MATERNAL METHYL NUTRIENTS, OBESITY PROGRAMMING, AND NEONATAL

ANTHROPOMETRIC OUTCOMES

by

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Abstract

Maternal folate, riboflavin, betaine, choline, and vitamins B-6 and B-12 (B-12) concentrations, also known as methyl nutrients, have an interrelated role in fetal growth and DNA methylation. To date, the relationship between individual maternal methyl nutrient concentrations and neonatal anthropometric outcomes have shown conflicting results, while the interrelationship of maternal methyl nutrient concentrations and their association with DNA methylation levels of fetal growth and obesity-related genes in the offspring is unknown.

The overall goal of my thesis was to provide novel evidence of the interrelationship of maternal methyl nutrients during early pregnancy i.e., <20 weeks of gestation and their association with neonatal anthropometric outcomes and fetal growth and obesity programming in Canadian mother-offspring dyads. To address this goal, firstly, the relationship between concentrations of betaine, a methyl donor nutrient, and total homocysteine (tHcy), an intermediate metabolite of methylation reactions, was tested in 723 pregnant women at early pregnancy. Betaine was inversely associated with tHcy (β =-0.21µmol/L; 95%CI -0.34, - 0.07μ mol/L). Furthermore, the relationship between maternal methyl nutrient patterns and neonatal anthropometric outcomes was explored. Methyl nutrient patterns were mainly characterized by maternal B-12 biomarkers and betaine. Only second-trimester B-12 pattern was inversely associated with head circumference (HC) (β =-0.13cm; 95%CI -0.24, -0.03cm) and HC z-score (β =-0.09; 95% CI -0.09, -0.01). Lastly, whether DNA methylation levels of CpG sites associated with IGF-2, HIF-3a, RXRA, LEP, LEP-R, DNMT-1, DNMT-3A, and DNMT-3B genes, measured in infants, differed by maternal red blood cell (RBC) folate concentration ≤ 1360 nmol/L and RBC folate >1360 nmol/L was tested, and whether these CpG sites were associated with maternal methyl nutrient patterns. Infant DNA methylation levels did not significantly differ

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by maternal folate concentration and were not significantly associated with maternal methyl nutrient patterns. In summary, these results indicated that betaine and total B-12 were the main drivers of maternal methyl nutrients patterns in these folate-replete populations. However, the lack of association between maternal methyl nutrient patterns with neonatal anthropometric outcomes and DNA methylation levels of fetal growth and obesity-related genes in infants suggests the need for a better understanding of the role of these nutrients in fetal programming and growth.

Lay Summary

Maternal B-vitamins, choline, methionine, and betaine, also known as methyl nutrients, participate in interrelated pathways required for fetal growth and provision of methyl groups for DNA methylation. DNA methylation is an important biological process, especially during early pregnancy, because it contributes to 'program' gene expression for later in life. However, the relationship of combined maternal methyl nutrient concentrations with DNA methylation levels of genes related to fetal growth and obesity, and with the baby's size at birth is unknown. The overall goal of my research was to investigate the association of combined methyl nutrient concentrations at early pregnancy with baby's birth size, and DNA methylation levels of genes related to fetal growth and obesity. My findings suggest that B-12 and betaine characterized maternal methyl nutrients patterns; however, there no association between maternal methyl nutrient patterns and baby's birth size and DNA methylation of fetal growth and obesity genes.

Preface

This dissertation was prepared according to the requirements for a Ph.D. thesis by The University of British Columbia (UBC) Faculty of Graduate and Postdoctoral Studies. This research was the result of a collaborative effort. The sample analyses and data collection presented in this dissertation were conducted in the UBC Nutritional Biomarker Laboratory led by Dr Yvonne Lamers, the Newborn Screening Laboratory at the British Columbia (BC) Children's Hospital, and the Pathology Laboratory at St Paul's Hospital in Vancouver, BC, as well as at the Li Ka Shing Centre for Research at the University of Alberta in Edmonton, AB, and the University of Calgary in Calgary, AB. Maternal and neonatal data included in Chapters 2 and 3 were retrieved from the BC Perinatal Data Registry. DNA methylation data included in Chapters 4 and 5 were analyzed at the UBC Department of Medical Genetics. All projects and associated methods were approved by the UBC / Children's and Women's Health Centre of BC Research Ethics Board (certificates H17-03141 and H15-00820).

A version of Chapter 2 has been published. Maria F Mujica-Coopman, Amy Tan, Theresa H Schroder, Graham Sinclair, Hilary D Vallance and Yvonne Lamers. Serum betaine and dimethylglycine are higher in South Asian compared with European pregnant women in Canada, with betaine and total homocysteine inversely associated in early and mid-pregnancy, independent of ethnicity. J Nutr. 2019 Dec 1;149(12):2145-2155. I was responsible for design, and data analysis, as well I wrote the first version of the manuscript. Dr. Theresa H Schroder, former PhD student in Dr. Yvonne Lamers' lab, and I with assistance by staff members and undergraduate research students performed the sample analyses. Amy Tan led the acquisition of maternal and neonatal data from the BC Perinatal Data Registry. Dr. Graham Sinclair, Dr. Hilary D Vallance and Amy Tan contributed to data interpretation and manuscript revisions. Dr. Yvonne

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Lamers was the supervisory author on this project and was involved throughout the project in study design, concept formation, method development, and manuscript composition. Preliminary results of this chapter were presented at the 11th International Conference on Homocysteine & One-Carbon Metabolism, Aarhus, Denmark (2017), and at the BC Children's Hospital Research Institute Research Trainee Day, Vancouver, Canada (2018).

A version of Chapter 3 is being prepared for submission to a peer-reviewed journal. I was responsible for the study design, and data analysis, as well I wrote the first version of the manuscript. Dr. Theresa H Schroder, former PhD student in Dr. Yvonne Lamers' lab, and I with assistance by staff members and undergraduate research students performed the biomarker analyses. Amy Tan led the acquisition of maternal and neonatal data from the BC Perinatal Data Registry. Amy Tan, Dr. Graham Sinclair, Dr. Andre Mattman, and Dr. Hilary D Vallance contributed to data interpretation and manuscript revisions. Dr. Yvonne Lamers was the supervisory author on this project and was involved throughout the project in concept formation, method development, and manuscript composition. Preliminary results of this chapter were presented at the Folic Acid, Vitamin B12, and One-Carbon Metabolism FASEB Science Research Conference, Halifax, Canada (2018), at the Nutrition 2018 Annual Meeting of the American Society for Nutrition, Boston, United States (2018), and at the Canadian National Perinatal Research and DoHAD Meeting, Mont-Tremblant, Canada (2019).

For Chapter 4, I was the lead investigator and I lead the data analysis. Dr. Catherine Field (University of Alberta), Dr. Rhonda Bell (University of Alberta), Dr. Nicole Letourneau (University of Calgary) and Dr. Deborah Dewey (University of Calgary) of the Alberta Pregnancy Outcomes and Nutrition (APrON) Team were co-investigators in this project. Staff in Dr. Catherine Field's lab had previously performed the folate biomarker analysis in the Li Ka

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For Chapter 5, I was the lead investigator and I lead the data analysis. Dr. Catherine Field, Dr. Rhonda Bell, Dr. Nicole Letourneau and Dr. Deborah Dewey of the APrON Team were coinvestigators in this project. Staff in Dr. Yvonne Lamers' lab and I performed the B-6, riboflavin, choline, betaine and related biomarker analysis. Serum total vitamin B12 was measured at the BC Children's Hospital Pathology Laboratory. Biomarker data for folate and holotranscobalamin had been previously collected by staff in Dr. Catherine Field's lab in the Li Ka Shing Centre for Research at the University of Alberta. DNA methylation analyses had been conducted by staff at Dr. Michael Kobor's lab in the UBC Department of Medical Genetics. Dr. Yvonne Lamers was the supervisory author on this project and was involved throughout the project in concept formation, method development, and chapter composition. Preliminary results of Chapter 5 were presented at the 12th International Conference on One-Carbon Metabolism, B-Vitamins and Homocysteine, Tarragona, Spain (2019).

