin B_{12} is provided as a supplement in natural health products, including multivitamins.

1.3 Vitamin B_6

Vitamin B_6 is also one of a water soluble B-vitamins. It comprises of three related pyridine derivatives; pyridoxine (PN) which is an alcohol form, pyridoxal (PL) an aldehyde form and pyridoxamine (PM) containing an amino group. Their metabolically active forms consist of 5-phosphate esters. These forms of vitamin B_6 are interconvertible in human metabolism and among all forms pyridoxal 5-phosphate (PLP) is the major form that is used by vitamin B_6 dependent enzymes in over hundred metabolic reactions (Figure 1.3).


CH2-OH
HO
CH2-OH
H3C
N
1
2
CH2-OH
HO
OH
CH2-O-P=O
OH
H3C
N
3
Pyridoxine
Pyridoxine 5-phosphate
O=C-H
HO
CH2-OH
H3C
N
1
2
O=C-H
HO
OH
CH2-O-P=O
OH
H3C
N
3
Pyridoxal
Pyridoxal 5-phosphate
CH2-NH2
HO
CH2-OH
H3C
N
1
2
CH2-NH2
HO
OH
CH2-O-P=O
OH
H3C
N
Pyridoxamine
Pyridoxamine 5-phosphate

1 Pyridoxine kinase; 2 Phosphatase**;** 3 Pyridoxine phosphate oxidase

Figure 1.3: Structure of vitamin B_6 derivatives

1.3.1 Dietary Sources

1.3.1.1 Naturally occurring vitamin B_6

Vitamin B_6 is widely distributed in foods in both its free and bound forms. According to Canada's Food Guide, excellent sources of vitamin B_6 are fruits and vegetables followed by meats, fish and poultry.

1.3.1.2 Vitamin B_6 from supplements

Vitamin B_6 present in multivitamin has been used to treat nausea and vomiting in early pregnancy for decades, commonly in conjunction with other medications such as metoclopramide or doxylamine.

1.4 Common factors in folate, vitamin B_{12} and vitamin B_6 metabolism

Folate is required to carry one carbon unit transfer reactions in the body. 5-Methyltetrahydrofolate (MTHF) with single glutamate moiety is the form of the folate capable of entering cells and is substrate for vitamin B_{12} dependent enzyme methionine synthase. Homocysteine, which is a non-protein amino acid accepts methyl group from 5-MTHF by methionine synthase and biosynthesize methionine and tetrahydrofolate (THF). THF is potential substrate for glutamation that ensures

folate cellular retention. High concentration of serum homocysteine is associated with several conditions like atherosclerosis, thromboembolisis and neurodegenerative disorders as well as pregnancy related complications e.g. pre-eclampsia, abruption placentae, intrauterine growth retardation, fetal death, preterm birth and NTD (1-7). Remethylation of homocysteine to methionine is one way to reduce homocysteine and desulfuration of homocysteine is the other way. Transsulfuration occur in the cells by vitamin B_6 dependent enzyme cystathionine β-synthase and synthesize cysteine (Figure 1.4).

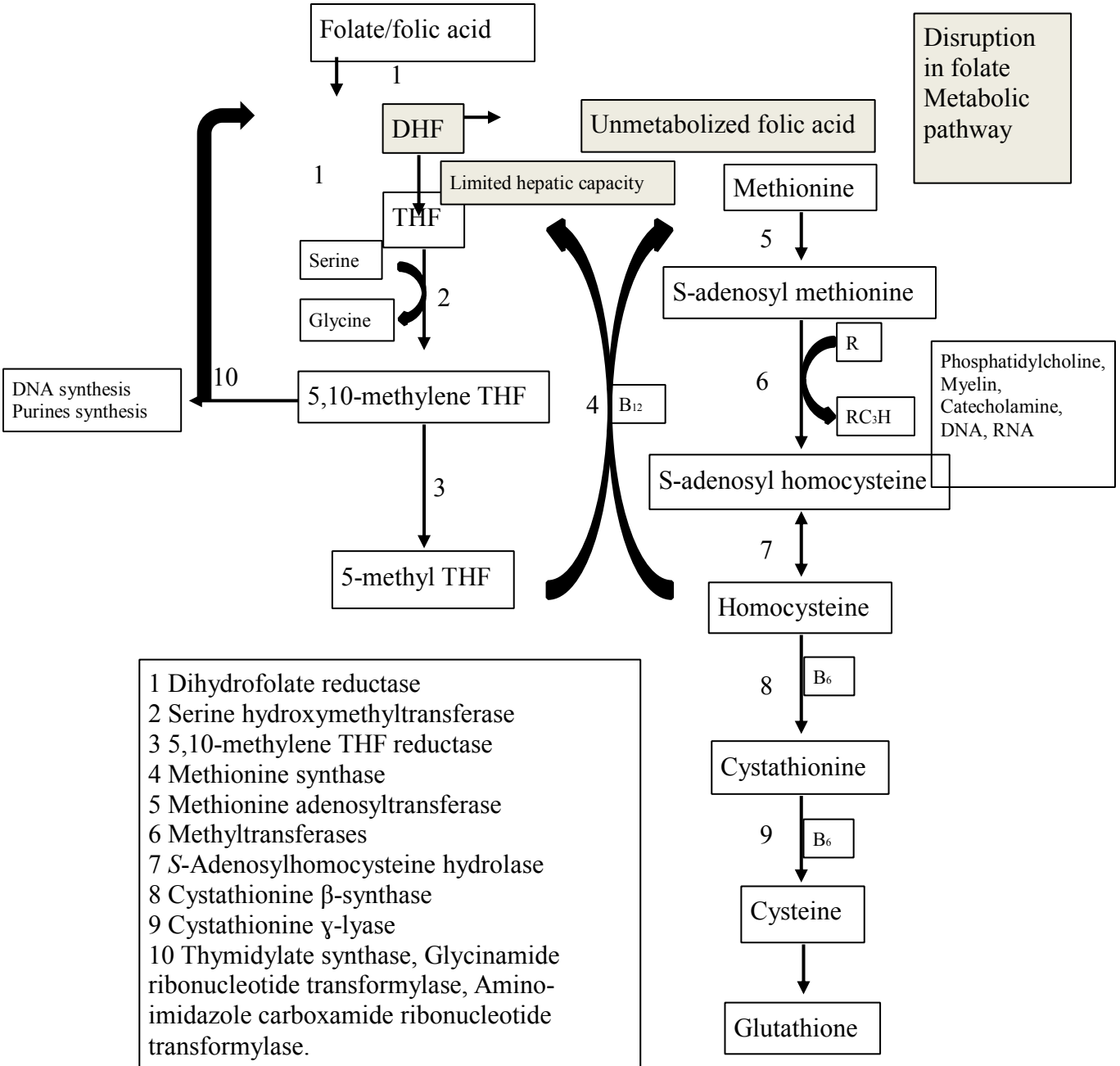

Folate/folic acid
1
DHF
Unmetabolized folic acid
Disruption in folate Metabolic pathway
1
Limited hepatic capacity
Methionine
THF
5
Serine
2
S-adenosyl methionine
Glycine
R
6
Phosphatidylcholine, Myelin, Catecholamine, DNA, RNA
DNA synthesis Purines synthesis
10
5,10-methylene THF
4
B12
RC3H
S-adenosyl homocysteine
3
7
5-methyl THF
Homocysteine
8
B6
Cystathionine
9
B6
Cysteine
Glutathione
1 Dihydrofolate reductase
2 Serine hydroxymethyltransferase
3 5,10-methylene THF reductase
4 Methionine synthase
5 Methionine adenosyltransferase
6 Methyltransferases
7 S-Adenosylhomocysteine hydrolase
8 Cystathionine β-synthase
9 Cystathionine ɣ-lyase
10 Thymidylate synthase, Glycinamide ribonucleotide transformylase, Amino-imidazole carboxamide ribonucleotide transformylase.

Figure 1.4: Metabolic reactions related to homocysteine metabolism involving folate (THF), vitamin B_{12} and vitamin B_6. DHF: dihydrofolate, THF: tetrahydrofolate, R: methyl group acceptor.

1.5 Recommendations

Educational practices related to NTD risk prevention have not been effective as they were intended as the majority of women (20% to 70%) of child bearing age still do not take folic acid containing supplements (8-11). Therefore in Canada, folic acid fortification program was implemented in 1998 with the aim of providing approximately 100 μg of folic acid per day through enriched white flour and pasta and cornmeal. The reference value used for folate, vitamin B_{12} and vitamin B_6 intake is termed as Dietary Reference Intakes (DRI) which is the quantitative evaluation of nutrients to be used for assessing and planning healthy people's diet (12). It includes; the Recommended Dietary Allowance (RDA) which reflects the intake that meets the requirements of approximately 98% of healthy people of a particular age group and gender. Because the RDA fulfills the needs of most people, it is not suitable to state that intakes below the RDA are inadequate. Also, it should not be used in assessment or planning of dietary intake of groups of people. On the other hand, the Estimated Average Requirement (EAR) is sufficient to meet the daily requirement of half of the people of a particular age group and gender. The EAR can be used in the assessment of dietary inadequacy in groups of people. However, it should not be considered recommended intake as it is estimated to only meet the requirements of half of the

people. When research data is not sufficient, Adequate Intake (AI) is used to assess the intake which is obtained from estimated intakes associated with adequate status of people. A Tolerable Upper Intake Level (UL) is also determined for some nutrients. The UL is the highest average daily intake that poses no adverse health effects. Red blood cell folate (RBCF) concentration, which reflects liver and tissue stores, has been used to assess the folate status to estimate the RDA or EAR (13). Additionally, RDA or EAR for folate are expressed as Dietary Folate Equivalents (DFE) micrograms per day in order to include both folate naturally in foods and synthetic folic acid in fortified foods. This does not apply to UL where only intakes of synthetic folic acid are considered. The DFE is calculated by multiplying quantity of synthetic folic acid with1.7 and then added with folate quantity obtained naturally. As folic acid when consumed with a meal is ~85% bioavailable (14) compare to ~50% bioavailability from natural resources (15), this 1.7 conversion factor obtained from the ratio of 85/50. The RDA of pregnant women for folate intake is 600 µg/day (EAR 520 µg/day and 450 µg/day during postpartum) is 1.5 times higher the RDA for non-pregnant women (RDA 400 µg/day; EAR 320 µg/day). The RDA recommended for folate intake during pregnancy is higher due to the additional pregnancy requirements. It is also recommended that folic acid intake should not exceed 1000 µg/day due to potential masking of vitamin B_{12} deficiency. The RDA for vitamin B_{12} is 2.4 µg/day (EAR 2.0 µg/day) which increased during pregnancy (2.6 µg/day; EAR 2.2 µg/day and 2.4 µg/day during postpartum) due to additional fetal needs (13). The RDA of vitamin B_{12} is established based on serum/plasma vitamin B_{12} deficiency identified as neurological, cognitive and haematological manifestations which

occurred at serum/plasma B_{12} < 148 pmol/L or vitamin B_{12} depletion which was established based on biological markers inadequacy (elevated MMA or homocysteine) occurred when serum/plasma B_{12} is < 221 pmol/L (13). There is no evidence that excess vitamin B_{12} intake has any detrimental health effects, therefore no UL is established for vitamin B_{12}. For vitamin B_6, a cutoff of PLP < 20 nmol/L has been used to established the EAR for vitamin B_6 (13). The RDA for pregnant women is set as 1.9 mg/day (EAR 1.6 mg/day and 1.7 mg/day during postpartum) (13). An UL for vitamin B_6 intake of 100 mg/day has also been established based on some reports describing that large doses of pyridoxine (1 to 6 g/day) can pose severe sensory neuropathy with some indication of adverse effects at doses of 500 mg/day (13).

1.6 Literature Review

1.6.1 Folic acid supplementation during pregnancy

During pregnancy, folate requirements increase to meet growth and synthetic demands of mother and fetus. The incidence of NTD decreases 50% to 70% in mothers taking folic acid supplementation periconceptionally (16) and more recently was associated with reductions in other negative birth outcomes such as abruptio placentae, spontaneous abortions, preterm delivery, low infant birth weight, fetal growth retardation, congenital heart defects and orofacial clefts (7). Since pregnancy is associated with increased demands of folate due to the developing embryo, any disruption in folate metabolism, intake or genetics could have an impact on pregnancy

outcomes. The main sources of folate are folate from dietary sources (natural folate), folic acid from fortified foods and supplements.

It is recommended that all women of childbearing age consume a daily supplement containing 400 μg folic acid daily (Health Canada 2009). Women with a previous NTD-affected birth are recommended to consume 4 mg/day folic acid (17). However, few women seek medical or nutritional advice before attempting pregnancy, many women do not have their first prenatal care visit until well into the first trimester (after the crucial weeks of neural tube development) and only 58% of Canadian women reported to take prenatal supplements containing folic acid 3-months prior to pregnancy (9). It is perhaps not surprising that folate status may be less than optimal in women in the first trimester of pregnancy.

Literature showed that multivitamin supplement use can be influenced by economic status, educational background, ethnicity and unplanned pregnancy (18-20). Tam and colleagues (2005) surveyed 383 pregnant women in Toronto about the usage and knowledge of using folic acid supplements periconceptionally. They found that only 28% of the women took folic acid containing multivitamin periconceptionally. They also found that unplanned pregnancy is a major factor associated with not taking a multivitamin supplement during early pregnancy (adjusted relative risk (RR), 1.5; 95% confidence interval (CI), 1.4-1.6) (21). In another part of Canada (Vancouver area), childbearing (n = 148) women aged 18-45 years were interviewed about the intake and knowledge of preventive role of folate. Folate intake was estimated using semi-quantitative food frequency questionnaire. It was found that only 26% of the women capable of being pregnant were meeting the requirements of folic acid intake

(supplemental folic acid 400 µg/day) and only 25% of the women had the knowledge that folic acid use can prevent NTD (11). From these studies, it can be assumed that women of childbearing age could be at risk of NTD if their pregnancy is unplanned or if they do not have knowledge about the preventive role of folic acid against NTD during early pregnancy, a time when most of the women have not confirmed that they are pregnant.

1.6.1.1 Folic acid fortification

To address the need for optimal folate status at conception, fortification of cereal grains, including flour, with folic acid became mandatory in Canada in 1998. The goal was to provide an additional 100 µg (25% of the recommendation) of folic acid in diets of women of childbearing age. After fortification, the rates of NTD decreased considerably in Canada (22). De Wals et al. (2007) evaluated the changes in the rate of NTD before and after folic acid fortification implementation in Canada. They collected live births, stillbirths, and terminations of pregnancies data from seven provinces of Canada from 1993 to 2002. Among 1.9 million births, 2446 NTD cases were recorded. The prevalence of NTD decreased from 1.58 to 0.86 per 1000 births after fortification (46% reduction; 95% CI; 40-51). The rate of reduction was highest in the regions where the prevalence of NTD was highest before fortification. Geographical differences in the rate of NTD disappeared after fortification began.

Roebothan and colleagues (2007) estimated the intake of folic acid through fortified foods in a random sample of young women (18 to 34 years; n = 302) and

seniors (65 to 74 years; n = 337) who were residents of Newfoundland and Labrador. The information on dietary intake was collected using 24-hour recalls. They found that estimated mean dietary intake of folic acid contributed by fortified food increased; 226 to 247 DFE µg/day for young women and 252 to 267 DFE µg/day for seniors. Additionally, they found that enriched white flour was the main contributor of the dietary folic acid (23). Shakur et al. (2010) determined the prevalence of folate intake below EAR or above the UL among Canadians and also examined the supplemental dose that provided childbearing women with 400 µg folic acid/day. The dietary intake data information was collected using 24-hour recalls and supplement intake was collected for 30 days prior from Canadian Community Health Survey 2004 (n = 35107) . They found that less than 1% women of childbearing age in Canada were meeting the recommendations of folate intake through diet alone and when supplement intake was combined, only 17% of the women consumed above 400 µg/day of folic acid. Data modelling analysis revealed that supplements providing 325 to 700 µg of folic acid would enable women to achieve recommended folate intakes while not exceeding the UL (10).

In another study Shakur et al. (2012) estimated the intake of various micronutrients including folate, vitamin B_{12} and vitamin B_6 in Canadian population to determine the prevalence of nutrient adequacy from diet among the supplement users and non-users. Dietary intakes were recorded using 24-hour recalls and supplement intake information collected for previous 30 days in the Canadian Community Health Survey 2.2 of 34,381 respondents. The results of the study showed that prevalence of intakes below EAR of folate, vitamin B_{12} and vitamin B_6 from diet alone was low in

this population and there was no significant difference in the prevalence of dietary intake below the EAR between supplement users and non-users. Additionally, supplement use increased the proportion of women (10%) with intakes of folic acid and some other micronutrients (vitamin A, vitamin C, niacin, iron, zinc and magnesium) above the UL (24). Both studies concluded that supplement use did not have a very large impact on reducing the prevalence of low folate intakes in Canadians. Interestingly, among women of childbearing age, only 15% to 30% of the women in these studies reported consuming a daily supplement containing folic acid.

Sherwood et al. (2006) estimated the dietary folate intake of a small group (n = 61) of predominantly university educated pregnant and lactating women who were participating in a randomized control trial. They used 3-day weighted food records for collecting dietary intake information from the women. They found that 36% of the pregnant women (36 weeks) and 32% of the lactating women (4 weeks and 16 weeks postpartum) had estimated folate intakes below the estimated average requirements (EAR) during pregnancy (520 µg/day) and lactation (450 µg/day). However, when they combined reported supplement intake with the dietary intake of folate in their analyses, the percentage of women with folate intake below EAR declined to 0% and women with intake above the upper limit reached 67% during pregnancy. Similarly, during lactation considering the supplement use, the percent of women with folate intakes below the recommendation reached to zero. Women in this study were assigned to three groups at delivery; placebo (control), L-MTHF (416 µg/d), folic acid (400 µg/day). Interestingly, none of the women in any group consume intake above the UL of folate. Based on their findings, they recommended that increasing

the folic acid fortification content twice the current levels could reduce potential inadequacy to as low as 1% during pregnancy and 3% during lactation (25). Also, some health care practitioners have supported the recommendation to doubling the folate content in fortified products to bring maximum benefits for childbearing women in reducing the risks of NTD (26,27).

