ASSOCIATION OF MATERNAL FOLATE AND VITAMIN B12 STATUS WITH BIRTHWEIGHT AND GESTATIONAL AGE AT BIRTH IN MOTHER-NEWBORN DYADS RESIDING IN BRITISH COLUMBIA

by

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Abstract

Birthweight and gestational age at birth have been inversely associated with chronic disease risk in later life. The vitamins folate and vitamin B12 (B12) have interdependent metabolic functions that are essential for fetal growth. Maternal folate and B12 concentrations during pregnancy have been positively associated with birthweight and gestational age at birth; however, the findings from the literature are inconsistent. The objective of this research was to evaluate the association of maternal serum folate and B12 biomarker concentrations, combined and individually, with birthweight and gestational age at birth.

This retrospective cohort study included biobanked non-fasting serum samples and data from 674 apparently healthy pregnant women of South Asian and European ethnicity residing in Lower Mainland, British Columbia (BC). Maternal serum samples, collected in the first and second trimesters of pregnancy, were retrieved from the BC Prenatal Genetic Screening Program and analysed for folate and B12 biomarker concentrations. Birth outcome data were retrieved from the BC Newborn Screening Program. The association of folate and B12 biomarker concentrations with birth outcomes was assessed using multiple linear regression models with adjustment for confounding factors, including infant sex, ethnicity and maternal age.

The prevalence of low birthweight, preterm and small-for-gestational-age were 1.9%, 8.9% and 0.88%, respectively. The combined maternal folate and B12 status, in either trimester, was not associated with birthweight or gestational age at birth. Maternal B12 biomarker concentrations individually, in either trimester, were not or only weakly associated with birth outcomes. Second-trimester maternal folate concentrations of the second, third and fourth quartile group (Q2=55.5-69.3, Q3=69.3-87.8, Q4≥87.8 nmol/L, respectively) were associated with an approximate 0.6-week (i.e., 4-day) increase in gestational age at birth, compared to the

reference group (Q1≤55.5 nmol/L) (95% CI: 0.28, 1.02, p=0.02; 95% CI: 0.23, 0.95, p=0.03, 95% CI: 0.16, 0.89, p=0.005, respectively).

In conclusion, early pregnancy folate and B12 status were neither combined nor individually associated with birthweight in this sample. Due to the vitamins' importance in fetal growth and development, the association between maternal folate and B12 status and birth outcomes warrants further investigation in a population with a higher prevalence of low birthweight and preterm birth.

Lay Summary

A newborn's birthweight is a possible indicator for health and disease risk in later life. Many factors, including the mother's vitamin levels, may influence the growth of the unborn baby. This research aimed to study whether the mother's folate and vitamin B12 (B12) status are associated with her newborn's birthweight and the duration of her pregnancy. First- and second-trimester blood samples from 674 apparently healthy pregnant women living in Lower Mainland, British Columbia (BC), and information on their newborns were retrieved from the BC Prenatal Genetic Screening Program and the BC Newborn Screening Program, respectively. The prevalence of low birthweight was 1.9%. Mother's folate and B12 status were not associated with birthweight and the duration of her pregnancy in this sample. More research is needed to determine whether early pregnancy folate and B12 status are associated with birth outcomes in a population with a higher prevalence of low birthweight.

Preface

This thesis has been prepared in partial fulfillment of the requirement for the degree of Master of Science in Human Nutrition. I prepared this thesis under the direction and supervision of Dr. Yvonne Lamers (primary). Dr. Lamers acted as the primary supervisor in all aspects of this research project and provided feedback to the proposal and thesis drafts. Dr. Jennifer Black, Human Nutrition Program, and Dr. Patricia Janssen, School of Population and Public Health, served as supervisory committee members. Dr. Black and Dr. Janssen provided feedback on the research proposal, and on the thesis draft.

My work was part of a larger project, for which the research team comprised myself, graduate students, research assistants, and undergraduate research students. I was part of the team that was responsible for aspects related to sample deidentification and data entry. The lead graduate student of the research project was Theresa Schroder. Theresa Schroder and Dr. Yvonne Lamers were responsible for all major areas of study design and study coordination. Dr. Hilary Vallance and Dr. Graham Sinclair from the Newborn Screening Laboratory, BC Children's Hospital, Vancouver BC, contributed to the study design, biospecimen retrieval, and data interpretation.

Biospecimen retrieval and deidentification procedures were supported by co-op students, undergraduate students and Work Learn students, as well as technicians and other staff members of the Newborn Screening Laboratory at BC Children's Hospital and of the Human Nutrition and Vitamin Metabolism Laboratory, led by Dr. Yvonne Lamers, at The University of British Columbia, Vancouver BC. Biomarker analysis was conducted by Theresa Schroder and Benny Chan with support from the co-op student, Work Learn student, and undergraduate students in the Human Nutrition and Vitamin Metabolism Laboratory and supervised by Dr. Yvonne

Lamers. Dr. Andre Mattman of St Paul's Hospital, Vancouver BC, contributed to biomarker analysis and data interpretation. Dr. Benjamin Jung oversaw the serum total vitamin B-12 analysis in the Pathology Laboratory at BC Children's Hospital.

I performed all statistical analyses, in consultation with Dr. Yvonne Lamers and with Dr. Arianne Albert, a biostatistician at the Women's Health Research Institute. I disseminated preliminary results of this work in March 2017 in the form of an oral presentation at the UBC Faculty of Land and Food Systems Graduate Student Conference, for which I received the People's Choice Award, and as a poster presentation in June 2017 at the 17th BC Children's Hospital Research Forum. This study was approved by the University of British Columbia / Children's and Women's Health Centre of British Columbia Research Ethics Board (UBC C&W REB), Vancouver BC, (#H15-00820).

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List of Abbreviations

AGA= appropriate-for-gestational-age
B12 = vitamin B12
BMI= body mass index
BW= birthweight
CI= confidence interval
G = gram(s)
GA= gestational age
HoloTC= holotranscobalamin
LC-MS/MS= liquid chromatography-tandem mass spectrometry
LGA= large for gestational age
MMA= methylmalonic acid
NTD= neural tube defect
RBC= red blood cell
RDA = recommended dietary allowance
SA= South Asian or South Asia, *depending on context*
SD = standard deviation
SGA= small for gestational age
tHcy= total homocysteine
Wk = week(s)

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I gratefully acknowledge the laboratory assistance of Benny Chan, Pablo Elizondo, Dr. Joanna Suliburska, Matthew Saunders and Monja Sandler. Undergraduate volunteers Prineet Ghuman, Alex Wang, Stephanie Chen and Mirah Valdes provided invaluable help in tube labeling and data entry. I am also indebted to Dr. Arianne Albert from the Women's Health Research Institute for her statistical expertise, support, and input. In addition, I thank the two co-op students Mandeep Takhar and Roy Chen, and the undergraduate research assistant Ori Nevares, for their help in specimen retrieval and deidentification. I would also like to thank Dr. Hilary Vallance, Dr. Graham Sinclair, and the staff of the BC Newborn Screening Laboratory for their contributions to the study and the sample retrieval. I would like to acknowledge the support of the Ministry of Education in Saudi Arabia for providing funding throughout my Master's degree. I would like to express my sincere thanks to my colleagues in the Lamers' lab, for supporting me throughout the entire process, and especially to Theresa Schroder, who has been a great inspiration. Finally, special thanks are owed to my parents and siblings, who have encouraged me throughout my years of education.

Dedication

I would like to dedicate this thesis to my mother,

Kadria Kamal,

Who has a special place in my heart.

Thanks to her for raising me with the love for learning and a passion for education.

Chapter 1: Introduction

Birthweight and gestational age at birth are important predictors of infant morbidity and mortality. Both have been inversely associated with chronic disease risk in later life. Low birthweight infants have a higher risk of cardiovascular disease compared to normal birthweight infants (Leeson et al. 2001). Additionally, preterm newborns have a higher risk of respiratory illness and learning disabilities compared to term infants (Beck et al. 2010).

Maternal nutrient adequacy is critical for both the mother and the developing child (Dror, 2012; Fall et al., 2003). Early pregnancy is an important period for nutrient adequacy because rapid fetal development and cellular differentiation occur. During pregnancy, maternal adequacy in energy, protein intake and micronutrient status are critical to supporting the growth and development of the fetus (Gadgil et al., 2014; Simpson, Bailey et al., 2010). Studies have shown that micronutrient deficiencies during pregnancy are associated with adverse health outcomes in the mother, and with also impaired fetal development (Fall et al., 2003; Sande et al., 2013).

Folate and vitamin B12 (B12) are water-soluble vitamins that play crucial roles in maternal health and normal fetal development and growth. Folate and B12 deficiencies have been associated with low birthweight, intrauterine growth restriction, preterm birth, and poor neonatal growth, all of which are linked to higher prevalence of morbidity and mortality (Furness et al., 2013; Simpson et al., 2010). Folate and B12 function together in one-carbon metabolism that contributes to DNA synthesis, formation of phospholipids, and numerous methylation reactions which are critical during embryonic and fetal development (Ahmed, Afsana, Mahbooba Akhter, Shafia Sharmin, Shamim Ara et al., 2011; Combs, 2012; Dror and Allen, 2012; Furness et al., 2013; Obeid et al., 2005; Sande et al., 2013).

Maternal folate and B12 concentrations have been positively associated with birthweight and gestational age at birth (Bergen et al. 2012; Relton 2005; Afsana et al. 2012; Pagan et al. 2002); however, the findings are inconsistent (Pagan et al. 2002; Furness et al. 2013). Some studies did not find an association between maternal folate and B12 concentrations and birthweight and gestational age at birth, while others found a positive association. Also, there is a limited number of studies on the association between maternal folate and B12 concentrations and gestational age at birth. Recently, concerns have been raised regarding a potential adverse effect of high maternal folate status and a high folate / low B12 imbalance on pregnancy outcomes (Gadgil et al. 2014). In Canada, there seems to be a high prevalence of high folate and low vitamin B12 concentrations during pregnancy (Fayyaz et al. 2014; Visentin et al. 2016). There is a lack of research on the association of maternal folate and B12 status combined with birth outcomes; in addition, most of the current evidence focuses on B-vitamin status during late pregnancy. A better understanding of the association between maternal folate and vitamin B12, and birth outcome will enhance information currently available and help reduce the prevalence of low birthweight and preterm birth. The results of this study will also be significant for future intervention studies focusing on the importance of balanced folate and B12 status in pregnancy.

My research focused on early pregnancy, which is a critical period for neonatal development. I also looked into the imbalance between folate and B12, since it may be of concern in Canadian pregnant women and their offspring, and their association with birth outcomes. The main goal of my thesis was to evaluate the association of maternal serum folate and B12 biomarker concentrations individually with birth outcomes, including birthweight and gestational age at birth. Another goal of my thesis was to investigate the association of maternal folate and B12 status combined with birthweight and gestational age at birth, as well as to

investigate the association of the change in maternal folate and B12 biomarker concentrations in early pregnancy (between the first and second trimester) with birthweight and gestational age at birth.

Chapter 2: Literature Review

This chapter will provide background on: **[1]** the definition, related health outcomes, prevalence, and potential confounding factors of birth outcomes, including birthweight, gestational age at birth and appropriate-for-gestational-age; **[2]** the role of folate and vitamin B12 (B12) in pregnancy; **[3]** the interrelation of folate and B12 in one-carbon metabolism; **[4]** biomarkers for folate and B12 status assessment and related cut-offs; **[5]** folate and B12 status in Canadian reproductive-aged and pregnant women; **[6]** the importance and definition of pregnancy stages, followed by the changes in maternal folate and B12 biomarker concentrations during pregnancy; and **[7]** existing evidence on the association of folate and B12 with birth outcomes.

2.1 Birth Outcomes

Birth outcomes are some of the indicators of infant health status. In an unhealthy situation during pregnancy, such as unhealthy diet, adverse birth outcomes may occur. Adverse birth outcomes include small for gestational age (SGA), low birthweight and preterm birth, and may lead to higher risk of neonatal morbidity and mortality (Beck et al. 2010; McCowan & Horgan 2009; Lundgren & Tuvemo 2008; Goldenberg et al. 2008). These adverse birth outcomes may overlap; low birthweight is highly associated with preterm birth and SGA (Wardlaw 2004). However, each adverse birth outcome has its own risk factors and aetiologies. These adverse birth outcomes may be inversely associated with maternal folate and B12 status.

2.1.1 Birthweight – Definition, Classification, Related Health Outcomes

"Birthweight" refers to the infant's weight at delivery. The normal birthweight of an infant is defined by the World Health Organization (WHO) as between 2500 and 4200 grams (g), while low birthweight (LBW) is defined as less than 2500 grams (g) (Kramer 1987). Very low birthweight is defined as less than 1500 g, and extremely low birthweight is less than 1000 g

(Wardlaw 2004). Low birthweight is associated with neonatal morbidity and chronic diseases in later life, such as cardiovascular diseases, hypertension, type 2 diabetes (Pilgaard et al. 2010; Moore et al. 1996; Barker 1995; Leeson et al. 2001). The association between low birthweight and the higher risk of cardiovascular disease has been suggested to be due to the metabolic changes that occur with the delayed growth in utero (Barker 1995; Leeson et al. 2001). Birthweight is also considered to be an important predictor of infant survival, health, and development (Wardlaw 2004).

The prevalence of low birthweight in Canada has been slightly increasing in recent years. In 2000, the prevalence was 5.6% and it increased to 6.3% in 2014. The reasons behind the increasing prevalence of low birthweight throughout the years may be due to the improvements in medical facilities that contribute to the prevention of stillbirth. Canada's infant mortality prevalence decreased from 5 to 4.7 between 2010 and 2014 (Statistics Canada 2017). In recent years, birth rates among those aged 30 and over have increased in comparison to a younger age (Milan 2015).

Mother age is negatively associated with birthweight, as it has been found that the prevalence of low birthweight was reported to be higher among mothers aged 35 to 49 years (7.6%), than among mothers aged 20 to 34 years (5.8%) in 2010 (Government of Canada 2016). There are many other potential factors associated with birthweight, such as nutrition (e.g. maternal folate levels) (Relton 2005), infant sex (Kumar et al. 2013), birth order (Bacci et al. 2014), parity, gestational age (Ohlsson & Prakeshkumar 2008), maternal weight and ethnicity (Ronnenberg et al. 2007). It has been found that female infants have 45 g lower birthweight than male infants of the same gestational age (Kumar et al. 2013), firstborn infants are lighter than subsequent infants (mean difference 89 g) (Bacci et al. 2014), and twins weigh 100 g less than

singletons (Buckler & Green 1994). In Canada, the percentage of low birthweight was higher among females. Over the period of 1979 to 2010, the prevalence varied between 5.1% and 5.7% for males, and between 5.9% and 6.7% for females (Government of Canada 2016).

Pre-pregnancy body mass index and maternal weight gain during pregnancy are found to be closely associated with birthweight. Mothers with pre-pregnancy body mass index indicating underweight had an increased risk of LBW (OR:1.47, 95% CI: 1.27, 1.71, $p< 0.001$) compared to mothers with body mass index of normal weight (Yu et al. 2013). Additionally, maternal weight gain during all trimesters is significantly associated with birthweight; each one of kg increase in the mother's weight was associated with a significant increase of 18, 32.8, and 17 g in birthweight, in the first, second and third trimesters, respectively, with the adjustment for maternal age, height, parity, pre-pregnancy body mass index (BMI), infant sex and gestational age (Abrams & Selvin 1995).

An ethnic group is often referred to as a group of people whose members are identified through a common trait. This includes common heritage, culture, ancestry and religion. Thus, ethnicity has two influencing factors: biological factors (e.g. genetic factor, body size), and cultural or lifestyle factors (e.g. diet, smoking, alcohol, physical activity, parity, maternal height, weight and socioeconomic status) (Kramer 1987).

The ethnicity factor association with birthweight is well documented. Fetal growth differs significantly between different ethnic groups (Troe et al. 2007). The reasons behind ethnic differences in fetal growth and body composition are not fully understood. These differences might be due to genetic factors (Smith et al. 1976) or that ethnicity is associated with socioeconomic status and dietary pattern (Kramer 1987). Maternal weight is an important predictor of birthweight (Smith et al. 1976). Adult South Asians have a lower weight and a

smaller stature than Europeans (Deurenberg et al. 2002; Davies et al. 1982). The maternal shorter stature and weight is likely to be responsible for the smaller size of South Asian infants. South Asians including Indians were found to have infants with lower birthweight (mean weight of 3020 g) and a higher prevalence of low birthweight (11.5%) than the Malays (3080 g and 8.1%) and the Chinese (3130 g and 6.1%), in a cross-sectional study of 187 mothers and their infants (Alvear & Brooke 1978).

Studies have also shown that South Asian infants have a lower birthweight compared with those of white European ethnicity (Alvear & Brooke 1978; Davies et al. 1982). A study conducted in the UK found that the average birthweight of Indian infants [weight difference= 344 (95% CI: 329, 360) g] was lower than the birthweight of European infants (Wells et al. 2013). In British Columbia, Canada, the birthweight of South Asian infants was 254.6 g lower on average than the birthweight of European infants (Mean (SD) BW of 3452.5 (399.1) and 3687.6 (410.5g) respectively, at 40 weeks of gestation) $p< 0.001$) (Janssen et al. 2007). The importance of ethnicity differences has been emphasized by the improvement in the identification of short-term neonatal morbidity risk when using ethnicity-specific birthweight distributions (Hanley & Janssen 2013).

2.1.2 Gestational Age at Birth – Definition, Classification, Related Health Outcomes

Gestational age is the term used to describe the estimated pregnancy duration or the age of a fetus. The normal gestational age at birth can range from 38 to 42 weeks, while preterm is defined as a live birth occurring prior to 37 completed weeks or 259 days of gestation (World Health Organisation 2016). Late preterm is a live birth occurring in a period of less than 37 weeks until 32 weeks of gestation; very preterm is less than 32 weeks until the 28 weeks, and extremely preterm is less than 28 weeks of gestation (World Health Organisation 2016). Preterm

birth is associated with neonatal morbidity and also with long-term negative health consequences, such as cerebral palsy, learning disabilities, sensory deficits, and respiratory illnesses. It is also considered an important predictor of neonatal mortality (Beck et al. 2010).

The prevalence of preterm births in Canada was 8.1% in 2006–2007 and 7.9% in 2010–2011 (CIHI 2012). There are many potential factors associated with preterm birth, such as multiple pregnancies (World Health Organisation 2016), infant sex (Zeitlin 2002), ethnicity (Schempf et al. 2007; Beck et al. 2010), the use of fertility medication, *in vitro* fertilization (Jackson et al. 2004), maternal body mass index (Hendler et al. 2005), and maternal age and parity (Schempf et al. 2007). Other potential factors increasing the risk of preterm delivery are maternal medical conditions, such as pre-eclampsia (adjusted odds ratio=5.07), chronic diabetes (adjusted odds ratio=2.54) and gestational diabetes (adjusted odds ratio=1.28) (Goldenberg et al. 2008; Rosenberg et al. 2005).

2.1.3 Growth Rate Indicators – Definition, Classification, Related Health Outcomes

The growth rate indicators can be referred to as weight per gestational age; appropriate for gestational age (AGA) is the term used to describe an infant whose birthweight is within the normal range of their gestational age at birth (between the 10^{th} and 90^{th} percentile on the intrauterine growth chart). An infant whose size is large for gestational age (LGA) has a birthweight above the 90^{th} percentile on the intrauterine growth chart. Small for gestational age (SGA) is the term used to describe an infant whose birthweight falls below the 10^{th} percentile of the standard weight for that gestational age. It can be used as an indicator of fetal growth restriction (Mahan et al. 2012; Williams et al. 1982; Kierans et al. 2006).

SGA can be a consequence of intrauterine growth restriction, defined as reduced growth of a fetus during pregnancy. SGA birth is associated with long-term negative health

consequences, such as obesity (higher BMI, greater fat mass), hypertension, non-insulin diabetes mellitus, cardiovascular disease and metabolic syndrome in adulthood (Meas et al. 2008; Chatelain 2000). It is associated with non-severe neurological dysfunction, lower intelligence, poor academic performance, low social competence and behavioral problems in childhood and in young adulthood (Lundgren & Tuvemo 2008).

The prevalence of SGA births in Canada was 8.4% in 2000–2002 and 8.7% in 2010–2012 (Canadian Socio-Economic Information Management System (CANSIM) 2012). There are many potential maternal factors associated with birthweight for gestational age, such as ethnicity (Hanley & Janssen 2013), smoking (McCowan & Horgan 2009), weight gain (Ricci et al. 2010), pre-eclampsia, gestational hypertension (Groom et al. 2007), and chronic hypertension (Wolfe et al. 1987). The population-based birthweight distributions in British Columbia (Kierans et al. 2006) does not consider for differences between ethnic groups, that may result in the misclassification of infants as having abnormal growth for their gestational age. Thus, using ethnicity-specific growth charts is better for identifying the risk of short-term neonatal morbidity (Hanley & Janssen 2013), with regard to the differences in fetal growth between ethnic groups.

2.2 Folate and B12

Folate and B12 are water-soluble vitamins and are considered essential micronutrients throughout the lifespan. Folate and B12 have an interdependent role in one-carbon metabolism that includes the formation of DNA and other critical cell components. The following section will describe folate and B12 in terms of function, metabolism, dietary sources, deficiency symptoms, recommendations, and suggested roles in healthy pregnancies.