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List of Abbreviations

AGA	Appropriate for gestational age
AGRP	Agouti-related protein
APrON study	Alberta Pregnancy Outcomes and Nutrition study
B-6	Vitamin B-6
B-12	Vitamin B-12
BC	British Columbia
BADH	Betaine aldehyde dehydrogenase
BCPDR	British Columbia Perinatal Data Registry
ВНМТ	Betaine-homocysteine S-methyltransferase
BL	Birth length
BMI	Body mass index
BECs	Buccal epithelial cells
BW	Birth weight
CB	Cord blood
CBS	Cystathionine β -synthase
CGL	Cystathionine γ-lyase
CH ₃	Methyl group
CHDH	Choline dehydrogenase
CpG	5'-C-phosphate-G-3'site
CV	Coefficient of variation
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase

DMG	Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT-1	DNA methyltransferase 1
DNMT-3A	DNA methyltransferase-3A
DNMT-3B	DNA methyltransferase-3B
DNMT-3L	DNA methyltransferase-3L
DNMT-10	DNA methyltransferase-10
DOHaD	Developmental Origins of Health and Disease
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FGR	Fetal growth restriction
fMet-RNA	Formylmethionyl-tRNA
FMN	Flavin mononucleotide
FSA	Forward Sortation Area
FTHFS	Formate-tetrahydrofolate ligase
GA	Gestational age
GCS	Glycine cleavage system
GD	Gestational diabetes
GNMT	Glycine N-methyltransferase
GUSTO	Growing Up in Singapore Towards Healthy Outcomes study

HC	Head circumference
HIF	Hypoxia inducible factor
HoloTC	Holotranscobalamin
HTN	Hypertensive disorder of pregnancy
IGF-1	Insulin growth factor-1
IGF-2	Insulin growth factor-2
IQR	Interquartile range
Kg	Kilogram
LBW	Low birth weight
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LEP	Leptin gene
LGA	Large for gestational age
Mg	Milligram
MoBa	Norwegian Mother and Child Cohort Study
MUAC	mid-upper arm circumference
MS	Methionine synthase
α-MSH	Melanocyte-stimulating hormone
5-MTHF	5-methyltetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEST	Newborn Epigenetics STudy

NTD	Neural tube defect
OR	Odds ratio
PC	Phosphatidylcholine
PCA	Principal component analysis
PCA 1	First trimester principal component analysis
PCA 2	Second trimester principal component analysis
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine-N-methyltransferase
PI	Ponderal index
PL	Pyridoxal
PLP	Pyridoxal 5'-phosphate
PMNS	Pune Maternal Nutrition Study
POMC	Pro-opiomelanocortin
PREFORM	PREnatal Folic acid exposure on DNA methylation study Perinatal Services BC
R	Methyl group acceptor
RBC	Red blood cell
RXRA	Retinoid X-receptor α
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SDH	Sarcosine dehydrogenase
SE	Standard error

SGA	Small for gestational age
SHMT1	Cytosolic serine hydroxymethyltransferase
SHMT2	Mitochondrial serine hydroxymethyltransferase
SOGC	Society of Obstetricians and Gynaecologists of Canada
SWS	UK Southampton Women's Survey
tHcy	Total homocysteine
THF	Tetrahydrofolate
Total B-12	Total vitamin B-12
TS	Thymidylate synthase
UK	United Kingdom
U.S.A.	United States of America
WHO	World Health Organization
Wks	Weeks

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Chapter 1: INTRODUCTION, LITERATURE REVIEW, AND OBJECTIVES AND HYPOTHESES

1.1 Introduction

Pregnancy is characterized as a vulnerable period of life due to the increased rate of cellular differentiation and proliferation (1), where maternal factors, including nutritional status, have a crucial role in ensuring a healthy pregnancy and optimal fetal development (2,3). Maternal B-vitamin adequacy, for example, adequate folate concentration, has been associated with a lower risk of adverse pregnancy outcomes such as small for gestational age (SGA) and preeclampsia (4-6). B-vitamins such as folate, riboflavin, vitamin B-6 (B-6) and vitamin B-12 (B-12) participate, with betaine, choline, and methionine, in one-carbon metabolism. In onecarbon metabolism, methyl groups and other one-carbon units are transferred through the folate and methionine cycles to ultimately contribute to nucleotide formation and cellular proliferation (7). Additionally, methylation reactions are central to one-carbon metabolism as is the remethylation of homocysteine, an intermediary metabolite of one-carbon metabolism, to methionine, and the subsequent formation of S-adenosyl methionine (SAM), the universal methyl donor, which is key for deoxyribonucleic acid (DNA) methylation (7). Due to their interdependent role in one-carbon metabolism and methylation reactions, the aforementioned nutrients are often described as methyl nutrients.

The essential role of methyl nutrients, for example that of folate, in the early stages of pregnancy (i.e., <20 weeks of gestation) has been described since the 1990s. Findings of two clinical trials published in the early 1990s showed that periconceptional supplementation with 4000µg of folic acid/day, which is the synthetic form of folate, from at least 1 month before until

2 months after conception, reduced the risk of a neural tube defects (NTD) recurrence in 70% in pregnant women with prior NTD-affected pregnancy (8), whereas periconceptional folic acid supplementation with a dose of 800µg/day reduced the primary occurrence of NTDs by nearly 70% (9). In 1998, the Institute of Medicine recommended that all women capable of becoming pregnant consume 400µg of folic acid/day from supplements or fortified foods (10). The current recommendation for periconceptional folic acid supplementation in Canada states that all women with a low risk of NTD capable of being pregnant should consume 400µg of folic acid/day from 2-3 months before conception until lactation (11). However, the most common dose of folic acid in prenatal supplements available on the market is 1000µg of folic acid (12,13). The high dose and prolonged use of prenatal supplements have led to high folate status in Canadian childbearing aged (14,15) and pregnant (16) women. It has been suggested that the use of high-dose folic acid supplements throughout pregnancy may be associated with adverse health outcomes in the offspring such as dermatitis, asthma or allergic diseases; however, the currently available evidence is only associational and not conclusive (17).

Potential mechanisms underlying the association between prenatal folic acid supplementation and pregnancy outcomes, for example the prevention of NTDs, are not fully understood. Given the role of folate as the key methyl donor, it has been suggested that folate plays an essential role in the provision of methyl groups for DNA methylation reactions and thereby in the programming of the neural tube closure (18,19). DNA methylation is one of the most stable epigenetic mechanisms, which has a crucial role in gene expression (18). Alterations of DNA methylation levels during early stages of development (e.g., in gametogenesis and embryogenesis stages) may lead to long-term health consequences in the offspring (20,21). Recent studies have suggested that DNA methylation of fetal growth and obesity related-genes

may underlie the relationship between maternal methyl nutrient status and neonatal outcomes (20,22), such as birth weight (BW), birth length (BL), head circumference (HC), and body composition. However, limited evidence is available regarding the relationship of maternal methyl nutrient status and DNA methylation levels of fetal growth and obesity-related genes in the offspring. A prospective study conducted in North American pregnant women showed that red blood cell (RBC) folate concentration, a long-term indicator of folate status, in the second quartile, measured at 12 weeks of gestation was positively associated with DNA methylation levels of the insulin growth factor-2 (*IGF-2*) gene in the offspring, which is a well-known imprinted gene that plays a key role in fetal growth programming (22). Furthermore, it has been reported that pre-pregnancy and maternal dietary methyl nutrient intake (e.g., folate and betaine) is associated with DNA methylation levels of obesity-related genes such as leptin gene (*LEP*) in the offspring (23,24); however, the potential relationship between maternal methyl nutrient status and DNA methylation levels of obesity-related genes (i.e., *LEP*) in the offspring is currently unknown.