Given that only 58% of the Canadian women take a supplement containing folic acid prior to conception (9), increasing the folate fortification levels would be beneficial for this targeted group. However, exposing whole population to the higher intakes of folic acid cannot be over looked in this scenario. Some of the health concerns associated with high folic acid intake are masking symptoms of vitamin B_{12} deficiency and the resultant neurological disruption (28-30), progression and risk of cancer development (31,32), effect on immune function (33) and changes in epigenetic regulations (34,35) .

1.6.1.2 Association between a high intake of folic acid and vitamin B_{12} deficiency on maternal/infant health

Negative effects on Vitamin B_{12} status is one of the potential concern associated with folic acid fortification in the food supply as it exposes the entire population to a higher intake of folic acid. The impaired function of the vitamin B_{12} dependent enzyme methionine synthase (MS) during vitamin B_{12} deficiency traps folate as 5-MTHF, which produces a functional folate deficiency (Figure 1.4). The haematological signs of vitamin B_{12} deficiency are related to inhibition of DNA synthesis resulting in megaloblastic anemia whereby neurological manifestations

occur due to impairment in methylation resulting in hypomethylation of myelin protein in brain. High folic acid intake masks the vitamin B_{12} deficiency by providing cells with sufficient tetrahydrofolate, restoring DNA synthesis and correcting haematological disturbances. But folate supplementation cannot correct the neurological manifestations of vitamin B_{12} deficiency.

Negative effects of the high blood folate concentrations had been reported in elderly population with low vitamin B_{12} status. Morris et al. (2007) examined the relationship between serum folate and vitamin B_{12} status relative to anemia, macrocytosis, and cognitive impairment in senior participants (n = 1459) in the 1999-2002 US National Health and Nutrition Examination Survey (NHANES). Participants with low vitamin B_{12} status (< 148 pmol/L) and normal folate status had a 70% increased risk of cognitive impairment. The risk was even higher for those with high serum folate (> 59 nmol/L) and low vitamin B_{12} status for cognitive impairment (Odd ratio (OR): 5.1; 95% CI: 2.7-9.5) and anemia (OR: 5.2; 95% CI: 2.5-11.0) compare to those who had normal vitamin B_{12} and folate concentrations. However, in individuals with normal vitamin B_{12} status, high folate status was also associated with a reduced risk of cognitive impairment (28). This negative association could be explained by the mechanism in which folic acid might act as an antagonist in folate metabolism during the conversion to dihydrofolate (Figure 1.4). Polyglutamated dihydrofolate inhibits thymidylate synthase and further the formation of dTMP required for DNA synthesis. Dihydrofolate also impedes the enzymes of purine synthesis which are folate dependent. Additionally, it can halt MTHFR activity which can cause decrease in methionine synthesis. This could lead to a worse situation in individuals with poor

vitamin B_{12} status. Thus, consumption of folic acid found in fortified foods or supplements may have associated with either inhibit or aid normal DNA synthesis depending on vitamin B_{12} status. In other prospective study (Morris 2005) conducted in USA between 1993 to 2002 on elderly people (n = 3718; age 65 years and over), folate intake was assessed using Food Frequency Questionnaires (FFQ) to estimate dietary and supplement intake of past year. Folate intake in the highest quintile (median 742 μg/day) was associated with a significant cognitive decline ($P = 0.002$) when compared to estimated intake in the lowest quintile (median 186 μg/day). The rate of cognitive decline was even higher for those with folic acid supplementation of > 400 μg/day compared with nonusers ($\beta = -0.03$, $P < 0.001$). Additionally, in a multiple adjusted model including folate intake, the 25% slower cognitive decline was observed only in older people (average age 80 years) who took a vitamin B_{12} supplement (20 μg/day) compare to those whose vitamin B_{12} intake was only from diet (RDA 2.4 μg/day) (36).

The risk factors associated with high folate intake and low vitamin B_{12} status during pregnancy have been found in some of very recent studies and highlight the need to assess the status of vitamin B_{12} as an independent risk factor for NTD. In three independent nested case-control groups of two population based cohorts, serum vitamin B_{12} concentrations were measured at approximately 15 weeks of gestation during a time when folic acid supplementation or fortification was not common among Irish women. Blood samples were drawn from unaffected women and cases that have a NTD-affected pregnancy in addition to women who had a previous NTD-affected pregnancy but their current pregnancy was unaffected (in study group 2

only). In all three study groups, women with NTD-affected pregnancy, current or previous, had 13% to 19% lower B_{12} concentrations. Those women, who were in the lowest B_{12} quartiles, had two to threefold higher adjusted odds ratio for having a NTD-affected pregnancy. However, the RBCF status was significantly lower in cases of study group 3 ($P < 0.0001$) and marginally ($P = 0.079$) in study group 2. There was no RBCF values available for study group 1, however, average serum folate was not significantly different between case and control subjects ($P = 0.42$) (37). The results of this study suggest that NTD affected pregnancies was partially explained by the lower RBCF status.

In another case-control study conducted in Netherlands, blood samples were drawn from 45 mothers and their children with spina bifida and from 83 control mothers and their children to examine the relationship between spina bifida and the concentrations of serum folate and RBCF, serum vitamin B_{12}, whole blood vitamin B_6, and total plasma homocysteine. In mothers of cases (infants with spina bifida), the vitamin B_{12} concentration was 21% lower compared with control mothers. Vitamin B_{12} concentration $\leq$ 185 pmol/L was associated with a 3.5 fold (95% CI 1.3-8.9) increased risk of having a child with spina bifida. They found no significant association in risk of spina bifida and folate, vitamin B_6 and homocysteine status between case and control mothers (38).

In other population based case-control study (422 unaffected pregnant women and 89 women with infants with NTD in Ontario), serum holotranscobalamin (holoTC) was measured at 15 to 20 weeks of gestation for the determination of vitamin B_{12} status. A tripling in NTD risk in women in lowest quartile for vitamin B_{12} status

compare to highest (adjusted OR 2.9; 95% CI 1.2-6.9) was observed (39). Ray and colleagues (2008) have reported vitamin B_{12} deficiency among 10622 women (age 15 to 46 yeas) in Ontario. The results of their study reported that approximately 1 in 13 Canadian women of reproductive age and 1 in 20 pregnant women during early pregnancy have biochemical vitamin B_{12} deficiency (serum B_{12} < 125 pmol/L) even after a decade of folate fortification (40).

House et al. (2000) have conducted a cross-sectional study. Folate, vitamin B_{12} and homocysteine status was assessed in 1424 pregnant women in Newfoundland. Blood samples were collected between 1996 and 1997 from pregnant women at approximately16 weeks of gestation. The results showed that approximately 44% of the women had deficient or marginal vitamin B_{12} status (< 160 pmol/L), whereas 23% of the women had deficient or marginal RBCF status (< 420 nmol/L). Serum homocysteine concentrations were inversely associated with RBCF but not with vitamin B_{12} status (41).

The Pune Maternal Nutrition Study from India reported potential effects of high folate and low B_{12} status during pregnancy. They assessed RBCF, vitamin B_{12}, homocysteine and MMA during the 18^{th} and 28^{th} week of gestation and examined their association with the women's offspring's anthropometry, body composition and insulin resistance at 6 years of age. They found that two-thirds of the women had low vitamin B_{12} status (< 150 pmol/L) and only one woman has low RBCF concentration. They found that infants born to mothers having higher RBC folate and lower plasma B_{12} were at higher risk of adiposity and insulin resistant at 6 years of age suggesting an imbalance of these two nutrients during fetal development may have potential

programming effects on later body composition. The health outcomes of mothers were not investigated in this study (42).

Dwarkanath et al. (2013) assessed the relationship between imbalanced vitamin B_{12} and total folate (food + supplement) intakes with small-for-gestational-age (SGA) infants' outcomes in a prospective cohort study of 1838 pregnant women in India. They gathered dietary information using FFQ for the preceding three months during each pregnancy trimester. During first trimester, low vitamin B_{12} intake (<1.2 µg/day) and low folate intake (<500 µg/day) were independently associated with SGA risks. However, in second trimester in a subgroup of women with high supplemental folic acid intake (> 1000 µg/day), those with the lowest vitamin B_{12}: folate ratio had increased risk of SGA (adjusted relative risk (RR); 2.73, 95% CI; 1.17-6.37). Similarly, they found that low vitamin B_{12} status (<150 pmol/L) was negatively associated with SGA risk in women with high folic acid intake during second trimester, with every one unit decrease in plasma B_{12} concentration was associated with 1% increase in SGA risk (43). Gadgil et al. (2014) examined the association between folate, vitamin B_{12} and homocysteine status with neonatal anthropometrics including birth weight, length, head circumference, abdominal circumference, mid arm circumference, chest circumference, triceps skin-fold and sub-scapular skin-fold thickness in a cross-sectional observational study. They found that maternal (n=49; 36 weeks of gestation) plasma folate and vitamin B_{12} were not associated with neonatal anthropometrics independently. However, a higher folate to vitamin B_{12} ratio was associated with neonatal anthropometrics (correlation coefficient; birth weight: -0.512, birth length: -0.424, head circumference: -0.469 and chest circumference: -

0.514, $P < 0.05$). They also observed an association of high folate to vitamin B_{12} status ratio with an increase in plasma homocysteine concentration (correlation coefficient: 0.349; $P < 0.05$) (44). Based on these findings and others, it has been suggested that implementing vitamin B_{12} fortification would be more beneficial than additional folic acid fortification. One of the aims of current study was to estimate the intake and status of vitamin B_{12} in pregnant women in Alberta during pregnancy and postpartum who have normal and high folate status.

1.6.1.3 Unmetabolized folic acid

Folic acid is the oxidized and is a non-coenzymatic form of folate obtained from fortified foods and supplements. To be used metabolically, it must be converted into 5-MTHF. The first step in this process is initiated in the liver by the enzyme dihydrofolate reductase (DHFR) (Figure 1.4). Due to limited activity of DHFR in liver, a high dose of folic acid (260 to 280 µg) can result in the appearance of unmetabolized folic acid in blood (45-47). Fohr et al. (2002) and Sweeney et al. (2007) have found unmetabolized folic acid in the fasting plasma samples after daily folic acid supplementation of 400 µg taken for 8 to 14 weeks by adults (48,49). The metabolic consequences of unmetabolized folic acid are not known but there is growing body of literature suggesting possible adverse health outcomes in animal model studies (50,51). Unmetabolized folic acid can compete with coenzymatic forms of folate for uptake by transporters, binding proteins and enzymes and can impede folate metabolism (52-54). Lucock et al. (2004) raised concerns regarding unmetabolized folic acid which can theoretically compete with folate coenzymatic

forms for DHFR and can cause intracellular folate deficiency (55). Ashokkumar et al. (2007) found a significant down-regulation of both intestinal and renal folate uptake processes with long term high dose supplementation of folic acid to rats. This was associated with significant reductions of hRFC, PCFT/HCP1 and FR expression (56). Together these studies suggest that supplementation of folic acid at high doses for extended periods of time may lead to disruption in folate metabolic pathway and may lead to an intracellular folate deficiency.

1.6.1.3 .1 Unmetabolized folic acid and pregnancy

Several epidemiological studies have identified high intakes of folic acid are associated with increased concentrations of folic acid in blood. Sweeney et al. (2009) examined the concentrations of unmetabolized folic acid in non-fasting blood samples of adult (n = 50), fasted blood samples of women (n = 20) after caesarean section and from their infants' umbilical cord in Ireland to predict the effects of voluntary folic acid fortification on status. They found circulating unmetabolized folic acid in majority of their subject, both in fasted (mean 0.39 nmol/L; 1.31% of total plasma folate) or non-fasting states (mean 0.722 nmol/L; 2.25% of total plasma folate). They predicted the level could be as high as 12% if mandatory fortification was put into policy in their country (57). Obeid et al. (2010) assessed serum concentrations of unmetabolized folic acid in women (n = 87) and in umbilical cord (n = 29) blood at delivery in 24 mother-infant pair in Germany. They measured serum concentrations of total folate, tetrahydrofolate (THF), 5-MTHF, formyl-THF, 5,10-methenyl THF,

and folic acid. They found that women who took 400 µg folic acid daily (n = 25) had significantly higher total folate, 5-MTHF, and formyl-THF concentrations compare to the women who did not take supplements (n = 61). They also found free folic acid concentrations > 0.20 nmol/L in approximately half of the pregnant women and the cord serum samples. However they were not able to find an association between maternal blood or cord blood folic acid concentrations and supplement use (58). The study was conducted in Germany which is a country with no mandatory folic acid fortification in the food supply. However, many breakfast cereals and juices were reported to be fortified with folic acid and this may have contributed to unmetabolized folic acid concentrations not differing in the supplemented and non-supplemented group in their study (58).

Kalmbach et al. (2008) examined the effect of folic acid fortification on circulating concentrations of folic acid and 5-MTHF in a cross-sectional cohort study in USA. They used plasma samples from fasting subjects (n = 3532) before and after fortification. They also gather information regarding dietary intake using FFQ. They collected the information from subjects about dietary intake of past year along with their use of vitamin and mineral supplement. The median concentration of folic acid in plasma significantly increased among both non-supplement and supplement users after fortification. Among non-supplement users, the prevalence of high circulating folic acid (85^{th} percentile) increased from 9.4% to 19.1% (median from 0.25 to 0.50 nmol/L; $P < 0.001$) and from 15.9% to 24.3% (median from 0.54 to 0.68 nmol/L; $P = 0.001$) among supplement users after fortification (59).

Bailey et al. (2010) measured unmetabolized folic acid concentrations and examined the relationship with food and supplemental folate intake and status in subjects aged ≥ 60 years. They used the data from NHANES 2001–2002 which is a cross-sectional, nationally representative survey (n =1121) in the USA. They found the concentrations of unmetabolized folic acid (mean ± SD 4.4 ± 0.6) in 38% of the population. The subjects with detectable concentration of unmetabolized folic acid (UMFA+) had a significantly higher proportion of folic acid supplement users compare to the subjects without of unmetabolized folic acid. Additionally, they found that UMFA+ subjects also have higher supplemental and total folic acid intakes and also significantly higher serum folate concentrations. However, there was no significant difference in RBCF, homocysteine and MMA concentrations in both groups. Additionally, they found only moderate relationship between total folic acid intake and UMFA concentrations ($r^2 = 0.07$) and suggesting more research is required to determine the association of other factors, such as genetic variations in folate metabolizing genes (60).

The difference in determining the unmetabolized folic acid concentration in the studies above could be partly explain by the current clinical methods used or limit of detection (LOD) used in their assays. In addition to LOD, individual variations and the regular use of supplements containing folic acid. Bailey and Ayling (2009) examined the DHFR activity in relation to folic acid used in human liver samples (n = 6). The results of their study predicted that folic acid intake above the UL either from supplements or fortified foods would increase the unmetabolized folic acid concentrations in blood (52). Pfeiffer et al. (2004) determined the various folate

vitamers (5-MTHF, THF and folic acid) using isotope-dilution tandem mass spectrometric method coupled to liquid chromatography (LC/MS/MS) as a candidate reference method. They reported that plasma folate concentrations > 50 nmol/L were more likely to be associated with increased unmetabolized folic acid concentration compare to the folate concentration < 50 nmol/L (from 2.3% to 15.7%) (61).

Given the possibility that the higher exposure to folic acid could be related to adverse health outcomes, more studies are warranted to understand the impact on health of the routine consumption of high daily amounts of folic acid. Pickell et al. (2011) investigated the impact of high intake of folic acid given before and during pregnancy and its effects on fetal mouse development. They found that giving 20-fold higher folic acid to the pregnant mice resulted in 10 fold higher plasma folate which was further associated with greater risk of embryonic delay and growth retardation. They also found that the high folic acid intake prevented adverse health outcomes in mice with MTHFR deficiency (the enzyme responsible for conversion of 5-MTHF to THF). They suggested that the elevated concentrations of unmetabolized folic acid may act as a direct inhibitor of folate enzymes and transporters (51). It was also suggested that this inhibition could reduce the availability of folate to embryo or inhibit the enzymes in the embryo (51), thereby creating deficiency symptoms. Achon et al. (2000) examined the effects of long term folic acid supplements in a rat model study. They divided rats in four groups of (virgin vs. pregnant) and the experimental diet administered (folic-acid supplemented, 40 mg/kg diet vs. control, 2mg folic

acid/kg diet). They found that there were reductions in body weight and vertex-coccyx length in fetuses from supplemented dams compare to the controls (50).

Some negative impacts of a daily high folic acid intake during pregnancy have been suggested in humans. In a recent Spanish cohort, the relationship between periconceptional folic acid supplementation use and infant birth out comes was examined. Pregnant women (n = 786) were recruited during 1-13th weeks of gestation. They found that babies born to high folic acid user group (intakes more than 1mg/day from supplements + food) had significantly lower mean birth length compared with the babies of non-user group (only dietary folate intake) (β = -0.53; 95% CI -0.96-0.09) after adjusting it for maternal demographics and energy intake. For birth weight, mothers of high and moderate user group (intakes less than 1mg/day) had lower birth weight babies as compared to non-user group, however, this difference was not significant. This study may have significantly overestimated the dietary intakes as food composition information was obtained from the US Department of Agriculture food composition tables, where the mandatory folic acid fortification has increased the dietary content of folate (~ 225 DFE μg) compared to Spain where there is no mandatory folic acid fortification. Additionally, maternal blood biomarkers of folate status (plasma folate or RBCF) were not measured nor was unmetabolized folic acid measured, which would be needed to confirm the association with high folic acid and stunting in the offsprings (62).

Increased intakes of folic acid after fortification and further recommendations of supplement intake during pregnancy warranted more investigations and monitoring to determine the status of pregnant Canadian women, in terms of mother and infant

health. One objective of this study was to measure plasma and RBC folate concentrations and relate these to reported dietary intake and supplementation during pregnancy.