2.2.1 Folate – Function, Metabolism, Deficiency

Folate, formerly called vitamin B9, is a water-soluble vitamin that acts as a coenzyme in one-carbon transfer reactions. The folate-dependent one-carbon metabolism involves the synthesis of thymidylate (thymidine nucleotides) and purines, which are essential precursors for DNA synthesis and cell division. The coenzyme of folate occurs in the oxidation level of methanol (5-methyltetrahydrofolate (5-MTHF)), formaldehyde (5,10-methylenetetra-hydrofolate), or formate (5- or 10-formyl-tetrahydrofolate or 5,10-methenyltetrahydrofolate). It also occurs in the interconversions of amino acid, including the catabolism of histidine to glutamic acid and in serine, glycine and the conversion of homocysteine to methionine (Shane 2008; Simpson et al. 2010; Chen et al. 2015).

Folate is the form that occurs naturally in food and can be found in dark green leafy vegetables, orange juice, legumes (e.g. black beans, kidney beans), nuts, asparagus, and strawberries (Simpson et al. 2010). Folic acid is the man-made, fully oxidized folate form used in supplements, fortified food products, and pharmaceuticals. Folic acid is chemically stable and is absorbed more easily than dietary folate (Shane 2008; Simpson et al. 2010). However, after being absorbed by the gastrointestinal tract and liver, folic acid needs to be converted to the metabolically active form 5-MTHF in order to act as a substrate (Furness et al. 2013). The bioavailability of folic acid taken within a meal is 85%, whereas the bioavailability of folate from food is 50% (Simpson et al. 2010; Shane 2008).

Low maternal folate status increases the risk of neural tube defects (NTDs) in pregnancy (Kirke et al. 1993). NTDs can result in brain defects (anencephaly) and spinal malformation (spina bifida) (Simpson et al. 2010). It has been shown in a number of experimental and observational studies that folic acid supplementation lowers the NTD occurrence (Czeizel &

Dudás 1992; MRC Trial 1991). This was the rationale for the implementation of folic acid fortification programs. In Canada, by 1998, folic acid fortification of a large variety of cereal products became mandatory and this resulted in a 50% reduction in NTDs (Shane 2008; De Wals et al. 2007).

The consumption of folic acid above 0.2 mg may lead to unmetabolized folic acid in the systemic circulation (Furness et al., 2013; Kelly,1997). This can affect the normal homeostatic regulation of folate by interfering with the enzymes and cellular transport, such as carrier proteins and binding proteins (Obeid, 2010). Moreover, it is associated with potential adverse effects, such as low natural killer cell cytotoxicity in elderly women (Troen et al. 2006). It can also cause reduced response to antifolate drugs used against malaria, rheumatoid arthritis, psoriasis and cancer (Smith et al. 2008).

The supplemental folic acid recommendation is at least 400 µg/day for three months prior to pregnancy and throughout pregnancy, and this amount has been shown to be effective in reducing the risk of adverse pregnancy outcomes (Czeizel & Dudás 1992). In cases where there has been a previous pregnancy with NTD or a family history of NTDs, women are recommended to take daily multivitamins and a total intake of 400 µg/day folic acid for three months prior to pregnancy and throughout the first trimester, followed by multivitamins containing 400 µg/day of folic acid for the rest of the pregnancy (Wilson, 2015). The Recommended Dietary Allowance (RDA) for pregnancy is 600 µg/day dietary folate equivalents (DFEs) (Institute of Medicine, 1998).

2.2.2 Role of Folate in Healthy Pregnancies

The requirement of folate increases during pregnancy due to its critical role in phases of rapid cell growth and development (Lamers 2011). The maternal folate is actively transported to

the fetus to accommodate synthesis of DNA, RNA, amino acids, and other compounds for fetal and maternal tissue growth (Chen et al. 2015). Additionally, serum and RBC folate concentrations decrease in pregnancy as a result of physiological changes, dilution of serum folate secondary to a 50% increase in blood volume, increased folate catabolism and decreased folate absorption (Cikot et al. 2001)

The role of folate as a superoxide scavenger in antioxidant defenses can influence placental implantation and vascular remodeling (Bergen et al. 2012). Hence, low maternal folate status during pregnancy can result in megaloblastic anemia (Simpson et al. 2010; Bailey 2009), neural tube defects, recurrent pregnancy loss, stillbirth (Bailey 2009; Simpson et al. 2010; Molloy et al. 2014) and higher risk of pre-eclampsia (Bergen et al. 2012; Wen et al. 2016).. Folate may also contribute to the prevention of intrauterine infection, thus reducing the risk of preterm birth (Goldenberg et al. 2008). Moreover, women with low folate concentration (below 9.2 nmol/L) have lighter placentas by 19 to 25 g and lower birthweight by 53–125 g compared to women with high folate concentration (above 25.9 nmol/L) (Bergen et al. 2012).

There have been concerns regarding potential adverse effects of high maternal folate status on pregnancy outcomes, through the combination of sources from food fortification and supplementation. Potential adverse health outcomes of maternal high folate concentration, including increased insulin resistance in offspring and greater adiposity were determined in two prospective observational cohort studies that were conducted in India. The Parthenon study looked at children aged 5, 9 and 13 years, and found that high maternal folate concentrations in the third trimester were associated with higher insulin resistance in the children at 9.5 [β 0.10 (95% CI: 0.01, 0.2) p= 0.03] and 13.5 years of age [β 0.10 (95% CI: −0.01, 0.2) p= 0.03] (Krishnaveni et al. 2014). The Pune Maternal Nutrition (PMN) study found that high maternal

RBC folate concentrations at 28 weeks of gestation were associated with higher insulin resistance and greater adiposity in children at 6 years of age (both $p< 0.01$) (Yajnik et al. 2008). In a rat model study, maternal high folic acid intake was associated with lower birthweights (310 mg mean difference in body weight, $p< 0.001$) (Achón,1999) and growth-restricted embryos (2.5 mg mean difference in embryonic weight, $p< 0.1$) (Pickell et al. 2011).

The physiological implications of high maternal folate concentration during pregnancy is unknown, although it has been suggested that high folic acid intake levels above 0.2 mg folic acid can cause the presence of plasma unmetabolized folic acid, which can disrupt normal folate metabolism (Kelly et al. 1997; Lucock 2004; Lamers 2011a).

2.2.3 Vitamin B12 – Function, Metabolism, Deficiency

Vitamin B12 or "cobalamin" is a water-soluble vitamin that functions as a coenzyme for the methylmalonyl-CoA mutase in the odd-chain fatty acid and energy metabolism in mitochondria, and for methionine synthase in conjunction with 5-MTHF in the remethylation of homocysteine to methionine in the cytosol. Methionine is a precursor of *S*-adenosylmethionine (SAM), the key methylation agent in human metabolism. SAM is the methyl group donor for DNA methylation and functions in epinephrine synthesis. Given its role in methionine regeneration, B12 is essential for the formation of hemoglobin and for normal neurodevelopment (Combs, 2012; IOM, 1998; Pepper, 2011; Sande et al., 2013).

B12 is found only in animal food. Vegetarians are at risk of B12 deficiency as a result of restrictions in the consumption of animal-derived foods, such as fish, meat, poultry, eggs, and dairy products (IOM, 1998; Simpson et al., 2010). Therefore, vegetarians should consider taking B12 containing dietary supplements (Pepper, 2011). The dietary bioavailability of vitamin B12

depends on the amount of B12 in the food consumed. The absorption rate of food-bound B12 is approximately 50% that of crystalline B12 (Shane 2008; Simpson et al. 2010).

B12 deficiency can cause megaloblastic anemia and neurological abnormalities that include peripheral neuropathy characterized by cognitive disturbances, dementia, paresthesia, and ataxia and increases the risk of NTDs in newborns (Dror & Allen 2012; Simpson et al. 2010). The RDA for adults is 2.4 μg/day of B12. The RDA is 2.6 μg/day for pregnant women to meet the needs of the fetus (Institute of Medicine, 1998; Pepper & Black, 2011; Shane, 2008).

2.2.4 Role of Vitamin B12 in Healthy Pregnancies

B12 is considered a critical nutrient in fetal growth and development. It also maintains normal folate metabolism necessary for cell multiplication during pregnancy and specifically in the rapidly dividing placental and fetal tissues (Sande et al. 2013). During pregnancy, total B12 concentrations decline (Murphy et al. 2007). This decrease can be partially explained by active transport of vitamin B12 to the fetus and by increased metabolic rate.

The risk of B12 deficiency in pregnant women can be due to inadequate dietary intake of B12. There may also be a physiological decline of maternal B12 concentrations as a result of Crohn's disease or Celiac disease or gastric bypass surgery which affects the B12 absorption efficiency (Green et al. 2017). B12 deficiency can be associated with preeclampsia, fetal growth restriction (Yajnik et al. 2005; Afsana et al. 2012), recurrent abortion (Hübner et al. 2008), neonatal neurological symptoms (Jadhav et al., 1963), neonatal megaloblastic anemia, neural tube defects (Shane 2008) and preterm labor.

In a case-control study of 434 women aged 21–34 years, the risk of preterm birth (n = 29) has been reported to be 60% higher among B12 deficient women (< 258 pmol/L) compared to women with B12 ≥ 258 pmol/L in the pre-conception period (Ronnenberg et al. 2007). Among

Irish women with an average of 15 weeks of gestation, women with B12 concentrations <250 ng/L were associated with a 2.5 to 3 times higher risk of having an NTD-affected pregnancy compared to those women with B12 concentrations >250 ng/L (Molloy et al. 2009). In a prospective cohort study in India, maternal serum B12 of lower concentration (median: 157 pg/ml compared to 304 pg/ml in the 1st trimester; 153 compared to 285 pg/ml in the 2nd trimester; 150 pg/ml compared to 246 pg/ml in the 3rd trimester) had a significantly higher risk of intrauterine growth retardation in all three trimesters (adjusted odds ratios: 5.98, 9.28 and 2.81 for trimesters 1–3, respectively) (Muthayya et al. 2006).

2.2.5 Interrelation of Folate and Vitamin B12 Metabolism

Folate and B12, with B6 and B2, participate in one-carbon metabolism that is required for cellular biosynthesis reactions, including DNA synthesis and methylation, amino acid synthesis, such as methionine and glycine, purines and pyrimidines, and methylation of nucleic acids, proteins, and lipids. Different forms of folate serve as methyl donors and acceptors. For example, a one carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form 5,10-methylene-THF. 5,10-methylene-THF can then be used for the synthesis of thymidine which is incorporated into DNA. The folate form 10-formyl-THF is necessary to synthesize purines that are building blocks of RNA and DNA. 5,10-methylene-THF can be reduced to 5-MTHF. This step requires FAD (riboflavin precursor).

Folate in the 5-MTHF form and vitamin B12 have an interdependent role in the homocysteine remethylation reaction forming methionine. Methionine is the precursor of *S*-adenosylmethionine (SAM), which is primary methylation agent in human metabolism. SAM has the ability to change gene expression through epigenetic mechanisms involving DNA

methylation. Vitamin B6 is a coenzyme in the one-carbon transfer reactions of glycine and serine to THF and in the transsulfuration pathway converting homocysteine to cysteine (**Figure 1-1**).

In light of their interdependent roles in one-carbon metabolism, reduced levels of folate, B12, B6 and B2 impair cell division and methylation activity, which can negatively affect fetal growth and development (Chen et al. 2015; Stover 2004).

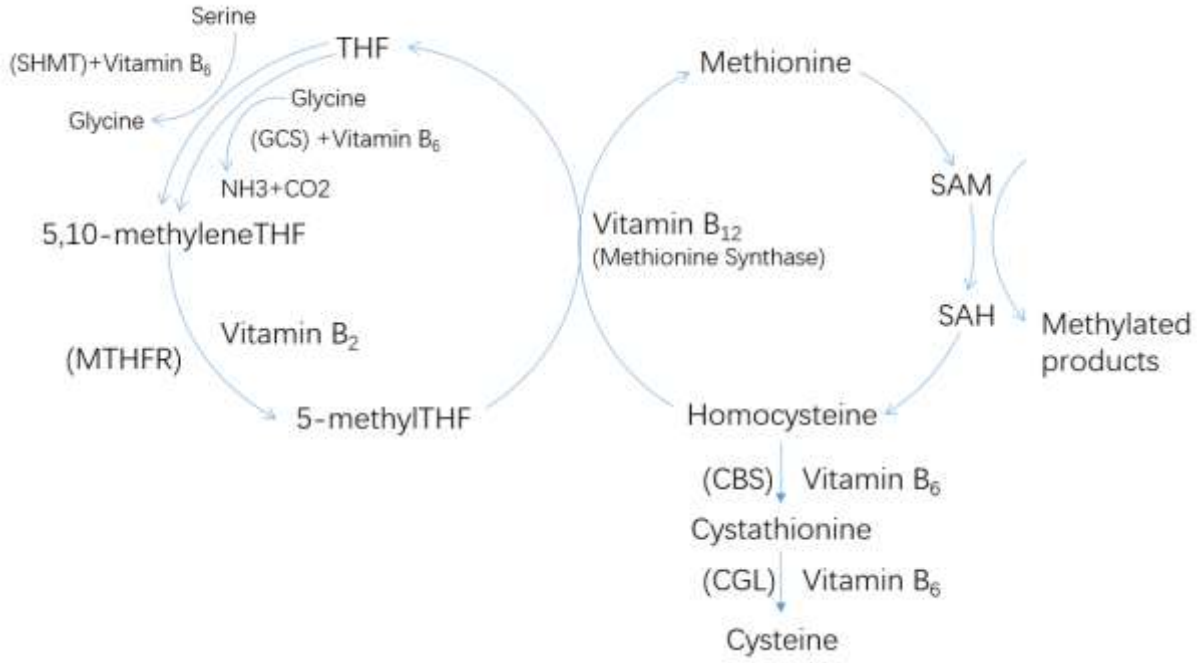

Figure 1-1: Schematic of One-Carbon Metabolism.

This figure shows that the B-vitamins folate, B12, B6, and B2 (riboflavin) act as coenzymes in specific pathways, which is indicated in parentheses after the enzyme names.

Abbreviations: CBS, cystathionine-β-synthase; CGL, cystathionine-γ-lyase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; TH4-folate, Tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

The metabolic interrelation between folate and B12 is strong, and deficiency of either will cause similar hematological changes. This can be a reason for the misdiagnosis of vitamin B12 deficiency when consuming high doses of folic acid. The intake of high-dose folic acid in B12

deficiency was shown to treat megaloblastic anemia and thus, due to the lack of hematologic symptoms, can interfere with the diagnosis of the neurological symptoms of B12 deficiency. This effect is referred to as "masking" of vitamin B12 deficiency. In either folate or B12 deficiency, the megaloblastic changes occur in the bone marrow and other replicating cells as a result of lacking 5,10-methylenetetrahydrofolate (Institute of Medicine, 1998). Folic acid after uptake is metabolized to 5,10-methylenetetra-hydrofolate and will, therefore, prevent or treat megaloblastic anemia. Neurological symptoms appear later in development and therefore, in the case of delayed or undiagnosed B12 deficiency, neurological complication will worsen over time and may be irreversible at a later stage (Institute of Medicine, 1998; Simpson et al., 2010; Stover, 2004).

B12 has a key role in maintaining normal folate metabolism which is essential for the cell multiplication in placental and fetal tissues. Together they function in developing embryos as a coenzyme in DNA synthesis and numerous methylation reactions (Molloy et al. 2014). Thus, the maternal imbalance between folate and B12 has shown more possible adverse outcome in offspring compared to maternal inadequacy status in either folate or B12 individually. The imbalance between folate and B12 is usually defined as having high folate concentrations with low B12 concentrations, quantiles. One study found that maternal combination of high RBC folate (>1144 nmol/L) and low plasma B12 concentrations (<114 pmol/l) during late gestation had the most insulin resistance in children of 6 years of age (Yajnik et al., 2008). Another recent study has found that maternal combination of high folate (range from 44.5 to 58.5) and low B12 concentrations (<221 pmol/L) during late gestation had a higher risk of gestational diabetes mellitus [OR: 1.97 (1.05, 3.68)] (Lai et al. 2017). This suggests the importance of the balanced maternal one-carbon metabolism in fetal growth and programming of diabetes risk. The possible

physiological mechanism may be that with low B12, folate will be trapped as 5-methyltetrahydrofolate, preventing the recycling of methionine from homocysteine which results in the reduction of protein synthesis and lean tissue deposition. Additionally, increased lipogenesis may occur because of methylmalonyl-CoA elevation by inhibiting carnitine palmitoyltransferase and thus β-oxidation (Ruderman et al. 1999). Another possible mechanism is the epigenetic regulation involving DNA methylation of hepatic gene expression that is demonstrated in animal models (Lillycrop et al. 2005). Nevertheless, the risk of insulin resistance is inversely, strongly associated with birthweight and appropriate-for-gestational-age (Deshpande et al. 1999; Geremia & Cianfarani 2004; Hernández & Mericq 2011; Gupta et al. 2007).

2.3 Biomarkers and Related Cut-offs for Folate and Vitamin B12 Status Assessment

Folate and B12 status can be measured by a variety of methods and biomarkers. In the following section folate and B12 will be described for functional and direct biomarkers, status assessment methods, and biomarker cut-off values.

2.3.1 Biomarkers and Related Cut-offs for Folate Status Assessment

Folate status is determined by measuring serum or plasma folate and/or RBC folate concentrations. Serum/plasma folate is influenced by short-term dietary intakes of almost 6 to 8 hours, thus fasting plasma sample is suggested. RBC folate concentration changes slowly over time and is considered an indicator of long-term folate status of 120 days (Gregory et al. 2000). Blood folate concentrations can be measured by microbiological assay (Molloy & Scott 1997). It is a widely-used measurement that has been applied for many years (Horne & Patterson 1988) and has long been considered the ‘gold standard’ as it allows the quantification of all forms of folate compared to immunoassays (Lamers, 2011). The alternative method is the separation and

quantitation of different folate forms by liquid chromatography tandem mass spectrometry (Pfeiffer, 2004). Different forms of folate include 5-methyltetrahydrofolate, 5-formyl-tetrahydrofolate, tetrahydrofolate, and 5,10-methenylte- tetrahydrofolate. Additionally, liquid chromatography tandem mass spectrometry can assess folic acid including the unmetabolized folic acid in serum. While this assay is suggested to be more accurate with respect to quantifying folate forms (Stamm et al. 2013), it involves higher cost, special equipment and expertise (Lamers, 2011).

Clinical deficiency or chronic folate deficiency is related to the classical symptoms of deficiency including megaloblastic anemia. While the suboptimal status is a subclinical nutrient deficiency that is related to an increased risk of chronic diseases, such as cardiovascular disease (Institute of Medicine 1998; Wald et al. 2003). The cut-off of folate in pregnancy is uncertain, since folate concentration declines during the pregnancy period (de Benoist 2008). However, the cut-offs of folate for adults have been used for pregnancy in many studies (**Table 2-1**).

Table 2-1: Biomarker Cut-Off Values to Categorize Folate Status for Adults

Analyte	**Deficient**	**Suboptimal Status ("possible deficiency")**	**Adequate**	**High**
Serum folate	<7 nmol/L [a]	7-14 nmol/L	> 14 nmol/L [b]	> 46 nmol/L [c]
RBC folate	<305 nmol/L [a]	305 – 340 nmol/L	>340 nmol/L [b]	>1360 nmol/L [c]

References: [a](Institute of Medicine 1998), [b] (World Health Organisation 2015), [c](Pfeiffer et al. 2007).

Plasma total homocysteine (tHcy) is suggested as a functional biomarker of folate status. Studies have shown that tHcy increases with the low level of folate indices. Although it is a very sensitive indicator to the changes in folate intake, it lacks the specificity since it is influenced by other B-vitamins, such as B12 and B6 (Gregory et al. 2000). The cut-off value indicating deficiency is tHcy >13-15 μmol/L (Institute of Medicine 1998).

2.3.2 Biomarkers and Related Cut-offs for Vitamin B12 Status Assessment

Total B12 is the most commonly used direct indicator for B12 status assessment. Holotranscobalamin (holoTC) is also a direct indicator and could be more useful because it is the circulating form of B12 taken up by tissues (Simpson et al. 2010; Institute of Medicine 1998). HoloTC has been shown to be a more sensitive indicator than total B12 concentration for B12 status assessment during pregnancy (Morkbak, 2007).

Total B12 and holoTC are measured most commonly using microparticle-enzyme immunoassays designed for the AxSYM analyzer immunoassays (Abbott Laboratories, Abbott Park, Illinois). The AxSYM is an immunochemical automated random-access analyzer. Serum Cobalamin microparticle-enzyme immunoassay operates on the principle of cobalamins binding to intrinsic factor (IF) coated onto a microparticle that is subsequently bound to a matrix cell. By adding 4-methylumbelliferyl phosphate to the matrix, the unbound IF will be saturated with 4-methylumbelliferyl phosphate and produce the fluorescent product methylumbelliferone. Thus, the concentration of cobalamin bound to the microparticles is quantified by the detection of photon emission (Briefs et al. 1998).

The holoTC microparticle-enzyme immunoassay operates on utilizing a monoclonal antibody against holoTC. The HoloTC antigen binds to microparticles that then bind to a matrix. The second step is similar to serum cobalamin assay, where the quantification of excess antibody is done by using fluorescence of 4-methylumbellifuerone (Brady et al. 2008).

Plasma tHcy and methylmalonic acid (MMA) are functional indicators of B12 status and inversely associated with B12 status. Plasma MMA is the most specific functional biomarker as tHcy is affected by folate status in addition to other B-vitamins. Plasma MMA is

measured by mass spectrometry (Blom, Van Rooij, and Hogeveen 2007; Obeid et al. 2005; Schroder, Quay, and Lamers 2014; Simpson et al. 2010) (**Table 2.2**).