Evidence from pregnancy studies has presented conflicting results of the relationship between individual maternal methyl nutrients (e.g., maternal folate concentration) with neonatal anthropometric outcomes (i.e., BW, BL and HC). Maternal RBC folate concentrations in early (i.e., <20 weeks of gestation) and late (i.e., \geq 20 weeks of gestation) pregnancy have been positively associated with BW (25–27), whereas only one of several studies has shown a positive association between early-pregnancy plasma folate concentrations and BW (28). Early and late pregnancy total homocysteine (tHcy) concentration, an intermediate metabolite of the remethylation reaction in one-carbon metabolism, has been negatively associated with BW and HC (25,28–33). Early and late-pregnancy plasma folate, B-12, and B-6, in the form of pyridoxal

5'-phosphate (PLP), concentrations have shown no significant association with neonatal anthropometric outcomes (34–38), while late-pregnancy betaine concentration has been negatively associated with BW and neonatal fat mass (39). However, the relationship between combined maternal methyl nutrient status and neonatal anthropometric outcomes has not been explored yet.

Given the interrelated role of methyl nutrients in one-carbon metabolism which is key for nucleotide synthesis and the provision of methyl groups for methylation reactions, a better understanding of the role of early pregnancy methyl nutrient status on DNA methylation levels of obesity and fetal growth-related genes in the offspring and neonatal anthropometric outcomes is still required. This review provides an overview of the one-carbon metabolism and related methylation pathways. In addition, it presents current evidence of the variation of maternal methyl nutrient concentrations throughout pregnancy. Furthermore, this review provides a summary of the role of methyl nutrients in early pregnancy, and the current evidence on the relationship between maternal methyl nutrient diet, maternal methyl nutrient status and DNA methylation of fetal growth and obesity-related genes. Finally, the current research related to the relationship between individual maternal methyl nutrient concentration (e.g., maternal folate concentration) and neonatal anthropometric outcomes is discussed.

1.2 Literature review

This literature review aims to provide background information on the role of B-vitamins, methionine, choline and, betaine, also known as methyl nutrients, in one-carbon metabolism, followed by an overview of methyl nutrient concentrations during pregnancy. Furthermore, the literature review presents a summary of the current knowledge of the role of methyl nutrients on

fetal development, and evidence of the relationship between maternal methyl nutrients status and DNA methylation levels of fetal growth and obesity-related genes is provided. Finally, the relationship between maternal methyl nutrients status and neonatal anthropometric outcomes is discussed, along with the rationale behind the research project.

1.2.1 B-vitamins, one-carbon metabolism, and methylation pathways

Folate, B-12, B-6, riboflavin, betaine and choline, also known as methyl nutrients, have interdependent roles in maternal and fetal metabolism, because of their interrelated functions in one-carbon metabolism. Their interrelated pathways are essential for the formation of phospholipids and protein, DNA and neurotransmitter synthesis, membrane synthesis and cell formation, and methylation reactions including DNA methylation. In brief, folate, B-12 and B-6 are essential for the formation of DNA, and have important roles in cognitive and neurological development (40,41). Additionally, folate, B-12, riboflavin and B-6 participate in protein and lipid metabolism (7,42,43). Choline is an essential nutrient, and a key component of cell membrane structure and neurotransmitter synthesis and lipid metabolism, which makes it a crucial factor in fetal neurodevelopment during early pregnancy (44,45). Furthermore, betaine, a nutrient obtained from choline oxidation and diet, is a methyl donor nutrient and the most important osmolyte in cells playing a protective role in embryonic cells (46–48).

Specifically, in addition to other functions, the aforementioned B-vitamins, betaine, and choline participate and interact in multiple one-carbon metabolism reactions, which are compartmentalized in the nucleus, mitochondria and cytosol of human cells (49). In the cytosol, the one-carbon metabolism reactions are part of interdependent biosynthetic pathways, which have as primary functions 1) nucleotide synthesis (i.e., purine and thymidylate synthesis), and 2)

remethylation of homocysteine to methionine (Figure 1.1). In the cytosol, folate functions as a co-substrate in the transfer of one-carbon units (7,50). Folate enters the one-carbon metabolism primarily as 5-methyltetrahydrofolate (5-MTHF); however, it can also enter as tetrahydrofolate (THF). Folic acid, the synthetic form of folate, requires conversion to dihydrofolate (DHF) and subsequently to THF by the enzyme dihydrofolate reductase upon digestion. THF can be converted to 10-formyl-THF for purine synthesis, or to 5,10-methylene-THF for the conversion of deoxyuridylate to thymidylate. 5,10-methylene-THF is also the precursor of 5-MTHF and is converted to 5-MTHF by the enzyme methylenetetrahydrofolate reductase (MTHFR) with riboflavin serving as cofactor in the form of flavin adenine dinucleotide (FAD) (41).

5-MTHF transfers its one-carbon unit to homocysteine to regenerate methionine, one of the most important methyl donor nutrients. This remethylation reaction requires B-12 as a cofactor (40). Methionine is the precursor of *S*-adenosylmethionine (SAM), the primary methylation agent in DNA and protein methylation. Variations in intracellular SAM concentration may lead to alterations in DNA methylation and consequently, in the regulation of gene expression (51). SAM is required in over 100 transmethylation reactions forming methylated products and *S*-adenosylhomocysteine (SAH). The latter is subsequently cleaved to homocysteine, which is either remethylated to methionine or enters the transsulfuration pathway to form cysteine by the action of the enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CGL), using B-6, in the form of PLP, as a cofactor (42). Furthermore, riboflavin in its flavin mononucleotide (FMN) form is key for the conversion of B-6 into its coenzyme PLP form. Cysteine is a key nutrient for glutathione and taurine synthesis, both of which have a crucial role in intracellular redox balance (52).

It has been reported that approximately 40% of cellular folate is located in the mitochondria (53). The primary functions of one-carbon metabolism in the mitochondria are 1) to generate one-carbon units for the cytosol one-carbon metabolism, 2) to produce glycine, and 3) to produce formylmethionyl-tRNA (fMet-tRNA), which is used for protein synthesis (7,50). In the mitochondria, the folate form THF is converted to 5,10-methylene-THF by several catabolic reactions that involve serine, glycine, and dimethylglycine (DMG), as well as the condensation of THF with formaldehyde (7). After 5,10-methylene-THF is converted to 5,10-methenyl-THF and, subsequently, oxidized to 10-formyl-THF. Later, 10-formyl-THF is converted to fMet-tRNA and formate (7,54), with formate being the source of one-carbon units for one-carbon metabolism in the cytosol (7,55).