1.6.1.4 Folate and cancer

Epidemiological evidence showed the protective role of dietary folate against colorectal cancer (CRC) (63). A long term folate intake ≥ 800 μg/day compared to < 250 μg/day was associated with a lower risk of colorectal cancer in an observational study (RR= 0.69, 95% CI: 0.51-0.94) (64). However, recent research indicated that it might be limited to dietary folate intake and not supplemental folic acid (65). On the contrary, there has been evidence that chronically high intake of folic acid is associated with pre-cancerous colorectal neoplasm (66). Cole et al. (2007) determined the safety and efficacy of folic acid supplementation for colorectal adenomas in a double-blind, placebo-controlled, randomized clinical trial. Participants who have recently diagnosed with colorectal adenomas (n = 1021) were randomly assigned to receive 1 mg/day folic acid (n = 516) or placebo (n = 505) and also received aspirin (81 or 325 mg/day) or placebo. They found that folic acid treatment was associated with higher risks of having 3 or more adenomas and of non-colorectal cancers (31). In Norway, a country without folic acid fortification, a study was conducted by Ebbing and colleagues (2009) to assess the effects of supplementation with B-vitamins on cancer outcomes in 2 randomized controlled trials (1998 to 2005). Patients with ischemic heart disease (n = 6837) were either treated with B-vitamins or placebo. Folic acid (0.8 mg/day) was given to 1708 participants with vitamin B_{12} (0.4

mg/g) and vitamin B_6 (40 mg/day) or the same treatment without vitamin B_6 to 1703 participants. The results of their study suggested that patients treated with folic acid and vitamin B_{12} without vitamin B_6, had an increased risk of cancer (67). In support of a possible association of folic acid and cancer, in both US and Canada, after folic acid fortification, the incidence of CRC has increased (4 to 6 additional case per 100,000 individuals) (68). Also in Chile, an increased risk of colon cancer was reported after mandatory fortification was implemented.

1.6.1.5 Folate and immune function

Toen et al. (2006) determined the relationship between dietary folate and supplemental folic acid with natural killer cell (NK) activity. The study population comprised of healthy postmenopausal women (n = 105) recruited for an exercise intervention trial. Folate intake data was collected using FFQ and supplement intake information was collected in face to face interviews. Unmetabolized folic acid was found in 78% of the fasted blood samples. Unmetabolized folic acid, 5-MTHF and total plasma folate concentrations were associated with NK cytotoxicity activity. Natural killer cell cytotoxicity in blood was approximately 23% lower among women with unmetabolized folic acid ($P = 0.04$) which was even stronger in women age $\geq$ 60 years. The result of the study indicated that folic acid may be linked to disrupt to at least one important function of the innate immune system and this could be one of a possible explanation of association of risk of cancer with higher intakes of folic acid (33).

1.6.1.6 Folate and epigenetic regulations

The role of folate in the pathogenesis of chronic diseases via changes in epigenetics has gained a lot of attention recently. Folic acid supplementation appears to be associated with increased global and site-specific DNA methylation in humans and rodents. Sie et al. (2013) determined the effects of folic acid supplementation (5 mg/kg diet) during pregnancy and post-weaning on DNA methylation in rat offsprings. They found that maternal and post-weaning folic acid supplementation significantly increased global and gene-specific DNA methylation in rat offsprings at 14 weeks of age (34). Hoyo et al. (2011) evaluated the association between maternal folic acid supplementation before and during pregnancy and DNA methylation at two differentially methylated regions (DMR) which regulate insulin-like growth factor 2 (IGF2) expressions in infants. They collected information on folic acid intake one year prior to and during pregnancy from women (n = 438) using self-administered questionnaires. They found that methylation levels at the H19 DMR decreased in umbilical cord blood samples with increasing maternal folic acid intake (2.8% to 4.9%, $P < 0.05$; before and during pregnancy) compare to women reporting no folic acid supplement intake before or during pregnancy (69).

1.6.2 Folate status during early pregnancy

In a recent Canadian Health Measure Survey, folate status of a nationally representative sample of Canadians (n=5248) including a subset of women of childbearing age (n=1162), was investigated by measuring RBCF (70). Less than 1% folate deficiency < 305 nmol/L was observed, while 40% of the population were

found to have high RBCF concentrations (>1360nmol/L). However, among the women of childbearing age, 22% of them still had RBCF concentrations below the level considered optimal for the prevention of NTD i.e. < 906 nmol/L (71,72). This suggests that the availability of folate supplements and fortification of food supply has not eliminated the risk of insufficient folate status in a proportion of women who do not take a supplement and who might become pregnant (70). In another cross-sectional study in Canada, it has been reported that folate deficiency is not present in women of childbearing age (n = 95), yet only 14% of the women in this study reached the RBCF concentrations that were > 906 nmol/L, the optimal concentration for NTD prevention (73). Bar-Oz et al. (2008) examined RBCF status of Ontario women of childbearing age. They gathered data (including age, gender, RBCF, serum folate, hemoglobin, mean cell volume and pregnancy test) from four general practice laboratories and two hospital laboratories in Toronto for the years 1995 and 1997 (pre-fortification), 1998 (start of fortification) and from 2000 to 2006 (post-fortification). For the analysis, they examined a subset of women at ages 14–45 years that were non-anemic and normocytic (MCV 75 to 94 fL). They reported that in the year 2006, 40% of the women of childbearing age and 36% of pregnant women had RBC folate levels below 900 nmol/L which made them sub-optimally protected against NTD (74). One of the purposes of this thesis was to estimate the intake and status of folate of pregnant women during early pregnancy.

1.6.3 Assessment of blood folate concentration

Folate status is routinely assessed by measurement of folate concentrations in plasma/serum and red blood cells (RBC). While serum folate is considered to be the earliest and most sensitive indicator of negative folate balance, it is influenced, transiently by recent dietary intake of folate. RBC folate concentration is considered to be an index of longer term folate status. RBC folate concentrations are also a common biochemical measure of folate status and are believe to reflect hepatic folate stores. Folate uptake into the RBC only occurs during the early stages of reticulocyte development in the bone marrow. Mature RBC membrane is not permeable to folate during the 120 days life span of the RBC, thus when measured early in pregnancy the concentration in RBC relates to folate status of women entering pregnancy.

Measurement of RBC folate remains the mainstay for establishing folate status early in pregnancy and in screening for folate deficiencies during the first months of conception. The gold-standard method to assess folate status has been microbial assay, in which *Lactobacillus rhamnosus* is cultured in a folate-depleted culture medium to which hemolysed whole blood from the subject is added to a folate deficient culture; this method was introduced in 1986 (75). This method requires that whole blood be collected, treated with ascorbic acid and frozen and requires that the aliquots are analyzed within two to three months. This assay can be confounded by antibiotic use, which is not uncommon during pregnancy (76). There are a number of newer techniques available to measure folate using a variety of detection systems including radioisotopic, enzyme linked, and chemiluminescent tags that measure

folate captured by a binding protein. These techniques offer the advantage of automation and the ability to perform these measures immediately on a small blood sample, thereby eliminating the need to store samples. This reduces the potential for folate to be degraded during storage, which is important because folate is susceptible to degradation by temperature, light, and oxidation. For this reason we proposed to use an automated ion-capture binding assay with fluorescent detection in the APrON study to measure RBCF concentrations.

1.6.4 Significance of Vitamin B_{12}

Some others factors should be taken into account that may be important in the etiology of NTD for prevention of the disease. For example, deficiency of vitamin B_{12} could be an independent risk factor for NTD in a folate sufficient population. Vitamin B_{12} is metabolically related to folate (Figure 1.4). Molloy et al. (2009) assessed the vitamin B_{12} status of pregnant women in 3 nested case-control groups of Irish women. They found that women with serum B_{12} concentration below 200 ng/L were at risk of having a NTD affected pregnancy three times more than those with above 400 ng/L. They also indicated that women should have serum B_{12} concentrations above 300 ng/L during early pregnancy in order to be protected against the 20% to 25% of natural physiological decline in B_{12} concentration associated with increasing gestation. They suggested that fortification of grain products with vitamin B_{12} might be the appropriate food vehicle to prevent NTD (37). Multifactorial etiology of NTD was also confirmed by the study of Groenen et al. (2004). They conducted a case-

control study of the women with NTD affected pregnancy versus normal pregnancy. They found approximately 4-fold increased risk of spina bifida in women with serum B_{12} concentration ≤ 185 pmol/L. The vitamin B_{12} concentration was 21% lower compare to the women with unaffected pregnancy. Additionally, they did not observe any significant changes in serum folate, RBCF, PLP and homocysteine in both groups (38).

In one study in Canada, Ray et al. (2007) assessed the vitamin B_{12} and folate status among NTD affected pregnancies (n = 89) and compare it with unaffected pregnancies (n = 422). They found approximately 34% of all NTD were related to low maternal B_{12} status as measured by serum holoTC < 55pmol/L (which was the lowest quartile). Whereas, they found no significant difference in serum folate concentration in cases and controls (39). They also suggested vitamin B_{12} fortification along with folate might be beneficial to prevent NTD (39). Although these studies are very supportive of vitamin B_{12} fortification in the food supply of women, randomized control trials to demonstrate that increasing vitamin B_{12} intake prevents NTD has not been done. Additionally, vitamin B_{12} intake was not determined in many of these studies and it is not known how voluntary supplementation contributes to vitamin B_{12} intake during pregnancy. One of the objective of this study was to estimate intake of vitamin B_{12} through diet and supplements in pregnant women during pregnancy and postpartum and assess the status.

1.6.5 Assessing vitamin B_{12} status during pregnancy

For the assessment of vitamin B_{12} concentration, several biomarkers have been used including plasma vitamin B_{12}, plasma holoTC and MMA. Ray et al (2008) measured the vitamin B_{12} concentration of 10622 women of childbearing age and pregnant women in Ontario. They found that 1 in 20 women had serum vitamin B_{12} deficiency (< 125 pmol/L) during early pregnancy (40). Vitamin B_{12} also known as cobalamin is bound to proteins transcobalamin and haptocorrin in serum. Assessment of serum vitamin B_{12} concentration indicated biologically available vitamin B_{12}-transcobalamin (called as holotranscobalamin; holoTC) complex and metabolically inert complex of vitamin B_{12}-haptocorrin (holohaptocorrin; holoHC). Morkbak et al. (2007) examined the longitudinal changes in holoTC and holoHC in healthy pregnant Danish women during 18^{th}, 32^{nd} and 39^{th} weeks of pregnancy and 8-weeks postpartum. They found that cobalamin concentration decreased 50% with increasing gestation. However, concentration of holoTC remained unchanged (77). They have suggested that decline in cobalamin concentration is due to changes in holoHC and not due to holoTC (77). They have also measured the concentration of MMA which is a functional biomarker for vitamin B_{12} deficiency, and found no change in MMA concentration at any time point of pregnancy and postpartum (77). Based on these findings they suggested that measurement of holoTC is a better indicator of assessing vitamin B_{12} deficiency during pregnancy. One of the objectives of this study was to determine the vitamin B_{12} status by measuring concentrations of holoTC in pregnant women of APrON 1^{st} cohort during early pregnancy.

1.6.6 Significance of Vitamin B_6

Vitamin B_6 is important for its role in disposing of excess homocysteine (Figure 1.4), and is a very important cofactor in several other metabolic reactions in the body. Hyperhomocysteinemia is a major NTD risk factor that is associated with disrupting neural tube closure. Folate, vitamin B_{12} and vitamin B_6 are all largely involved in homocysteine metabolism and could be involved in the etiology of NTD. Chandler et al. (2012) assessed the micronutrients status of pregnant women particularly of those micronutrients involved in one carbon metabolism including vitamin B_6 in a case-control study. The study included 954 NTD-affected pregnant women (cases) and 6268 controls. It was found that higher intakes of folate, vitamin B_6 and some other micronutrients were associated with decreased risk of anencephaly and spina bifida (78). In another case-control study, Gu et al. (2012) determined the concentrations of homocysteine, folate, vitamin B_{12} and vitamin B_6 to examine the relationship with NTD. The concentration of plasma homocysteine was higher (15.1 ± 7.8 μmol/ L vs. 8.5 ± 4.0 μmol/ L, $P < 0.001$) and of plasma folate (9.7 ± 8.1 μg/ L vs. 15.0 ± 8.1 μg/ L, $P < 0.001$) and plasma vitamin B_{12} (181.3 ± 107.7 ng/ L vs. 394.3 ± 386.3 ng/ L, $P < 0.001$) was lower significantly in cases. Additionally they have found a marginal difference of plasma vitamin B_6 concentration (48.7 ± 16.5 mg/L vs. 42.0 ± 10.5 mg/L, $P = 0.051$) between cases and control group. In the study, they found that folate and vitamin B_{12} were inversely while vitamin B_6 was directly related to homocysteine concentration. The increased concentration of vitamin B_6 in cases could be the compensation of lower folate and vitamin B_{12}. However, it cannot

be elucidated from the results of this study (79). The synergistic role and level of vitamin B_6 and its association with NTD needs further research.

1.6.7 Vitamin B_6 status and significance during pregnancy

In 2002, Baker and colleagues analyzed the blood of 563 pregnant women during each trimester and determined blood concentrations of 11 vitamins including vitamin B_6. They reported deficiency for vitamin B_6 as assessed by plasma concentration of total vitamin B_6 (phosphorylated and unphosphorylated vitamin B_6) during all trimesters (80). Pyridoxal which is an unphosphorylated form of vitamin B_6 is actively transported across placenta. However, the transport in at greater rate towards the fetus (81).Whereas transfer of PLP is much smaller and status of PLP is not affected by recent dietary intake of vitamin B_6 (82). Therefore, we speculate that determination of deficiency of vitamin B_6 during pregnancy by assessing total plasma vitamin B_6 could overestimate actual status relevant to what is available to the fetus. Thus, the determination of plasma PLP is a more useful tool for assessing vitamin B_6 status during pregnancy.

Vitamin B_6 status has been studied recently with respect to pregnancy and birth outcomes. Ronnenberg et al. (2007) assessed the plasma PLP, vitamin B_{12}, folate and homocysteine status in women during preconception (n = 364) who conceived at least once during the prospective observation period (1996 to 1998) and examined their relationship with early pregnancy loss. Adjusted for demographics and other vitamins, women in third and fourth quartile of PLP status had higher relative

hazards of conception compare to those in the first quartile (adjusted HR 2.2; 95% CI 1.3-3.4 and adjusted HR 1.6; 95% CI 1.1-2.3 in third and fourth quartile respectively). Similarly, they also found that women in fourth quartile of PLP status had significantly lower odds of early pregnancy loss compare to those in the first quartile (adjusted OR 0.5; 95% CI 0.3-1.0). Vitamin B_6 status appears to have impact on early pregnancy loss and probability of conception (83). Takimoto et al. (2011) assessed the association between infant birth outcomes (birth-weight, birth-length and head circumference) and blood biomarkers of B-vitamins (serum folate, pyridoxal, vitamin B_{12} and plasma homocysteine). Demographic and 24-hour recalls along with the information of supplement use was obtained from pregnant women (n = 42) during third trimester of pregnancy. The dietary intake of folate was not different among folic acid supplement user group compare to non-users. The rate of serum folate deficiency was very low (one in supplement user and two in non-user group). However, serum vitamin B_{12} and pyridoxal deficiency is not uncommon. They found that infants' birth-weight was lower in folic acid supplement user women compare to non-users (2894 ± 318 g vs. 3154 vs. 230 g; P=0.01) with two infants born with low birth-weight (< 2500g) in folic acid supplement user group and none in non-users. There was no significant difference in infants' birth-length, however; head circumference was lower in folic acid supplement users than non-users (84). Further analysis revealed that birth-weight was negatively correlated with folic acid supplement use and serum pyridoxal concentrations (84). It can be speculated that nutrient imbalance such as excess folic acid in presence of deficiency of other nutrient

like vitamin B_{12} and vitamin B_6 can alter the metabolic pathway of homocysteine and can have impact on infant birth outcomes (84).

CHAPTER 2: Research Plan

2.1 Statement of the Problem

Folate, vitamin B_{12} and vitamin B_6 are essential nutrients and inter-linked metabolically in one carbon metabolism. In Canada, food fortification with folic acid and prenatal folic acid supplementation recommendation are aimed to reduce the incidence of Neural tube defects (NTD). However, not all the incidence of NTD appears to be explained by folate deficiency. Therefore, the possible role of insufficiencies in other B-vitamins including vitamin B_{12} and vitamin B_6 in the etiology of NTD has recently gained attention. Additionally, folic acid supplementation greater than requirement and the capacity to metabolize and excrete has potential to increased exposure to high levels of unmetabolized folic acid. Research has suggested that high doses of folic acid can saturate the activity of dihydrofolate reductase (DHFR), a liver enzyme required to convert folic acid to its biologically active form 5-methyltetrahydrofolate. Unmetabolized folic acid in plasma has the potential to disrupt folate metabolism. Methionine synthase, a vitamin B_{12} dependent enzyme is required for the remethylation of homocysteine into methionine. Methionine undergoes a methylation cycle providing methyl group to several methylation reactions that are vital for the body. Deficiency of vitamin B_{12} can disrupt the methylation cycle. Increased availability of folate by a cell can mask vitamin B_{12} deficiency by providing the cell enough tetrahydrofolate to restore DNA synthesis and exacerbating the neurological manifestations of vitamin B_{12} deficiency. High concentrations of homocysteine are associated with several pregnancy related complications including pre-eclampsia, miscarriages and NTD. In most, accumulated

homocysteine is disposed of by remethylation into methionine or by transsulfuration to cysteine by vitamin B_6 depended enzyme cystathionine β-synthase. The imbalance of these B-vitamins has the potential to not only affect the mother's health but also has a potential to affect fetal life by epigenetic dysregulation (DNA hypomethylation or hypermethylation). Women in Canada can be exposed to high levels of folic acid through the use of folic acid containing supplements in addition to that obtained through mandatory folic acid fortification of flour. There is a limited understanding of intake of these B-vitamins in the perinatal period. The monitoring of the status of folate and other related vitamins like vitamin B_{12} and vitamin B_6 is needed.

2.2 Rationale

2.2.1 Estimating the dietary and supplemental intake of folate, vitamin B_{12} and vitamin B_6

Previous studies have shown that dietary intake of folate and vitamin B_{12} in women of childbearing age and during pregnancy is not sufficient to meet increased pregnancy demands (10,24,25). Therefore, there are debates over the potential benefits of increasing the fortification of foods with folic acid and vitamin B_{12}. We hypothesized that Canadian women are meeting the vitamin intake recommendations by taking supplements in addition to their diet during pregnancy and postpartum. We also hypothesized, that based on the use of supplements in this cohort, women may be exceeding current recommendations for folic acid and this will be reflected in RBCF concentrations.