Table 2-2: Biomarker Cut-Off Values to Categorize Vitamin B12 Status for Adults

Analyte	Deficient	Suboptimal status	Adequate	Other
Total B12	<148 pmol/L [a]	148-221 pmol/L[b]	>221 pmol/L	
Holotranscobalamin	<35 pmol/L [c]		≥35 pmol/L	<55 pmol/L [d] Associated with NTD risk
Methylmalonic acid	>260 nmol/L[e]	>210 - 260 nmol/L	<210 nmol/L [g]	

References: [a] (de Benoist 2008), [b](Molloy et al. 2009; Allen 2009), [c](Brady et al. 2008; Herrmann & Obeid 2003), [d](Ray et al. 2007), [e](Hølleland et al. 1999), [g](Pfeiffer et al. 2005).

2.4 Folate and Vitamin B12 Status in Canadian Women

This section will describe the current state of knowledge about folate and B12 status in reproductive-aged and pregnant women in Canada.

2.4.1 Reproductive-aged Women in Canada

Periconceptional adequacy in folate and B12 is crucial for the prevention of NTD, poor pregnancy outcomes and pregnancy loss. Folate and B12 status in women of reproductive age have been investigated in some studies. In Canada, folate status has increased among general population because of the mandatory folic acid fortification policy and use of supplements (Colapinto et al. 2011). In studies based on Canadian Health Measures Survey among reproductive-aged Canadian women, only 22% of reproductive-aged women showed RBC folate concentrations below the optimal level for minimizing NTD risk (< 906 nmol/L) (Colapinto et al. 2011), while folate deficiency was not found to exist, and B12 deficiency prevalence was reported to be low (6.1%) (MacFarlane et al. 2011). A recent study investigating B12 deficiency in South Asian and European reproductive-aged women in Metro Vancouver, stated that the prevalence of B12 deficiency is 14% (Quay et al. 2015) (**Table 2-3**).

Table 2-3: Prevalence of Vitamin B12 Deficiency (%) in Adult Women in Canada, and in a Sample of Reproductive-Aged Women Residing in Metro Vancouver.

Reference	Biomarkers	Status	Prevalence	Study Sample
(MacFarlane et al. 2011)	Serum total B12	Deficient (<148pmol/L)	104 (6.1%) *n*=1708	Adult women aged 20–79 years; participants from the Canadian Health Measures Survey
		Suboptimal status (148-220 pmol/L)	340 (20%) *n*=1708	
(Quay et al. 2015)	Serum total B12	Deficient (<148 pmol/L)	29 (14%) *n*=204	Reproductive-aged women 19–35 years; convenience sample; Metro Vancouver
		Suboptimal status (148-220 pmol/L)	41 (20%) *n*=204	
	Plasma MMA	Deficient (>210 nmol/L)	37 (20%) *n*=187	
	Serum holoTC	Deficient (<35 pmol/L)	16 (8.0%) *n*=199	

2.4.2 Pregnant Women

There has been an increasing number of pregnancy cohort studies in Canada that reported on the folate and B12 status in healthy pregnancies. In 2014, the Alberta Pregnancy Outcome and Nutrition (APrON) study found that only 3 out of 122 pregnant women in their first trimester and no women in their second and third trimesters had an RBC folate concentration (<305 nmol/l) that indicated deficiency (Fayyaz et al. 2014).

On the other hand, high RBC folate (>1360 nmol/L) was observed in approximately half of the pregnant women during all the trimesters; with an approximate 45% during the first trimester, increasing to 62% and 59% during the second and third trimester, respectively (Fayyaz et al. 2014) (**Table 2-4**). This is similar to the PREFORM (Prenatal Folic Acid Exposure on DNA Methylation in the Newborn Infant) prospective cohort study that was conducted in Toronto, where they did not find any of the 368 Canadian pregnant women in the study with serum and RBC folate concentrations that indicated folate deficiency in early pregnancy (12–16

wk of gestation), nor at delivery (28–42 wk of gestation). Furthermore, maternal plasma unmetabolized folic acid (≥0.2 nmol/L) was 97% during early pregnancy which indicates that pregnant women are probably consuming excessive amounts of folic acid (Plumptre et al. 2015). This is a concern, as it has been found that excess folic acid intake may negatively affect fetal development in mice (Mikael et al. 2013). A prospective cohort study conducted in Vancouver, BC, also reported none of the women to have plasma folate concentrations indicative of folate deficiency in the second and third trimesters of pregnancy (Wu et al. 2013).

Canada is regarded as a folate-replete population, likely due to the mandatory folic acid food fortification policy and the high use of prenatal supplements in pregnant women. The food fortification policy mandates the fortification of grain and cereal products with folic acid (Colapinto et al. 2011; MacFarlane et al. 2011). The prevalence of women using B vitamin–containing supplements is 92.8% and 89.0% in early and late pregnancy, respectively (Masih et al. 2015). In addition to the prevalent use of prenatal supplements, the folic acid dose in prenatal supplements is regarded as high, with a median supplemental folic acid intake of 1000 μg/day (Masih et al. 2015). Folic acid supplementation is commonly used throughout pregnancy; in the APrON cohort study, folic acid-containing supplements consumption was 94%, 97%, and 94% during the first, second, and third trimesters, respectively (Gómez et al. 2013).

The prevalence of B12 deficiency, defined as having plasma holoTC concentration <35 pmol/L, in the APrON study was less than 1% in the first and second trimesters (**Table 2-4**); B12 status was not assessed in the third trimester (Fayyaz et al. 2014). This might not be a representative sample of Canadian pregnant women since it was based on voluntary enrollment and most of the participants had high socioeconomic status.

Table 2-4: Folate and B12 Status in Pregnant Women in the APrON Study.

Vitamin Status	**Biomarker and cut-off used**	**1st Trimester** *n*=122	**2nd Trimester** *n*=520	**3rd Trimester** *n*=446
Prevalence of folate deficiency, *n* (%)	RBC folate (<305 nmol/L)	3 (2.5)	0	0
	Plasma folate (<7 nmol/L)	0	0	-
Prevalence of high folate status, *n* (%)	RBC folate (>1360 nmol/L)	55 (45)	320 (62)	265 (59)
Prevalence of vitamin B12 deficiency, *n* (%)	Plasma holoTC (<35 pmol/L)	1 (0.8)	6 (1.2)	-
(Fayyaz et al. 2014)				

Abbreviations: RBC: red blood cell; holoTC: holotranscobalamin.

In 2008, a population-based case-control study investigated maternal B12 status in Ontarian pregnant women at around 28 days of gestation (Ray et al. 2008). The study focused on B12 deficiency as a risk factor for NTD. Hence, they determined the prevalence of B12 deficiency (defined as total B12 <125 pmol/L) at 28 days gestation or less (*n*=1224), and after 28 days gestation (*n*=2490); 28 days period of gestation is the estimated time of the embryonic neural tube closure. They found that the prevalence of maternal serum total B12 concentration <125pmol/L was nearly doubled after 28 days gestation 10.1% (*n*=252), compared to 5.2% (*n*=56) before 28 days of gestation. This might be due to the hemodilutional effect of pregnancy on serum total B12 (**Table 2-5**). About 1 in 20 Ontarian pregnant women may be B12 deficient in the first trimester (Ray et al. 2008).

The B12 biomarker concentrations decrease throughout pregnancy, thus the prevalence of maternal B12 deficiency (using adult cut-offs) seems to increase in the second and third trimesters in comparison to the first trimester (**Table 2-5**) (Wu et al. 2013; Visentin et al. 2016; Jeruszka-Bielak et al. 2017). The differences in the prevalence between the studies demonstrated

in **Table 2-5**, may be partially explained by the disparity in the place (provinces) and time (early vs late pregnancy) the studies were conducted, and the biomarkers that were used. A study conducted in Metro Vancouver investigated maternal B12 status in late pregnancy between 20 and 35 gestational weeks by using the two biomarkers plasma total B12 and MMA (Jeruszka-Bielak et al., 2017). Most participants were of European descent (47%) and of high education (57% had a university degree). The median (IQR) plasma total B12 and MMA concentrations were 215 (160, 283) pmol/L and 140 (110, 187) nmol/L, respectively. In line with the previous studies conducted during the late pregnancy period, the study found that a high prevalence (18%) of B12 concentrations indicate deficiency (Plasma total B12<148 pmol/L) using non-pregnant adult cutoffs (**Table 2-5**).

Table 2-5: Prevalence of Vitamin B12 Deficiency *n* (%) in Pregnant Women in Toronto and Vancouver, Canada.

Reference	**Biomarkers**	**Status**	**2nd Trimester 12-16 week**	**3rd Trimester 28–42 week**
(Visentin et al. 2016) Toronto	Total B12	Deficient (<148pmol/L)	54 (17%) *n*=322	105 (38%) *n*=275
		Suboptimal status (148-220 pmol/L)	113 (35%) *n*=322	118 (43%) *n*=275
	MMA	Deficient (>271 nmol/L)	6 (1.9%) *n*=315	14 (5.3%) *n*=267
(Wu et al. 2013) Vancouver	Plasma total B12	Deficient (<148 pmol/L)	27 (10%) *n*=264	51 (23%) *n*=220
		Suboptimal status (148-220 pmol/L)	55 (21%) *n*=264	77 (35%) *n*=220
(Jeruszka-Bielak et al., 2017) MetroVancouver	Plasma total B12	Deficient (<148 pmol/L)	58 (18%) *n*=320	
		Suboptimal status (148-220 pmol/L)	106 (33%) *n*=320	
	MMA	Functional deficiency (>370 nmol/L)	6 (1.9%) *n*=320	

Abbreviations: MMA, methylmalonic acid.

In conclusion, it seems that B12 biomarker concentrations are low among pregnant and reproductive-aged women in Canada. On the other hand, folate level seems to be high or adequate among most of them. In light of the observed associations between high folate: low B12 status and adverse health outcomes in the mother and offspring (Gadgil et al. 2014; Dwarkanath et al. 2013), the imbalance between folate and B12 status in Canadian pregnant women may be of concern.

2.5 Changes in Maternal Folate and Vitamin B12 Biomarker Concentrations during Pregnancy

Pregnancy can be divided into two stages: early and late pregnancy. Early pregnancy is the period of less than 24 weeks of gestation when ectopic pregnancies and complications of abortion can occur. Late pregnancy is the period equal to or greater than 24 weeks of gestation when antepartum, intrapartum and postpartum hemorrhages can occur (Butrick et al. 2012). Most of the rapid fetal development occurs in the early stages of pregnancy. A high rate of cellular differentiation occurs, leading to the initial development of the heart, brain, spinal cord, body structure and organ systems. The development of all major organ systems happens within the first 10 weeks of gestation. This period is also the estimated time of the embryonic neural tube closure, and during this time neural tube defects may occur (Daly et al. 1995). The majority of miscarriages and other fetal pathologies also occur in early pregnancy (Graham et al. 2015).

Vitamin adequacy is critical in early pregnancy, as evidenced by the prevention of adverse health outcomes, such as neural tube defects, with maternal multivitamin supplementation in early pregnancy (Milunsky et al. 1989). Maternal adequate nutrient intake,

such as the intake of folate and B12, may be of importance in promoting early placental development (Seagraves et al. 2013).

Throughout pregnancy, most of the B-vitamins and their circulating metabolites change. Plasma total B12 concentration gradually decreases by about 100 pmol/L during pregnancy (Green et al. 1975; Cikot et al. 2001; Bruinse & van den Berg 1995; Murphy et al. 2007). This gradual decline in maternal total B12 concentration may be due to alterations in the concentration of cobalamin-binding proteins, and the active placental transport of B12 to the fetus.

Plasma holoTC declines from preconception until the 8th week of gestation, then it remains constant throughout the rest of pregnancy (Murphy et al. 2007). Plasma MMA slightly reduces at the 8th week of gestation, then gradually increases from the 20th week of gestation throughout the rest of pregnancy (Murphy et al. 2007; Green et al. 2017). Serum folate concentrations significantly decrease, while erythrocyte folate concentrations showed only minor and less dramatic decline (Bruinse & van den Berg 1995). tHcy concentrations slightly decrease in the first trimester and remain approximately constant in the second and third trimesters (from 20 weeks of gestation) (Cikot et al. 2001).

The changes in folate, B12 biomarkers and tHcy throughout pregnancy may be explained by many different physiological factors, such as the increase in circulating blood volume, plasma volume (Bruinse & van den Berg 1995; Picciano 2003), renal vitamin excretion (Bruinse & van den Berg 1995; Picciano 2003; Cikot et al. 2001), and vitamin catabolism and tissue retention (Bruinse & van den Berg 1995). Further suggested explanations are hormonal influences and haemodilution (Picciano 2003; Cikot et al. 2001).

2.6 Association of Maternal Folate and Vitamin B12 Status with Birth Outcomes

This section will mainly discuss the published studies that investigated the association of maternal folate and B12 status using biochemical indicators during pregnancy with birth outcome measured as birthweight, gestational age, and small for gestational age. Studies have looked at different timing of maternal blood sampling. The first subsection, 2.6.1, will be about studies that only investigated the stages of early pregnancy, followed by the second subsection, 2.6.2, that includes studies focusing on the late pregnancy period. **Table 2-6** is a summary of studies which investigated both early and late pregnancy periods.

Table 2-6: Summary Table of Published Studies on the Association of Maternal Folate and Vitamin B12 Status with Birth Outcomes.

Author (Year)	Design/ Method	Sample/ Setting Vitamin status	Measurement / Biomarkers results	Adjustment variables for statistical analysis	Association findings
Studies investigating 1 time point of pregnancy in the order of trimesters					
Relton et al., (2005)	prospective T1 11.5 (5.8 wk)[a]	UK *n*=614	**IV:** - RBC folate: [418(178) ng/ml][a] - total B12: [324(132) pg/ml][a] *converted [239.11(97.42) pmol/L]* [a] **DV:** BW: [3.43(0.47) kg][a]	age, parity, infant sex, GA at birth.	**-Folate** conc. positively associated with **BW (kg)** [*b*=0.25, (CI:0.08,0.42), R^2=0·021, *p*= 0.005]. -B12 conc. was not associated with BW [*b*=0.03, (CI: 20.05, 0.12), R^2=0.001, *p*= 0.41].
Bergen et al., (2012)	prospective cohort T1 T2 13.2 (11.4–16.2 wk,)[b]	Netherlands (59.3% European) *n*=5805	**IV:** - total B12: [169 (98–298) pmol/L][b] - plasma folate: [15.8 (7.3–30.6) nmol/L][b] - tHcy: [6.9 (5.3–9.4) µmol/L][b] **DV**: - BW: (3421.2 ± 563.1g) - SGA (<5th centile, 5.2%) - GA: [40.1 (38.0-41.7)wk.][b] (<37wk, 3.6%)	age, parity, education, geographical origin, folic acid supplement, smoking, alcohol, caffeine.	-High **tHcy** conc. (≥8.3 µmol/L) were associated with **LBW** (difference 110 g, *p*< 0.001) increased risk of **SGA** (OR: 1.7, *p*= 0.006) compared with lowest quintile (≤5.8 µmol/L). -Low **folate** conc. (≤9.2nmol/l) were associated with **LBW** (difference 125 g, *p*< 0.001), increased risks of **SGA** (OR: 1.9, *p* = 0.002), & **prematurity** (OR: 2.2, *p*= 0.002) compared with highest quintile (≥25.9 nmol/L). -No associations with B12.
Furness et al., (2013)	prospective T2 18–20 wk	South Australia *n*=137	**IV:** - plasma tHcy: [4.6 (CI: 4.4,4.9) mmol/L] - RBC folate: [652 (CI: 613,692) nmol/L] - serum folate: [26.5 (CI: 24.9,28.2) nmol/L] - total B12: [239 (CI: 215, 265) pmol/L] **DV:** BW	centiles were used to adjust for maternal height, weight, parity, ethnicity, infant sex, GA at birth	**-Folate** & **tHcy** were positively & inversely associated with subsequent customised BW centiles (RBC folate, r = 0.310, *p*= 0.015; tHcy, r = -0.273, *p*= 0.044; respectively).

Author (Year)	Design/ Method	Sample/ Setting Vitamin status	Measurement / Biomarkers results	Adjustment variables for statistical analysis	Association findings
Chen et al., (2015)	cohort T3(26–28 wk)	Singapore (Chinese 54%, Malay 27%, Indian 19%) *n*=999 -low folate 3% (<13.6nmol/L),16 % for B12 (<148 pmol/L)	**IV:** -plasma folate: [34.4 (24.5–44.6) nmol/L][b] -plasma total B12: [209(167–258) pmol/L][b] **DV:** -BW: [3101(449) g][a] -GA: [38.6(1.4) wk][a]	age, ethnicity, infant sex, birth order, height, pre-pregnancy BMI, weight gain, education, GDM, gravidity.	-Folate conc. is positively associated with GA [β=0.12 (CI: 0.02, 0.21) *p*=0.017]. -B12 conc. had a trend toward positive association with GA [β=0.09 (CI:0.00, 0.19), *p*=0.05] -B-6 [β=0.05 (CI: 20.04, 0.14), *p*=0.23] was not association with GA. -Folate [β=20.5 (CI: 27.8, 48.9) *p*=0.16],& B12 [b=21.2 (CI: 229.1, 26.6) *p*=0.93] were not associated with BW.
Yajnik et al. (2005)	prospective T3 (28 wk)	Pune, India *n*=80 70% (B12 <150 pmol/L), none folate deficient (<283nmol/L).	**IV:** RBC folate plasma total B12 tHcy **DV:** BW	maternal height, weight, GA at birth, infant sex	Maternal plasma tHcy conc. (µmol/L) was inversely associated with BW (β= -280.3, *p*= 0.027) -B12 conc. not related to BW. RBC **folate** conc. was positively related to BW (r=0.3, *p*< 0.01).
Navarro, J., et al., (1984)	case-control T3 (at delivery) GA: (26-36, 37-42 wk) in case & control groups, respectively.	France *n*=26 vs 32	**IV:** - Folate: [7 (1.8), 21 (2.8) ng/ml][a] - total B12: [400 (122), 422 (79) pg/ml][a] *converted [295.2 (90), 311.4 (58.3) pmol/L][a]* **DV:** BW: (1100-2480, 5650-3980g) in case & control groups, respectively.		- **Folate** of LBW is significantly lower than control (*p*< 0.001, r = 0.62). -B12 had no difference between low and control group.
Lindblad et al. (2005)	prospective T3 (at delivery)	South Asia, Lahore, Pakistan *n*=128	**IV:** - serum folate: [14 (2–91) nmol/L][b] - serum total B12: [102 (23–317) pmol/L] [b] - serum tHcy: [10.3 (4.3–23) umol] [b] **DV:** GA: (preterm delivery)		- High tHcy quartile had increased risks for premature delivery [OR: 2.5, (CI:1.1, 6.2)] as compared to low quartile.

Author (Year)	Design/ Method	Sample/ Setting Vitamin status	Measurement / Biomarkers results	Adjustment variables for statistical analysis	Association findings
Hogeveen et al.,(2010)	prospective study T3 (30-34 wk) GA: [39.7 (38.4–40.7 wk)][b]	Netherlands *n*=366	**IV:** - MMA: [0.16 (0.13–0.22) µmol/L][b] - tHcy: [5.5 (4.5–6.7) µmol/L][b] - folate: [9.1 (6.1–16.4) nmol/L][b] - plasma total B12: [179 (134–219) pmol/L][b] **DV:** BW: [3425 (3075–3855) g][b]	GA at birth, smoking, infant sex	- tHcy [β=–12 (CI: –82 ,58)], B12 [β=–37 (CI: –100,29)], MMA [b9.2 (CI: –51, 70)], & folate [β=28 (CI: –78, 133)] were not associated with BW.
Gadgil et al., (2014)	observational full-term pregnant women T3(36 wk.)	Pune, India *n*=49 -82% high folate, (>3–12 ng/ml); 35% low B12 (<150 pg/ml *(110.7pmol/L)*); 39% high tHcy (>9.5 µmol/L)	**IV:** - plasma folate: [17.8 (15.1,18.95 ng/ml)][b] - total B12: [196.9 (138.6, 261.4 pg/ml)][b] *converted1[45.3(102.3, 192.9) pmol/L][b]* -tHcy: [08.92 (07.05, 10.36 mmol/L)][b] **DV:** BW: (2748±575 g)[a]	age, maternal size, parity, GA at birth, SES, education, infant sex	- Plasma folate (r=–0.167, *p*= 0.423) & B12 (r=0.217, *p*= 0.297) were not associated with BW. - The imbalance in folate & B12 showed as **folate to B12 ratio** was **inversely** associated with BW (r=0.512, *p*= 0.009)
Halicioglu et al.,(2012)	cross-sectional full-term pregnant women T3	Izmir, Turkey *n*=208 47.6% B12, (≤160 pg/ml; *(118pmol/L)*); 17.3% folate deficiency	**IV:** total B12: (163 pg/ml)[d] *converted (120.3pmol/L)[d]* Folate: (8 ng/ml)[d] **DV:** BW:(3357±466g)[a`]		- Low B12 group (≤160 pg/ml) [BW: 3416.3±504.1g][a] & normal group(>160pg/ml;*118pmol/L*) [BW:3295.2±416.6 g][a], *p*= 0.06 had no significant difference in BW. - Low folate group (≤5 ng/ml) [BW: 3289.7±427.96g] [a], and normal group (>5 ng/ml) [BW: 3375.5±474.5g] [a]; *p*= 0.24 had no significant difference in BW.
McCulloug h et al., (2016)	Prospective observational (at enrollment) T1 (64%) T2 (33%)	USA (Black 42%, White 30%, Hispanic 28%) *n*= 496	**IV:** total B12: (Q1:322.5 vs Q4: 575.5 ng/L) *converted (Q1:238 vs Q4:424.7 pmol/L)* tHcy: (Q1:4.40 vs Q4:6.0 umol/L)	ethnicity, GW at blood draw & birth, marital status, parity, income,	- B12 (β =1.85g, *p*= 0.28), tHcy (β =-87.21, *p*= 0.10) were not associated with BW.