In addition to B-vitamins, choline and betaine also participate in several reactions of the one-carbon metabolism. In the liver, free choline can be used for the synthesis of phosphatidylcholine (PC) by the CDP-pathway. Once PC is formed it can be used for the synthesis of sphingomyelin, a critical compound of the membrane in the brain. Furthermore, the liver-enzyme phosphatidylethanolamine-N-methyltransferase (PEMT) can synthesize PC from phosphatidylethanolamine (PE) through three transmethylation reactions using SAM (56). Additionally, free choline can be released from the synthesized PC by the action of the enzymes phospholipase and phosphodiesterase. In the mitochondria, free choline can be oxidized to produce betaine, which is the methyl donor for the folate and B-12 independent remethylation pathway occurring in the liver and kidneys (57). In the mitochondria and cytosol, betaine donates a methyl group to homocysteine, obtaining methionine and DMG as metabolic products, by the action of the betaine-homocysteine *S*-methyltransferase (BHMT) (44,49), while in the mitochondria, DMG can be converted to sarcosine and subsequently, to glycine by the action of

the mitochondrial enzymes dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SDH), respectively (57). In summary, folate, B-12, B-6, and riboflavin, in addition to choline, betaine, and methionine participate in interrelated pathways that are crucial for nucleotide formation, cellular division, tissue differentiation and methylation reactions which make them key nutrients for the provision of methyl groups for stable epigenetic mechanisms such as DNA methylation (42,58,59).



Figure 1.1 Schematic of one carbon metabolism

5-MTHF, 5-methyltetrahydrofolate; BADH, betaine aldehyde dehydrogenase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-β-synthase; CGL, cystathionine gamma-lyase; CH₃, methyl group; CHDH, choline dehydrogenase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; DNMT-1, DNA methyltransferase-1; DNMT-3, DNA methyltransferase-3; FAD, flavin adenine dinucleotide; fMet-RNA, formylmethionyl-tRNA; FMN, flavin mononucleotide; FTHFS, formate-tetrahydrofolate ligase; GCS, glycine cleavage system; GNMT, Glycine N-methyltransferase; MS, methionine synthase; MTHFR; methylenetetrahydrofolate reductase; NADP, nicotinamide adenine dinucleotide phosphate hydrogen; PLP, pyridoxal-5'-phosphate; R, methyl acceptor; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SDH, sarcosine dehydrogenase; SHMT1, cytosolic serine hydroxymethyltransferase; SHMT2, mitochondrial serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase.

1.2.2 Maternal methyl nutrient concentrations during pregnancy

Periconceptional folic acid supplementation, defined as folic acid supplementation from at least 1 month before until 2 months after conception, has been recommended to prevent the occurrence of NTDs (60). Results of two clinical trials published in the early 1990s, showed that periconceptional folic acid supplementation ($4000\mu g/day$) reduced the risk of NTDs in 70% in all pregnant women with a prior NTD-affected pregnancy (8), whereas periconceptional folic acid supplementation ($800\mu g/day$) reduced the primary occurrence of NTDs by ~100% (9). In 1998, the Institute of Medicine indicated that all women capable of becoming pregnant should consume $400\mu g$ of folic acid/day from supplements or fortified foods (10). However, in North America, only 50% of pregnant women were reported to consume folic acid supplements in the periconceptional period (61), and 49% had an unplanned pregnancy (62). Because of the early development of the neural tube of the fetus (i.e., the neural tube closes before 4 weeks of gestation), mandatory folic acid fortification ($150\mu g/100g$) of white flour and pasta was implemented in Canada in 1998 (63) as a public health strategy to increase the consumption of folic acid intake in women.

Longitudinal studies conducted in pregnant women who do not regularly consume folic acid supplements and from countries without mandatory folic acid fortification have shown that plasma and RBC folate concentrations, which are short- and long-term indicators of folate status, respectively, are higher at early pregnancy (i.e., <20 weeks of gestation) compared to late pregnancy (i.e., >20 weeks of gestation) (64,65). For example, Velzing et al. (65) reported that plasma folate concentration increased from 16.4 nmol/L at 9 weeks of gestation to 19.6 nmol/L at 16 weeks of gestation in West African pregnant women. However, it is known that after this early

period, plasma, serum and RBC folate concentrations decline across weeks of gestation in late pregnancy, with this change being more pronounced in women who only consume folic acid supplements in the first trimester (64,66).

A different situation has been reported in pregnant women who take folic acid supplements beyond the first trimester (i.e., > 12 weeks of gestation), and reside in countries with mandatory folic acid fortification (e.g., Canadian population). Specifically, in Canada, the Society of Obstetricians and Gynaecologists of Canada (SOGC) recommends that all women who could become pregnant should take folic acid supplements ($400\mu g/day$) starting 2-3 months before pregnancy until the end of lactation (11); however, it has been reported that most Canadian pregnant women take $1000\mu g/day$ of folic acid at some time during the first three months of pregnancy, which has been associated with high folate concentration (i.e., RBC folate >1360 nmol/L) (16,67,68).

A study conducted in pregnant women with all of them reporting prenatal multivitamin supplement use in Vancouver, British Columbia (BC) showed no changes in plasma folate concentration between early (35.9 nmol/L, 16 weeks of gestation) and late pregnancy (35.8 nmol/L, 36 weeks of gestation) (36). In pregnant women living in Toronto, RBC folate concentration increased from 2423 nmol/L at 12-16 weeks of gestation to 2862 nmol/L at delivery in folic acid supplement users (93%), while RBC folate concentration decreased from 2343 nmol/L to 2262 nmol/L in non-folic acid supplement users (7%) (16).

Maternal B-6, measured as PLP, and total vitamin B-12 (total B-12) concentrations continuously decline from the first to the third trimester of pregnancy (36,69–73). In Canada, the PREnatal Folic acid exposure on DNA methylation in the newborn infant (PREFORM) study showed that maternal PLP concentration dropped from 107 nmol/L (95% CI 98-116 nmol/L) at
early pregnancy (i.e., 12-16 weeks of gestation) to 58 nmol/L (95% CI 53-62 nmol/L) at delivery (37-42 weeks of gestation) (71), while maternal total B-12 concentration decreased from 219 pmol/L (95% CI 210-229 pmol/L) to 169 pmol/L (95% CI 162-176 pmol/L) (72). Similarly, Wu et al. (36) found that maternal total B-12 significantly declined from 259 pmol/L (IQR 199-351 pmol/L) at 16 weeks of gestation to 206 pmol/L (IQR 150-284 pmol/L) at 36 weeks of gestation. In a Spanish pregnancy study, maternal total B-12 concentration decreased from 293 pmol/L in the preconception period to 267 pmol/L, 230 pmol/L and 198pmol/L at 8, 20 and 32 weeks of gestation, respectively (73).

Similar to PLP and total B-12 concentrations during pregnancy, maternal betaine concentration has also been shown to significantly decrease during early pregnancy (i.e., <20 weeks of gestation) (64,65). Velzing et al. (65) found that maternal betaine concentration decreased from 16.3 μ mol/L to 10.3 μ mol/L between 6 to 16 weeks of gestation. Consistently, betaine concentration dropped from ~21 μ mol/L to ~13 μ mol/L between <12 to 24-27 weeks of gestation in Spanish pregnant women (64). Data on maternal riboflavin status is limited. To date, only two studies, both conducted in the Netherlands, have reported maternal riboflavin status. In 1995, a pregnancy study in Caucasian women showed that whole blood riboflavin concentration slightly increase during the last weeks of pregnancy (74). Later, Cikot et al. (69) also found that plasma riboflavin concentration increased in late pregnancy (i.e., 32 weeks of gestation). However, further pregnancy studies are still needed to understand changes in plasma and whole blood riboflavin concentrations across trimesters of pregnancy.