2.2.2 Determining the red blood cell/plasma concentrations of folate, vitamin B_{12} and vitamin B_6

Literature have shown that folic acid supplementation/fortification can prevent 50% to 70% of NTD (22), and the remaining 30% to 50% could possibly be due to a deficiency of other related B-vitamins like vitamin B_{12}. Increased availability of folic acid poses a risk in masking vitamin B_{12} deficiency. Additionally, a recent Canadian study has reported that vitamin B_{12} deficiency is not uncommon among women of childbearing age and pregnant women despite mandatory folic acid fortification in the food supply (40). In this study, total vitamin B_{12} in plasma was measured. However, the transfer of biologically inactive vitamin B_{12} is greater towards fetal direction. Therefore, we proposed to assess vitamin B_{12} status using plasma holoTC (metabolically active vitamin B_{12}) which is believed to be more reflective of true vitamin B_{12} status during pregnancy. Very few studies have reported vitamin B_6 status, important in the etiology of NTD during pregnancy. We proposed to determine plasma pyridoxal 5-phosphate concentration, a biomarker of vitamin B_6 status, during pregnancy.

2.3 Purpose

The purpose of this thesis was:

(a) To determine the status of folate, vitamin B_{12} and vitamin B_6 in pregnant women.

(b) To determine the daily intakes of folate, vitamin B_{12} and vitamin B_6 from food and supplements in women during pregnancy and early postpartum (3-months postpartum).

2.4 Objectives

CHAPTER 3: Folate, vitamin B_{12} and vitamin B_6 status of pregnant women in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort

The specific objectives were as follows:

(a) To evaluate folate status and its relationship to supplementation

(b) To assess the relationship between folate status and the status of vitamin B_{12} and vitamin B_6 in a cohort of pregnant women.

2.4.1 Research Questions

(a) What is the RBCF and plasma folate status of women during pregnancy?

(b) What proportion of women achieve the RBCF status known to prevent the risk of NTD during early pregnancy?

(c) Do women have RBCF status above the reference range?

(d) Does folic acid intake associated with RBCF and plasma folate status?

(e) What proportion of women meet vitamin B_{12} and vitamin B_6 status reference ranges in a folic acid fortified/supplemented environment?

CHAPTER 4: Supplement use is required to meet recommendations of folate, vitamin B_{12} and vitamin B_6 in women during pregnancy and 3-months postpartum in Alberta

The specific objectives were as follows:

(a) To estimate the intake of folate, vitamin B_{12} and vitamin B_6 from food and supplements during pregnancy and 3-months postpartum.

(b) To examine the contribution of different food sources towards these vitamins in women during pregnancy and postpartum.

(c) To determine the proportion of women not achieving estimated average requirement by food alone or by food + supplement.

2.4.2 Research Questions

(a) Do women adhere to current recommendations of folate, vitamin B_{12} and vitamin B_6 during pregnancy and 3-months postpartum?

(b) What proportion of the women are consuming folate above the UL during pregnancy and 3-months postpartum?

(c) Are women at risk of inadequate intakes of vitamin B_{12} and vitamin B_6 in folic acid fortified/supplemented environment?

(d) What is the contribution of food towards total intake (food + supplement) of folate, vitamin B_{12} and vitamin B_6?

(e) What are the major food sources contributing to intake of folate, vitamin B_{12} and vitamin B_6?

(f) What proportion of women are at risk of inadequate intake of folate, vitamin B_{12} and vitamin B_6 from food sources?

(g) What proportion of women are at risk of inadequate intake of folate, vitamin B_{12} and vitamin B_6 from food + supplement?

CHAPTER 5: Final Discussion and Conclusions

It contains a summary of the major findings in the thesis and conclusions and future directions.

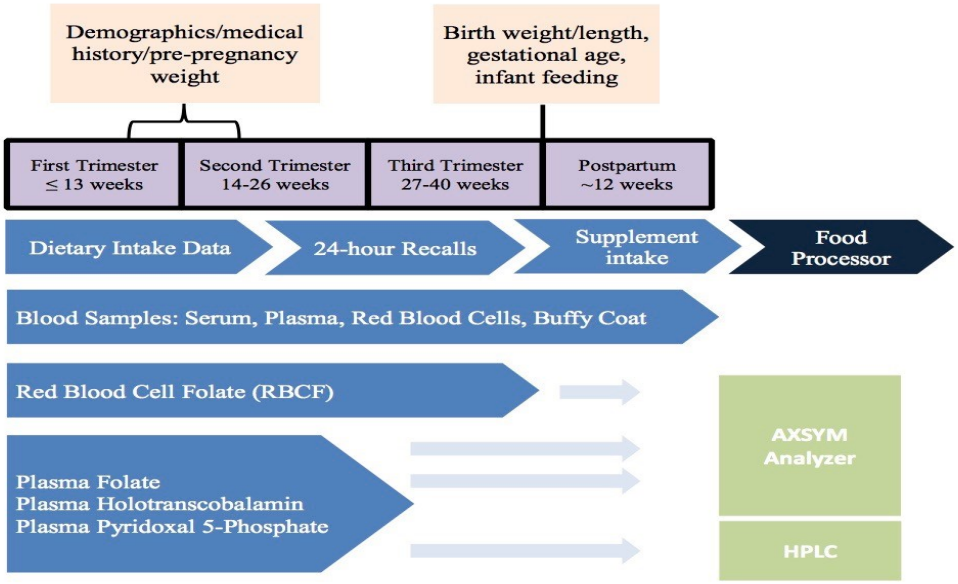


Figure 2.1: Study Design; Alberta Pregnancy Outcomes and Nutrition (APrON); Number of participants = 599

CHAPTER 3: Folate, Vitamin B_{12} and Vitamin B_6 Status of Pregnant Women in the Alberta Pregnancy Outcomes and Nutrition (Apron) Cohort[1]

3.1 Introduction

Folate, vitamin B_{12} and vitamin B_6 are essential for early embryonic development and impact health outcomes later in life. In Canada, folic acid fortification of cereal grains became mandatory in 1998. This policy resulted in a 46% reduction in the prevalence of neural tube defects (NTD) in Canada (22) and an improvement in the folate status of the general population (10,85). According to Alberta Congenital Anomalies Surveillance System Eighth Report: 1980 – 2007 (2009), the current rate of NTD in Alberta is 0.07 per 1000 total births; a rate that is similar to current rates in other provinces of Canada. Folate deficiency is now rare in the Canadian population (< 1%); 40% of Canadians have high folate status (70). The implications of high folate status on health are not well understood; however, high daily intakes of folic acid (above 1000 μg/day) have been reported to negatively impact birth outcomes, particularly birth length (62).

[1]A version of this chapter has been accepted for publication as; Folate, vitamin B_{12} and vitamin B_6 status of a group of high socio-economic status women in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort. Faiqa Fayyaz, Flora Wang, René L. Jacobs, Deborah L. O'Connor, Rhonda C. Bell, Catherine J. Field. Journal of Applied Physiology, Nutrition and Metabolism. 2014.

Offspring of pregnant rodents fed folic acid at 20-fold the recommended intake exhibited embryonic delays, growth retardation and reduced fetal body weight and length (50,51). Supplemental intakes of folic acid result in the appearance of unmetabolized folic acid in blood, presumably due to the limited capacity of hepatic dihydrofolate reductase to convert folate to dihydrofolate (57,86). It has been suggested that the presence of unmetabolized folic acid could inhibit normal folate metabolism by competing with coenzymatic form of folic acid for transporters and binding proteins (52-54). Although the rates of folate deficiency are low, only 25% of Canadian women of childbearing age (Canadian Health Measure Survey sample Cycle 1; n = 1162) reported taking a folic acid supplement and 22% were not achieving folate status sufficient to minimize NTD risk (70) . A significant proportion of Canadian women may have sub-optimal folate status during the critical period of neural tube closure, a period where many women are unaware of being pregnant.

Other concerns associated with high folic acid intakes included negative impact on masking vitamin B_{12} deficiency and the resultant neurological disruption (28-30), progression and development of cancers (87,88), immune function (89) and epigenetic regulation disruption (34,35). Because of the interaction between folic acid and vitamin B_{12} (90), it has been suggested that 34% of all NTD occurring post-folic acid fortification may be caused by low vitamin B_{12} status (39,40,91,92). Vitamin B_6 status during pregnancy is also of potential concern, as a low plasma pyridoxal 5-phosphate (PLP) has been associated with increased risk of spontaneous abortion and preterm birth (83,93).

Folic acid fortification policies in Canada have reduced the incidence of NTD significantly, thus one might question whether increasing folic acid fortification would further reduce these rates or whether research should be aimed at identifying other factors that might contribute to the rate of NTD in the country. Adequate folate, vitamin B_{12} and vitamin B_6 intake is essential for maternal health and fetal development. Status of these nutrients during early pregnancy in Canadian women is not known. The objectives of the current study were (*i*) to evaluate folate status and its relationship to supplementation and (*ii*) to assess the relationship between folate status and the status of vitamin B_{12} and vitamin B_6 in a cohort of pregnant women.

3.2 Materials and Methods

3.2.1 Study design and subjects

The present study employed the first cohort (n=599) of the Alberta Pregnancy Outcomes and Nutrition (APrON) study. The recruitment and methods of the APrON study have been described in detail (94). Subjects were enrolled in the APrON study between June 2009 and June 2010 from Edmonton and Calgary and were ≥ 16 years old, able to read and write in English, and ≤ 27 weeks gestation. Written consent was obtained from all women prior to enrolment, and ethical approval for the study was obtained from the Health Research Ethics Boards at the University of Alberta (Pro 00002954) and the University of Calgary (E22101).

Women recruited at ≤ 13 week of gestation (first trimester, n = 138) were assessed during three trimesters; those recruited between 14-26 weeks of gestation

(second trimester, n = 581) were assessed during their second and third trimesters (27-40 weeks, n = 533). Pre-pregnancy information (mental/medical history, physical activity and socio-demographics) was gathered during the first visit. Maternal characteristics including maternal age at study entry (17-30 years or 31-45 years), pre-pregnancy body mass index (BMI; underweight, normal weight, overweight or obese), household income/year (less than 20 000, 20 000 to 39 000, 40 000 to 69 000, 70 000 to 99 000 or ≥ 100 000), education level (≤ high school/diploma/certificate or ≥ high school/university study), ethnicity (Caucasian or other; Native/Asian/Latin American/African American), smoking (never or ever), previous pregnancy (yes or no), marital status (married/common law partner or other; single/divorced) and planned pregnancy (yes or no) were also obtained from women during their first visit and were considered as covariates. Information regarding folate intake from foods and supplementation was obtained from 24-hour recall and supplement intake questionnaires (SIQ), which were completed at each visit under the supervision of trained personnel. Participants' consumption of folic acid-containing multivitamins (type and quantity) during the previous 24 hour period was recorded (95).

3.2.2 Folate, vitamin B_{12} and vitamin B_6 measurements

Biochemical analyses were carried out as previously described (94). Briefly, non-fasting blood samples were taken at each visit, processed for serum, plasma, buffy coat and red blood cells, aliquotted, and stored at -80 °C. For red blood cell folate (RBCF) analysis, a hemolysate was prepared directly after blood sampling. The ion-capture method of analysis confers a number of analytical benefits over the traditional microbiological assay including ease of automation and small sample size

requirement. The accuracy and reproducibility of blood folate analyses was assessed by repeated measurements of a whole blood standard reference material with a certified value (29.5 nmol/L) (Whole blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). Repeated analysis of this standard in our laboratory yielded an interassay CV of < 10%.

Folate deficiency was defined as RBCF concentration ≤ 305 nmol/L (96). Due to lack of internationally recognized range of sub-optimal folate status for NTD risk reduction, we used the cutoff of 305 to < 906 nmol/L for sub-optimal folate status based on the findings of a nested case-control study in a prospective cohort of pregnant women (71). This study (n = 81 cases, 266 controls) conducted between 1986-1990 demonstrated a continuous inverse dose-response relationship of NTD risks with maternal RBCF concentration with its highest incidence at RBCF concentration below 340 nmol/L (6.6 per1000 live births) to lowest risk at 906 nmol/L (0.8 per 1000 live births). For high RBCF status we used a cutoff of >1360 nmol/L, reflecting the 97th percentile from NHANES 1999-2004 (97). Plasma folate and plasma holotranscobalamin (holoTC) concentrations were determined using the AXSYM analyzer as per manufacturer's instructions. For plasma folate status, we used a standard cutoff of < 7 nmol/L for deficiency (96) and > 46 nmol/L for above the normal range which was previously defined for non-pregnant women (97). The reference value used for normal holoTC was 35 to 140 pmol/L (98,99) previously defined for non-pregnant women. Plasma pyridoxal 5-phosphate was determined using an HPLC assay kit (Eagle Biosciences Inc, Nashua, NH, USA). Final plasma concentrations were determined using the calibrator as a reference. The reference

range for normal vitamin B_6 status was 20 to 220nmol/L as previously defined for non-pregnant women (96).

3.2.3 Statistics

All statistical analyses were conducted using SPSS version 20.0 (IBM SPSS for Windows, version 20.0, Chicago, IL); $P < 0.05$ were considered significant. Due to data not being normally distributed, median and 95% confidence interval (CI) were used to characterize RBCF, plasma folate, plasma holoTC and plasma PLP. The Mann-Whitney U tests were used to compare means of continuous variables and the Chi-square test was used for categorical variables. Friedman's test was used for data grouped by RBCF status categories to determine longitudinal changes in RBCF status. RBCF and plasma folate concentrations were expressed as mean ± SD grouped by folic acid supplementation above or below 1000 µg/d. Multiple linear regression analysis was used to determine the independent association between folic acid supplements intake and RBCF and plasma folate concentrations. First trimester RBCF and plasma folate values were not included in this analysis because of the very small number of samples available for comparison. The data were adjusted for folate intake from diet in all cases and also for maternal covariates which were found to be significantly associated with blood folate values differ in the bivariate analysis (Table 3.2).

3.3 Results

3.3.1 Subjects

Study participants were predominantly Caucasian, married, held university or post-graduate degrees, and had high family annual income ($100 000+). The high socio-economic status of the current study group is not surprising as Alberta Official Statistics (2014) represented the similar statistics. The majority of participants were multiparous and had a planned pregnancy. Full details of cohort demographics are available in Tables 3.1.

3.3.2 Folate status of women

Blood samples were available from 122, 520 and 446 women in their first, second and third trimesters, respectively for determination of folate status (Figure 3.1). Median RBCF concentration was significantly higher in the second and third trimesters (1504 nmol/L and 1462 nmol/L, respectively) compared with the first trimester (1280 nmol/L) ($P < 0.05$, Table 3.3). Only 3 of 122 women in their first trimester, and no women in their second and third trimesters had a RBCF concentration corresponding to overt folate deficiency (< 305 nmol/L) (Table 3.3). However, in the first trimester, 24% of women had an RBCF concentration below the value which has been suggested to minimize risk of NTD (< 906 nmol/L, Table 3.3). Approximately 45%, 62% and 59% of women had RBCF concentrations above the normal range (> 1360 nmol/L) during their first, second and third trimesters, respectively (Table 3.3). All women fell within the normal range (7-46 nmol/L) for plasma folate in all trimesters.

3.3.3 Change in folate status during pregnancy

The proportion of women with an RBCF concentration < 906 nmol/L decreased, and the proportion of women with a RBCF concentration > 1360 nmol/L increased over time compared to first trimester ($P < 0.001$, Figure 3.2 and Table 3.3). In the group of women who were recruited in their first trimester and provided samples in all three trimesters, there was a significant increase in RBCF concentration over time (data not shown). Using the women enrolled in their second trimester into the cohort, RBCF concentration were found to increase in the third trimester for the women who identified as having sub-optimal status (305 to < 906 nmol/L) or status in normal range (906 to 1306 nmol/L) in the second trimester ($P < 0.05$, Figure 3.3). For women who were classified as having RBCF above the normal range in second trimester, there was a 10% decrease in RBCF concentration in the third trimester. However, all of these women remained with RBCF concentration above the normal range (Figure 3.3).

3.3.4 Impact of folic acid supplementation on folate status

We analyzed the mean RBCF and plasma folate concentration in women who supplemented with folic acid above the Tolerable Upper Intake Level (UL; 1000 µg/d) compared to women who reported taking a daily supplement below the UL ($P < 0.05$, Table 3.4). Although it would have been logical to select 600 µg/d (the current recommendation for intake during pregnancy), almost the entire cohort was taking a

supplement that contained at least 600 µg/d. The mean ± SD (median) intake of women whose supplemental intake was ≤ 1000 µg/d was 861 ± 252 (1000) µg/d and 882 ± 240 (1000) µg/d and women whose intake was > 1000 µg/d was 2633 ± 1912 (2000) µg/d and 2528 ± 1825 (1999) µg/d during second and third trimesters, respectively. We found that both RBCF and plasma folate were significantly higher in women who supplemented with folic acid above the UL compared to women who reported taking a daily supplement below the UL ($P < 0.05$, Table 3.4). Median intake of folate from diet during pregnancy is represented in Table 3.5. Median intake of folate from diet was 269 µg/day, 299 µg/day, 306 µg/day during first, second and third trimesters respectively (Table 3.5). After adjusting for folate intake from diet and maternal covariates which were significantly differ (Table 3.2), the effect of supplemental folic acid dose on RBCF or plasma folate remained significant (Table 3.4).

3.3.5 Vitamin B_{12} and vitamin B_6 status

HoloTC concentrations were within the normal range (35 to140 pmol/L) in 88 - 91% of the women (depending on the trimester). Seven women (one in her first trimester and six in their second trimester) were found to have holoTC concentrations that would classify them vitamin B_{12} deficient (<35 pmol/L). More than 80% of the women had plasma PLP concentrations in the reference range during their first and second trimesters of pregnancy, and approximately 17% in the first trimester and 13% in the second trimester had plasma PLP concentrations above 220 nmol/L. Due to

high proportion of women with plasma folate, holoTC and PLP in the reference range in the first and second trimesters, these biomarkers were not measured in samples collected during third trimester.

3.4 Discussion

Although folate deficiency was rare (3%) in the APrON cohort, 24% of women in their first trimester had RBCF concentrations below the concentrations considered to minimize the risk of NTD (71,72). The proportion of women in this category declined substantially in the second and third trimesters. High RBCF was observed in 45% to 62% of the cohort (depending on trimester); the significance of this is currently unknown. We observed a very low prevalence of vitamin B_{12} and vitamin B_6 deficiency (less than 1% of the cohort).