Author (Year)	Design/ Method	Sample/ Setting Vitamin status	Measurement / Biomarkers results	Adjustment variables for statistical analysis	Association findings
	T3 (3%) [12[c]; range (4±32.5wk.)]		**DV:** BW: (3294±541g)[a]	pre-pregnancy BMI, smoking.	
Prospective studies investigating more than 1 time point of pregnancy					
Dwarkanath et al.,(2013)	prospective observational cohort -T1 (8.9-14.5wk) -T2 (22.8-25.8wk)	India *n*= 316	**IV:** - plasma total B12: (Q1:118 vs Q3:284, Q1:108 vs Q3:245 pmol/L)[b] - RBC Folate:(Q1:325.12 vs Q3:890, Q1:449.49 vs Q3:1027.84 nmol/L)[b] in T1, T2, respectively **DV:** SGA: (30%)	age, education, parity, weight at T1, energy intake	- **B12** status in T1 [OR: 1.43 (CI: 1.02, 2.17)] and T2 [OR: 1.45 (CI: 0.92, 2.27)] & **folate** status in T2 [OR: 1.54 (CI: 0.97, 2.44)] were inversely associated with **SGA**. - Folate status in T1[OR: 1.30 (CI:0.80, 1.92)] was not associated with SGA.
Pagán et al., (2002)	-cohort -non- fasting serum sample -T2 (18 wk) -T3(30 wk)	USA (53% African American, 47% Caucasian) *n*=285	**IV:** - serum folate: [52(37), 51(40) nmol/L][a] - total B12: [357 (131), 285(100) pmol/L][a] - tHcy: [5.0 (1.8) 5.1(2.2) µmol/L][a] at T2 & T3, respectively **DV:** BW: (28% LBW)	ethnicity, infant sex pre-pregnancy BMI, smoking, GA at birth.	- **Folate** at T3 was positively associated with BW (b=2.1, *p*= 0.024), whereas this association was not significant at T2 wk (b=1.5, *p*= 0.161). - B12 (b=–0.2, *p*= 0.521; b=0.2, *p*= 0.53), & tHcy (b=–10.6, *p*= 0.69; b= –7.2, *p*= 0.67) at T2 & T3, respectively; were not associated with BW.
Yajnik et al., (2014)	assessment of two cohorts (PMNS) (18±2 &28±2 wk)	Pune, Mysore/ India *n*=526 -Low folate was rare in both but	**IV:** - RBC folate: [958 (734, 1261) nmol/L][b] - plasma folate: [34.4(16.8,51.2) nmol/L][b] - plasma total B12: [122 (94, 164), 162 (123, 223) pmol/L][b]	infant sex, GW of blood drawl	- **tHcy** conc. was inversely associated with BW in PMNS: [–22 g/SD (CI: –50, 5)] Parthenon: [–57 g (CI: –92, –21)]; meta-analysis: [–40 g (CI: –62, –17)]. - **Folate** conc. was positively associated with BW [PMNS: β=22.8 g/SD, (CI: 4.7,40.9), *p*= 0.01]; [Parthenon: β=8.2 g/SD, (CI: –7, 23.8), *p*= 0.3].

Author (Year)	Design/ Method	Sample/ Setting Vitamin status	Measurement / Biomarkers results	Adjustment variables for statistical analysis	Association findings
	& Parthenon (at 30±2 wk) -fasting blood sample -T2 -T3	70% & 43% of low B12	- tHcy: [8.6 (6.7,10.8)[b] ,6.0 (5.1,7.1) mmol/L][b] **DV:** - BW: [2642(379), 2871 (443) g][a] - GA: [39.0 (1.7), 39.1 (1.7) wk][a] - in PMNS & Parthenon, respectively		- B12 conc. was not associated with BW [PMNS: β=5.1 g/SD, (CI: –14.5, 24.6), *p*= 0.6]; [Parthenon: β=1.6 g/SD, (CI: –13.9, 17.1), *p*= 0.8]. - tHcy conc. inversely associated with GA in Parthenon: [β=0.17 wk/SD, (CI: 0.32, –0.02), *p*= 0.02], & in combined cohort effect [β= –0.14 (CI: –0.24, –0.04) *p*= 0.009], but not in PMNS: [β= –0.10 (CI: –0.25, 0.04) *p*= 0.16]
Muthayya et al.,(2006)	-prospective observational -T1 (9.6-16.2 wk) -T2 (22.1-26.1 wk) -T3 (32.7-35.1 wk)	Bangalore, India. *n*=410	**IV:** - serum total B12: (Q1:157 vs Q3:304, Q1:153 vs Q3:285, Q1:150 vs Q3:146 pg/ml) *Converted (Q1:115.9 vs Q3:224.4, Q1:112.9 vs Q3:210.3, Q1: 110.7vs Q3: 107.8 pmol/L)* - RBC folate: (Q1:518 vs Q3:1028, Q1:634 vs Q3:1235, Q1:509 vs Q3:1228 nmol/L) in T1, T2, T3, respectively **DV**: SGA: (28.6%)	age, education, parity, weight at T1.	- B12 conc. during all trimesters; [OR:5.98 (CI: 1.72, 20.74)]; [OR:9.28 (2.90, 29.68)]; [OR:2.81(1.01. 7.87)] were inversely associated with the risk of SGA. - Folate conc. during all trimesters; [OR:1.72(CI:0.66-4.48)]; [OR:1.17(CI:0.39-3.50)]; [OR:2.03(CI:0.46-8.92)] were not associated with the risk of SGA. (in T1, T2, T3, respectively).

Abbreviations: BW: birthweight; CI: 95% confident interval; conc.; concentration DV: dependent variable; GA: gestational age at birth; GDM: gestational diabetes mellitus; GW: gestational weeks; IV: independent variable; LBW: low birthweight; MLR: multiple linear regression; MMA: methylmalonic acid N/A, not available; SES: socioeconomically status; SGA: small for gestational age; T1: first trimester; T2: second trimester; T3: third trimester; tHcy: total homocysteine; &: and; wk: weeks of gestation;

[a] Values are expressed as mean (SD).

[b] Values are expressed as median (IQR).

[c] Values are expressed as mean.

[d] Values are expressed as median.

2.6.1 Early Pregnancy Period

The studies that investigated the early pregnancy period (< 24 weeks of gestation) were three studies of prospective cohort design. All three studies found that folate but not B12 is associated with birthweight after adjustment for maternal age, parity, infant sex and gestational age at birth (Bergen et al. 2012; Relton 2005; Furness et al. 2013). Bergen et al. (2012) is the largest study conducted in early pregnancy. Its strength lies not only in the large sample size (*n*=5805), but also in the many birth outcomes that were investigated. The authors of the study found that low folate concentration [lowest quintile (≤9.2 nmol/L)] was associated with lower birthweight (difference 125 g; $p< 0.001$), higher risk of SGA (OR 1.9; $p= 0.002$) and prematurity (OR 2.2; $p= 0.002$), compared with the highest quintile (≥25.9 nmol/L). Additionally, high tHcy concentration [highest quintile (≥8.3 µmol/l)] was associated with low birthweight (difference 110 g; $p< 0.001$), and increased risk of SGA [odds ratio (OR) 1.7; $p= 0.006$] compared with lowest quintile (≤5.8 µmol/L). There is a limited number of studies on the association of maternal folate and B12 biomarker concentrations in early pregnancy with birth outcomes

2.6.2 Late Pregnancy Period

Most of the studies that investigated the late pregnancy period (> 25 weeks of gestation), found that neither folate nor vitamin B12 are associated with birthweight (Chen et al. 2015; Yajnik et al. 2005; Lindblad et al. 2005; Hogeveen et al. 2010; Gadgil et al. 2014; Halicioglu et al. 2012; Takimoto et al. 2007). Those studies were conducted in different countries; the Halicioglu et al. (2012) study was in Turkey and similar results were also found in Japan (Takimoto et al. 2007), Singapore (Chen et al. 2015), and Netherlands (Hogeveen et al. 2010). Although Gadgil et al. (2014) stated that neither folate nor vitamin B12 are associated with birthweight, which concurred with the previously mentioned studies, he added that the combined

association of folate and vitamin B12 was associated with the neonatal anthropometrics. This was demonstrated by an imbalance in the maternal blood levels with an increasing ratio of folate to B12 which was associated with an increase in plasma total tHcy concentrations (p= 0.014, R=0.349), resulting in lower birthweight (p= 0.009, r = -0.512) (Gadgil et al. 2014). This was in line with another study, that found that the imbalance between folate and vitamin B12 dietary intake had an increased risk of SGA outcome (Dwarkanath et al. 2013). To my knowledge, these are the only two studies that investigated the imbalance between maternal folate and B12 with birthweight and small for gestational age.

Unlike most of the studies conducted in late pregnancy, Ahmed et al. (2011) noticed from his cross-sectional study of 150 pregnant women in Bangladesh that maternal serum concentrations of both folate and B12 individually are positively associated with birthweight. However, the blood samples collection for this study took place after delivery. Most maternal vitamin serum concentrations of normalize to the preconceptional value shortly after delivery (Cikot et al. 2001; Bruinse & van den Berg 1995). The change in maternal vitamin biomarker concentrations may explain the possible reason for finding the association with both vitamins.

In a case-control study, maternal folate status (p< 0.001, r = 0.62) of a low birthweight group was significantly lower than the control group of normal birthweight; yet, no difference was found in B12 (Navarro et al. 1984). Similar results were found in a prospective cohort study in which maternal plasma total B12 concentration was not associated with birthweight, and RBC folate concentration was positively associated (r =0.3, p< 0.01) with birthweight (Yajnik et al. 2005). Additionally, maternal plasma tHcy concentration (μmol/L) was inversely associated with birthweight (β= -280.3, p= 0.027) (Yajnik et al. 2005). These studies had a small sample size and did not control for some important confounding factors, such as maternal age.

Two cohort studies investigated maternal folate and B12 biomarker concentrations individually with gestational age at birth in the late pregnancy period (>25 weeks of gestation). A cohort study in Singapore investigated the association of maternal plasma folate and B12 concentration individually at 26–28 weeks of gestation in relation to gestational age at birth. The study revealed that maternal plasma folate concentration was positively associated with gestational age at birth [β=0.12 wk (CI: 0.02, 0.21) *p*= 0.017], but maternal plasma B12 concentration was not associated with gestational age at birth [β=0.09 wk (CI:0.00, 0.19), *p*= 0.05] (Chen et al. 2015). The prospective cohort study conducted by Lindblad et al. (2005) investigated the association of maternal concentrations of folate, B12, and tHcy at delivery with preterm delivery, in the city of Lahore, Pakistan. A higher risk of preterm delivery (OR 2.5, 95% CI: 1.1, 6.2) was found among women with the highest quartile of tHcy (Lindblad et al. 2005).

To my knowledge, there are more studies conducted in the late pregnancy period than in early pregnancy, most of which investigated birthweight only, as an outcome, but not gestational age at birth.

2.6.3 Early and Late Pregnancy Period

Studies that investigated early and late pregnancy adjusted for gestational weeks at blood collection or analyzed each trimester individually. The study that adjusted for gestational weeks of blood draw was an assessment of two cohorts in India for gestational weeks of 18±2, 28±2, and 30±2 weeks, that analyzed for folate, B12, and tHcy. The study revealed that maternal tHcy concentration was inversely associated with birthweight in the Pune Maternal Nutrition Study (PMNS): [–22 g/SD (CI: –50, 5)], Parthenon: [–57 g (CI: –92, –21)], and the meta-analysis: [–40 g (CI: –62, –17)]. Interestingly, folate concentration was positively associated with birthweight in both cohorts [PMNS: β=22.8 g/SD, (CI: 4.7,40.9), *p*= 0.01; Parthenon: β=8.2 g/SD, (CI: –7,

23.8), *p*= 0.3]. However, B12 concentration was not associated with birthweight in either cohort [PMNS: β=5.1 g/SD, (CI: –14.5, 24.6), *p*= 0.6; Parthenon: β=1.6 g/SD, (CI: –13.9, 17.1), *p*= 0.8]. In regards to gestational age at birth as an outcome, tHcy concentration was inversely associated with gestational age at birth in Parthenon: [β=0.17 wk/SD, (CI: 0.32, –0.02), *p*= 0.02], and in the meta-analysis [β= –0.14 (CI: –0.24, –0.04) *p*= 0.009], but not in PMNS: [β= –0.10 (CI: –0.25, 0.04) *p*= 0.16] (Yajnik et al. 2014).

A prospective observational study that adjusted for gestational weeks of blood draw which took place in the USA, investigated the maternal B12 and tHcy at enrollment with birthweight (McCullough et al. 2016). The study revealed that neither B12 (β =1.85g, *p*= 0.28), nor tHcy (β =-87.21g, *p*= 0.10) were associated with birthweight after adjusting for many confounding factors, such as marital status, income, and pre-pregnancy BMI (McCullough et al. 2016). Thus, both of the studies that investigated early and late pregnancy by adjusting for gestational weeks of blood draw stated that maternal B12 concentrations are not associated with birthweight (Yajnik et al. 2014; McCullough et al. 2016).

Some studies that investigated early and late pregnancy analyzed each trimester individually. The first study evaluated the association of maternal folate and B12 concentrations at 18 and 30 weeks of gestation with birthweight. The results indicated that a 10 nmol/L increase in folate concentration at week 30 of gestation corresponded to a 21g increase in birthweight. This association was not significant at week 18 of gestation, nor was it significant for B12 at either time point (Pagán, 2002). In contrast, another study found that low maternal total B12 concentration, but not folate, was an important determinant of the risk of SGA in all trimesters (Muthayya et al. 2006). The difference in results might be due to the heterogeneity in ethnicity (McCowan & Horgan 2009), and the use of different folate biomarkers, serum folate versus RBC

folate. Serum folate is considered an indicator for the circulating folate while RBC folate is considered an indicator of long-term folate status of 120 days (Lamers 2011b).

2.7 Summary and Rationale

The evidence supports an association between maternal low folate and high tHcy concentrations and risk of low birthweight. However, data regarding maternal B12 status and risk of low birthweight remain equivocal. The inconsistent results might be due to the differences in the timing of maternal blood sampling, fasting-fed state at blood sampling and the selection of biomarkers. Additionally, some studies used birthweight alone instead of birthweight in relation to gestational age, e.g., SGA vs. LGA. The studies were conducted in different countries which include variations in ethnicity, fortification policies, and recommendations for and practice of prenatal supplement use.

Food fortification policy and prenatal supplement use differ among different countries. Mandatory versus voluntary food fortification can influence maternal folate and B12 intake and thus influence maternal folate and B12 status (Picciano 2003; Allen et al. 2006). Ethnicity is a confounding factor for maternal B-vitamin status, given that ethnicities may differ in their lifestyles, including their dietary habits, and in birth outcome measures including infant's size (Quay et al. 2015; Hanley & Janssen 2013).

There is inadequate information on the association between maternal folate and B12 status and gestational age at birth. Further, data on maternal folate and B12 in early pregnancy are limited. In conclusion, most of the studies were conducted in the third trimester and investigated birthweight. The majority of studies analyzed one direct B12 biomarker, plasma or serum total B12, but scarcely holoTC or the functional indicator MMA.

There is a lack of research investigating the association of maternal folate and B12 status combined with birth outcome. There seems to be a high prevalence of high folate and low B12 status in Canadian pregnant women (Fayyaz et al. 2014; Visentin et al. 2016). Therefore, I

investigated the association of maternal folate and B12 status combined, on birth outcome. Additionally, there is a lack of research focusing on the association between maternal folate and B12 status in early pregnancy and birth outcome, including gestational age at birth. Thus, the objective of this research is to evaluate the association of maternal serum folate and B12 concentrations in early pregnancy, combined and individually, with birthweight and gestational age at birth.

Chapter 3: Study Design and Methodology

This retrospective cohort study utilized residual maternal non-fasting serum samples, as well as maternal and neonatal data, which had been routinely collected during the BC Prenatal Genetic Screening Program and the BC Newborn Screening Program. This thesis, as described herein, is a secondary analysis that included 674 mother-newborn dyads with available birth outcome data, to determine the associations of maternal folate and B12 status in early pregnancy with birth outcomes, specifically birthweight and gestational age at birth. The primary objective of the original study, as described in detail by Theresa Schroder (Schroder et al. 2017), was to determine the difference in serum total B12 concentrations between pregnant women of South Asian and European ethnicity residing in BC, Canada.

3.1 Study Objectives

The study objectives were:

1) To determine the association of maternal folate and B12 biomarker concentrations individually with birthweight and gestational age at birth;

2) To investigate the association of maternal folate and B12 status combined with birthweight and gestational age at birth;

3) To investigate the association of the change in maternal folate and B12 biomarker concentrations between the first and second trimesters of pregnancy with birthweight and gestational age at birth.

The related hypotheses were as follows:

1) I hypothesized that the maternal serum concentration of each direct indicator of folate and B12 status is positively associated, and each functional indicator inversely associated, with birthweight and gestational age at birth; i.e., the higher a woman's circulating folate concentration, the longer is her pregnancy duration and the higher is the birthweight of her newborn, after controlling for maternal age, ethnicity and infant sex.

2) I hypothesized that the ratio between maternal folate and total B12 concentrations is inversely associated with birthweight and gestational age at birth; i.e., if a woman shows a high folate to B12 ratio, she will have a shorter pregnancy duration and an infant with lower birthweight than a woman with a lower folate to B12 ratio, after controlling for maternal age, ethnicity and infant sex.

3) I hypothesized that the change between first and second trimester of pregnancy in maternal serum concentration of each direct indicator of folate and B12 status is positively associated, and the change between the first and second trimester of each functional indicator is inversely associated, with birthweight and gestational age at birth; i.e., if a woman shows a greater decrease in circulating folate and B12 concentrations between first and second trimester of pregnancy, she will have a shorter pregnancy duration and an infant with lower birthweight than a woman with a smaller decrease in circulating folate and total B12 concentrations, after controlling for maternal age, ethnicity and infant sex; and, if a woman shows a greater increase in circulating folate and B12 concentrations between first and second trimester of pregnancy, she will have a longer pregnancy duration and an infant with higher birthweight than a woman with a smaller increase in circulating folate and total B12 concentrations, after controlling for maternal age, ethnicity and infant sex.

3.2 Study Design

In this retrospective cohort study, biobanked maternal serum samples were retrieved from the BC Prenatal Genetic Screening Program ("Perinatal Services BC (2016) BC Prenatal Genetic Screening Program"). Maternal non-fasting blood samples collected at 11±2 and 17±2 weeks of gestation for the BC Prenatal Genetic Screening Program were stored in the BioBank in the BC Children's Hospital (Vancouver, BC), and de-identified by trained personnel of the BC Newborn Screening and Biochemical Genetics Laboratories prior to transferring to the research team's laboratory (UBC Nutritional Biomarker Laboratory). In addition, maternal data, including maternal age and ethnicity were retrieved from medical records. Other maternal data that might influence birth outcomes, including maternal weight and parity, were not available for this secondary analysis.

Consecutive maternal serum samples were retrieved according to inclusion and exclusion criteria. Neonatal characteristics of the matched newborns, including infant sex, birthweight, and gestational age at birth were derived from the BC Newborn Screening Program (Perinatal Services BC, Vancouver BC). Gestational age at maternal blood collection was confirmed by ultrasound using crown-rump length, while gestational age at birth was calculated based on the gestational age during the second trimester visit and infant birth date. Birthweight was collected from medical records, for which it had been measured with a digital scale by the medical staff.

<u>Inclusion and exclusion criteria for maternal and neonatal data and specimen:</u>

The inclusion criteria were South Asian or Caucasian (for consistency "Caucasian" is here referred to as European) ethnicity (self-reported), 19 to 45 years of age, and resident in BC, Canada. These criteria were used to determine the difference in serum total B12 concentrations

between pregnant women of South Asian and European ethnicity residing in BC, Canada (Schroder et al. 2017).

Exclusion criteria were medical conditions, including human immunodeficiency virus (HIV), diabetes mellitus type I or II; assisted conception and/or pregnancy following *in vitro* fertilization or intracytoplasmic sperm injection; intravenous therapy or oral steroid medication during pregnancy; history of Down Syndrome, Trisomy 18, or open neural tube defect; and multiple pregnancy.

Sample Size:

We retrieved 748 maternal second trimester serum samples and 686 matched first trimester serum samples of apparently healthy women. The sample size was larger in the second trimester because second trimester samples were pulled first. The reasons for unmatched first trimester samples and/or for women not having participated in the first trimester blood collection are unknown. The maternal biomarkers were analyzed sequentially in the following order: total B12, holoTC, MMA with MCA, tHcy and folate. This order of analysis was also dependent on the adequacy of serum volume (minimum serum volume required for analysis: total B12=120 µl, holoTC=200 µl, MMA with MCA=50 µl, tHcy=50 µl and folate=50 µl) (**Figure 1-2**).

Data exclusion was conducted after receiving the results of all serum biomarker analyses. Maternal serum total B12 concentrations above the upper limit of the analytical measurement range of 1107 pmol/L (n=3) were excluded from data analysis. The limited serum sample volume did not allow for a repeat, dilute analysis. Because only three samples had total B12 concentrations greater than 1107 pmol/L, the data were excluded and not treated as censored data. Maternal biomarker data of one woman with potential renal impairment was excluded;

potential renal impairment was assessed based on the criteria serum 2-methylcitric acid (MCA) > serum MMA concentrations with MMA > 210 nmol/L (Allen et al. 1993).