Potential mechanisms underlying the decline in maternal folate, betaine, B-6 and total B-12 concentrations across trimesters are likely related to the increased maternal-fetal nutrient transport (75), as well as hemodilution due to an increase of maternal blood volume (1,76). An increase in glomerular filtration has been reported during pregnancy, and this may accelerate folate and betaine catabolism and clearance (77,78). In addition, an increase in activity of a nonspecific tissue alkaline phosphatase occurs during pregnancy, which suggests that PLP is converted to pyridoxal (PL), decreasing PLP concentration (79,80). Furthermore, inadequate dietary folate and B-6 intake may also affect folate and B-6 related biomarker concentrations (81–83). In Canada, folic acid supplement use has been indicated as the main predictor of folate concentration in childbearing-aged women (15); therefore, the extended use of folic acid supplements throughout pregnancy likely explains the increase of maternal folate status in Canadian pregnant women (16,67,68).

In contrast to the pregnancy-related changes of circulating folate, B-6, total B-12 and betaine concentrations, maternal free choline concentration increases across gestational weeks (65). From the totality of water-soluble choline forms, i.e., free choline, phosphocholine and glycerophosphocholine, and lipid-soluble choline forms, i.e., PC and sphingomyelin, only free choline and PC are transported to the fetus. In humans, cord blood free choline concentration was reported to be 3-4 times higher than maternal plasma free choline concentration, which suggests a preferential transport of free choline from the mother to the fetus (84). The increase of maternal free choline concentration throughout gestation may be explained by the stimulated endogenous synthesis of choline. It has been reported that the fetus has a limited capacity to synthesize PC by the CDP-pathway (REF), and is thus dependent on the maternal choline supply. The PEMT coding gene contains estrogen response elements whereby estrogen can modulate PEMT expression (85), which may promote the synthesis of PC under increasing estrogen concentration, such as pregnancy (86). A recent randomized controlled trial showed that in pregnant women consuming a supplemented diet that contained 480 or 930 mg of total choline/day (i.e., 100 or

550 mg of choline chloride plus 380 mg of dietary choline, respectively), their total choline intake, i.e., the sum of the water-soluble, lipid soluble and supplemental forms of choline, was preferentially used for PC synthesis by the PEMT and cytidine diphosphate-choline pathways (87). Subsequently, PC can be converted to free choline, which may partially explain the increasing free choline concentration across trimesters.

1.2.2.1 Interaction between folate-B12 and betaine dependent homocysteine remethylation pathways during pregnancy

High maternal plasma concentration of tHcy, the intermediary metabolite of the methionine cycle, in early (i.e., <20 weeks of gestation) and late pregnancy (i.e., >20 weeks of gestation) has been associated with adverse pregnancy outcomes such as preeclampsia (88), SGA (89), premature delivery (90), and impaired neural cell development of the fetus (91).

Maternal plasma folate concentration, as well as folic acid supplementation, are negatively associated with plasma tHcy concentration in early and late pregnancy (65,92). Recently, betaine has become a nutrient of interest due to its role as a methyl donor in the alternative folate-independent homocysteine remethylation pathway, and especially during early fetal development, under low folate concentration (64,93) or when folate-dependent pathways are impaired (94), betaine is used as a methyl donor nutrient. In contrast, it has been suggested that under folate-replete conditions, and due to the ubiquity of the folate-dependent remethylation pathway, folate is the most used methyl donor nutrient for homocysteine remethylation (64,66).

Results from a study conducted in a sample of Spanish pregnant women from a nonfolate-replete population, showed that plasma betaine concentration predicted tHcy concentration in pregnant women with low folate concentration (defined as folate concentration <11.4 nmol/L)

during late pregnancy (>24 weeks of gestation) (64). Similar results were shown by Wallace et al. (66) in a study conducted in pregnant women living in the Republic of Seychelles, an archipelago located in the Indian ocean; betaine concentration at 28 weeks of gestation was negatively associated with tHcy concentration only in women with low methionine concentration (defined as methionine concentration <23.8 μ mol/L at 28 weeks of gestation). Different results were reported for the relationship between maternal betaine and tHcy concentrations in pregnant women from folate-replete populations. A pregnancy study conducted in Vancouver by Wu et al. (36), in which 80% of the participants self-identified as Europeans, showed no significant association between betaine and tHcy concentrations at 16 and 32 weeks of gestation.

Potential explanations for the inverse association between tHcy and betaine concentrations under low folate and methionine concentrations are likely related to the upregulation of BHMT under low folate conditions and tandem-regulation of methionine in BHMT activity (93,95). While, the extended use of folic acid supplementation throughout pregnancy and lactation in Canadian pregnant women (16,68) and the potential subsequent inhibitory effect on the expression of BHMT in the liver (96), as well as the suggested spared use of betaine as a methyl donor (97,98) resulting from high folate status (i.e., RBC folate concentration >1360 nmol/L) (16,67), may explain the lack of a significant association between betaine and tHcy concentrations in this folate-replete population.

Current knowledge from pregnancy studies of the association of circulating betaine and tHcy concentrations is limited to relatively few pregnancy studies. Most of the studies were conducted in populations from non-mandatory folic acid fortified countries in which ~70% of the participants used periconceptional folic acid supplementation, and thus where pregnant women presumably consume smaller amounts of folic acid-fortified foods, than in countries where

mandatory folic acid fortification is implemented and there is a higher compliance of folic acid supplement use in childbearing-aged and pregnant women, and mandatory folic acid fortification is implemented. Additionally, only one study has explored the relationship between maternal betaine and tHcy concentrations at early and late pregnancy in pregnant women from a mandatory folic acid fortified population, specifically in Vancouver, Canada. However, this study only explored the relationship between betaine and tHcy concentrations at 16 weeks of gestation and included a sample of pregnant women of primarily (80%) European ethnicity.

According to Statistics Canada, the South Asian population made up the largest ethnic minority in BC in 2011 (99). A recent study published by Jeruszka-Bielak et al. (100) found that pregnant women of South Asian ethnicity residing in Metro Vancouver had a higher risk of B-12 deficiency compared to those of European ethnicity, in late pregnancy (second or third trimester). Whether low maternal total-B12 concentration impairs the use of folate in the folate-B-12 dependent remethylation pathway of homocysteine to methionine and promotes the use of betaine as a methyl donor is unknown in this population. To date, it is also unknown whether maternal betaine concentration may differ between European and South Asian pregnant women residing in Canada. Results of the 2007-2009 Canadian Health Measures Survey indicated that total B-12 but not folate concentration was a significant predictor of tHcy concentration in adults. However, the national data did not include pregnant women (101). Given the role of betaine as a methyl donor in the remethylation pathway which is critical for the synthesis of SAM, the universal methyl donor, further pregnancy studies exploring the relationship between early-pregnancy betaine and tHcy concentrations in populations with high folate status are needed to understand if betaine becomes a key nutrient for the remethylation of homocysteine to methionine in folate-replete populations.

1.2.3 Role of methyl nutrients in early stages of pregnancy

In the first trimester of pregnancy (Figure 1.2), a crucial physiological process - the differentiation of three embryonic germ layers (i.e., endoderm, mesoderm, and ectoderm) - occurs and this determines the structure and function of fetal organs (1). In addition, from the extraembryonic mesoderm, placenta is formed. In vitro and animal studies have shown that betaine preferentially accumulates in fertilized eggs by a specific transporter, the sodium/iminoacid transporter-1 (102,103), while the role of betaine as a methyl donor during early stages of development has been confirmed by the presence of BHMT activity during the blastocyst stage in a mouse model (47). In mice, the inhibition of folate intracellular transport increases in utero mortality due to the early damage and failure of hematopoietic organs, and the use of folic acid supplementation prevented the offspring death (104). Additionally, it has been observed that a folic acid deficient diet in mice from embryonic day to 17-19 days impaired mitosis and cellular differentiation, and increased cellular apoptosis in brain (105). Findings of a study conducted in female mice showed that the consumption of a folate deficient diet (i.e., 0mg of folic acid/kg) before and during gestation was associated with a lower fetal/placental weight ratio (REF), and inhibited the mTOR intracellular placental pathway (REF), which is associated with cellular growth (REF). Additionally, a maternal folate deficient diet decreased the activity of the placental amino acid transporter (i.e., system A and L) (106), which suggest that maternal folate status is also key for an adequate placenta development.