Our results are in contrast to a relatively recent Canadian study. Ray et al. (2008) investigated vitamin B_{12} status of 10,622 Ontarian women (of child bearing age or pregnant) and reported that 7% of the women of child bearing age and 5% of women <28 days of gestation had a serum vitamin B_{12} concentration below 125 pmol/L during critical period of neural tube closure. They suggested that this was the result of women not preparing nutritionally for pregnancy by taking prenatal multivitamins. The incongruous findings between the studies may be partly explained by the differences in demographics between the cohorts; most of the pregnancies in the APrON cohort-1 were planned and the majority of women were in a very high socio-economic status group. The retrospective, cross-sectional study of Ray et al. (2008)

was likely more varied in its demographics since cases were recruited based on concomitant analysis of human beta-gonadotropin compare to the current study where the women recruited were volunteers. The discrepancies among the two studies may also be explained by the difference in the status biomarker selected; where serum B_{12} concentration was chosen in the previous study, the current study employed holoTC as a measure of vitamin B_{12} status. HoloTC is the biologically active fraction of vitamin B_{12} (representing 30% of total plasma B_{12}) and both holoTC and methylmalonic acid (a functional biomarker of vitamin B_{12}) have been reported to provide a better index of true cobalamin status than the measurement of total vitamin B_{12} (Herrmann et al. 2003). During pregnancy total cobalamin (vitamin B_{12}) concentration decreases up to 50% over the gestation period; however, concentration of holoTC remained unchanged (77,100). Therefore, total B_{12} may overestimate the proportion of individuals with low vitamin B_{12} status and holoTC may be a better indicator of vitamin B_{12} deficiency during pregnancy.

It is recommended that all women of child-bearing age consume a daily supplement containing 400 μg of folic acid (101,102) as demands for folate during pregnancy do not appear to be met in the vast majority of women through their self-selected diets (103). We observed that 24% of the cohort had sub-optimal RBCF status during the first trimester. This is most likely due to insufficient preconception folate intake as only 36% of the women in this cohort reported taking a supplement that contains folic acid in the year prior to pregnancy (unpublished results). This finding was also observed in the Canadian Health Measure Survey which reported that 22% of women of child-bearing age had sub-optimal folate status (70). In

another study, 36% of pregnant Ontarian women had a RBCF concentration below 906 nmol/L (74). In the present study, women who had low or sub-optimal folate status during early pregnancy had significantly higher RBCF at the second and third trimester (Figure 3.2). This is most likely due to supplemental intake of folic acid throughout pregnancy; the women in APrON cohort reported taking a folic acid-containing supplement at 94%, 97% and 94% during the first, second and third trimesters respectively (95). In the present study, women with less than optimal RBCF status had non-fasting plasma folate in the reference range, suggesting that women were indeed taking folic acid supplements. In support of this, a small but significant difference in plasma folate concentration was observed in women taking a supplement that contained folic acid at levels above the UL compared to those taking a supplement below this. In the current study plasma folate concentrations in a non-fasting blood sample do not appear to be a good indicator for assessing folate status (Table 3.3) especially during early pregnancy when women may have lower status but have begun to take maternal supplements.

Approximately, 45% of the APrON women had RBCF concentration above 1360 nmol/L during the first trimester and the proportion of women in this category increased over time (62% and 59% during the second and third trimesters, respectively). The impact of very high folate status on fetal development and maternal health is not well elucidated; however, animal studies have demonstrated risks associated with very high intakes of folic acid during gestation. Feeding pregnant dams twenty times higher folic acid than recommended resulted in reductions in birth weight and fetal length (50) and increased the risk of embryonic delay and growth

retardation (51). It has been suggested that the detrimental effects of high folic acid intake may be due to a disruption in normal folate metabolism by the presence of unmetabolized folic acid in the plasma (55). The level of supplementation used in these animal studies was undoubtedly much higher than that of the women in the APrON study. The physiological implications of high RBCF concentrations during pregnancy in women are not known. Recently, it was reported that women who ingested high doses of folic acid supplements (above the UL) during early pregnancy were at significantly higher risk of delivering a baby with small for gestational age-height (OR 5.33, 95% CI 2.08, 13.7) (62). Folate in the form of tetrahydrofolate is also an essential cofactor for DNA methylation and plays an important role in regulating gene expressions. Sie et al. reported that maternal folic acid supplementation 2.5 times higher than the dietary requirements significantly decrease global and site-specific hepatic DNA methylation in weanling rat off-springs (34). Collectively, these suggest that consuming high levels of folic acid through supplements can have profound impact on fetal development through epigenetic changes. The functional outcomes of these changes warrant further research, particularly in light of the high prevalence of high folic acid supplementation in a folic acid fortified food environment and this is the future goal of APrON cohort.

3.5 Conclusions

Although our findings are not generalizable to the entire population due to the high socio-economic status and recruitment of volunteers, our findings suggest that

additional education about prenatal folic acid supplementation could be useful to this group of women that would generally be identified as having 'low nutritional risk'. In summary, there was no evidence of deficiency of folate and vitamin B_{12} and vitamin B_6 in the APrON cohort. However, the prevalence of sub-optimal folate status during early pregnancy and the high folate status in over half the women during pregnancy, suggests that folate status is not appropriate and further education on supplement use is needed. Future studies are required to examine potential health risks associated with marginally sub-optimal status early in pregnancy and high folate status in early and late pregnancy.

3.6 Acknowledgments

The APrON study was funded by a team grant from Alberta Innovates Health Solutions and additional support for folate analysis was provided by a grant from the Women and Children's Health Research Institute. Faiqa Fayyaz was supported by the Food and Health Innovation Initiative Fund. We would like to thank the APrON study team and participants for helping make this study a success. We would also like to acknowledge Paige Sorochan and Susan Goruk for technical assistance and Anne Gilbert, Sarah Loehr and Andrea Deane for assisting with dietary intake data collection.

Table 3.1: Characteristics of the women enrolled in APrON cohort-1

Characteristics	Percentage (%)
Age (year)	N = 597
17-30	46
31-45	54
Pre-pregnancy BMI (kg/m^2)	N = 572
Underweight	3
Normal	65
Overweight	18
Obese	14
Household income (CAD)	N = 550
Less than $20, 000	2
$20, 000 to $39, 000	4
$40, 000 to $69, 000	14
$70, 000 to $99, 000	25
$100, 000+	55
Education level	N = 557
High school/university study	69
Less than high school/diploma/certificate	31
Ethnicity	N = 556
Caucasian	87
Other	13
Smoking	N = 557
Never	68
Ever	32

Previous pregnancy	N = 561
Yes	60
No	40
Marital status	N = 560
Married	97
Other	3
Planned pregnancy	N = 562
Yes	81
No	19

Note: BMI, body mass index.

Table 3.2: Characteristics of women enrolled in APrON cohort-1 according to RBCF and plasma folate

	RBCF (nmol/L)				Plasma folate (nmol/L)	
Maternal Characteristics	Second trimester		Third trimester		Second trimester	
	Mean ± SD	*P*	Mean ± SD	*P*	Mean ± SD	*P*
Age (years)						
17-30†	1447 ± 409		1475 ± 406		35 ± 4	
31-45	1526 ± 400	<0.05	1502 ± 377	0.443	36 ± 4	0.120
Pre-pregnancy BMI (kg/m^2)						
Underweight	1480 ± 449		1339 ± 317		37 ± 4	
Normal†	1475 ± 396		1488 ± 376		36 ± 5	
Overweight	1492 ± 413		1488 ± 384		35 ± 4	
Obese	1597 ± 436	0.325	1550 ± 494	0.236	35 ± 5	0.049
Household income						
Less than $20 000	1041 ± 364		1282 ± 354		31 ± 7	
$20 000 to $39 000	1412 ± 402		1536 ± 415		36 ± 4	
$40 000 to $69 000	1484 ± 393		1505 ± 384		36 ± 5	
$70 000 to $99 000	1507 ± 370		1495 ± 408		36 ± 4	
$100 000+†	1505 ± 423	<0.05	1492 ± 385	0.751	36 ± 4	0.150
Education level						
High school/university study†	1497 ± 397		1476 ± 380		36 ± 4	
Less than high school/diploma/certificate	1484 ± 427	0.804	1538 ± 410	0.094	36 ± 5	0.872

Ethnicity						
Caucasian†	1506 ± 409		1490 ± 390		36 ± 4	
Other	1378 ± 378	<0.05	1506 ± 387	0.848	36 ± 5	0.300
Smoking						
Never†	1488 ± 411		1494 ± 388		36 ± 5	
Ever	1506 ± 394	0.859	1466 ± 399	0.441	35 ± 4	0.430
Previous pregnancy						
Yes	1514 ± 407		1491 ± 396		36 ± 5	
No†	1457 ± 408	0.136	1494 ± 381	0.832	36 ± 4	0.639
Marital status						
Married†	1494 ± 405		1494 ± 390		36 ± 4	
Other	1392 ± 464	0.393	1478 ± 364	0.776	33 ± 6	0.110
Planned pregnancy						
Yes†	1505 ± 399		1485 ± 388		36 ± 4	
No	1438 ± 437	0.206	1534 ± 395	0.335	35 ± 4	0.145

Note: RBCF, red blood cell folate; BMI, body mass index.

† Reference group. $P < 0.05$ was significant, obtained from Kruskal Wallis test or Mann-Whitney test.

Table 3.3: Folate, vitamin B_{12} and vitamin B_6 status among pregnant women enrolled in the Alberta Pregnancy Outcomes and Nutrition (APrON) study cohort-1

	First trimester		Second trimester		Third trimester	
	N	Median (95% CI)	N	Median (95% CI)	N	Median (95% CI)
RBCF (nmol/L)						
<305	3	250 (238,305)	0	-	0	-
305 to <906	29	573 (520,773)	44	745 (707,835)	29	811 (746,860)
906 to1360	35	1114 (1034,1208)	156	1191 (1150,1234)	152	1186 (1141,1231)
>1360	55	1740 (1594,1903)	320	1740 (1686,1764)	265	1730 (1680,1752)
Total	122	1280 (1114,1393)[a]	520	1504 (1450,1568)[b]	446	1462 (1421,1529)[b]
Plasma folate (nmol/L)						
<7	0	-	0	-		ND
7 to 46	128	36 (35,36)	534	36 (35,36)		ND
>46	0	-	0	-		ND
Total	128	36 (35,36)[a]	534	36 (35,36)[b]		ND
Plasma holoTC (pmol/L)						
<35	1	26	6	31 (17,34)		ND
35 to140	108	87 (81,94)	472	81 (77,83)		ND
>140	14	256 (176,298)	43	232 (211,272)		ND
Total	123	92 (84,100)[a]	521	83 (80,85)[b]		ND
Plasma PLP (nmol/L)						
< 20	0	-	1	14		ND

20 to 220	99	84 (73,94)	457	67 (63,72)		ND
>220	20	359 (298,458)	70	302 (279,314)		ND
Total	119	94 (82,112)[a]	528	76 (70,83)[b]		ND

Note: RBCF, red blood cell folate; holoTC, holotranscobalamin; PLP, pyridoxal 5-phosphate; 95% CI; 95% confidence interval for median; ND, not determined. $P < 0.05$ obtained from Wilcoxon Signed Ranks test and letters not similar are significantly different.

Table 3.4: Folate status in pregnant women according to supplemental intake of folic acid.

Folic acid supplement (µg/d)	**≤1000**		**>1000**		***P****	**β Coefficient**	**95% CI**	***P***
	N	mean ± SD	N	mean ± SD				
RBCF (nmol/L)								
Second trimester	387	1404 ± 1	115	1536 ± 1	0.006	101.25	11.62, 190.87	0.027†
Third trimester	347	1409 ± 1	86	1557 ± 1	0.003	150.82	57.53, 244.11	0.002
Plasma folate (nmol/L)								
Second trimester	400	35 ± 1	116	36 ± 1	0.001	1.20	0.24, 2.17	0.015

Note: µg/d; micrograms per day, 95% CI; 95% confidence interval for β-coefficient. *$P < 0.05$ was significant, obtained from Mann-Whitney tests for pairwise comparisons. Multiple linear regression analysis was conducted to adjust the data for folate intake from diet and further for maternal covariates significantly different. † adjusted for maternal age, income and ethnicity.

Table 3.5: Dietary intake of women during three trimesters of pregnancy

	N	First trimester	N	Second trimester	N	Third trimester
Folate (µg/d)	129	269 (240, 303)	563	299 (281, 310)	498	306 (290, 318)
Vitamin B_{12} (µg/d)	129	3.7 (3.3, 4.0)	567	4.0 (3.7, 4.2)	502	4.1 (3.8, 4.4)
Vitamin B_6 (mg/d)	129	1.8 (1.6, 2.0)	567	1.8 (1.7, 1.9)	502	1.9 (1.8, 2.0)

Note: The results are presented as median (95% CI). µg/d; micrograms per day, mg/d; milligrams per day, 95% CI; 95% confidence interval for median.

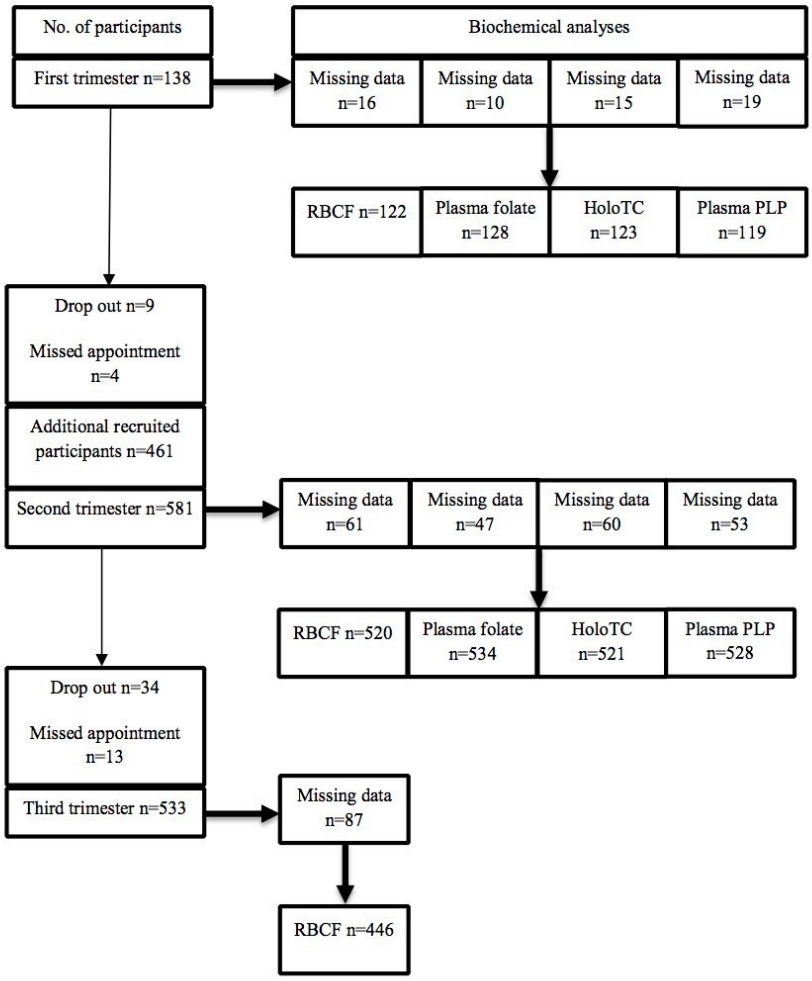


Figure 3.1: Women recruitment in APrON cohort-1 during each pregnancy visit for biochemical analyses

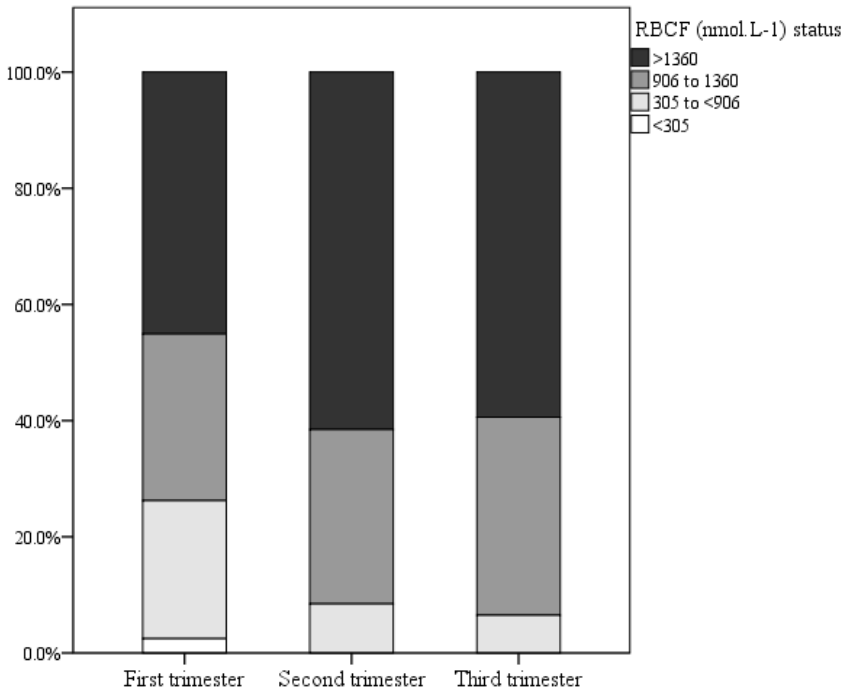


Figure 3.2: Proportion of women divided by RBCF status. The percentage of women in the <305 nmol/L and 305 to < 906 nmol/L status ranges decreased and for >1360 nmol/L range increased significantly over time ($P < 0.001$ from chi-square analysis)

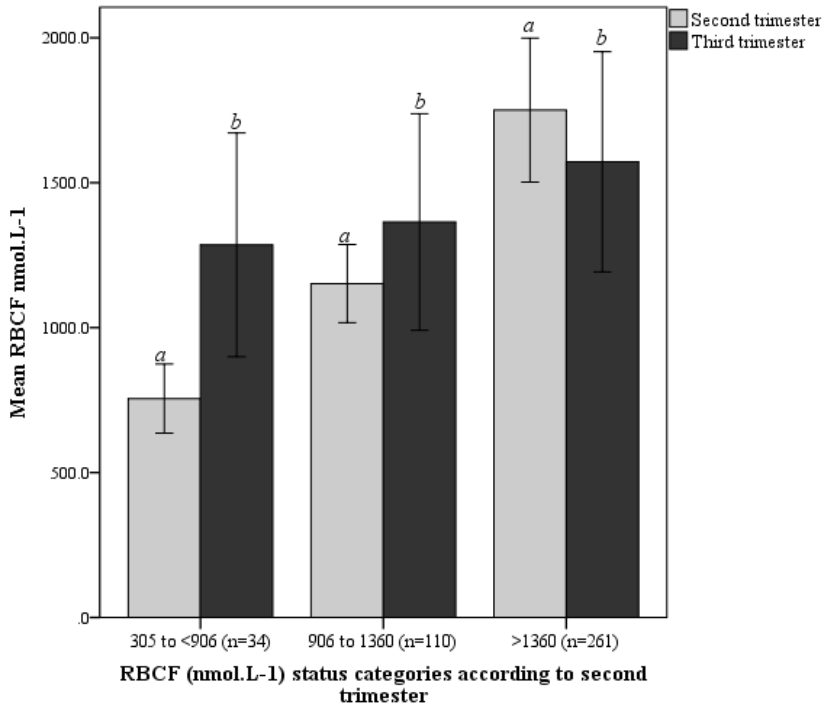


Figure 3.3: Changes in mean RBCF concentration between trimesters two and three. Bars are mean ± SD for the women who were recruited in their second trimester and provided samples in their second and third trimesters. The x-axis represents the reference range for RBCF in which women were classified in second trimester. Within each RBCF reference range, bars that do not share a letter are significantly different ($P < 0.05$, obtained from Friedman's test).