Data from mother-newborn dyads with missing information on both birthweight and gestational age at birth were excluded (*n*=70). These data were unavailable for one of the following reasons: 1) giving birth outside of the study's catchment area (i.e., BC); 2) having a stillbirth; 3) inability to link maternal to newborn specimen, or 4) unknown causes (**Figure 1-2**). Thus, a sample size of in total 674 mother-newborn dyads was included in this secondary analysis.

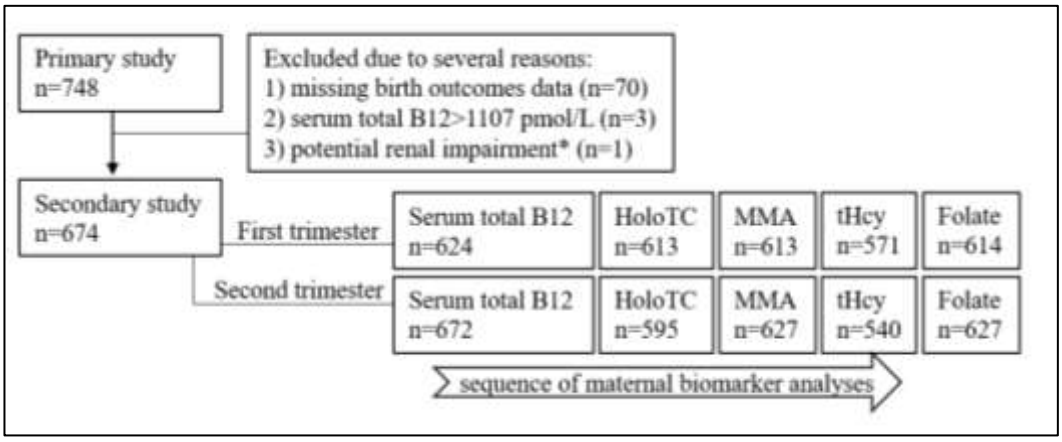

Figure 1-2: Flowchart of Study Sample Size and Maternal Folate and B12 Biomarker Analyses.
* Renal impairment was based on the criteria: 2-methylcitric acid (MCA)>MMA with MMA > 210 nmol/L

3.3 Analytical Methods for Biomarker Quantitation

Maternal serum samples were analysed for direct and functional indicators which are, sequentially, total B12, holoTC, MMA, folate and tHcy between January 2016 and September 2016. Circulating B12 concentrations were assessed by measuring the direct indicators of total B12 and holoTC using fully automated immunoassays (Access by Beckman Coulter Inc. and Architect by Abbott Technologies, respectively) according to manufacturers' protocols at the pathology laboratories at BC Children's Hospital and St. Paul's Hospital, respectively.

The inter-assay CV for four total B12 control samples (Bio-Rad, mean concentration of 93·6, 245, 335, 407 pmol/L) ranged from 2.4 to 7.1% (analysed weekly over 4 months; n 18). Manufacturer (Abbott Laboratories) control samples for holoTC were run once per batch for the three batches of study samples, yielding mean concentrations of 46 (SD 1·5) pmol/L (CV: 3·3%) and 16 (SD 2·0) pmol/L (CV: 12 %) for the high and low control sample, respectively. The upper limits of the analytical measurement range for the total B12 and holoTC assays were 1107 and 128 pmol/L, respectively.

In addition, the functional indicators MMA and serum tHcy were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analyses were conducted in the UBC Nutritional Biomarker Laboratory (Dr. Lamers' lab). Simultaneously with MMA, MCA was quantified using MCA (Sigma-Aldrich) as standard and d3-MMA (Cambridge Isotopes Ltd) as an internal standard. Results are reported as the sum of all stereoisomers. The inter-assay CV for an in-house control sample was 7% for MMA and 14% for MCA (analysed over 19 d); the intraassay CV was <5% for all analyses.

Serum tHcy was analysed by using stable isotope dilution LC-MS/ MS. Samples were reduced with dithiothreitol and protein precipitation, then injected into an LC system (Agilent 1260; Agilent Technologies). Compounds were separated by a normal phase column (Fortis H2O, 2·1×150mm, 5 μm; Fortis Technologies). The mobile phase consisted of (A) 0·2% heptafluorobutyric acid in water and (B) 0·2% heptafluorobutyric acid in acetonitrile using a gradient run (A:B 95:5 (v/v) to 20:80 (v/v)). The affluent was directed into an MS/MS system (API4000; SCIEX Pte). Quantification was performed with seven-point calibration curves (1·14–114 μmol/l) made using L-homocysteine (Sigma-Aldrich) as calibrator and d4-homocysteine (Cambridge Isotopes Ltd) as an internal standard. The inter-assay CV for an in-

house control sample was 9·4% (analysed over 17 d). Two external quality control samples (ClinChek 23082 and IRIS Technologies International) were quantified with every analysis and were within their acceptable range of 9·0 (SD 1·8) and 25·9 (SD 5·1) μmol/l, respectively.

Folate concentration in serum was indirectly assessed using the *Lactobacillus casei* microbiological assay in 96-well microtiter plates. The method uses *Lactobacillus casei*, a bacterial strain that has a growth response in the presence of all folic acid derivatives. Thus, the concentration of folate is equal to the turbidity of the media (Molloy, 1997; O'Broin, 1992). For the inter-assay CVs for folate samples: The National Institute for Biological Standards and Control reference material 95/528 (NIBSC 95/528) and an in-house serum control samples were included with each of the 22 runs. The analyses yielded folate contents of 28.3 nmol/L (13 ng/mL) (inter-assay CV: 8.8%) for the NIBSC 95/528 control sample (manufacturer value: 13 ng/mL), and of 42.8 nmol/L (inter-assay CV: 10.1%) for the in-house serum control sample.

3.4 Statistical Analyses

3.4.1 Descriptive Statistics

All statistical analyses were performed using R statistical software (version 1.0.153). Normality was assessed visually (i.e., histogram, QQ plots) and using Shapiro-Wilks tests. All variables were found non-normally distributed; thus, descriptive statistical analyses were conducted to provide basic summaries of each variable as medians with interquartile ranges (IQR).

The prevalence of low birthweight and preterm birth were indicated by <2500 g and <37 weeks of gestation; respectively. The prevalence of SGA (birthweight<10th percentile), AGA and LGA were calculated according to the data from Janssen et al. (2007). The growth charts

measurements in Janssen et al. (2007) were for full-term infants of South Asian and European ethnicity and grouped by infant sex. The distribution of the intrauterine growth charts in Janssen et al. (2007) did not include preterm infants as they are already in an abnormal intrauterine environment. The data available from Janssen et al. (2007) only included weeks of gestation and not days, thus I used a monotone piecewise cubic interpolation method to make the SGA curve smooth by the R function "splinefun" (Fritsch & Carlson 1978). This SGA curve was then used to calculate the prevalence of SGA.

Birth outcome data were compared between women of South Asian and European ethnicity, because ethnicity is a strong confounding factor (Wells et al. 2013) and the two ethnic groups were of equal sample size which made the comparison suitable to detect. Additionally, South Asian populations are the largest visible ethnic minority group in Canada (Statistics Canada 2013). The prevalence of low birthweight and preterm birth between the ethnicities was compared by using the Fisher's exact test, i.e., a non-parametric test for frequency distribution in independent samples. Biomarker concentrations between trimesters were compared by using a non-parametric test for dependent samples, i.e., the Wilcoxon signed rank test. The two-sided p-value of less than 0.05 was considered significant.

3.4.2 Association Analyses

3.4.2.1 Folate and Vitamin B12 Biomarkers Individually

1) To determine the association between maternal folate and B12 biomarker concentrations individually with birthweight and gestational age at birth

A) Simple Linear Regression Analysis

Simple linear regression was used to test the association between maternal folate and B12 biomarker concentrations individually on each birth outcome, i.e., birthweight and gestational age at birth.

Example: Linear model: e.g. Birthweight = $\beta o + \beta 1$(folate)

B) Multiple Linear Regression Models Construction

The associations of maternal serum folate and B12 biomarker concentrations with birthweight and gestational age at birth were assessed using multiple linear regression models after adjustment for potential confounders. Multiple linear regression models were created with birthweight and gestational age at birth as the dependent variables. Multiple linear regression was used for description purposes, to determine how much of a change in maternal folate and B12 biomarker concentrations may predict a change in the outcome variable, such as birthweight and gestational age at birth. Confounding factors included ethnicity, maternal age, and infant sex.

South Asian ethnicity has been shown to be a predictor of low birthweight as described in the literature review (Chapter 2, section 2.1.1). To test whether ethnicity is an effect modifier, I conducted statistical tests including interaction term and stratified analysis. Effect modification would occur if the association of maternal folate and B12 biomarker concentrations with birth outcomes differed between the two ethnic groups (South Asian and European). The interaction terms of ethnicity x serum folate and ethnicity x B12 biomarkers (total B12, holoTC, MMA) were conducted for each biomarker individually in a regression model with birthweight as outcome variable. **Table 3-1** shows that the results of the interaction terms ethnicity x serum

folate, ethnicity x total B12, ethnicity x holoTC, and ethnicity x MMA with birthweight were not statistically significant. These results indicate that the association of maternal folate and B12 biomarker concentrations with birthweight do not significantly differ by ethnicity. The stratified analysis showed that the model residual standard error decreased when using ethnicity as a confounding factor compared to the model residual standard error of separate models for each ethnicity. This indicates that using ethnicity as a confounding factor in a regression model results in a better model than stratifying the two ethnic groups into separate regression models. In summary, the insignificant interaction term and the reduced residual standard error indicated that there is no effect modification for the ethnicity factor. Thus, separate linear regression models for each ethnic group were not used to assess the association between maternal folate and B12 biomarker concentrations with birthweight; instead, ethnicity was used as a confounding factor.

Table 3-1: Effect Modifier Testing for Ethnicity with Birthweight as Outcome Variable

Biomarker	**Independent Variable**	**Regression Coefficient**	***p*-value**
Serum folate	Intercept	3698	< 0.001
	Folate	-1.11	0.226
	Ethnicity SA	-317	0.001*
	(Folate x Ethnicity SA)	0.79	0.55
Serum total B12	Intercept	3579	< 0.001
	Total B12	-0.22	0.29
	Ethnicity SA	-292	0.0007*
	(Total B12 x Ethnicity SA)	0.101	0.76
HoloTC	Intercept	3607	<0.001
	HoloTC	-0.966	0.262
	Ethnicity SA	-387.7	0.0002*
	(HoloTC x Ethnicity SA)	1.445	0.211
MMA	Intercept	3517	<0.001
	MMA	5777	0.991
	Ethnicity SA	247.5	0.002
	(MMA x Ethnicity SA)	002.2	0.997

Regression coefficients, unstandardized beta (*b*), denoting the mean change in the dependent variable (birthweight or gestational age at birth) for one unit of change in the independent variable (e.g., serum total B12 concentration), are presented with accompanying

95% confidence intervals. The coefficient of determination, R^2 values, represents the proportion of the variation in the outcome explained by the independent variables in the regression model and it indicates how well the model fits the data.

Stepwise linear regression was avoided in the regression analyses since this study had few confounding factors available and the stepwise process tends to inflate effect sizes. The regression models included confounding factors that were biologically plausible as described in the literature review (Chapter 2, section 2.1). Thus, to test my hypotheses on the associations of maternal serum folate and B12 biomarker concentrations with birthweight and gestational age at birth, multiple linear regression models were conducted while adjusting for the confounding factors, in the following order for birthweight and gestational age at birth as an outcome, respectively:

Birthweight = βo + β1 maternal folate or B12 biomarker concentration for each trimester individually + ethnicity + maternal age + infant sex+ gestational age at birth.

Gestational age at birth = βo + β1 maternal folate or B12 biomarker concentration for each trimester individually + ethnicity + maternal age + infant sex.

The number of women with a concentration of holoTC above the upper limits of the analytical measurement range (128 pmol/L) was considerable (n=118 and n= 75 for the first and second trimester, respectively), thus these women were not excluded. Multiple linear regression models were conducted for maternal serum holoTC concentrations by using discontinuous regression with categorical variables for the censored data indicating whether holoTC

concentrations were below or above the upper analytical limit of 128 pmol/L. The censored data are the data that are above the upper limit of the analytical measurement, e.g. holoTC=128 pmol/L.

C) Regression analysis diagnostic protocol

Influential data points were identified by visual assessment of residuals versus leverage plot by looking for influential data points outside of a dashed line known as Cook's distance (high Cook's distance scores). Assumptions of linearity and constant variance (homoscedasticity) were visually assessed by residuals versus fitted and scale-location plots. The assumption of normality was assessed by Q-Q plots.

3.4.2.1.1 Quartiles Analyses

Biomarker concentrations were divided into quartiles and subsequently used as categorical variables to assess the association of maternal folate and B12 biomarker concentrations (as quartiles) with birthweight and gestational age at birth in the first and second trimesters. The quartile groups were conducted by using first quartile (25^{th} percentile), median and third quartile (75^{th} percentile) as cut-off values. The lowest quartiles were used as a reference. The associations were calculated using adjusted linear regression analyses as explained in sub-section (3.5.2.1), part (B, multiple linear regression models construction).

This approach was chosen to explore the potential nonlinearity of the association and to examine the association across the quartiles, especially the highest and lowest quartiles, since the maternal folate and B12 biomarker concentrations were of limited variation as tested in the descriptive statistic by inter quartile range. Quartiles were used due to the large sample size,

making it possible to split the sample into four equal groups. The values are represented as Q1, Q2, Q3, Q4, and these were used to divide the sample size accordingly. Quartiles were used rather than biomarker cut-off values because only non-pregnant adult cut-offs are available; pregnancy-specific biomarker cut-offs are lacking. Additionally, using non-pregnant adult biomarker cut-off values results in a large variation in the sample size between the categorical groups. For example, no woman had folate concentration that indicates deficiency (plasma/serum folate <7 nmol/L), whereas 85% of the women had high folate status (plasma/serum folate >46 nmol/L).

3.4.2.2 Folate and Vitamin B12 Imbalance

2) To investigate the association of maternal folate and B12 status combined with birthweight and gestational age at birth.

The ratio of serum folate to serum total B12 concentration in each trimester was calculated as follows: Ratio = (Serum folate $_{\text{trimester 1}}$ / serum total B12 $_{\text{trimester 1}}$) *1000 *conversion factor of 1000.

The ratio approach was chosen to determine whether the imbalance of folate and total B12 is associated with the birth outcomes, including birthweight and gestational age at birth. Thus, the higher the ratio, the higher the imbalance is between folate and total B12 concentrations; e.g., a woman with a ratio of 73.6 (folate: 40.1 nmol/L and total B12: 545 pmol/L) has less imbalance than a woman with a ratio of 319 (folate: 77.9 nmol/L and total B12: 244 pmol/L).

Then a simple linear regression model and a multiple linear regression model were conducted in a similar process as that stated in section 3.5.2.1 (part A, simple linear regression analysis and part B, multiple linear regression models construction). In addition, I grouped the folate to B12 ratio into quartiles and assessed their association with birth outcomes, including birthweight and gestational age at birth in a similar process as stated in section 3.5.2.1.1. The interaction term was also performed to test the joint association of maternal folate and total B12 on each birth outcome individually (birthweight and gestational age at birth).

3.4.2.3 Changes in Folate and Vitamin B12 Biomarker Concentrations

3) To investigate the association of the change in maternal folate and B12 biomarker concentrations between first and second trimester of pregnancy with birthweight and gestational age at birth

The changes of maternal folate and B12 biomarker concentrations were calculated by: (change in biomarker concentration per week of gestation)

$$\frac{biomarker\ concentration\ (T2) - biomarker\ concentration\ (T1)}{gestational\ age\ at\ blood\ collection\ (T2) - \ gestational\ age\ at\ blood\ collection\ (T1)}$$

HoloTC data with values above the upper limit of the analytical measurement range of 128 pmol/L (n=118 and 75 for the first and second trimester, respectively) were excluded from this analysis due to unavailability of an exact value.

Multiple linear regression models, including the change in biomarker concentration as the independent variable and birthweight or gestational age at birth as the outcome variable were

conducted in a similar process as stated in section 3.5.2.1 (part B, multiple linear regression models construction).

3.5 Sample Size and Power Calculation

The sample size calculation of the original study (Schroder et al. 2017) was based on its primary study objective which was to detect the difference in mean serum total B12 concentration between South Asian (μ_2) and European (μ_1) pregnant women (t-test) (**Table 3-2**).

Equation 1: Sample size calculation.

$$\boldsymbol{n = \frac{2\sigma^2 \times (Z_\beta + Z_{\alpha/2})^2}{(\mu_1 - \mu_2)^2}}$$

Table 3-2: Statistical Variables for Sample Size Calculation.

Statistical Variable	
Power (1-β)	0.80
Confidence Level (α)	0.05
Mean $_{European}$ (serum total B12) (μ_1) [pmol/L]	177*
Mean $_{South\text{-}Asian}$ (serum total B12) (μ_2) [pmol/L]	142
Standard Deviation (σ) [pmol/L]	74*
Sample Size (n)†	71
Effect Size (Cohen's *d*)	0.47

*based on (Hure et al. 2012)
†per group

The secondary analysis study objective presented in this thesis was to determine the association of maternal folate and B12 biomarker concentrations with birthweight and gestational age at birth. In the existing literature, more studies have investigated the association between maternal folate status and birthweight (Van Uitert & Steegers-Theunissen 2013), compared to B12 status and birthweight. In regards to the birth outcome variables, birthweight was the most frequently selected indicator used as a proxy for fetal growth. Thus, my power calculation was based on the association of folate with birthweight.

Based on the literature, the significant association between maternal folate and birthweight has a regression coefficient, unstandardized beta (b_1), of approximately 0.25 g (Relton 2005; Pagan et al. 2002; Yajnik et al. 2014). Calculating the power with G*Power 3 software (Erdfelder et al. 2009) of the current sample size (n= 674) based on $b_1 = 0.25$ by t-test, with a 5% level of significance, resulted in 98% power. When calculating the required sample size, based on $b_1 = 0.25$ by t-test, with a 5% level of significance and 90% power, the total sample size required was 442 mother-newborn dyads. Thus, my sample size of 674 mother-newborn dyads was powered to determine a significant association between maternal folate and birthweight.

Chapter 4: Results

The results are presented in three main sections:

Section 4.1 presents maternal and neonatal characteristics and a comparison of maternal folate and B12 biomarker concentrations between trimesters. This section also includes the prevalence of pregnant women classified as folate deficient or with high folate status and the prevalence of pregnant women classified as B12 deficient or with suboptimal B12 status according to non-pregnant adults' cut-offs for the respective biomarkers.

Section 4.2, presents the results of univariate and multiple linear regression analyses. These results allow the assessment of the association between maternal folate and B12 biomarker concentrations individually and combined as folate to B12 ratio, in the first and second trimester, with birth outcomes (i.e., birthweight and gestational age at birth). **Subsection 4.2.1**, details the results of using quartiles of maternal folate and B12 biomarker concentrations in a multiple linear regression model. This allows the assessment of the association between maternal folate and B12 biomarker concentrations as categorical variables in the first and second trimesters with birth outcomes.

Section 4.3 presents the results of multiple linear regression analyses to assess the association between the change in maternal folate and B12 biomarker concentrations, between first and second trimester of pregnancy, and birth outcomes.

4.1 Maternal and Neonatal Characteristics (Age, Sex)

The characteristics of the mothers and newborns are summarized in **Table 4-1**. The sample size was almost equal for women of European ethnicity (52.2%) and South Asian ethnicity (47.8%). The median age of all women was 31 years (IQR: 28, 35). The median (IQR) gestational weeks at the two study time points were 11.4 (IQR: 10.6-12.3) and 16.1 (IQR: 15.1-

17.1) weeks of gestation, respectively. There were no significant differences in maternal age and gestational age at birth between women of European and South Asian ethnicity. Overall, the prevalence of low birthweight (<2500g) and preterm birth (<37weeks of gestation) were 1.93% and 8.9%, respectively, in the sample. The prevalence of preterm birth was not significantly different between women of European and South Asian ethnicity (p= 0.33); however, the prevalence of low birthweight was significantly higher among women of South Asian ethnicity compared with those of European ethnicity (p= 0.0097).

Since the prevalence of low birth weight differed between the two ethnic groups, I have used the ethnic-specific growth charts created by Janssen et al. (2007), for term infants (gestational age at birth ranging from 37 to 41 weeks). The prevalence of SGA and LGA was 0.88% (n=5) and 2.84% (n=16) of newborns, respectively. Most of the infants, 96.3% (n=542), were AGA in the sample of in total 605 infants with gestational age at birth ranging from 37 to 41 weeks. Due to the very low prevalence of SGA infants, a logistic regression to investigate the association of folate and B12 status with SGA and AGA as categorical outcome variables was deemed inappropriate.

Table 4-1: Maternal and Neonatal Characteristics [1]

Characteristic	All
Maternal	
Sample size *n* (%)	674
Age (y)	31 (28, 35)
Age range (min-max)	(19-44)
Gestational age at birth (wk)	39.0 (38.0, 40.0)
Gestational age at birth range (min-max)	(27.6-42.9)
Gestational weeks (1st trimester visit) median (IQR)	11.4 (10.6, 12.3)
Gestational weeks (2nd trimester visit) median (IQR)	16.1 (15.7, 17.1)
Neonatal	
Male sex *n* (%)	335 (49.7)
Birthweight (g) median (IQR)	3385 (3090,3698)
[2]Low birthweight *n* (%)	13 (1.93)
[3]Preterm birth *n* (%)	57 (8.9)

[1]Values are presented as median (inter-quartile range), or *n* (%).
[2] Low birthweight defined as <2500g, [2]Preterm birth defined as <37 weeks of gestation. **Abbreviations**: y=years, wk=weeks, g=grams.