Furthermore, Lambrot et al. (107) showed that a low paternal dietary folate intake in mice negatively impacted the fertility rate and the implantation of the embryo into the uterus, and increased the presence of anatomical abnormalities such as hydrocephalus in the litter. In

addition, results of the study indicated that a folate-deficient paternal diet altered the sperm epigenome at the DNA methylation level. In humans, it is well-known that folate has a vital role at early stages of pregnancy. Periconceptional folic acid supplementation has been shown to significantly decrease the occurrence of NTDs in the offspring (8,9). Furthermore, findings of a study conducted by Daly et al.(108) indicated that women with RBC folate concentration <340 nmol/L, measured ~15 weeks of gestation, have at least eightfold more risk of NTDs compared to those with RBC folate >906 nmol/L. Neural tube closure occurs before 4 weeks of gestation; therefore, these findings indicate that folate is involved in early stages of cellular differentiation and nervous system development.

Similar to the role of folate, a dietary choline-deficient diet (i.e., 0g chloride choline /kg from embryonic day to 17-19 day) in pregnant mice decreased cellular proliferation of progenitor cells and significantly increased the apoptosis cellular rate in the brain of the offspring, compared to offspring of pregnant mice that received a choline-supplemented diet (i.e., 4.95g choline chloride/kg) (109). Additionally, it has been reported that offspring of dams who received a choline-deficient diet (i.e., 0.3g choline/kg) for 6 weeks had a higher incidence of ventricular septal defects, compared to offspring of dams receiving a control diet (110). Regarding the extra embryonic tissue, choline supplementation (5.6g of choline chloride/kg diet) during pregnancy has also been shown to reduce the expression of proinflammatory factors such as tumor necrosis factor- α and interleukin-8 in a sex-dependent manner, in placenta tissue of murine (111). Additionally, choline supplementation decreased the transcription of the glucose transporter-1 and increased the transcription of the glucose transport-3 and DHA transporters, suggesting that maternal choline supply is important not only for embryonic development, but also for an adequate supply of other critical nutrients, such as DHA, to the embryo and fetus (112).

Furthermore, in humans, findings from a case-control study conducted in North American women indicated that lower serum total choline concentration (i.e., <2.71 mmol/L), which represents the sum of the choline esters converted to free choline, measured between 15-18 weeks of gestation, was associated with a higher risk of NTDs compared to those with higher serum total choline concentration (i.e., \geq 3.21 mmol/L)(113), which also suggest a potential critical role of total choline concentration at early stages of development.

Riboflavin also has a key role in early embryogenesis. Embryos from dams fed a riboflavin-deficient diet for 6 weeks had a significantly higher occurrence of heart defects such as ventricular septal defects and lower ventricular wall thickness, compared to those receiving a control diet (110). These results were consistent with the findings of a pregnancy case-control study, in which the mothers of children with congenital heart disease had a significantly lower dietary riboflavin intake (1.32 mg/day) compared to the control group (1.41 mg/day) (114). Possible mechanisms underlying the relationship between riboflavin-deficient status and heart abnormalities are associated with the coenzyme role of riboflavin in the forms of FAD and FMN. Both FAD and FMN participate in the mitochondrial electron transport chain. It has been previously reported that alterations in mitochondrial DNA may cause defects in cardiac muscle (115,116). Additionally, DNA methylation may potentially underlie the relationship between riboflavin and embryo development. A recent study conducted by Azzi et al. (117) showed that usual dietary riboflavin intake during the year before pregnancy was positively correlated (r=0.02, P<0.05) with the DNA methylation index of differentially methylated regions (DMR) of ZAC1 gene, a gene involved in fetal growth and metabolism, in leukocytes of cord blood (118,119).

In addition to folate, choline, betaine and riboflavin, the essential amino acid methionine is also vital during early stages of embryogenesis. At the 2 cell-embryo stage, 190-350 fmoles/per embryo/hour of methionine are up taken by the fertilized egg (3-4% are converted to SAM) while at morula stage this amount increases to 650 fmoles/embryo/hours (5% converted to SAM)(120). The decrease in methionine uptake and high homocysteine concentration (by the action of an inhibitor of the methionine cycle) impairs embryo development suggesting that adequate SAM and methionine concentrations are crucial for blastocyst development (120). Similar of the role of methionine, in vitro and animal studies have also shown that B-12 and B-6 are critically needed for early embryonic development. The disturbance of the uptake of B-12 by the genetic deletion of cubilin, a peripheral membrane protein expressed in epithelial cells such as intestinal cells, in mice was shown to result in embryonic lethality (121). Additionally, homozygous cubilin knockout embryos were developmentally delayed and had abnormal organ differentiation (121). Furthermore, findings of a retrospective pregnancy study showed that first-trimester total B-12 concentration was inversely associated with DNA methylation levels measured of angiogenesisrelated genes (e.g., VEGF), measured in placenta, which suggest that maternal B-12 may be involved in the programming of placental development(122).

In bovine cells cultured with 250 μ M of pyridoxine, a form of B-6, showed higher blastocyst formation and cell division rate compared to the control (i.e., without pyridoxine) (123). Also, B-6 supplementation significantly decreased the apoptotic rate in early stages of cellular division suggesting that B-6, among the other methyl nutrients, is vital for adequate early fetal development.



Figure 1.2 Schematic of pregnancy stages and DNA methylation¹

¹Black line represents *de novo* DNA methylation in embryo stage; blue dotted line represents *de novo* DNA methylation of the male genome; red dotted line represents *de novo* DNA methylation of the female genome.

1.2.4 Suggested role of DNA methylation in embryonic and fetal programming

According to the Barker's fetal programming theory established in the 1950s, and later expanded to the Developmental Origins of Health and Disease (DOHaD) theory, environmental and maternal lifestyle factors such as maternal nutritional status, maternal smoking, alcohol consumption, drug use, and maternal weight during critical periods of development such as pregnancy, can have long-term health consequences in the offspring (124).

Findings from retrospective cohort studies indicated that low birth weight (LBW) was associated with death related to ischemic heart disease later in life (125,126). Furthermore, offspring of survivors of the Dutch famine in 1944 who were exposed to intrauterine famine (i.e., maternal energy intake ~600 kcal/day), showed higher rates of obesity and cardiovascular diseases as adults (127). Additionally, offspring of pregnant women who were exposed to famine had a lower BL and higher ponderal index (PI) compared to offspring of pregnant women who were not exposed to famine (128).