CHAPTER 4: Supplement Use is Required to Meet Recommendations of Folate, Vitamin B_{12} and Vitamin B_6 in Women During Pregnancy and 3-Months Postpartum in Alberta

4.1 Introduction

Folate plays an important role in one-carbon metabolism, methylation reactions and amino acid metabolism. It is crucial during pregnancy and in situations of inadequacy it can negatively influence pregnancy outcomes (104-109). In Canada universal folic acid fortification of white flour and enriched pasta and corn meal was implemented in 1998 and it has been associated with improved folate intake and status of Canadians (110,111). Additionally, women of childbearing age in Canada are recommended to consume multivitamin supplements containing 400 μg of folic acid daily (17). It is not known, however, if Canadian women during pregnancy and lactation are meeting the current dietary recommendations for folate and the related B-vitamins (vitamin B_{12} and vitamin B_6).

Recent research suggests that Canadians are achieving folate status goals, but 40% have red blood cell folate concentrations above the reference range (>1360 nmol/L) (70). Presumably this is due to high intake of supplemental folic acid. Moreover, ~40% of Canadians reported taking a daily vitamin/mineral supplement (112,113). High doses of folic acid can mask vitamin B_{12} deficiency and can also aggravate neurological disruptions associated with vitamin B_{12} deficiency (28-30,114,115) and that has raised concerns about the other B-vitamins. Women with low vitamin B_{12} status are also at risk of Neural Tube Defects (NTD) (39,116). In a

cohort of nutritionally low risk, high socio-economic status (SES) women "Alberta Pregnancy Outcomes and Nutrition" (APrON), we found a reported high use (~90%) of supplements containing folic acid (95). Recent literature has confirmed that diet alone is not sufficient to fulfill the daily recommended requirements of many micronutrients (8,25,117,118). Additionally, the results of other studies showed that a considerable proportion of pregnant women (24%) (119) during early pregnancy and of women of childbearing age (22%) (70) are not achieving folate status considered optimal for NTD risk prevention (71,72). These findings suggest that the use of supplements may be essential in achieving nutritional adequacy of folate. However, it may result in a large proportion of women consuming intakes above the Tolerable Upper Intake Level (UL) of folic acid (1000 μg/day) (25), thus, creating a potential nutrient imbalance that may account for status observed above the reference ranges (119). Information on this is required by health professionals to appropriately counsel women on prudent supplement use.

The food and supplemental intake of folate, vitamin B_{12} and vitamin B_6 in healthy Canadian women during pregnancy and lactation is not known. The objectives of the current study were (1) to estimate the intake of folate, vitamin B_{12} and vitamin B_6 from food and supplements, (2) to examine the contribution of different food sources towards these vitamins of women during pregnancy and 3-months postpartum and (3) to determine the proportion of women unable to achieve estimated average requirement of these B-vitamins by food alone or by food + supplement.

4.2 Materials and Methods

4.2.1 Study design and subjects

Pregnant women (n = 599) were recruited in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort-1 from Edmonton and Calgary. Information on the APrON cohort has been published previously (94). Briefly, inclusion criteria for women were; residents of Edmonton or Calgary, Alberta, ≤27 weeks of gestation, ≥16 years of age and able to speak and write in English. During the first visit of each woman recruited, a detailed questionnaire was obtained to collect information on demographics (age, weight, height immediately prior to pregnancy, number of weeks of gestation, education level, marital status, parity, ethnicity, planned pregnancy and household income) and medical history or health concerns. The dietary data were collected for the present study (June 2009-2010) as part of a prospective cohort study to estimate folate, vitamin B_{12} and vitamin B_6 intakes during pregnancy and 3-months postpartum. The study ethics approved by the Health Research Ethics Boards at the University of Alberta and the University of Calgary. Written consent was obtained from the participants prior to enrolling in the study.

4.2.2 Dietary assessment of folate, vitamin B_{12} and vitamin B_6

The food intake information was collected during each pregnancy visit (first trimester; ≤13 weeks, second trimester; 14-26 and third trimester; 27-40 weeks of gestation) and 3-months postpartum. For the current study, 24-hour recalls were used to collect participants' food and beverage intake for the previous day. Face-to-face interviews were conducted by dieticians and trained research assistants and food

models and common kitchen measurement tools were used to help in defining the portion size. The food intake was coded and analyzed using ESHA Food Processor SQL version 10.5.0. (ESHA Research 1987-2010). Information on supplement intake was obtained using a supplement intake questionnaire (SIQ). With some adaptations for pregnant women, the SIQ was developed specifically for the APrON study which is based on the questionnaires used before (120,121). Details have been published elsewhere (95). Briefly, the SIQ questions were developed to collect information regarding Natural Health Products (NHP). Women were asked to provide information on dosage and frequency of current intake of NHP. A detailed file for each woman was created and correction factors were applied for days of week, weeks of trimester and dosages at each trimester and 3-months postpartum. The file was linked to a NHP database to determine the supplements' contribution to folate, vitamin B_{12} and vitamin B_6 total intake. The daily intake from supplements was summed with food intake data obtained from the 24-hour recalls to estimate the total daily intake of the nutrients. The Estimated Average Requirement (EAR) cut-point method (12) was used, as described in Dietary Reference Intake reports to assess nutrient intake adequacy in groups. This method requires that the distribution of requirement should be symmetrical after which it can be used to estimate the proportion of population with usual intake below the EAR. In the current study, usual intake of folate, vitamin B_{12} and vitamin B_6 during pregnancy was determined using dietary intake data of each pregnancy trimester. However, there were no repeated measures available for postpartum time period, thus usual intake cannot be determined. Due to folic acid fortification of food items, it is recommended to express the EAR of folate as Dietary

Folate Equivalents (DFE) (13) which is used to adjust the increased bioavailability of synthetic folic acid in fortified foods. It is calculated as; natural folate in food (µg) + 1.7(synthetic folic acid in food). Our database did not calculate the DFE value for some of the food items and thus we represented our food folate data as µg/day instead of DFE µg/day. The EAR of folate is 520 µg/day and 450 µg/day (13), for vitamin B_{12} is 2.2 µg/day and 2.4 µg/day (13) and for vitamin B_6 is 1.6 mg/day and 1.7 mg/day during pregnancy and postpartum respectively (13).

4.2.3 Estimation of major food sources and contribution to food intake of folate, vitamin B_{12} and vitamin B_6

Food sources were categorized into groups to assess the contribution of each food source to folate, vitamin B_{12} and vitamin B_6 food intake assessed by 24-hour recalls. Food sources were classified based on the food categories defined by Canadian Nutrient File (CNF) and United States Department of Agriculture (USDA). Similar food groups were combined to form larger categories (n = 7). Grain/baked products includes cereal grains, pasta and baked products; Fruits and vegetables includes fresh, frozen or canned fruits and vegetables; Meat and alternatives includes poultry, fish, shellfish, meats, legumes, eggs; Milk and products includes milk or powdered milk, soy/almond beverages, yogurt, cheese; Beverages includes tea, coffee, alcohol, juices; Fast foods includes fast foods and convenience foods; Miscellaneous includes fats and oils, soups, sauces, gravies, sweets.

The relative contribution of each food category was calculated for all three pregnancy trimesters and 3-months postpartum together. The amount of folate, vitamin B_{12} and vitamin B_6 consumed in each food category is reported as percent of total food intake of respective B-vitamins.

4.2.4 Statistics

The estimated nutrient intake was calculated and analyzed using SPSS version 20.0 (IBM SPSS for Windows, version 20.0, Chicago, IL); $P < 0.05$ was considered significant. The data were analyzed for normality using Kologrov-Smirnov test. Median, 95% confidence interval (CI) and Chi-square analysis was conducted to describe the data as intakes were not normally distributed. The difference of dietary intake of the B-vitamins among pregnancy time points and 3-months postpartum and intraindividual variability of nutrient intake during pregnancy was determined by Wilcoxon Signed Rank tests.

4.3 Results

4.3.1 Subjects

Baseline characteristics of participants are presented in Table 4.1. The majority of women were Caucasian, married, held university or post-graduate degrees, had high family annual income ($100,000+), multiparous and had a planned pregnancy (Table 4.1).

4.3.2 Folate, vitamin B_{12} and vitamin B_6 intake

Table 4.2 presents the estimated median intake of folate, vitamin B_{12} and vitamin B_6 during pregnancy and 3-months postpartum. The estimated median (95% CI) intakes of folate were 1228 µg (1154-1294 µg), 1294 µg (1273-1315 µg), 1302 µg (1277-1320 µg) and 1135 µg (1085-1169 µg) during first, second and third trimester and 3-months postpartum respectively. The number of women with estimated folate intakes below EAR was almost negligible during pregnancy, but this proportion increased to 20% at 3-months postpartum. For vitamin B_{12}, very few women's estimated intake was below the EAR during pregnancy (Table 4.2). However, the number of women with estimated intake of vitamin B_{12} below the EAR increased to 5% at 3-months postpartum. Similar trends for vitamin B_6 intake were found during pregnancy with 10% of the women not meeting the EAR during postpartum (Table 4.2). The median intake of folate, vitamin B_{12} and vitamin B_6 did not differ among trimesters of pregnancy; however, they significantly declined ($P < 0.05$) at 3-months postpartum (Table 4.2). Figures 4.1a-c represents the estimated mean intake of the B-vitamins from food, supplements and total intake of the women during pregnancy and 3-months postpartum. The estimated mean intake of folate through food alone was below the EAR during all time-points. However, for vitamin B_{12} and vitamin B_6, the estimated mean intake through food alone was meeting the EAR recommendations. Depending on the time-points; food contributed 20% to 27% of folate, 11% to 21% of vitamin B_{12} and 18% to 20% of vitamin B_6 to the total intake of the B-vitamins (Figure 4.1a-c).

4.3.3 Intra-individual variability

In order to determine changes in intake of the B-vitamins during pregnancy, estimated intake data from women where there were food records available for each of the three trimesters of pregnancy was analyzed (Table 4.3). Because the data were not normally distributed, Wilcoxon Signed Rank test and Spearman correlation coefficient was used to analyze the data. There was a strong correlation between the three pregnancy time-points intake data (coefficient of correlation for folate r = 0.5, for vitamin B_{12} r = 0.6 and for vitamin B_6 r = 0.7; $P < 0.01$) and there was no significant difference in mean intake between trimesters.

4.3.4 Risk of not meeting current recommendations of folate, vitamin B_{12} and vitamin B_6

Using the EAR cut-point method (12), the proportion of women whose usual intake was below the EAR from food alone and from food + supplement (total intake) for folate and vitamin B_{12} and vitamin B_6 during pregnancy was calculated. Approximately 92%, 17%, 33% of the women did not meet the current recommendations through food alone during pregnancy for folate, vitamin B_{12} and vitamin B_6 respectively (Figure 4.2a-c). While, the proportion of women not meeting the current recommendations after including intake from supplements was 5%, 1%, 2% for folate, vitamin B_{12} and vitamin B_6 respectively (Figure 4.2a-c). The odds of not meeting recommendation were approximately 25, 19, 20 times higher through

food alone compare to food + supplement (for folate: OR 24.9; 95% CI 17.4, 35.7; $P < 0.001$, for vitamin B_{12}: OR 19.0; 95% CI 10.8, 33.5; $P < 0.001$, for vitamin B_6: OR 19.6; 95% CI 13.3, 29.0; $P < 0.001$). Additionally, more than 90% of the cohort reported taking a daily folate, vitamin B_{12} and vitamin B_6 supplement (95) during pregnancy but this declined significantly (~10%) when asked at 3-months postpartum (data not shown; Chi square analysis; $P < 0.01$).

4.3.5 Food sources of folate, vitamin B_{12} and vitamin B_6

Food source categories and relative contribution analysis showed that grains/baked products (37%) followed by fruits and vegetable (26%) were the major contributors of food folate intake in the cohort (Table 4.4). For vitamin B_{12}, milk and milk products (44%) and meat and alternatives (40%) were the major food sources (Table 4.4). For vitamin B_6 fruits and vegetables (31%) and meat and alternatives (25%) were the major food sources consumed (Table 4.4).

4.4 Discussion

The results of this study present estimated daily intakes of folate, vitamin B_{12} and vitamin B_6 through food and supplements of a large cohort of highly educated and high SES pregnant women residing in Alberta. Overall, the percentage of women with intake below the EAR of folate, vitamin B_{12} and vitamin B_6 was negligible in the cohort, primarily due to their reported use of daily supplements. However, ~80% of the women during pregnancy and 59% during postpartum had intakes of folate that exceeded UL (1000 µg/day) (13). The findings support our previous results (119)

showing that approximately half of the women during each trimester of pregnancy have high red blood cell folate concentration that exceeded the top of the reference range (>1360 nmol/L) in the APrON cohort.

According to Health Canada (17) and Canada's Food Guide, the excellent sources of folate in the diet are vegetables such as okra, asparagus, spinach, green salads and beans per one food guide serving, followed by baked products made from enriched flour based on the amounts estimated from CNF. Although food and beverages only contributed ~20% (Figure 4.1a) to total folate intake, grain/baked products (37%) followed by the fruits and vegetables (26%) were the major food sources of folate. Our results are consistent with previous Canadian studies (25,73). According to Canada's Food Guide, the highest source of vitamin B_{12} is meat and alternatives followed by the milk and milk products. Our results indicated that 44% of vitamin B_{12} in food came from milk and milk products in the cohort and 40% of meat and alternatives contributed to total food vitamin B_{12} intake. This is likely due to the high consumption of milk in this cohort (122). Canada's Food Guide identifies fruits and vegetables and meat and alternatives as the best dietary sources of vitamin B_6. Consistent with this, fruits and vegetables accounted for 31% and meat and alternatives accounted for 25% of vitamin B_6 intake from food and beverages. According to our knowledge, this study is the first to report vitamin B_{12} and vitamin B_6 intake of healthy pregnant women in Canada.

Our results of risk analysis of vitamin inadequacy revealed that majority of the women had inadequate (< EAR) intake through food alone during pregnancy. The risk of not meeting current recommendations of daily intake without a supplement

was 25, 19, and 33 times higher for folate, vitamin B_{12} and vitamin B_6 respectively than diet with a supplement. This suggests that educating and facilitating the use of a daily supplement containing B-vitamins is important to reduce the risks of maternal and infant complications associated with poor status. In the current study, among these three vitamins the food inadequacy was highest for folate (92%) and lowest for vitamin B_{12} (17%) during pregnancy. Our results are in concordance with other Canadian studies that have suggested that food intake of folate is not sufficient to meet the daily intake recommendations (8,11,24,25,123). Sherwood et al. (2006) assessed the folate status of lactating women in a randomized control trial (n = 62). They found that 36% of women during pregnancy and 32% during postpartum had food folate intake below the EAR. Similar to the current study, they found that the incidence of dietary inadequacy disappeared when supplemental intake was summed up into total folate intake (25). Similarly, the results of another cross-sectional study showed that the folate intake of 23% women of childbearing age (n = 148) was below the EAR through food sources only (11). However, the percentage declined to 14% when supplemental intake was accounted for into total folate intake. The current study found that this is not only for folate but also for vitamin B_{12} and vitamin B_6 and supports the importance of ensuring and facilitating that pregnant and lactating women take a multivitamin supplement that contains all three of the B-vitamins. It should be noted that supplement use significantly declined to approximately 10% during 3-months postpartum; thus counselling is needed to ensure women continue to take a supplement during lactation.

Another consideration is the fact that taking a supplement put 59% to 85% (depending on the time-points) of the women at risk of consuming folate above the UL. A similar finding was reported by Sherwood et al. (2006) in pregnant and lactating women. The results of Sherwood et al. indicated that by combining the supplemental intake of folate with food intake, 67% of the women had intakes above the UL (25). Health Canada recommends that women of child-bearing age and during pregnancy should consume 400 µg folic acid/day through supplements (17). However, the results of the current study indicated that mean intake of folic acid from supplements exceeds the upper level (1000 µg/day) during pregnancy (Figure 4.1a). One of the concerns associated with high levels of folic acid intake is the appearance of unmetabolized folic acid and the aggravation in some pre-existing cancerous lesions (31,32). The other concern is the masking of vitamin B_{12} deficiency (28-30). Therefore, it has been suggested to introduce vitamin B_{12} fortification with folic acid to elude the masking effects of vitamin B_{12} deficiency. However, the results of our study showed that estimated total intake of vitamin B_{12} of APrON women was sufficient to meet the daily estimated average requirements. Inadequacy of vitamin B_{12} through food alone was also very low compared to food folate inadequacy, suggesting that most of the women in the APrON cohort were not at the risk of masked vitamin B_{12} deficiency.