Data on maternal folate and B12 biomarker concentrations in the first and second trimesters are summarized in **Table 4-2.** First and second trimesters median (IQR) serum folate concentrations were 66.9 (53.3; 81.8) and 69.3 (55.5; 87.0) nmol/L, respectively, which both fall into the classification of high folate status (serum folate >46 nmol/L). The second trimester serum folate concentration was significantly higher compared to the first trimester (p= 0.01). B12 biomarkers, including total B12, holoTC, and MMA concentrations were significantly different between the trimesters. Total B12 and holoTC were significantly lower in the second trimester compared to the first trimester. tHcy concentration significantly decreased between the first and second trimester (p< 0.0001).

Table 4-2: Maternal Serum Total Vitamin B12 (B12), Holotranscobalamin (HoloTC), Methylmalonic Acid (MMA), Total Homocysteine (tHcy), and Folate Concentrations During the 1st and 2nd Trimester of Pregnancy [1]

Biomarkers	1st Trimester	2nd Trimester	1st & 2nd Trimesters *p*-value[a]
Folate (nmol/L) [2]	66.9 (53.3; 81.8) *n*= 614	69.3 (55.5; 87.0) *n*= 627	0.01
Total B12 (pmol/L)	221 (160.0; 299.2) *n*= 624	203 (148; 272) *n*= 672	<0.0001
HoloTC (pmol/L)	83.8 (60.5; 114.9) *n*= 613	76.4 (54.9; 105.8) *n*= 595	<0.0001
MMA (nmol/L)	120 (100; 159) *n*= 613	114 (91.5; 137) *n*= 627	<0.0001
tHcy (µmol/L)	5.00 (4.45; 5.70) *n*= 571	4.38 (3.76; 5.13) *n*= 540	<0.0001

[1] Values presented as median (25th; 75th percentile), *n*.
Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.
tHcy, total homocysteine; holoTC, holotranscobalamin; MMA, methylmalonic acid.
[2] determined in non-fasting serum samples.
[a] Difference between trimesters, an asterisk indicates a significant difference in the biomarker concentrations between the trimesters with the use of Wilcoxon signed rank test.

Table 4-3 summarizes the prevalence of folate and B12 deficiency in the study sample and also the prevalence of those pregnant women who were classified as having high folate concentrations and deficient serum total B12 status simultaneously and also those classified as having a combination of high folate concentrations and deficient total B12 status. These categories were based on the biomarker cut-offs for non-pregnant adults, i.e., serum folate (<7 and >46 nmol/L) and total B12 (<148 and 148-221 pmol/L).

Folate status was not only adequate in all pregnant women in this study but considered high, with more than 80% of all women classified as having high folate status, indicated by serum folate >46 nmol/L in both trimesters. The prevalence of women classified as B12 deficient and with suboptimal status differed depending on the trimester. The prevalence of B12 deficiency, defined as having serum total B12 concentration < 148 pmol/L, was 15.8% and 18.7%, in the first and second trimester, respectively. Suboptimal B12 status prevalence, defined

as having serum total B12 concentration between 148-221 pmol/L, was 28.8% and 32.0%, in the first and second trimester, respectively. The prevalence of folate/B12 imbalance defined as having high folate status (i.e., serum folate >46 nmol/L) with B12 deficiency (i.e., total B12 <148 pmol/L) was 12.6% and 14.5% in the first and second trimester, respectively.

Table 4-3: Prevalence of Pregnant Women Classified as Folate Deficient or With High Folate Status, and as B12 Deficient or Having Suboptimal B12 Status, Using Non-Pregnant Adult Cut-Offs.[1]

Biomarkers	**Status**	**1st Trimester**	**2nd Trimester**
Folate[2] <7 nmol/L [a]	Folate deficient	0 (0)	0 (0)
7-14 nmol/L[b]	Folate suboptimal status	2 (0.3)	1 (0.15)
>46 nmol/L[c]	High folate status	517 (84.2)	543 (86.6)
Total B12 <148 pmol/L [d]	B12 deficient	99 (15.8)	126 (18.7)
148-221 pmol/L [e]	Suboptimal B12 status	180 (28.8)	216 (32.1)
Folate>46 nmol/L with total B12<148 pmol/L	High folate concentrations with B12 deficiency	79 (12.6)	96 (14.5)

[1] Values presented as *n* (%).
[2] Determined in non-fasting serum samples
References: [a] (Institute of Medicine 1998), [b] (World Health Organisation 2015), [c] (Pfeiffer et al. 2007), [d] (de Benoist 2008), [e](Molloy et al. 2009)

4.2 Association of Maternal Folate and Vitamin B12 Biomarkers with Birth Outcomes

This section states the results of the association of maternal folate and B12 biomarker concentrations in the first and second trimesters with birth outcomes, i.e., birthweight and gestational age at birth. Maternal folate and B12 biomarker concentrations as continuous variables were assessed using adjusted and unadjusted linear regression models. This section covers the first and second study objectives, which were: 1) to determine the association of maternal folate and B12 biomarker concentrations individually with birthweight and gestational age at birth, and 2) to investigate the association of maternal folate and B12 status combined with birthweight and gestational age at birth.

Birthweight

Adjusted linear regression analyses were conducted to determine the association of maternal folate and B12 biomarker concentrations with birthweight after adjustment for known confounding factors, including ethnicity, maternal age, infant sex and gestational age at birth, in the first and second trimesters of pregnancy. The results are presented in **Table 4-4**.

In the first trimester, maternal folate and total B12 showed a weak, inverse association with birthweight after adjustment for confounding factors (p= 0.042 and p= 0.021, respectively) (R^2=26% for both). The R^2 for the regression model of birthweight with only the confounding factors was 21%. This reveals that both folate and total B12 concentration, separately, explain additional 4% of the model variability for birthweight, after adjustment for the confounding factors, which slightly improves the fit of the model. However, 4% of the explained variability is a fairly small contribution, and most of the variability was explained by the confounding factors,

specifically ethnicity. Furthermore, maternal folate and total B12 were not significantly associated with birthweight after adjustment for confounding factors in the second trimester (p= 0.73 and p= 0.07, respectively).

There was a significant association between maternal holoTC concentration and birthweight in the second trimester (p= 0.042) after adjusting for confounding factors. Neither the folate to B12 ratio nor MMA or tHcy concentrations were associated with birthweight in either trimester, after adjustment for confounding factors. The interaction term for the association of folate and B12 combined with birthweight after the adjustment for confounding factors was also not significant (p= 0.26, and 0.55 for the first and second trimester, respectively). The unadjusted linear regression analyses that were conducted to determine the association between maternal folate and B12 biomarker concentrations and birthweight did not show any significant association in either trimester.

Table 4-4: Associations of Maternal Folate and B12 Biomarker Concentrations with Birthweight (in Grams) in 1st and 2nd Trimester[1]

Biomarkers		***b* (g)**	**95% CI**	**R^2**	***p*-value**
Confounding factors		n/a		0.22	
1st Trimester					
Folate (nmol/L)	unadjusted	-0.61	-1.96, 0.74	0.001	0.37
	adjusted[2]	-1.22	-2.41, -0.04	0.26	0.042*
Total B12 (pmol/L)	unadjusted	0.16	-0.17, 0.48	0.001	0.35
	adjusted[2]	-0.35	-0.64, -0.05	0.26	0.021*
Folate to B12	unadjusted	0.11	- 0.27, 0.06	0.003	0.20
	adjusted[2]	0.09	-0.064, 0.2	0.25	0.24
HoloTC (pmol/L)	unadjusted	0.53	-0.62, 1.69	0.001	0.36
	adjusted[3]	-0.77	-2.18, 0.63	0.26	0.28
MMA (nmol/L)	unadjusted	-0.33	-0.74, 0.086	0.004	0.12
	adjusted[2]	0.35	-0.06, 0.76	0.26	0.09
tHcy (µmol/L)	unadjusted	14.08	-21.33, 49.48	0.001	0.43
	adjusted[2]	11.84	-0.062,1.69	0.26	0.45
2nd Trimester					
Folate (nmol/L)	unadjusted	0.56	-0.84, 1.96	0.001	0.43
	adjusted[2]	-0.22	-1.53, 1.08	0.23	0.73
Total B12 (pmol/L)	unadjusted	0.20	-0.14, 0.54	0.002	0.25
	adjusted[2]	-0.29	-0.61, 0.03	0.023	0.07

Biomarkers		***b* (g)**	**95% CI**	**R^2**	***p*-value**
Folate to B12	unadjusted	0.032	-0.19, 0.13	0.0002	0.71
	adjusted[2]	0.11	-3.59, 0.27	0.28	0.13
HoloTC (pmol/L)	unadjusted	0.44	-0.77, 1.64	0.0008	0.48
	adjusted[3]	-1.39	-2.74, -0.047	0.25	0.042*
MMA (nmol/L)	unadjusted	-0.22	-0.64, 0.20	0.002	0.31
	adjusted[2]	0.15	-0.24, 0.54	0.23	0.45
tHcy (µmol/L)	unadjusted	-6.05	-38.4, 26.3	0.0003	0.71
	adjusted[2]	-16.0	-45.4, 13.4	0.20	0.28

Multiple linear regression analysis with birthweight as dependent variables, and folate, total B12, MMA, holoTC and homocysteine concentrations as independent variables.

Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine, g=grams.

Note: 95% CI; 95% confidence interval for *b*-coefficient

1 the association was tested using univariate and multivariate linear regressions

2 adjusted for: ethnicity, maternal age, infant sex, and gestational age at birth

3 adjusted for: ethnicity, maternal age, infant sex, gestational age at birth, and censored data.

* *p*-values were significant at the $p < 0.05$ level

Gestational Age at Birth

Adjusted and unadjusted linear regression analyses were conducted to determine the association between maternal folate and B12 biomarker concentrations and gestational age at birth after adjustment for confounding factors of gestational age at birth, including ethnicity, maternal age and infant sex, in the first and second trimesters of pregnancy. The results are presented in **Table 4-5**.

Maternal serum folate concentration and maternal folate to B12 ratio were not significantly associated with gestational age at birth in either the first or second trimester. The interaction term for the association of folate and B12 combined with gestational age at birth after the adjustment for confounding factors was also not significant (p= 0.9, and 0.8 for the first and second trimester, respectively). Maternal MMA concentrations in the first trimester showed a weak inverse association with gestational age at birth before and after adjusting for confounding factors (b=-0.002 wks, p= 0.009, and b=-0.002 wks, p= 0.036, respectively). In the second trimester, the unadjusted model showed that holoTC was significantly associated with gestational age at birth, but the effect size was small (b= 0.005, p= 0.029). The significant p-value was likely due to other confounding factors, which is explained by the non-significant association after the adjustment for confounding factors. After the adjustment for confounding factors, maternal total B12 and tHcy had weak, positive associations with gestational age at birth (b=0.0009 wks and p= 0.038, and b= 0.12 wks and p= 0.034, respectively).

Table 4-5: Association of Maternal Folate and B12 Biomarker Concentrations with Gestational Age at Birth (in Weeks) in 1st and 2nd Trimester[1]

Biomarkers		***b*** **(weeks)**	**95% CI**	***R²***	***p*-value**
Confounding factors		n/a		0.009	
1st Trimester					
Folate (nmol/L)	unadjusted	0.003	-0.002, 0.007	0.002	0.24
	adjusted[2]	0.002	-0.002, 0.007	0.014	0.33
Total B12 (pmol/L)	unadjusted	0.0001	-0.0001, 0.002	0.005	0.08
	adjusted[2]	0.0006	-0.0005, 0.002	0.012	0.28
Folate to B12	unadjusted	-0.0003	-0.0009, 0.0003	0.002	0.25
	adjusted[2]	-0.0002	- 0.0008, 0.0004	0.013	0.56
HoloTC (pmol/L)	unadjusted	0.004	-0.000007,0.008	0.015	0.52
	adjusted[3]	0.002	-0.004, 0.008	0.007	0.05
MMA (nmol/L)	unadjusted	-0.002	-0.004, -0.0005	0.012	0.009*
	adjusted[2]	-0.002	-0.003, -0.0001	0.018	0.036*
tHcy (µmol/L)	unadjusted	0.031	-0.091, 0.153	0.002	0.57
	adjusted[2]	0.035	-0.088, 0.16	0.0005	0.62
2nd Trimester					
Folate (nmol/L)	unadjusted	0.004	-0.001,0.009	0.004	0.12
	adjusted[2]	0.004	-0.0001,0.009	0.012	0.13
Total B12 (pmol/L)	unadjusted	0.001	0.00007, 0.003	0.007	0.17
	adjusted[2]	0.0009	0.0004,0.002	0.013	0.038*
Folate to B12	unadjusted	0.0001	-0.00007, 0.00006	0.00005	0.86
	adjusted[2]	0.00005	-0.0005, 0.00008	0.009	0.66

Biomarkers		***b*** **(weeks)**	**95% CI**	***R²***	***p*-value**
HoloTC (pmol/L)	unadjusted	0.005	0.0005, 0.009	0.008	0.029*
	adjusted[3]	0.004	-0.002, 0.009	0.018	0.19
MMA (nmol/L)	unadjusted	-0.002	-0.002, 0.001	0.00006	0.84
	adjusted[2]	0.0002	-0.001,0.002	0.010	0.82
tHcy (µmol/L)	unadjusted	0.13	0.013, 0.24	0.009	0.029*
	adjusted[2]	0.12	0.009, 0.24	0.018	0.034*

Multiple linear regression analysis with gestational age at birth as dependent variables, and folate, total B12, MMA, holoTC and tHcy concentrations as independent variables.

Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.

Note: 95% CI; 95% confidence interval for *b*-coefficient

1 the association was tested using univariate and multivariate linear regressions

2 adjusted for: ethnicity, maternal age and infant sex.

3 adjusted for: ethnicity, maternal age, infant sex, gestational age at birth, and censored data.

* *p*-values were significant at the $p < 0.05$ level

4.2.1 Association of Maternal Folate and B12 Biomarker Concentrations in Quartiles with Birth Outcomes

This subsection presents the results of maternal folate and B12 biomarker concentrations as quartiles (categorical variables), and their association with birthweight and gestational age at birth in the first and second trimester. It also includes maternal folate and B12 status combined (in the form of the ratio of serum folate/total B12 concentration) as quartiles (categorical variables) and their association with birthweight and gestational age at birth in the first and second trimester. The quartile groups were conducted by using first quartile (25^{th} percentile), median and third quartile (75^{th} percentile), of maternal folate and B12 biomarker concentrations. The first quartile was considered the reference group for the maternal folate and B12 biomarker concentrations in these analyses. The associations between maternal folate and B12 biomarker concentrations were calculated using adjusted linear regression analyses.

Birthweight

The association of maternal folate and B12 biomarker concentration quartiles with birthweight in the first and second trimesters assessed by multiple linear regression analyses, is summarized in **Table 4-6.** The analyses were completed by controlling for the confounding factors for birthweight, including ethnicity, maternal age, infant sex and gestational age at birth.

Maternal folate concentration in the first trimester was inversely associated with birthweight (*b*=-101.8 g, *p*= 0.03) when comparing the third quartile (66.9-81.8 nmol/L) with the reference group (≤53.3nmol/L), which is consistent with the continuous folate concentration analysis. Maternal tHcy in the first trimester was inversely associated with birthweight (*b*=-109.6 g, *p*= 0.02) when comparing the third quartile (5 – 5.7 µmol/L) with the reference group (≤5.7

μmol/L). Regarding vitamin B12 biomarker concentrations, there was a significant inverse association between birthweight and total B12 in the second quartile compared with the reference group. Maternal total B12 concentrations in the second quartile (Q2 =160-221 pmol/L and 148-221 pmol/L in the first and second trimester, respectively) was associated with a 103.9 and 103.5 g lower birthweight compared to the reference group (Q1 ≤160 pmol/L and ≤148 pmol/L in the first and second trimester, respectively) ($p< 0.05$). In the second trimester, maternal total B12 concentrations in the fourth quartile (Q4 ≥299 pmol/L) was associated with a 94.5 g lower birthweight compared to the reference group (Q1 ≤148 pmol/L) ($p= 0.045$). Maternal MMA and holoTC in quartile analysis were not associated with birthweight in either trimester. In conclusion, maternal serum folate and total B12 concentrations were inversely associated with birthweight in this study.

Table 4-6: Association of Maternal Folate and B12 Biomarker Concentration in Quartiles with Birthweight (in Grams) in 1st and 2nd Trimester[1]

Biomarkers	**Birthweight [1]**				**Biomarkers**	**Birthweight [1]**			
1st Trimester	**No. of subjects**	***b* (g)**	**(95%CI)**	***p*-value**	**2nd Trimester**	**No. of subjects**	***b* (g)**	**(95% CI)**	***p*-value**
Folate (nmol/L)					**Folate (nmol/L)**				
Q1 (≤53.3)	157	Reference			**Q1 (≤55.5)**	157	Reference		
Q2 (53.3-66.9)	152	14.41	-77.0, 105.8	0.75	**Q2 (55.5-69.3)**	158	-25.9	-120.4, 68.61	0.59
Q3 (66.9-81.8)	154	-101.8	-192.5, -11.1	0.03*	**Q3 (69.3-87.8)**	160	-25.9	-199.4, 67.61	0.58
Q4 (≥81.8)	154	-74.76	-165.5, 16.0	0.11	**Q4 (≥87.8)**	152	4.14	-89.97, 98.25	0.93
Total B12 (pmol/L)					**Total B12 (pmol/L)**				
Q1 (≤160)	153	Reference			**Q1 (≤148)**	168	Reference		
Q2(160-221)	156	-103.9	-195.8, -12.02	0.01*	**Q2 (148-221)**	169	-103.5	-194.6, -12.34	0.03*
Q3 (221-299)	159	-61.43	-154.3, -31.93	0.03*	**Q3 (221-299)**	166	-42.94	-135.1, 49.22	0.36
Q4 (≥299)	156	-128.3	-221.6, -34.92	0.19	**Q4 (≥299)**	170	-94.50	-186.7, -2.28	0.04*
Folate to B12					**Folate to B12**				
Q1 (≤199)	152	Reference			**Q1 (≤233)**	157	Reference		
Q2 (199-295)	152	-41.74	-133.0, 49.47	0.36	**Q2 (233-341)**	156	35.90	-57.28, 129.1	0.45
Q3 (295-441)	151	-55.01	-147.0, 37.01	0.24	**Q3 (341-491)**	156	36.82	-57.28, 130.8	0.44

Biomarkers		Birthweight [1]			Biomarkers	Birthweight [1]			
1st Trimester	**No. of subjects**	***b* (g)**	**(95%CI)**	***p*-value**	**2nd Trimester**	**No. of subjects**	***b* (g)**	**(95% CI)**	***p*-value**
Q4 (≥441)	152	13.75	-79.1, 107.3	0.77	**Q4 (≥491)**	156	71.01	-24.26, 166.3	0.14
HoloTC (pmol/L)					**HoloTC (pmol/L)**				
Q1 (≤60.5)	153	Reference			**Q1 (≤54.9)**	148	Reference		
Q2 (60.5-83.8)	152	-78.72	-171.6, 14.16	0.10	**Q2 (54.9-76.4)**	149	-40.55	-135.4, 54.26	0.40
Q3 (83.8-115)	154	-50.11	-141.4, 41.13	0.28	**Q3 (76.4-106)**	148	-56.76	-152.1, 38.54	0.24
Q4 (≥115)	154	-89.77	-182.3, 2.76	0.06	**Q4 (≥106)**	150	-77.12	-172.7, 18.43	0.11
MMA (nmol/L)					**MMA (nmol/L)**				
Q1 (≥159)	155	Reference			**Q1 (≥152)**	158	Reference		
Q2 (120-159)	153	-72.6	-166, 20.8	0.12	**Q2 (114-152)**	157	34.51	-9.09, 175.6	0.46
Q3 (100-120)	154	-28.4	-121.1, 64.3	0.54	**Q3 (91.5-114)**	156	46.17	-21.00, 164.2	0.33
Q4 (≤100)	151	-60.91	-154.3, 32.5,	0.20	**Q4 (≤91.5)**	156	-37.10	-55.54, 129.8	0.43
tHcy (µmol/L)					**tHcy (µmol/L)**				
Q1 (≥5.7)	142	Reference			**Q1 (≥5.15)**	133	Reference		
Q2 (5.0-5.7)	148	-109.6	-203.4, 15.81	0.02*	**Q2 (4.43-5.15)**	128	7.59	-91.47, 106.65	0.88

Biomarkers			Birthweight [1]		Biomarkers	Birthweight [1]			
1st Trimester	**No. of subjects**	***b* (g)**	**(95%CI)**	***p*-value**	**2nd Trimester**	**No. of subjects**	***b* (g)**	**(95% CI)**	***p*-value**
Q3 (4.40-5.0)	145	-83.4	-178.6, 11.72	0.08	**Q3 (3.68-4.43)**	159	40.66	-54.4, 135.8	0.40
Q4 (≤4.40)	136	-69.9	-166.6, 26.64	0.16	**Q4 (≤3.68)**	122	93.81	-8.11, 195.7	0.07

Multiple linear regression analysis with birthweight as dependent variables and, folate, total B12, MMA, holoTC and homocysteine quartiles in the first and second trimesters as independent variables. Q1 through Q4 represents the quartile distribution of the relative concentrations. Q1 is the reference value.
Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.
1 adjusted for ethnicity, maternal age, and infant sex.
* *p*-values were significant at the $p < 0.05$ level

Gestational Age at Birth:

The association of maternal folate and B12 biomarker concentration quartiles with gestational age at birth in the first and second trimester, assessed by multiple linear regression analyses, is summarized in **Table 4-7**. The analyses were done by controlling for confounding factors that affect gestational age at birth, including ethnicity, maternal age and infant sex. The first quartile was considered the reference group for the maternal folate and B12 biomarker concentration quartiles in these analyses.