While the mechanisms are not fully understood, epigenetic changes are suggested to be involved in fetal programming (20,129). Epigenetic changes refer to heritable DNA modifications, which do not change the DNA sequence (130). Many types of epigenetic mechanisms have been identified including methylation, acetylation, phosphorylation, as well as chromatin modifications (130). Results of the Dutch famine cohort study indicated that offspring who were exposure to intrauterine famine had significantly lower DNA methylation levels in CpG sites associated with *IGF-2*, a gene involved in fetal growth, compared to offspring that were not exposed to famine during the prenatal period (131). DNA methylation is a complex epigenetic mechanism, which involves the symmetrical incorporation of methyl groups into specific DNA strand sites (5-position of cytosine residues, defined as 5-methyl cytosine or 5mC)

within 5'-C-phosphate-G-3' (CpG) dinucleotides (132). CpG dinucleotides sites from CpG islands are usually unmethylated and are commonly located in the promoter region of the genes and other regulatory regions. Due to their proximity to the promoter region of the genes, CpG islands play a key role regulating gene expression, thus being crucial for tissue and pattern differentiation in early development. There are three different types of CpG sites: low, intermediate and high-density CpG sites. Each of these respond differently to DNA methylation (133). For example, the methylation of high-density CpG sites is not always associated with gene suppression, while the methylation of intermediate density CpG sites may result in gene silencing.

The transfer of the methyl group from SAM, the universal methyl donor in cells obtained from one-carbon metabolism, to a DNA strand is conducted by the enzyme DNA methyltransferase (DNMT) that comes in 3 isoforms, i.e., DNMT-1, DNMT-3A, and DNMT-3B (132). The DNA strand and the methyl group are attached by a covalent bond, which promotes the recruitment of methyl-CpG binding proteins. It has been suggested that the recruitment of CpG binding proteins inhibits gene expression by blocking the binding of transcription factors, as well as by modifying the histone structure.

An adequate DNA methylation process during the first stages of pregnancy is crucial since DNA methylation patterns are preserved across multiple cellular division cycles, regulating gene expression across the lifespan (58). During early stages of development (i.e., fertilization and implantation stages), demethylation and posterior remethylation of DNA strands occurs in the fertilized egg (Figure 1.2). The demethylation process differs between the paternal and maternal gametes. Shortly after fertilization, paternal and maternal gametes undergo active demethylation. It has been indicated that the ten-eleven translocated family of dioxygenase

enzymes convert 5mC to 5-hydroxy-methyl cytosine (5hmC), the oxidized form, and these are further converted to 5-formylcytosine and 5-carboxycytosine which undergo base excision repair (134,135) coupled with the action of thymine DNA glycosylase, generating abasic sites and regenerating unmodified cytosines (136,137). It has been shown that maternal gametes are protected from active DNA demethylation by the presence of the germ cell protein PGCs7/Stella, while in paternal gamete this protein only protects imprinted genes (138). However, more research is needed to fully understand the role of PGCs7/Stella in the protection of DNA methylation and demethylation at early stages of pregnancy. In maternal and paternal gametes, a passive demethylation process also occurs during the pre-implantation stage, mainly by the nuclear exclusion of *DNMT-1* (139). Additionally, *DNMT-1* has a lower affinity with 5hmC compared to 5mC, which may promote its exclusion from the nucleus, as well as a passive demethylation process (133).

De novo DNA methylation is conducted exclusively by *DNMT-3A* and *DNMT-3B* (18). A third *de novo DNMT-3L* has been described, apparently playing a role in the activation of *DNMT-3A* and *3B* (140). Subsequent DNA methylation reactions are conducted by *DNMT-1. DNMT-1o*, a genetic variant of *DNMT-1*, maintains the parent-specific DNA methylation patterns of imprinted genes during the demethylation and remethylation process, between the one-cell cycle to the eight-cell cycle stage (141). The crucial role of *DNMT-1* during early embryogenesis was confirmed in mice where the deletion of *DNMT-1* in the gastrulation stage was found to impair embryo development (133).

A recent study of Belgian pregnant women found that global DNA methylation measured in maternal samples (i.e., white blood cells) significantly increased from 6.2% in the first (11-13 weeks of gestation) to 6.7% in the second (18-22 weeks of gestation), and decreased to 6.4% in the third trimester (30-34 weeks of gestation) (142). Additionally, findings from the same study indicated that maternal dietary intake of methionine was positively associated with global DNA methylation (142). Furthermore, maternal dietary folate and total dietary choline intakes (i.e., the sum of free choline, glycerophosphocholine, PC, phosphocholine and sphingomyelin) estimated in the same group of Belgium pregnant women, were positively associated with DNA methylation levels of DNMT-1, measured in buccal cells of 3-mo infants (24). However, to date, there is no available information about the potential relationship between DNA methylation levels of DNMT-1 and DNMT-3 in the offspring and maternal methyl nutrient status at early pregnancy. As previously noted, pregnant women from mandatory folic acid fortified populations have significantly higher folate concentration compared to those from non-mandatory folic acid fortified populations (67,71). The potential excess supply of methyl donors before and in early pregnancy may promote DNA methylation alterations during fetal programming by modifying DNMT-1 and DNMT-3 methylation levels. The findings of cellular studies have indicated that the expression of DNMT-3A and DNMT-3B is regulated by nucleosome methylation (143,144). Thus, further studies exploring the relationship between maternal methyl nutrient status, as well as recommended folic acid use or voluntary multivitamin use, with DNA methylation levels of DNMT genes in mother-newborns dyads from mandatory folic-acid fortified populations are still needed.

1.2.4.1 Methyl nutrients, DNA methylation and obesity programming

B-vitamin imbalance may promote epigenetic changes that may promote the activation of obesity enhancing metabolic pathways. Evidence from animal studies has suggested that earlylife exposure (i.e. pre- and post-conception) to methyl nutrient imbalance may modify the phenotype in the offspring. Cooney et al. (145) found that compared to the control group, offspring of mice fed with increased amounts of methyl nutrients [i.e., choline (15 g), betaine (15 g), folic acid (15 mg), B-12 (1.5 mg) and methionine (7.5 g)] during pregnancy had a significantly different coat color and increased DNA methylation levels. These results were confirmed by Waterland et al. (20), who reported that offspring of mice that received a methyl nutrient-supplemented diet from 2 weeks before mating to lactation, showed higher DNA methylation levels of specific CpG sites, as well as a different coat color compared to the controls. Additionally, findings of the same study showed that the determination of DNA methylation levels in tissues derived from the three germ layers, i.e., endoderm (liver), mesoderm (kidney), and ectoderm (brain), of the mice indicated that DNA methylation is programmed in the embryogenesis stage (i.e., <8 weeks of gestation) (20). Although these epigenetic changes occurred in early embryogenesis, their influence on the phenotype, such as coat color, persisted during adulthood (20).

Maternal folate-deficient diets, as well as B-12 and sulfur amino acid (mainly methionine) restricted diets in animals, during the pre-pregnancy and prenatal periods, have shown to impact body composition, likely promoting body fat gain and obesity in the offspring (146,147). Maternal dietary methyl nutrient restriction promotes hypo- or non-methylation of CpG sites in liver tissues of offspring sheep, suggesting that DNA methylation may be involved in the expression of body composition-related genes (146). Despite that the fetal programming theory was based on famine or under-nutrition conditions (148), studies in mice have found that high doses of maternal folic acid impact weight gain, fat composition, as well as body weight control-related hormones, such as adiponectin in the offspring receiving a post-weaning control diet (149,150).