A limitation of the current study is that we did not use DFE for describing dietary folate intake data; thus we may have underestimated somewhat the contribution of food to total folate intake. This is likely a small difference as when we analyzed our data presented in Figure 4.1a, food folate contributed only ~20% to total

folate intake. Major sources of synthetic folic acid in food are the fortified cereals and grain products, which in our study contributed 37% to total food folate intake. Assuming all of the grain/baked products category provided synthetic folic acid, would result in a change to 50% to total food folate intake which might not make much difference in food folate inadequacy in APrON cohort.

4.5 Conclusions

In conclusion, the results of our prospective study showed that food alone is not sufficient to meet the current recommendations of folate, vitamin B_{12} and vitamin B_6 in a large proportion of apparently healthy women belonging to a high SES and considered nutritionally 'low risk' during pregnancy and 3-months postpartum. Supplements were required to ensure that the women met the recommendations for all three B-vitamins during early or late pregnancy and 3-months postpartum but placed them at risk for consuming folate above the UL. Based on the results of the current study, women should be counselled to consume a daily supplement containing 400µg of folic acid (680 µg DFE/day), vitamin B_{12} and vitamin B_6 with their diet to meet current population requirements of the B-vitamins of healthy pregnant and lactating women which is also Health Canada's recommendation but not putting them at risk for exceeding the UL of folate.

4.6 Relevance to Practice

Vitamin B_6 food sources are in consistence and of vitamin B_{12} and folate are not consistent with the major food sources described in Canada's Food Guide. Supplements required during pregnancy and postpartum to fulfill the dietary recommendations of folate, vitamin B_{12} and vitamin B_6 needs recognition in dietetic practice. Results of this study can be helpful for dietitians to acquire a more careful approach in using supplements containing folic acid more than 400 µg to help that total intake should not exceed UL.

4.7 Acknowledgement

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Table 4.1: Characteristics of women enrolled in Alberta Pregnancy Outcomes and Nutrition cohort-1.

Characteristics	Percentage (%)
Age (year)	N = 597
17-30	46
31-45	54
Pre-pregnancy BMI (kg/m^2)	N = 572
Underweight	3
Normal	65
Overweight	18
Obese	14
Household income (CAD)	N = 550
Less than $20, 000	2
$20, 000 to $39, 000	4
$40, 000 to $69, 000	14
$70, 000 to $99, 000	25
$100, 000+	55
Education level	N = 557
High school/university study	69
Less than high school/diploma/certificate	31
Ethnicity, 556	N = 556
Caucasian	87
Other	13
Smoking	N = 557
Never	68

Ever	32
Previous pregnancy	N = 561
Yes	60
No	40
Marital status	N = 560
Married	97
Other	3
Planned pregnancy	N = 562
Yes	81
No	19
Breast feeding	N = 557
>12 weeks	90
≤12 weeks	10

Note: Baseline characteristics data collected at first visit of each participant. BMI; body mass index, was calculated based on self-reported pre-pregnancy weight (kg) and height (m).

Table 4.2: Distribution of total dietary intake (food and supplement) of folate, vitamins B_{12} and vitamin B_6 during pregnancy and 3-months postpartum of the women enrolled in Alberta Pregnancy Outcomes and Nutrition (APrON) study cohort-1

	1st trimester		2nd trimester		3rd trimester		Postpartum	
	N (%)	**Median(95% CI)**	**N (%)**	**Median(95% CI)**	**N (%)**	**Median(95% CI)**	**N (%)**	**Median(95% CI)**
Folate (µg/day)								
< EAR (< 520)	8 (6)	391(120,517)	31 (5)	380(304,448)	25 (5)	358(260,385)	94 (20)	282(266,318)
EAR (520-1000)	29 (23)	768(679,858)	65 (12)	802(741,854)	49 (10)	756(675,813)	103 (21)	717(687,753)
> UL (> 1000)	89 (71)	1342(1269,1384)	464 (83)	1336(1318,1363)	421 (85)	1334(1318,1352)	286 (59)	1296(1270,1322)
Total number	126	1228(1154,1294)[a]	560	1294(1273,1315)[a]	495	1302(1277,1320)[a]	483	1135(1085,1169)[b]
Vitamin B_{12} (µg/day)								
< EAR (< 2.2)	3 (2)	1.5(1.3,2.1)	6 (1)	1.8(0.6,2.0)	4 (1)	1.5(1.2,1.9)	25 (5)	1.7(1.4,2.0)
EAR (≥ 2.2)	123 (98)	10.4(7.9,11.8)	558 (99)	10.6(9.6,11.5)	495 (99)	11.1(9.6,12.2)	461 (95)	8.3(7.7,9.1)
Total number	126	9.9(7.5,11.8)[a]	564	10.5(9.5,11.4)[a]	499	11.1(9.4,11.9)[a]	486	8.0(7.4,8.6)[b]
Vitamin B_6 (mg/day)								
< EAR (< 1.6)	5 (4)	1.1(1.1,1.5)	13 (2)	1.2(0.8,1.5)	11 (2)	1.2(0.8,1.4)	47 (10)	1.3(1.2,1.4)
EAR(1.6-100)	118 (94)	4.4(3.9,5.6)	541 (96)	5.0(4.6,6.2)	482 (97)	5.1(4.6,6.4)	431 (88)	4.2(4.0,4.7)
> UL (> 100)	3 (2)	112.6(104.5,136.7)	10 (2)	104.4(102.7,116.6)	6 (1)	105.1(103.0,135.5)	8 (2)	106.0(101.1,278.6)
Total number	126	4.4(3.9,5.5)[a]	564	5.0(4.6,6.1)[a]	499	5.0(4.6,6.1)[a]	486	4.0 (3.7,4.3)[b]

Note: N = number of women; SD=standard deviation; UL=Tolerable Upper Intake Level; EAR=Estimated Average Requirement according to Institute of Medicine guidelines for pregnant and lactating women. $P < 0.05$ obtained from Wilcoxon Signed Ranks test and letters not similar are significantly different.

Table 4.3: Comparison of intra-individual variability of the total folate, vitamin B_{12} and vitamin B_6 intake (food and supplement) of women during pregnancy

	1st trimester	2nd trimester	3rd trimester	Correlation coefficient*
Folate (µg/d)	1425 ± 1082 (1228)	1546 ± 1181 (1294)	1485 ± 1055 (1302)	r = 0.5
Vitamin B_{12} (µg/d)	41.3 ± 164.2 (9.9)	35.8 ± 139.6 (10.5)	32.3 ± 125.3 (11.1)	r = 0.6
Vitamin B_6 (mg/d)	9.8 ± 19.1 (4.4)	10.7 ± 16.4 (5.0)	10.1 ± 14.4 (5.0)	r = 0.7

Note: Data are presented as mean ± SD (Median). There was no significant difference among mean estimated intake during each trimester of pregnancy as assessed by Wilcoxon Signed Rank test. *$P < 0.01$ significant correlation between trimesters using Spearman correlation coefficient.

Table 4.4: Relative contribution of each food source to folate, vitamin B_{12} and vitamin B_6 dietary intake assessed by 24-hour recalls combined from pregnancy and 3-months postpartum.

	Contribution (%)*		
Food Sources	Folate	Vitamin B_{12}	Vitamin B_6
Grain/baked products	37	5	19
Fruits and vegetables	26	0	31
Meat and alternatives	10	40	25
Milk and milk products	9	44	9
Beverages	8	1	5
Fast foods	5	4	6
Miscellaneous	5	6	5

Note: Data are presented as percentage contribution of food source category. Food groups were classified based on the food categories defined by Canadian Nutrient File (CNF) and United States Department of Agriculture (USDA). Similar food groups were combined to form larger categories (n = 7). Grain/baked products includes cereal grains, pasta and baked products; Fruits and vegetables includes fresh, frozen or canned fruits and vegetables; Meat and alternatives includes poultry, fish, shellfish, meat, legumes, eggs; Milk and milk products includes milk or powdered milk, soy/almond beverages, yogurt, cheese; Beverages includes tea, coffee, alcohol, juices; Fast foods includes fast foods and convenience foods; Miscellaneous includes fats

and oils, soups, sauces, gravies, sweets. *Percent contribution was determined by calculating the total amount of folate, vitamin B_{12} and B_6 consumed for each food category and then dividing by total dietary intake of respective B-vitamins for all women.

Figure 4.1a

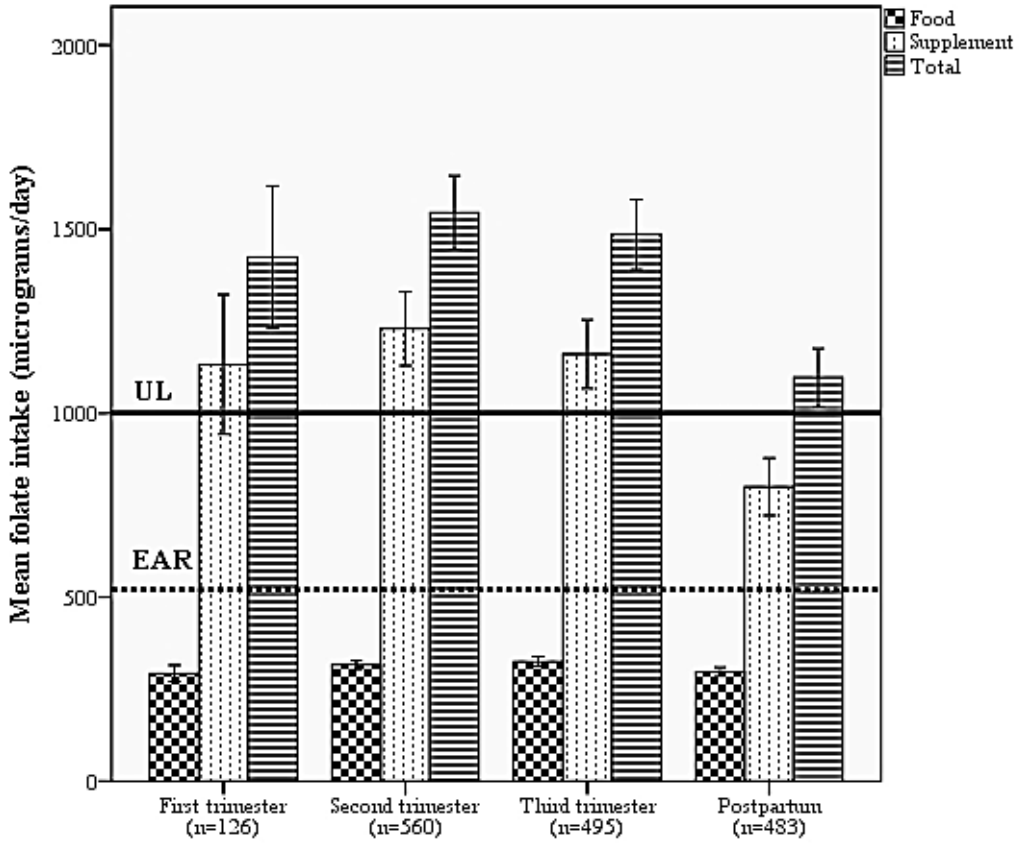
Food
Supplement
Total
Mean folate intake (micrograms/day)
2000
1500
1000
500
0
UL
EAR
First trimester (n=126)
Second trimester (n=560)
Third trimester (n=495)
Postpartum (n=483)

Figure 4.1b

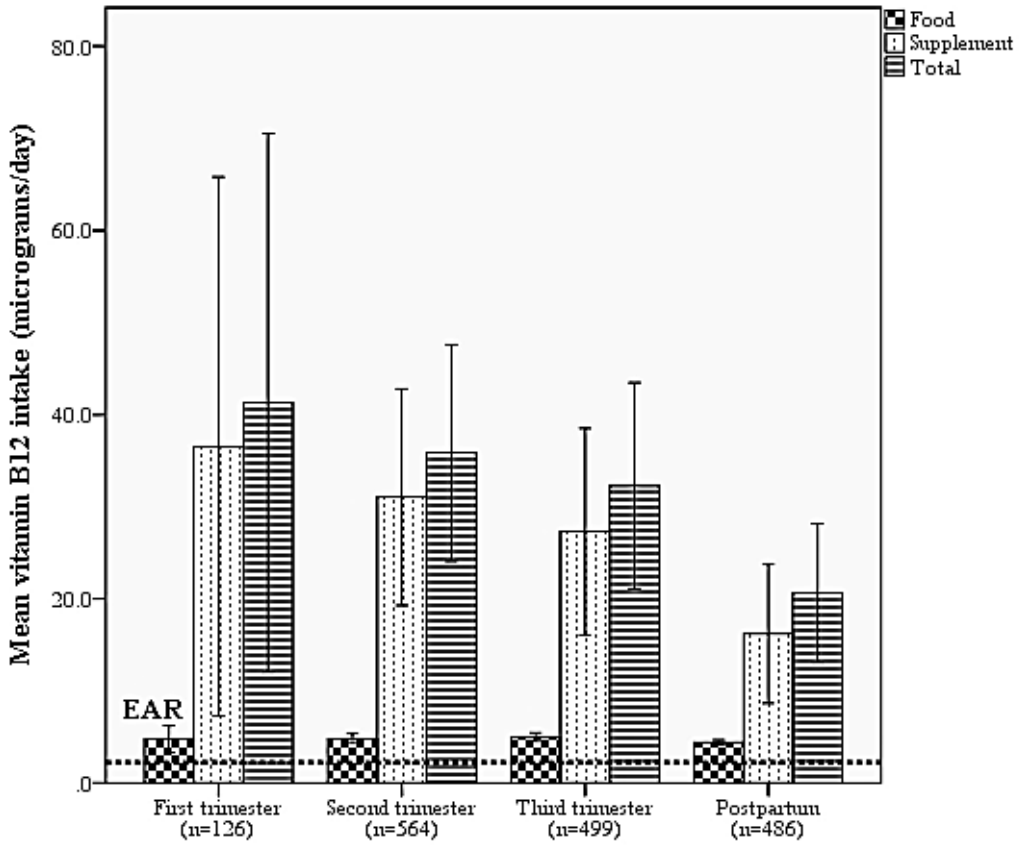
Food
Supplement
Total
Mean vitamin B12 intake (micrograms/day)
80.0
60.0
40.0
20.0
.0
EAR
First trimester (n=126)
Second trimester (n=564)
Third trimester (n=499)
Postpartum (n=486)

Figure 4.1c

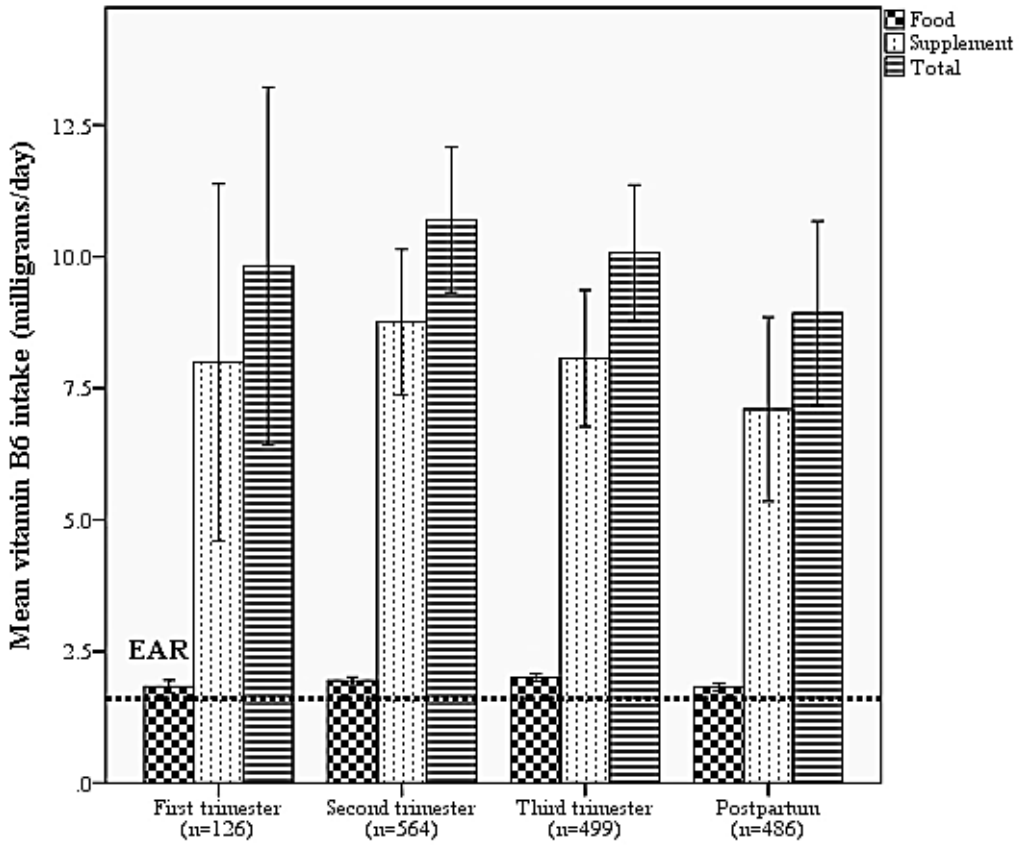

Figure 4.1a-c: Description of estimated mean ± SD of intake of (a) folate (b) vitamin B_{12} and (c) vitamin B_6 through food alone, through supplements and total intake

Figure 4.2a

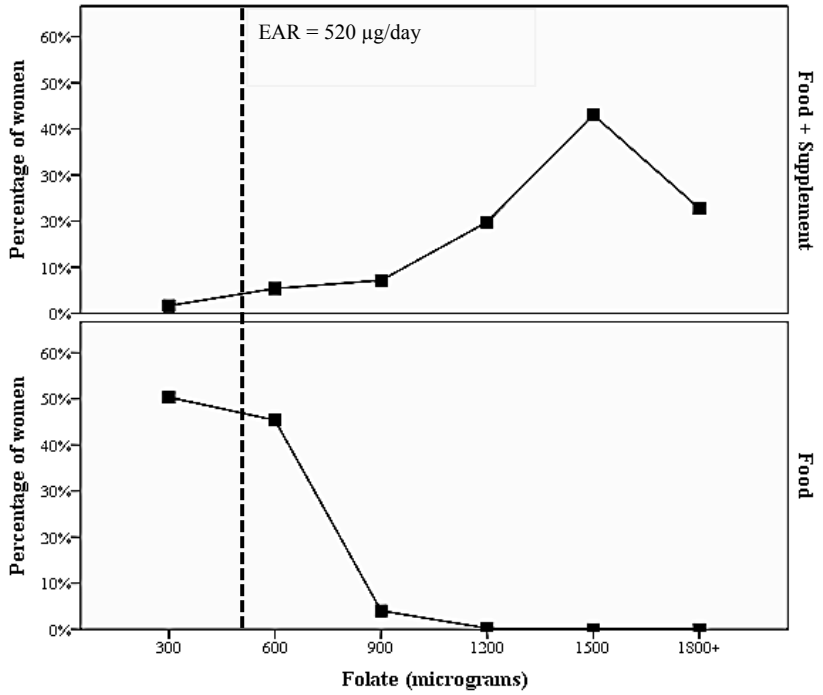
EAR = 520 µg/day
Percentage of women
60%
50%
40%
30%
20%
10%
0%
Food + Supplement
Percentage of women
60%
50%
40%
30%
20%
10%
0%
Food
300
600
900
1200
1500
1800+
Folate (micrograms)