In the first trimester, maternal folate and B12 biomarker concentrations did not show any significant associations with gestational age at birth. In addition, gestational age at birth did not differ significantly between mothers with different quartiles of folate to B12 ratio in either trimester.

In the second trimester, maternal total B12 concentration (Q3=220-299 nmol/L) was associated with higher gestational age at birth compared to the reference group (Q1≤148 nmol/L) (b=0.4 wk, p= 0.03). There was a 3-day increase in gestational age at birth for mothers in the third quartile of total B12 concentration compared to mothers in the lowest quartile for maternal total B12 concentration. There was a significant positive association between maternal folate and gestational age at birth in the second trimester. This association was found in the second, third and fourth quartile group compared to the reference group (p= 0.005, 0.001 and 0.005 respectively). Mothers in the second quartile (i.e., folate concentration between 55.5-69.3 nmol/L) had a 4.5-day increase in gestational age at birth compared to the mothers in the lowest quartile (i.e., folate concentration ≤55.5 nmol/L). Moreover, mothers in the third quartile (folate concentration between 69.3-87.8 nmol/L) had a 4-day increase in gestational age at birth compared to the mothers in the lowest quartile (folate concentration ≤55.5 pmol/L). Mothers in

the fourth quartile (i.e., folate concentration ≥87.8 nmol/L) had a 4-day increase in gestational age at birth compared to the mothers in the lowest quartile (i.e., folate concentration ≤55.5 nmol/L).

There was a significant positive association between maternal tHcy and gestational age at birth in the second trimester. This positive association was found in the lowest quartile group (Q4≤3.68 μmol/L) compared to the reference group (Q1≥5.15 μmol/L)(*b*=0.45 wk, *p*=0.02 respectively). However, this association was weak with a difference of a 3-day. In sum, maternal serum folate and total B12 concentrations had a very weak positive association with gestational age at birth in this study.

Table 4-7: Association of Maternal Folate and B12 Biomarker Concentrations in Quartiles with Gestational Age at Birth (in Weeks) in 1st and 2nd Trimester[1]

Biomarkers	**Gestational age at birth[1]**				**Biomarkers**	**Gestational age at birth[1]**			
1st Trimester	**No. of subjects**	***b*** **(weeks)**	**(95% CI)**	***p*-value**	**2nd Trimester**	**No. of subjects**	***b*** **(weeks)**	**(95% CI)**	***p*-value**
Folate (nmol/L)					**Folate (nmol/L)**				
Q1 (≤53.3)	151	Reference			**Q1 (≤55.5)**	152	Reference		
Q2 (53.3-66.9)	141	0.09	-0.27, 0.45	0.63	**Q2 (55.5-69.3)**	146	0.65	0.28, 1.02	0.0005*
Q3 (66.9-81.8)	147	0.27	-0.09, 0.62	0.14	**Q3 (69.3-87.8)**	153	0.59	0.23, 0.95	0.001*
Q4 (≥81.8)	148	-0.03	-0.38, 0.33	0.88	**Q4 (≥87.8)**	147	0.52	0.16, 0.89	0.005*
Total B12 (pmol/L)					**Total B12 (pmol/L)**				
Q1 (≤160)	147	Reference			**Q1 (≤148)**	158	Reference		
Q2 (160-221)	144	0.30	-0.67, 0.06	0.10	**Q2 (148-221)**	157	0.16	-0.20, 0.52	0.39
Q3 (221-299)	148	0.12	-0.25, 0.49	0.53	**Q3 (221-299)**	161	0.40	0.04, 0.77	0.03*
Q4 (≥299)	151	0.05	-0.32, 0.42	0.80	**Q4 (≥299)**	165	0.27	-0.09, 0.64	0.15
Folate to B12					**Folate to B12**				
Q1 (≤199)	149	Reference			**Q1 (≤233)**	154	Reference		
Q2 (199-295)	146	-0.06	-0.42, 0.30	0.75	**Q2 (233-341)**	148	-0.07	-0.44, 0.30	0.72
Q3 (295-441)	143	-0.01	-0.38, 0.35	0.94	**Q3 (341-491)**	147	-0.01	-0.39, 0.36	0.95
Q4 (≥441)	144	-0.07	-0.44, 0.30	0.72	**Q4 (≥491)**	147	-0.02	-0.40, 0.36	0.90

Biomarkers	Gestational age at birth[1]				Biomarkers	Gestational age at birth[1]			
1st Trimester	**No. of subjects**	***b*** **(weeks)**	**(95% CI)**	***p*-value**	**2nd Trimester**	**No. of subjects**	***b*** **(weeks)**	**(95% CI)**	***p*-value**
HoloTC (pmol/L)					**HoloTC (pmol/L)**				
Q1 (≤60.5)	149				**Q1 (≤54.9)**	141	Reference		
Q2 (60.5-83.8)	142	-0.19	-0.27, 0.54	0.31	**Q2 (54.9-76.4)**	141	-0.09	-0.46, 0.28	0.62
Q3 (83.8-115)	147	-0.04	-0.24, 0.58	0.85	**Q3 (76.4-106)**	140	0.002	-0.37, 0.38	0.98
Q4 (≥115)	144	0.23	-0.28, 0.53	0.22	**Q4 (≥106)**	142	0.33	-0.04, 0.70	0.08
MMA (nmol/L)					**MMA(nmol/)**				
Q1 (≥159)	147	Reference			**Q1 (≤91.5)**	152	Reference		
Q2 (120-159)	141	0.27	-0.09, 0.64	0.13	**Q2 (91.5-114)**	149	-0.23	-0.6, 0.15	0.23
Q3 (100-120)	147	0.28	-0.08, 0.65	0.12	**Q3 (114-152)**	148	-0.05	-0.42, 0.32	0.78
Q4 (≤100)	146	0.36	-0.004, 0.73	0.05	**Q4 (≥152)**	148	-0.02	-0.35, 0.39	0.90
tHcy (µmol/L)					**tHcy (µmol/L)**				
Q1 (≥5.7)	137	Reference			**Q1 (≥5.15)**	132		Reference	
Q2 (5.0-5.7)	144	-0.12	-0.49, 0.25	0.52	**Q2 (4.43-5.15)**	125	0.15	-0.23, 0.54	0.43
Q3 (4.4-5.0)	139	-0.29	-0.66, 0.078	0.12	**Q3 (3.68-4.43)**	150	-0.08	-0.45, 0.30	0.68
Q4 (≤4.4)	128	-0.023	-0.36, 0.40	0.90	**Q4 (≤3.68)**	117	0.45	-0.85, -0.05	0.026*

Multiple linear regression analysis with birthweight and gestational age at birth as dependent variables and, folate, total B12, MMA, holoTC and homocysteine quartiles in the second trimester as independent variables. Q1 through Q4 represents the quartile distribution of the relative concentrations. Q1 is the reference value.

Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.

1 adjusted for ethnicity, maternal age, and infant sex.

2 adjusted for ethnicity, maternal age, infant sex and, gestational age at birth

* *p*-values were significant at the $p < 0.05$ level

4.3 Association of Maternal Folate and Vitamin B12 Changes During Early Pregnancy with Birthweight and Gestational Age at Birth

Birthweight

Adjusted linear regression analyses to determine the change in birthweight as a result of the change in maternal folate and B12 biomarker concentrations between the first and second trimester are summarized in **Table 4-8.** The analyses were done after controlling for known confounding factors that affect birthweight. These included gestational age at birth, ethnicity, maternal age and infant sex. There was no significant association found.

Table 4-8: Association of Maternal Folate and B12 Biomarker Concentration Change Between the 1st and 2nd Trimester with Birthweight (in Grams) [1]

Biomarkers [2]	***b* (g)**	**95% CI**	**R^2**	***p*-value**
Folate (nmol/L per week)	3.49	-1.370, 8.353	0.26	0.16
Total B12 (pmol/L per week)	0.67	-0.980, 2.318	0.25	0.43
Folate to B12	0.054	-0.767, 0.875	0.25	0.89
HoloTC (pmol/L per week)	-2.36	-6.026, 1.315	0.24	0.21
MMA (nmol/L per week)	0.331	-1.080, 1.741	0.23	0.65
tHcy (µmol/L per week)	-18.9	-158.2, 120.4	0.23	0.79

Multiple linear regression analysis with birthweight as dependent variables, and folate, total B12, MMA, holoTC and homocysteine concentrations difference between the first and second trimesters as independent variables.
Note: 95% CI; 95% confidence interval for *b*-coefficient.
Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine, g=grams.
[1]the association was tested using Multiple linear regressions after adjustment for: ethnicity, maternal age, infant sex and gestational age at birth.
[2] units are expressed as change in biomarker concentration per gestational week, calculated as

$$\frac{\text{biomarker concentration (T2)} - \text{biomarker concentration (T1)}}{\text{gestational age at blood collection (T2)} - \text{gestational age at blood collection (T1)}}$$

Gestational Age at Birth

Adjusted linear regression analyses that were conducted to determine the change in gestational age at birth as a result of the change in concentration of maternal folate and B12 biomarker concentrations between the first and second trimester are summarized in **Table 4-9.** The analyses were completed after controlling the confounding factors for gestational age at

birth, including ethnicity, maternal age and infant sex. There was no significant association found.

Table 4-9: Association of Maternal Folate and B12 Biomarker Concentrations Changes Between the 1st and 2nd Trimester with Gestational Age at Birth (in Weeks) [1]

Biomarkers [2]	***b***	**95% CI**	**R^2**	***p*-value**
Folate (nmol/L per week)	0.00105	-0.0182, 0.0204	0.013	0.91
Total B12 (pmol/L per week)	0.00112	-0.0054, 0.0076	0.011	0.74
Folate to B12	0.00187	-0.0014, 0.0051	0.014	0.26
HoloTC (pmol/L per week)	0.01419	-0.0004, 0.0287	0.020	0.06
MMA (nmol/L per week)	0.00107	-0.0046, 0.0067	0.011	0.71
tHcy (µmol/L per week)	0.19817	-0.3369, 0.7333	0.013	0.47

Multiple linear regression analysis with gestational age at birth as dependent variables, and folate, total B12, MMA, holoTC, and homocysteine concentrations difference between the first and second trimesters as independent variables.

Abbreviations: holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.

Note: 95% CI; 95% confidence interval for *b*-coefficient

[1] the association was tested using Multiple linear regression after the adjustment for ethnicity, maternal age and infant sex.

[2] units are expressed as change in biomarker concentration per gestational week, calculated as

$$\frac{\text{biomarker concentration (T2)} - \text{biomarker concentration (T1)}}{\text{gestational age at blood collection (T2)} - \text{gestational age at blood collection (T1)}}$$

Chapter 5: Discussion

This chapter will begin with a summary, followed by the discussion of the findings including **[1]** the characteristics of the study sample and the biomarker concentrations in the first and second trimester of pregnancy, **[2]** the association of maternal folate and B12 biomarker concentrations as continuous and categorical variables with birthweight and gestational age at birth, and **[3]** the association of the change in folate and B12 concentration between the trimesters with birthweight and gestational age at birth. Thereafter, I provide **[4]** an elaboration of the study's strengths and limitations, **[5]** an outline of suggestions for future research to improve the current knowledge in this topic, and **[6]** conclusions of this research.

Summary:

Given that the data on the association between maternal folate and B12 biomarker concentrations in early pregnancy and birth outcomes are limited, I conducted a secondary analysis on a sample of 674 pregnant women of South Asian and European ethnicity residing in Lower Mainland, British Columbia. This retrospective cohort study evaluated the association of maternal serum folate and B12 biomarker concentrations, combined and individually, with the birth outcomes of birthweight and gestational age at birth. Additionally, the association of the change in maternal folate and B12 biomarker concentrations between first [median gestational week: 11.4 (IQR: 10.6-12.3)] and second trimester [16.1 (IQR: 15.1-17.1)] of pregnancy with birthweight and gestational age at birth was also investigated.

Following the ethnic-specific growth charts, only 0.88% (n=5) and 2.84% (n=16) of newborns were SGA and LGA, respectively. The prevalence of imbalance between maternal folate (>46 nmol/L) and B12 (148-221 pmol/L) was 23% and 25% in the first and second trimesters, respectively. The results of this thesis show that serum folate and B12 biomarker

concentrations in early pregnancy were not associated with birthweight in this study. The imbalance in serum folate and B12 concentrations was not associated with birthweight and gestational age at birth, in either trimester. In the second trimester, the second, third and fourth quartiles of maternal folate concentration (Q2=55.5-69.3, Q3= 69.3-87.8, Q4≥87.8 nmol/L) were associated with an approximate 4-day increase in gestational age at birth, compared to the first quartile (Q1≤55.5 nmol/L). The change in maternal folate and B12 biomarker concentrations between first and second trimester of pregnancy was not associated with birthweight and gestational age at birth. The findings of this current study enhance the information currently available on folate and B12 status in Canadian pregnant women, and is, to my knowledge, the first to look at the association of folate and B12 status with birth outcomes in Canadian pregnant women.

5.1 Characteristics of the Study Sample and Biomarker Concentrations in First and Second Trimester of Pregnancy

The study sample consisted of apparently healthy mother-newborn dyads residing in British Columbia, with maternal European or South Asian ethnicity. This sample most likely comprises of a healthy population, in light of the low prevalence of low birthweight (<2500g; 1.93%), preterm (<37 weeks of gestation; 8.9%), and SGA (0.88%) and also the limited variation in the folate and B12 biomarker concentrations.

The prevalence of SGA births in Canada was 8.7% in 2010–2012 according to national data by Statistics Canada (CANSIM 2012). The prevalence of SGA in this study sample was much lower with 0.88% of SGA newborns. The disparity in the prevalence might be due to the difference in the growth charts used to classify newborns with SGA. This study has used ethnic-

specific growth charts from a study conducted in BC, Canada (Janssen et al. 2007), while the Canadian data were categorized using Australian growth charts (CANSIM 2012). With respect to the differences in fetal growth between ethnic groups, using ethnic-specific growth charts was found to be a better indicator for the risk of short-term neonatal morbidity (Hanley & Janssen 2013). Studies suggested that infertility and infertility treatment such as *in vitro* fertilization may be risk factors for intrauterine growth restriction and thereby contribute to the risk of low birth weight and SGA (Kalra et al. 2011; Zhu et al. 2007). Because assisted conception and/or pregnancy following *in vitro* fertilization or intracytoplasmic sperm injection was an exclusion criterion for this study, the exclusion of specimen and data of women potentially at higher risk of SGA may also explain the disparity in the SGA prevalence between this study and the Canadian national data. The SGA prevalence of 0.88% in this study sample was much lower compared to that reported by previous studies investigating the association between maternal folate and/or B12 status with birth outcomes including a study conducted in the Netherlands reporting an SGA prevalence of 5% (Bergen et al. 2012) and two in India with SGA prevalences of 28% and 30% (Muthayya et al. 2006; Dwarkanath et al. 2013). According to my knowledge, none of the studies that investigated the association of maternal folate and B12 status with birth outcomes had an SGA prevalence similar to this study.

The variation in maternal folate and total B12 concentration in this study sample was limited. First- and second-trimester median (IQR) serum folate concentrations were 66.9 (53.3; 81.8) and 69.3 (55.5; 87.0) nmol/L, respectively, and those of total B12 were 221 (160; 299) and 202 (148; 272) pmol/L, respectively. The APrON study, a prospective pregnancy cohort study conducted in Alberta, reported median (95% CI) maternal plasma folate concentration of 36 (35, 36) nmol/L in the first and second trimester (Fayyaz et al. 2014). The PREFORM study, a prospective cohort

study conducted in Toronto, reported mean (95% CI) serum folate concentration of 51 (49, 54) nmol/L in pregnant women at 12–16 weeks of gestation. The PREFORM study reported similar total B12 concentration [mean: 219 (95% CI: 210, 229) pmol/L] (Visentin et al. 2016) compared to this study.

In contrast, maternal plasma or serum folate concentration of pregnant women in studies investigating the association between maternal folate and/or B12 with birth outcome conducted in Singapore (Chen et al. 2015) and India (Yajnik et al. 2014) was about half that of this study. The higher circulating folate concentration in this study may be partially explained by the staple food fortification with folic acid and the high prevalence and long duration of prenatal supplement use in Canada compared to other countries. The folate concentration of pregnant women in this study and a study conducted in the United States in 285 pregnant women at 18 and 30 weeks of gestation (Pagan et al. 2002) were similar, which may be explained by the prevalent use of prenatal folic acid supplements and the mandatory folic acid food fortification policy in both Canada and the United States (Crider et al. 2011).

In contrast to large difference in observed folate concentrations across studies, maternal serum total B12 concentration in this sample [median (IQR) 221 (160.0; 299.2) and 203 (148; 272) pmol/L, in the first and second trimesters, respectively] was somewhat comparable to those reported in studies conducted in the Netherlands [median: 169 (IQR: 98–298) pmol/L, in the first trimester] and Australia [mean: 239 (95% CI: 215, 265) pmol/L, in the second trimester] (Bergen et al. 2012; Furness et al. 2013), but higher compared to the results of a pregnancy study conducted in India [median (IQR) 122 (94, 164), 162 (123, 223) pmol/L, in the second and third trimesters, respectively] (Yajnik et al. 2014).

The results show that maternal folate and B12 biomarker concentrations changed during pregnancy. This study found that there was a small but significant increase in serum folate concentration between first and second trimester of pregnancy. In contrast, the results of a study in the Netherlands by Bruinse and van den Berg (1995) found that serum folate concentration significantly decreased during pregnancy (Bruinse & van den Berg 1995). The difference between the studies might be explained by the mandatory folic acid fortification policy and the high prevalence of prenatal supplement use in all trimesters of pregnancy in Canadian pregnant women (Masih et al. 2015). Serum tHcy concentration was slightly lower in the second trimester compared to the first trimester in this study (median difference = 0.62 µmol/L $p< 0.0001$), which is similar to the findings of a Dutch study as reported by Cikot et al (Cikot et al. 2001). In this study, serum total B12 and holoTC concentrations were significantly lower in the second trimester compared to the first trimester, which is in line with the decreasing circulating B12 concentrations across trimesters as reported by other pregnancy cohort studies (Murphy et al. 2007; Green et al. 2017).

The prevalence of pregnant women in this sample classified as folate deficient or with high serum folate concentration, and as B12 deficient or with suboptimal B12 status using non-pregnant adult biomarker cut-offs, was similar to previous studies conducted in Canada (Jeruszka-Bielak et al. 2017; Wu et al. 2013; Visentin et al. 2016). This study found that there is a high prevalence of suboptimal B12 status (serum total 148-221 pmol/L) among pregnant women in Canada: 29% and 32% in the first and second trimester, respectively. This is in agreement with some previous studies in Canada (Visentin et al. 2016; Wen et al. 2016; Jeruszka-Bielak et al. 2017). According to a Canadian population-based survey, the prevalence was 5% of B12 deficiency (148 pmol/L) and 19.7% of suboptimal deficiency (148–220 pmol/L)

in Canadian adults (MacFarlane et al. 2011). The decreasing serum total B12 concentration during pregnancy has been described before (Green et al. 1975; Cikot et al. 2001; Bruinse & van den Berg 1995; Murphy et al. 2007) and may be due to pregnancy-related physiologic changes, including hemodilution, and the increasing placental transport of B12 to the developing fetus. Canada's largest ethnic minority group is South Asian (Statistics Canada 2013) and South Asians are at higher risk of B12 deficiency (Jeruszka-Bielak et al. 2017). In this study, half of the pregnant women were of South Asian ethnicity and were shown to have lower B12 status during early pregnancy (Schroder et al. 2017).

There were no women classified as folate deficient (<7 nmol/L) in this study, which is in line with several previous studies in Canada (Fayyaz et al. 2014; Wu et al. 2013; Plumptre et al. 2015). On the other hand, the prevalence of high serum folate concentration (>46 nmol/L) in this study was more than 80% in the first and second trimesters. A high prevalence of high circulating folate concentration has also been observed in previous pregnancy cohort studies in Canada (Fayyaz et al. 2014; Plumptre et al. 2015). Almost 50% of pregnant women had high RBC folate concentration (>1360 nmol/L) throughout pregnancy (Fayyaz et al. 2014), and 97% had unmetabolized folic acid (≥0.2 nmol/L) in the early pregnancy period (Plumptre et al. 2015). Unmetabolized folic acid in the systemic circulation can occur when consuming more than 200 µg of folic acid (Furness et al., 2013; Kelly,1997). From this study and other studies in Canada, it seems that pregnant women in Canada have circulating folate concentrations that are in a range that is generally considered to be very high.

B12 plays a key role in maintaining normal folate metabolism, and their interdependent function is important for embryonic and fetal development (Molloy et al. 2014). The imbalance between maternal folate and B12 concentration may be of concern, because observational studies

showed an association between high folate combined with low B12 status and potential adverse outcomes, such as insulin resistance and low birthweight, in offspring of mothers with folate-B12 imbalance during pregnancy compared to those of mothers with either high folate or low B12 status individually (Yajnik et al. 2008; Gadgil et al. 2014). Maternal folate/B12 imbalance, defined as high folate and low B12 concentrations, during late gestation has been associated with a higher risk of insulin resistance in children at 6 years of age (Yajnik et al., 2008), and was inversely associated with neonatal anthropometrics, including birthweight, birth length, head circumference and chest circumference (Gadgil et al. 2014). The potential underlying mechanism for the association between high folate/low B12 imbalance with reduced fetal growth is not known. The hypothesised mechanism may be that high folate and low B12 may lower methionine synthase activity, which could affect homocysteine remethylation and further DNA methylation, and thereby potentially impact fetal growth (Smith et al. 2008). This study found that 12.6% and 14.5% of the women in the first and second trimester, respectively, had an imbalance between folate and B12, defined as having both high serum folate concentration (>46 nmol/L) and deficient levels of total B12 (<148 pmol/L). The high prevalence may be partially explained by the prevalent use of prenatal supplements in Canadian pregnant women and the high dose of folic acid in prenatal supplements, with a median supplemental folic acid intake of 1000 μg/day (Masih et al. 2015).