Figure 4.2b

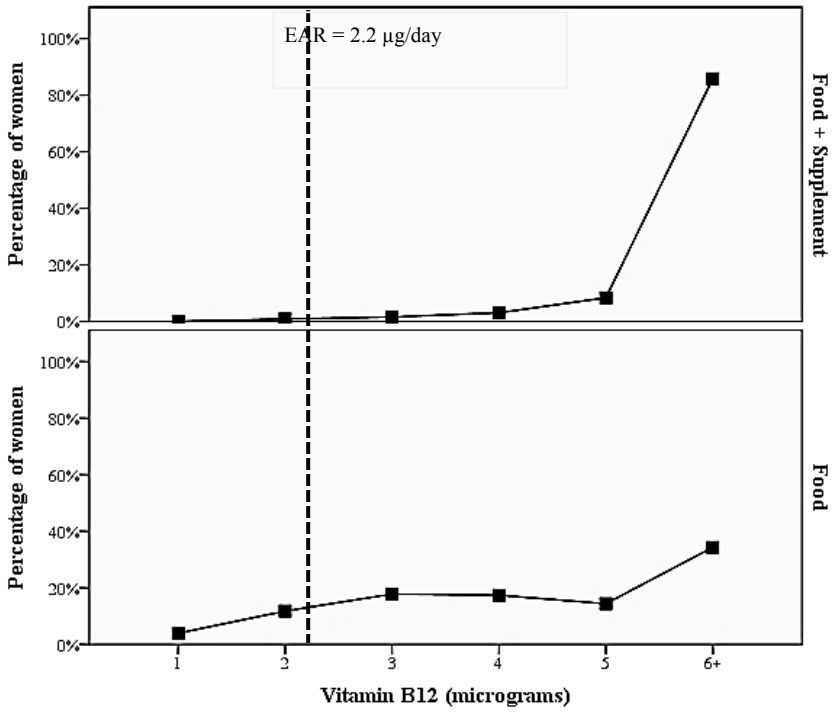
EAR = 2.2 µg/day
Percentage of women
100%
80%
60%
40%
20%
0%
Food + Supplement
Percentage of women
100%
80%
60%
40%
20%
0%
Food
1
2
3
4
5
6+
Vitamin B12 (micrograms)

Figure 4.2c

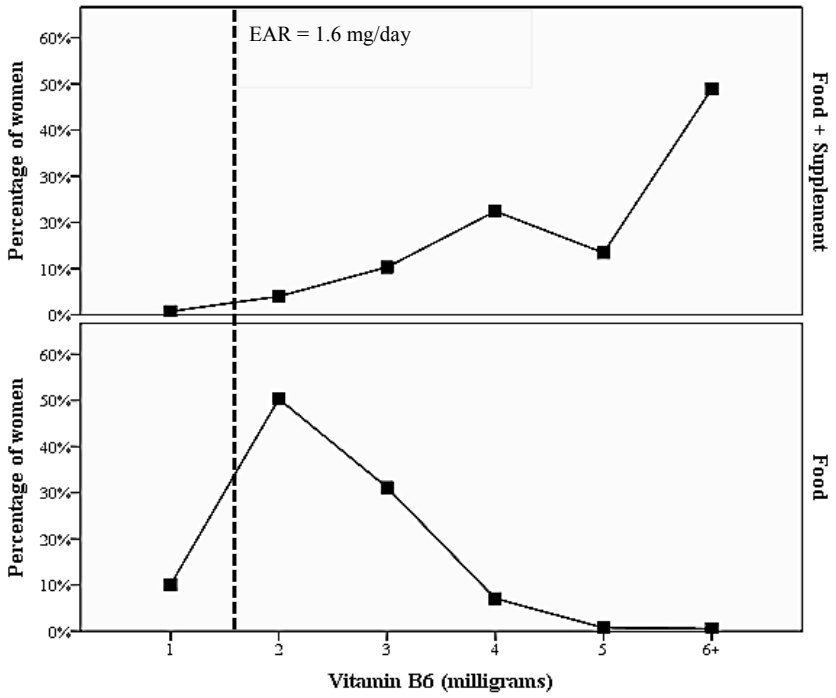

Figure 4.2a-c: Distribution of the estimated (a) folate, (b) vitamin B_{12} and (c) vitamin B_6 intake through food and food + supplement in women during pregnancy using data from APrON. Using the EAR cut-point method, the area below EAR line represents the proportion of women whose intakes are below EAR.

CHAPTER 5: Final Discussion and Conclusions

5.1 Review of study questions and conclusions

(a) What is the RBCF and plasma folate status of women during pregnancy?

For folate status analyses, 122, 520 and 446 blood samples were available during first, second and third trimesters respectively. Median (95%CI) RBCF concentration were 1280 nmol/L (1114 nmol/L, 1393 nmol/L), 1504 nmol/L (1450 nmol/L, 1568 nmol/L) and 1462 nmol/L (1421 nmol/L, 1529 nmol/L) during first, second and third trimesters respectively. The concentrations of RBCF during second and third trimester were significantly higher compare to first trimester ($P<0.05$). The median (95% CI) concentration of plasma folate was 36 nmol/L (35 nmol/L, 36 nmol/L) during first and second trimesters. All plasma folate values were within the normal range of reference.

(b) What proportion of women achieve the RBCF status known to prevent the risk of NTD during early pregnancy?

Based on the results available from first trimester RBCF analysis, only 3 out of 122 women had RBCF concentration corresponding to folate deficiency (<305

nmol/L) and none in second and third trimesters. However, 24% (29/122) of the women had RBCF concentration below the value considered optimal for NTD prevention (<906 nmol/L) during first trimester of pregnancy. The percentage declined to 8% in second trimester and 6% in third trimester. The results indicated that a quarter of the women of APrON cohort had sup-optimal folate status during early pregnancy.

(c) Do women have RBCF status above the reference range?

Approximately 45%, 62% and 59% of the women participated in APrON cohort-1 had RBCF concentrations above the cut-off of the normal range (>1360 nmol/L) during first, second and third trimesters respectively. The results indicated that a significant proportion of women had high folate status during pregnancy.

(d) Does folic acid intake associated with RBCF and plasma folate status?

During second trimester, mean RBCF concentration was 1404 ± 1 for women consuming supplement containing ≤ 1000 μg folic acid compare to 1536 ± 1 for women consuming >1000 μg of folic acid in supplements daily. For plasma folate, values were 35 ± 1 vs. 36 ± 1 for women consuming folic acid supplement

≤ 1000 µg and >1000 µg respectively. Though it was a small difference but significantly different. During third trimester, mean RBCF concentration was 1409 ± 1 vs. 1557 ± 1 for women consuming folic acid supplement ≤ 1000 µg and >1000 µg respectively. The RBCF and plasma folate concentrations were significantly higher in women who consume supplement containing >1000 µg/day compare to who consume ≤ 1000 µg/day of folic acid ($P<0.05$). The values remained significant after adjusting for dietary intake data of folate and maternal covariates which were statistically different in a bivariate analysis.

(e) What proportion of women meet vitamin B_{12} and vitamin B_6 status reference ranges in a folic acid fortified/supplemented environment?

Median (95% CI) plasma holoTC concentrations were 92 pmol/L (84 pmol/L, 100 pmol/L) and 83 pmol/L (80 pmol/L, 85 pmol/L) and plasma PLP were 94 nmol/L (82 nmol/L, 112 nmol/L) and 76 nmol/L (70 nmol/L, 83 nmol/L) in women during first and second trimesters respectively. Deficiency of holoTC and PLP was also rare (less than 1%) in the cohort. The results indicated that in APrON cohort there was not a risk of deficiency of vitamin B_{12} and vitamin B_6 in a folic acid fortified/supplemented environment.

(f) Do women adhere to current recommendations of folate, vitamin B_{12} and vitamin B_6 during pregnancy and 3-months postpartum?

Estimated Average Requirements (EAR) of folate is 520 µg/day and 450 µg/day, of vitamin B_{12} is 2.2 µg/day and 2.4 µg/day and of vitamin B_6 is 1.6 mg/day and 1.7 mg/day during pregnancy and postpartum respectively. Median intake of total folate (food + supplement) of women was 1228 µg, 1294 µg, 1302 µg and 1135 µg , of total vitamin B_{12} was 9.9 µg, 10.5 µg, 11.1 µg and 8.0 µg and of vitamin B_6 was 4.4 mg, 5.0 mg, 5.0 mg and 4.0 mg during first, second, third and 3-months postpartum respectively. Median intake of these B-vitamins significantly decreased at 3-months postpartum ($P<0.05$). The percentage of women with folate intake below EAR was 5% to 6% (depending on the trimester) during pregnancy which increased to 20% during 3-months postpartum. The percentage of women with vitamin B_{12} intake below EAR was 1% to 2% (depending on the trimester) during pregnancy which increased to 5% during 3-months postpartum. The percentage of women with vitamin B_6 intake below EAR was 2% to 4% (depending on the trimester) during pregnancy which increased to 10% during 3-months postpartum. The results showed that inadequacy of these B-vitamins were rare in the cohort; however, increased significantly in 3-months postpartum.

(g) What proportion of the women are consuming folic acid above the UL during pregnancy and 3-months postpartum?

The Tolerable Upper Intake Level (UL) for folate is 1000 μg/day. During pregnancy 71% to 85% of the women had their folate intake above the UL and 59% during 3-months postpartum.

(h) Are women at risk of inadequate intakes of vitamin B_{12} and vitamin B_6 in folic acid fortified/supplemented environment?

There was a very small proportion of women with intake below EAR for vitamin B_{12} (1% to 5%; depending on the time-point) and vitamin B_6 (2% to 10%; depending on the time-point) which indicated that women in APrON were not at risk of inadequacy of these B-vitamins in folic acid fortified/supplemented food environment.

(i) What is the contribution of food towards total intake (food + supplement) of folate, vitamin B_{12} and vitamin B_6?

Depending on the time-points; food contributed 20% to 27% of folate, 11% to 21% of vitamin B_{12} and 18% to 20% of vitamin B_6 to the total intake of the B-vitamins.

(j) What are the major food sources contributing to intake of folate, vitamin B_{12} and vitamin B_6?

Major contributors of food folate were grains/baked products (37%) and fruits and vegetables (26%); of vitamin B_{12} were milk and milk products (44%) and meat and alternatives (40%) and of vitamin B_6 were fruits and vegetables (31%) and meat and alternatives (25%) combined during pregnancy and 3-months postpartum in women participated in APrON cohort.

(k) What proportion of women are at risk of inadequate intake of folate, vitamin B_{12} and vitamin B_6 from food sources?

Based on the results of risk estimation of food intake only, 92%, 17% and 33% of the women were at risk of inadequate intakes of folate, vitamin B_{12} and vitamin B_6 respectively during pregnancy using EAR cut-point method.

(l) What proportion of women are at risk of inadequate intake of folate, vitamin B_{12} and vitamin B_6 from food + supplement?

The inadequacy risk analysis showed that only 5%, 1% and 2% of the women during pregnancy had their intake below the EAR when supplement intake summed with food intake. The odds of not meeting recommendations were approximately 25, 19, 20 times higher through food alone compare to food + supplement for folate, vitamin B_{12} and vitamin B_6 respectively. The results indicated that a significantly large proportion of women of APrON were at risk of not meeting recommendations through food alone and supplements were required to fulfill the requirements of the women.

5.2 Discussion

This thesis has studied the status of folate, vitamin B_{12} and vitamin B_6 in women during pregnancy and also examined the dietary intake of these B-vitamins in foods and supplements during pregnancy and 3-months postpartum. The main finding of the thesis is that approximately 50% of the women participated in this cohort had high folate status and folate intake primarily from supplements. Folate status was even higher for the women consuming folic acid supplements above the UL of 1000 µg. However, in spite of the high folate intake and status, a quarter of women during

early pregnancy had folate status below the cut-off which is considered optimal for NTD risk prevention.

In chapter 3 we assessed the RBCF in all three trimesters and plasma folate, vitamin B_{12} and vitamin B_6 status in first and second trimesters of pregnancy. We found that 24% of the women during first trimester had sub-optimal RBCF status which is likely to reflect preconception folate status of women. Our results are inconsistent with previous Canadian studies (70,74). However, women with sub-optimal folate status attained higher status with increasing gestation. This is most likely due to high rate of multivitamin supplement usage in the cohort as more than 90% of the cohort reported taking supplements (95). We have examined that plasma folate status was within normal ranges during early pregnancy which was reflective of the current usage of supplement and food intake and thus may not be a good indicator for assessing folate status in early pregnancy when women have sub-optimal status but stated taking a multivitamin supplement containing folic acid. On the other hand, most of the women had vitamin B_{12} and vitamin B_6 status within the reference ranges. Regarding vitamin B_{12} our results are in contrast to a recent study published in Canada (40). The difference in results could be partly explained by the method of recruitment used in the studies and biomarker used for the assessment of vitamin B_{12} status. We also found that over half of the cohort had RBCF above the reference range of 1360 nmol/L during pregnancy. The health implications of high folate status on pregnancy outcomes warrant further research.

Chapter 4 examined the estimated intake of folate, vitamin B_{12} and vitamin B_6 in food and supplements during pregnancy and 3-months postpartum. We found that a small proportion of women had these B-vitamins intake below the EAR during pregnancy and 3-month postpartum. The analysis of risk assessment of inadequate intakes indicated that a significant proportion of women cannot achieve their recommended intake through food alone during pregnancy. Our results are consistent with other Canadian studies which showed that food alone is not sufficient to meet the dietary recommendations (8,11,24,25). However, risk of not meeting the recommendation declined significantly when supplement intake was taken into consideration.

Another fact should also be taken into consideration is that taking a supplement placed 59% to 85% of the women at risk of consuming folate above the UL which is similar to another Canadian study (25). The health concerns associated with high folate intake is the appearance of unmetabolized folic acid and exacerbation of some pre-existing cancerous lesion (31,32). The other health concern is masking the vitamin B_{12} deficiency (28-30). The results of our study showed that women of APrON cohort did not have deficient vitamin B_{12} status nor did they have inadequate vitamin B_{12} intakes. However, multivitamin supplements are required not only for folate but for vitamin B_{12} and vitamin B_6 to achieve the recommended intakes. We also found that estimated intake of these vitamins declined significantly during 3-months postpartum which can be explained by the fact that approximately 10% of the

women stopped taking a multivitamin supplement and counselling is needed to ensure that women should continue taking a supplement during lactation.

According to our knowledge, we reported for the first time the daily intakes of vitamin B_{12} and B_6 and the sources in Canada. Excellent food sources of the food folate intake in this cohort were grain/baked products (37%) followed by the fruits and vegetables (26%) which is similar to other Canadian studies (25,73). For vitamin B_{12} excellent sources were milk and milk products (44%) which is likely due to the high consumption of milk in his cohort (124) and meat and alternatives (40%). For vitamin B_6 fruits and vegetables accounted for 31% and meat and alternatives accounted for 25% of intake from food and beverages.

5.3 Limitations

The findings of our study were based from the observations on women who were healthy with no history of chronic health problems, belonged to high socio-economic status, high education and predominantly Caucasians. The findings cannot be generalized to the whole population. But, it suggests that women of high socio-economic status who would generally be identified as 'low nutritional risk' needed additional education about prenatal folic acid supplementation.

Another limitation of the current study is the use of DFE. We did not use DFE for describing dietary folate intake data because the food data base we used (food processor) had incomplete data on the DFE content of many of the food items consumed by our cohort; thus we may have somewhat underestimated the contribution of food to total folate intake. Due to the reason we described our data as µg of folate in food sources. This is likely a small difference because food folate contributed only ~20% to total folate intake. Major sources of synthetic folic acid in food are the fortified cereals and grain products, which in our study contributed 37% to total food folate intake. If we assume that all of the grain/baked products category provided synthetic folic acid, the result would be changed to 50% of total food folate intake which might not make much difference in food folate inadequacy in APrON cohort.

Another limitation of the study is the small sample size in first trimester. Keeping in view the results our study where a quarter of women had sub-optimal folate status, having a large number of participants would be of more significance.

Based on our results, having women with sub-optimal folate status during early pregnancy and high folate status throughout pregnancy might be associated with some pregnancy and infant health outcomes. This is another limitation of our study. Also, we did not know of the genetic variations in women in regards to folate, vitamin B_{12} and vitamin B_6 related genes.

5.4 Recommendations for Future Studies:

This thesis examined the status and intake of folate, vitamin B_{12} and vitamin B_6 in pregnant Albertan women. Generally due to the homogenous nature of our participants, it cannot be extrapolated to other demographic and socio-economic status groups. It would be of great interest to use a nationally representative data to determine the subgroups at risks of inappropriate intakes and status.

It would be of great interest too to collect data before pregnancy such as for folate intake which can be associated with folate status early in the pregnancy. The pre-pregnancy dietary information was collected by using Food Frequency Questionnaires (FFQ) to obtain previous year's dietary information in APrON cohort. However, the data is still not available for the analysis and could be of great use once available.

Risks associated with high folate intake and status are the unmetabolized folic acid and masking of vitamin B_{12} deficiency. Future studies should aim prospectively on determination of unmetabolized folic acid and possible pregnancy and birth outcomes associated with it.

We also observed in our study that percentage of women using multivitamin supplements significantly drops during 3-months postpartum. Future studies can aim

to identify the factors associated with it and possible health outcomes with the determination of biochemical biomarkers. As majority of women had high folic acid intake and high RBCF status during pregnancy, future studies could be used to determine if the new cutoff for reference ranges in this population are needed or study associations with other heath outcomes such as immune function.

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