5.2 Association of Maternal Folate and Vitamin B12 Biomarker Concentrations with Birth Outcomes

Birthweight

An inverse, weak association of maternal folate concentration around 11 weeks of gestation with birthweight was found, after controlling for the available confounding factors. Every 10-nmol/L increase in maternal folate concentration was associated with a 12-g decrease in birthweight. The model explained 25.6% (R^2) of the variation in birthweight. The R^2 for the regression model of birthweight with only the confounding factors was 21%. It reveals that the contribution of folate to the model variability after adjustment for the confounding factors is 4% for birthweight, which slightly improves the fit of the model. However, 4% of the explained variability is a fairly small contribution, and most of the variability was explained by the confounding factors, and especially by ethnicity.

Similarly, maternal folate was associated with a 102-g decrease in birthweight, when comparing the third quartile (66.9-81.8 nmol/L) with the reference category (≤53.3nmol/L). However, maternal folate was not associated with birthweight when comparing the fourth quartile (≥81.8 nmol/L) with the reference category. This might be due to the small coefficient (*b*= -74.76), or the large variation in the 95% CI (-165.5, 16.0). There was no association of maternal serum folate concentration in the second trimester with birthweight. The findings of an association between maternal serum folate concentration in the first but not the second trimester and birth weight may be partially explained by the stages of fetal development in these trimesters; most of the rapid fetal development occurs in the early stages of pregnancy (Graham et al. 2015).

The inverse association between maternal folate concentrations and birthweight may be confounded, given that this study did not adjust for maternal weight which is an important confounding factor for birthweight (Abrams & Selvin 1995; Yu et al. 2013). However, limited previous studies have found an association between maternal high folate and low birthweight (Achón et al. 1999; Pickell et al. 2011; Van Uitert et al. 2014). The Cochrane Review by Lassi et al. (2013) stated that based on findings of 31 trials, there is no conclusive evidence of the association between folic acid supplementation during pregnancy and pregnancy outcomes such as preterm birth and low birthweight (Lassi et al. 2013).

One of the hypothesized mechanism for a potential adverse effect of high folate concentration and/or high folic acid intake is the appearance of unmetabolized folic acid in the plasma, at folic acid intake levels above 200µg, which may affect normal folate metabolism (Kelly et al. 1997; Obeid et al. 2010; Plumptre et al. 2015). In regards to folate status, this study is in line with a study conducted in Canadian pregnant women (Fayyaz et al. 2014), which found a high prevalence of women with high folate status (defined as having RBC folate >1360 nmol/L (Pfeiffer et al. 2007)). There is a potential concern of an excessive folic acid intake in Canadian women of reproductive age and pregnant women due to the high folic acid content in prenatal supplements (Mudryj et al. 2016); however, further research is required to study the association between high folic acid intake and birth outcomes.

Studies that found a positive association between maternal folate status in early pregnancy and birthweight differ in a few aspects compared to this study. A UK-based study conducted in 2005, using RBC folate as the folate status indicator, found that maternal RBC folate concentration near 11 weeks of gestation is positively associated with birthweight (b=0.25; 95% CI: 0.08, 0.42; p= 0.005). In addition to using RBC and not serum folate as a biomarker,

maternal RBC folate concentrations were within the normal ranges (160–640 ng/ml) and not considered 'high' (Relton 2005). A more recent study conducted in The Netherlands in 2012 also found a positive association between maternal serum folate and birthweight (Bergen et al. 2012). The maternal first-trimester serum folate concentration was much lower (median: 15.8, 90% range: 7.3–30.6 nmol/l) compared to that of this study sample (median: 66.9, IQR: 53.3; 81.8 nmol/l) (Bergen et al. 2012). The difference in folate concentrations between this study sample and those of the women in the UK and The Netherlands is likely because the latter two countries do not have mandatory folic acid fortification, unlike Canada that has staple food fortification with folic acid, and prenatal supplement use is not recommended throughout pregnancy but only until the end of the first trimester.

Regarding maternal B12 biomarker concentrations and their association with birthweight, first-trimester total B12 concentration was inversely associated with birthweight, when analyzed as a continuous variable (b= -0.346; 95% CI: -0.642, -0.052; p= 0.021). By taking into account nonlinearities in the association between maternal total B12 and birthweight, having total B12 concentration in the second or third quartile of total B12 (compared to the first and lowest quartile) was associated with having a newborn with lower birthweight. In the second trimester, the association was only significant in the categorical analysis between the second and fourth quartile (with the first, i.e., lowest quartile as the reference category), but not in the continuous analysis. This is in agreement with the meta-analysis of results from cohort studies mostly conducted in the second or third trimester, as there was no linear association between maternal total B12 concentration and birthweight (Rogne et al. 2017).

Maternal holoTC concentration (as a continuous variable) in the second trimester was inversely associated with birthweight (b= -1.39g; 95% CI: -2.74, -0.047; p= 0.042); but not

holoTC concentration in the first trimester. These weak inverse associations of maternal B12 biomarker concentrations, specifically total B12 and holoTC, with birthweight might be confounded by an uncontrolled factor, such as maternal weight. To note, maternal MMA concentration in the first and second trimester were not associated with birthweight.

Most of the previous studies investigating maternal total B12 concentration and birthweight did not find a significant association (Relton 2005; Bergen et al. 2012; Furness et al. 2013; Krishnaveni et al. 2014; Hogeveen et al. 2010; Halicioglu et al. 2012; McCullough et al. 2016; Pagan et al. 2002). Latter studies mostly used total B12 as a biomarker, except for Hogeveen et al. (2010), who used MMA in addition to total B12. In line with my findings, the study by Hogeveen et al. (2010) did not find an association between maternal MMA concentration and birthweight. Although I used many biomarkers for B12, including total B12, holoTC, and MMA, I missed some important confounding factors, such as maternal weight.

Due to folate and B12 interrelationship in one-carbon metabolism, B12 deficiency can result in folate being trapped as 5-methyltetrahydrofolate, inhibiting the remethylation of methionine from homocysteine. The imbalance between maternal folate and B12 concentration was not associated with birthweight. This null association was present in both the continuous and categorical analyses. This contradicts the outcomes by Gadgil et al. (2014) who found an inverse association between the imbalance in maternal folate and B12 status and birthweight ($p = 0.009$). Differences between the study by Gadgil et al. (2014) and this study includes the adjustment for several maternal confounding factors, including maternal size, age, parity, socioeconomic and educational status (Gadgil et al. 2014). Latter variables were available in this study. Also, while this study focused on maternal folate and B12 concentration in the first and second trimesters, the study by Gadgil et al. (2014) was conducted in pregnant women in their third trimester (36

weeks of gestation). It is possible that the association between an imbalance in maternal folate and B12 status and birthweight is more apparent in the late pregnancy period, since fetal maximal growth is in the last trimester (Higgins et al. 2000), or that perhaps the consequences of a maternal folate / B12 imbalance during pregnancy may only show during growth and development in later childhood. Latter was proposed by an observational study in India, in which the combination of maternal high RBC folate (>1144 nmol/L) and low plasma total B12 concentrations (<114 pmol/l) during late pregnancy predicted the highest level of insulin resistance in the women's 6-year-old children, compared to children of mothers with high RBC folate or low plasma total B12 individually (Yajnik et al., 2008).

Gestational Age at Birth

The most significant association in this study, with the largest coefficient, was found between second-trimester maternal folate concentration and gestational age at birth. Mothers in the second, third and fourth quartile (Q2: 55.5-69.3 nmol/L, Q3: 69.3-87.8 nmol/L, Q4: ≥87.8) had a 4.5, 4.1, 3.6-day increase in gestational age at birth, respectively, compared to the mothers in the lowest quartile (folate concentration ≤55.5 nmol/L). Only one study has previously investigated the association of maternal folate status and gestational age at birth, but the study only looked at late-pregnancy (26 to 28 weeks of gestation) folate status (Chen et al. 2015).The study reported that maternal folate concentration in the third trimester was positively associated with gestational age at birth (Chen et al., 2015), using folate concentration as a continuous variable. In contrast, this study did not find an association between maternal folate as a continuous variable and gestational age at birth. This contradiction can be due to differences in trimester of pregnancy and the maternal folate concentration, because the maternal folate concentration in this study sample [median: 69.3 (IQR: 55.5; 87.0 nmol/L)] was much higher

compared to that [median: 34.4 (IQR: 24.5–44.6 nmol/L)] of the cohort analyzed by Chen et al. (2015).

When investigating the association of maternal folate and B12 biomarker concentrations in the first trimester as continuous variables with gestational age at birth, I only found a significant association between first-trimester MMA concentration and gestational age at birth, suggesting a positive association between maternal B12 status (reflected by lower MMA concentration) and gestational age at birth. However, the association was very weak with an only 30-hour increase in gestational age at birth for every 10 nmol/L decrease in MMA concentration. This association also had a very small R^2=0.018, which indicates that the regression model was weak.

In the unadjusted model, holoTC concentration in the second trimester (as a continuous variable) was significantly associated with gestational age at birth (p= 0.029), but most likely other confounding factors were driving the association as there was no significant association after the adjustment for confounding factors. There was a weak, positive association of second-trimester maternal total B12 concentration (as a continuous variable) with gestational age at birth (b= 0.0008, p= 0.038). As a categorical variable as well, the maternal total B12 concentrations of the third quartile (Q3=220.3-299.2 nmol/L) were associated with a 3-day increase in gestational age at birth compared to the first quartile (Q1≤148 nmol/L) (p= 0.03). In conclusion, the association of maternal B12 biomarker concentration with gestational age at birth in this study sample was not a strong association. My findings of a weak association are somewhat consistent with previous reports describing no association between maternal B12 status and gestational age at birth (Chen et al. 2015; Bergen et al. 2012).

Maternal tHcy concentration in the second trimester (as either a continuous or a categorical variable) showed a positive association with gestational age at birth. Circulating tHcy concentration is a functional indicator for several B-vitamins including both folate and B12, although folate is the main determinant of tHcy concentration. It is not possible to conclude whether B12, and/or other B-vitamins and influencing factors can explain the association of tHcy with gestational age at birth. However, it seems that there is an uncontrolled confounding factor (e.g. parity) influencing the association between maternal tHcy and gestational age at birth (Schempf et al. 2007). Previous studies found an inverse association between maternal tHcy concentration and gestational age at birth, in an assessment of two cohort studies in India with 526 pregnant women in their second and third trimester: results showed an inverse association with the combined cohort effect of a 10-day increase for every 10 µmol/L decrease in maternal tHcy [β= –0.14 (CI: –0.24, –0.04) *p*=0.009] (Yajnik et al. 2014). Another study found that women with high tHcy concentration (i.e., > 23µmol/L) had an increased risk for premature delivery [OR: 2.5, (CI:1.1, 6.2)] (Lindblad et al. 2005).

The imbalance between maternal folate and B12 status (assessed as folate to total B12 ratio and the interaction term) was not associated with gestational age at birth. This null association was in both the continuous and categorical analyses and for biomarker concentrations of both trimesters. According to my knowledge, there is no previous study that has investigated the association of maternal imbalance of folate and B12 status with gestational age at birth. Although my study found that 12.6% and 14.5% of the women in the first and second trimesters of pregnancy, respectively, had an imbalance with both high folate (>46 nmol/L) and deficient B12 (<148 pmol/L), no association of maternal imbalance of folate and B12 status with gestational age at birth was found. This may be partially because of the low prevalence of

preterm births (<37 weeks of gestation, 8.9%) in my study, and the low variation in gestational age at birth [median: 39.0 (IQR: 38.0, 40.0)].

5.3 Changes in Folate and Vitamin B12 Biomarker Concentrations in Early Pregnancy

Pregnancy is a complex and dynamic physiological phase, in which numerous maternal changes occur. Maternal folate and B12 biomarker concentrations vary between different stages of pregnancy. The physiological changes occurring across trimesters of pregnancy, such as the increase in blood volume (Bruinse & van den Berg 1995; Picciano 2003), renal vitamin excretion (Bruinse & van den Berg 1995; Picciano 2003; Cikot et al. 2001), vitamin catabolism and tissue retention (Bruinse & van den Berg 1995), and hormonal influences and haemodilution (Picciano 2003; Cikot et al. 2001) may explain the change in maternal circulating B-vitamin concentrations throughout pregnancy. The pregnancy-related physiologic changes should be taken into consideration when studying the association of maternal folate and B12 biomarker concentrations with birth outcomes; e.g., most of the rapid fetal development occurs in the early stages of pregnancy (Butrick et al. 2012). An association between maternal folate and B12 biomarker concentrations and birth outcomes may suggest a different physiologic and metabolic linkage depending on the trimester of investigation.

One of the strengths of the retrospective cohort study design was the inclusion of samples from more than one time point of pregnancy, which facilitated the opportunity to explore the differences in biomarker concentrations between the first and second trimester. To my knowledge, all published studies investigated the association of maternal folate and B12 status for each trimester independently with birth outcomes. We were able to test the association of the

change in folate and B12 biomarker concentrations during early pregnancy with birth outcome because my study design allowed me to understand the timing and directionality of events.

In this retrospective cohort study, there was no association between the change in maternal folate and B12 biomarker concentrations between the first and second trimester and birth outcomes (birthweight and gestational age at birth). Due to the limitation of the study design and not being able to assess all potential confounding factors, such as parity, and also due to the fact that this sample of pregnant women was likely folate replete, a conclusion cannot be made. Nevertheless, the changes in folate and B12 concentration during pregnancy are important physiological changes that need to be taken into consideration for future studies assessing the association of these two important vitamins with birth outcomes.

5.4 Strengths and Limitations

A major strength of this study lies in the investigation of the combined association of folate and B12 on fetal growth, not just the association of either B-vitamin with birth outcomes, which is important because folate and B12 have interdependent roles in one-carbon metabolism. In addition, the study investigates the association of folate and B12 imbalance on birthweight and gestational age at birth. Also, most of the published studies to date did not look at gestational age at birth as an outcome. Gestational age at birth is an important indicator of infant health status and is also considered a predictor of neonatal mortality (Beck et al. 2010).

The study is also unique in assessing the combination of direct and functional indicators of folate and B12 status in early pregnancy and how they are related individually and independently with birthweight and gestational age at birth. Assessing more than one indicator

for B12 gives a better reflection of the maternal status than assessing only one indicator. It is also recommended to use at least one direct and one functional indicator (Yetley et al. 2011).

To my knowledge, this study is the first to investigate the association of more than two biomarkers of B12 in addition to folate with birth outcomes. This study is also the first in Canada, to investigate the association between folate and B12 imbalance in pregnant women and birth outcomes.

The strength of this study is the large sample size that was sufficient to detect an association of maternal folate and B12 concentration with birth outcomes and that offers a higher level of accuracy of the effect estimate. This study investigated the association of maternal folate and B12 biomarker concentrations in early pregnancy period with birth outcomes. Early pregnancy period is critical for fetal development and an adequate status of folate and B12 that are critical for cell growth and neurodevelopment is of great importance in this period. Moreover, the strength of the study is not only in investigating maternal folate and B12 status in early pregnancy but also in having more than one-time point, including the first and second trimester. To my knowledge, this is the first study to examine the association between tHcy, folate and B12 concentrations in early pregnancy with more than one pregnancy outcome in a Canadian population. Investigating the association of the folate and B12 imbalance in a Canadian population is important as there is a high prevalence of high folate concentrations in combination with low B12 concentrations.

Weaknesses of this research include the limitation of available maternal lifestyle factors, such as body mass index (Ronnenberg et al. 2007), alcohol consumption (McCowan & Horgan 2009), tobacco usage (McCowan & Horgan 2009) and socioeconomic status (Kramer 1987), that may have confounded the association with birth outcome. The lack of these variables prevented

appropriate statistical adjustments in the analyses. Additionally, my data did not include other anthropometric parameters, such as birth length, which has been associated with low folate and B12 concentrations in some studies (Ahmed et al., 2011; Torres-Sánchez, 2014). Moreover, I could not investigate the association of maternal folate and B12 concentrations with the risk of small for gestational age, low birthweight nor preterm birth since this study sample had very low incidences of these adverse birth outcomes.

Maternal samples were not collected in late pregnancy (i.e., third trimester) in addition to the early pregnancy period. Having this extra measurement could have provided more information about the changes in folate and B12 biomarker concentrations throughout pregnancy and the association of these biomarkers in late pregnancy with birth outcomes. Data on periconceptional folic acid and multivitamin supplement use, and dietary intake data were also missing.

The generalizability of the results is within South Asian and European ethnicities in Canada or even within the Lower Mainland, British Columbia, specifically. Since this study focuses on specific ethnic groups, the data cannot be extrapolated to Canadian pregnant women in general. The exclusion of women with medical conditions, including human immunodeficiency virus (HIV), diabetes mellitus type I or II; assisted conception and/or pregnancy following *in vitro* fertilization or intracytoplasmic sperm injection; intravenous therapy or oral steroid medication during pregnancy; history of Down Syndrome, Trisomy 18, or open neural tube defect; and multiple pregnancy, have limited the generalizability of my results to relatively healthy pregnant women only; thus, the estimates can be conservative.

The number of tests in my study is large since I have tested the association among the various biomarkers and at two time-points. Additionally, I have tested the association in the

biomarker concentrations as continuous and categorical variables (accumulative type 1 error). Yet, I did not adjust for multiple comparisons since this is an exploratory analysis. Thus, there is a probability that some of my findings were statistically significant just by chance.

Despite the use of multiple biomarkers, using non-fasting serum samples to reflect folate status is a limitation as serum folate concentration is influenced by short-term dietary intakes (Gregory et al. 2000), whereas RBC folate or fasting serum samples are considered better indicators of long-term folate status (Gregory et al. 2000). RBC or whole blood samples, however, were not available in this study.

5.5 Suggestions for Future Research

In light of the high prevalence of high maternal folate status and low B12 status in Canadian pregnant women, future studies are required to elucidate the association between high folate and low B12 combined in early pregnancy and adverse pregnancy outcomes, with adjustment for further known confounding factors that were not included in this current study, for example, maternal weight and parity.

Future work to build upon this research and to provide further understanding about the association of maternal folate and B12 biomarker concentrations with birth outcomes should include:

[1] Investigation of the association between folate and B12 imbalance and birth outcomes, with adjustment for known confounding factors, such as parity, pre-pregnancy weight and weight gain during pregnancy, due to the high folate and low B12 concentration among pregnant women in Canada.

[2] Studies with time points across all trimesters to investigate the association of maternal folate and B12 biomarker concentrations throughout pregnancy with birth outcomes, and to further understand the presence of the association of maternal folate and B12 biomarker concentrations with birth outcomes in different trimesters.

[3] Consideration of birthweight in relation to gestational age (e.g., SGA) rather than birthweight alone, as SGA is a better indicator of birth outcome.

[4] Studies that investigate the association of more maternal B-vitamins, such as vitamins B6 and B2 in combination with birth outcomes of birthweight and gestational age at birth, due to their interdependent functions in one-carbon metabolism.

Since optimal B-vitamin status is critical for fetal growth and development, randomized controlled trials would provide the highest level of evidence for the effect of different levels of B-vitamins on birth outcomes and thereby have a considerable impact on public health.

5.6 Conclusions

Through the work conducted for this thesis, it was found that in the second trimester, maternal folate concentration of the second, third and fourth quartile groups (Q2=55.5-69.3, Q3= 69.3-87.8, Q4 ≥87.8 nmol/L) compared to the lowest quartile (Q1≤55.5 nmol/L) were associated with an approximate 4-day increase in gestational age at birth. The analyses were done after the adjustment for confounding factors, including ethnicity, maternal age and infant sex.

In the first trimester, maternal tHcy concentration of the third quartile (5 – 5.7 μmol/L) compared to the lowest quartile (≤5.7 μmol/L) was associated with an approximate 110 g increase in birthweight. Maternal folate and B12 biomarker concentrations including MMA, holoTC, total B12 were not positively significantly associated with birthweight. Additional

potential confounding factors, such as maternal weight, height, and parity, may confound the association of maternal folate and B12 status with birthweight. The limited variation of the folate and B12 biomarker concentrations might explain the lack of association of maternal folate and B12 with birthweight in this sample.

My study found that the prevalence of an imbalance in maternal high folate (>46 nmol/L) and low B12 (<148 pmol/L) status was 12.6% in the first trimester and 14.5% in the second trimester. Future studies are needed to investigate the metabolic and/or physiologic consequences, if any, of an imbalance in folate and B12 status in early pregnancy.

In conclusion, early pregnancy folate and B12 biomarker concentrations were not associated with birthweight in this sample. The outcomes suggest that maternal folate concentration in the second trimester may be positively associated with gestational age at birth. The results add to the current evidence that there is a high prevalence of an imbalance in folate and B12 status in early pregnancy in Canadian pregnant women.

These findings complement current research on folate and B12 concentrations with birth outcomes, and offer an insight into these associations in the Canadian context. In light of their interdependent role in fetal growth and development, the association of maternal folate and B12 status with birth outcomes should be further assessed in a population with a higher prevalence of low birthweight, preterm birth, and SGA.

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