1.2.4.2 Obesity related genes

1.2.4.2.1 Retinoid X-receptor α gene

The gene retinoid X-receptor α gene (RXRA) is a gene that has been suggested to be involved in obesity programming (151). It has been previously described that RXRA gene depends of peroxisome proliferator-activated receptors, which are related to insulin sensitivity and adipose tissue metabolism (152,153). In children, adiposity has been related to the DNA methylation levels of RXRAchr9:136355885(151). The Princess Anne Hospital study and the Southampton Women's Survey (SWS) – both large cohort studies conducted in the United Kingdom (UK), explored the association of RXRA chr9:136355885 methylation measured in cord blood and adiposity of 9- and 6-year old children, respectively (151). In both cohorts, RXRA chr9:136355885 DNA methylation levels were positively associated with fat mass and percentage fat in children. A recent study conducted with Belgian pregnant women found that maternal dietary folate intake in the third trimester was negatively associated with DNA methylation levels of CpG sites associated with RXRA, measured in the cord blood of the newborns (23), whereas the duration of folic acid supplementation before conception was positively associated with DNA methylation levels in most of the studied RXRA CpG sites (23). Similar results were found in the same study in infants at 6 months of age, where maternal betaine and folate intake before pregnancy was positively associated with RXRA DNA methylation levels measured in buccal epithelial cells (BECs) of the infants (24), which suggest that the early exposure to maternal methyl donors intake (i.e., betaine or folate intake) may be involved in obesity programming of the offspring.

1.2.4.2.2 Leptin gene

In addition to the RXRA gene, DNA methylation levels of CpG sites associated with the leptin gene, LEP, has been associated with obesity programming. Leptin is a protein produced mainly by adipose tissue, which was first identified in an obese-mouse model (154). However, it has also been indicated that leptin is produced in the hypothalamus, placenta and stomach. Leptin is one of the most important hormones participating in energy balance and satiety (155). Specifically, leptin stimulates neurons of the arcuate nucleus of the hypothalamus which results in an anorexigenic (i.e., feeding inhibition) effect driven by the melanocyte-stimulating hormone $(\alpha$ -MSH) and amphetamine-related transcript, which is produced from pro-opiomelanocortin (POMC), and inhibits the production of orexigenic molecules (i.e., feeding inducers) such as peptides neuropeptide Y and agouti-related protein (AGRP) in different areas of the hypothalamus (156). The α -MSH acts as an agonist of melanocortin-4 receptor which inhibits food intake, while AGRP acts as an antagonist (157). Thus, the leptin-mediated effect on satiety results from its action in anorexigenic and orexigenic pathways. Morash et al. (158) reported that LEP is regulated by dietary intake (i.e., nutrient availability) in the brain. Additionally, it has been described that leptin stimulates lipid oxidation in skeletal tissue, which may promote a negative energy balance (159). Pre-pregnancy dietary folate intake (not reported in the study) in Belgium women has been negatively associated with the DNA methylation levels of the LEP measured in BECs in the infant (24). Additionally, second-trimester maternal folate intake [mean 263.2 (standard error 92.3) µg] was negatively associated with *LEP* gene methylation measured in cord blood (24), which may suggest that maternal methyl nutrient intake, and potentially, maternal methyl nutrient status are involved in obesity programming.

1.2.4.3 Hypoxia inducible factor gene

The expression of the hypoxia-inducible factor (*HIF*) can be induced by pathological and environmental conditions, for example, cancer and oxygen saturation, respectively. Under adequate oxygen conditions, the expression of the gene *HIF* is inhibited by molecular mechanisms such as hydroxyl-methylation and ubiquitination. While under low oxygen supply (i.e., hypoxic conditions), the expression of the *HIF* gene is promoted (160). *HIF* is activated under hypoxic conditions due to its high sensitivity to oxygen concentrations which leads to vascular and metabolic adaptations (161). Increased adipocyte hypertrophy is one of the most common metabolic alterations associated with obesity. This process leads to an insufficient vascular and oxygen supply, promoting hypoxic conditions in adipose tissue. Additionally, obesity is an important risk factor of obstructive sleep apnea, which is characterized by intermittent episodes of hypoxia (162). Furthermore, results of an animal study indicated that *HIF* is expressed in the hypothalamus. HIF senses the presence of glucose in the brain and regulate satiety by the POMC pathway, independent of leptin action (163).

In humans, the relationship between *HIF* and obesity was first described in adults. Among the three isoforms of *HIF*, (*HIF1a*, *HIF2a*, and *HIF3a*), *HIF3a* has been recognized as a gene potentially related to obesity. A recent large study that included three cohort studies, showed that DNA methylation levels at three specific CpG sites related to *HIF3a* (i.e., cg22891070, cg27146050, and cg16672562) determined in blood were positively associated with body mass index (BMI) in adults. Furthermore, DNA methylation levels of CpG sites related to *HIF3a* were negatively associated with *HIF3a* expression in adipose tissue (164). Results from the Nurses' Health Study (women aged 30-55 years) and the Health Professionals Follow-Up Study (men aged 40-75 years) showed, for the first time, a significant interaction between DNA methylation levels of the variant *HIF3a rs3826795* and dietary B-vitamin intake (165). Participants who had lower dietary riboflavin, B-12, and folate intake at the beginning of the study reported a smaller change in BMI after a 10-year follow-up. In contrast, participants with a higher dietary riboflavin, B-12, and folate intake had a greater increase in BMI over time (165).

Evidence of the relationship between maternal factors and DNA methylation levels of *HIF* in the offspring is limited. A recent study conducted by Mansell et al. (166) showed that gestational diabetes (GD) and weeks of gestation were positively associated with DNA methylation levels of the *HIF-3A* gene measured in cord blood, whereas preeclampsia and the rs3810298 *HIF-3A* variant were negatively associated. Whether maternal methyl nutrient concentrations are associated with DNA methylation levels of the *HIF-3A* gene has not been elucidated to date.

In summary, different genes have been identified as fetal-growth and obesity-related genes. Changes in DNA methylation levels of CpG sites related to these genes have been associated with changes of BMI and body fat in adults and children. *IGF-2, LEP*, and *RXRA* genes have shown variation in their DNA methylation levels in the offspring by maternal dietary methyl nutrient intake, whereas *HIF-3a* DNA methylation levels have shown variations by dietary intake only in adults. Given that early pregnancy is considered a critical plasticity window for fetal programming, future pregnancy studies exploring the relationship between early-pregnancy methyl nutrient status, including multiple direct biomarkers of maternal methyl nutrient status, and DNA methylation levels of obesity-related genes measured in the offspring are crucial to understand the potential role of methyl nutrients on obesity programming. Additionally, the inclusion of pregnant women from folate-replete populations is important to

explore if the exposure to a potential excess of methyl nutrients (e.g., high folate concentration) in early pregnancy may play a role in obesity programming through DNA methylation.

1.2.5 Maternal methyl nutrients and related metabolites and their relationship with neonatal anthropometric outcomes

1.2.5.1 Indicators of neonatal anthropometric outcomes

BW and BL are important predictors of mortality and morbidity in infants (167–169). Evidence from epidemiological studies has shown a positive relationship between BW and BL, and BMI and risk of obesity later in life (170–173). Additionally, BMI in infants at 2 months of age has been indicated as a significant predictor of adiposity at the age of 2 years (174). Findings from a meta-analysis conducted by Yu et al. (173) showed that BW >4000 g was associated with a higher risk of obesity in childhood and adulthood, compared to newborns with a BW <4000g. In addition, a large prospective study in which 4,376 newborns were followed up until 18 years of age, found that BL was positively associated with height at 16 years. Also, BL was positively associated with BMI in females, but not in men (172). Similar results were found in a large Brazilian prospective study (>5,200 participants) in which BL was positively associated with a higher BMI and adiposity at 11 years (175).

In addition to BW and BL, HC has been indicated as a key anthropometric outcome for the tracking of brain growth (176,177). Results of a retrospective study in which HC was measured by ultrasound between 16-24 weeks of gestation showed that having a small HC at the second trimester (i.e., <5th percentile) was associated with a higher risk of perinatal mortality and major malformations such as spina bifida, polycystic kidneys and aortic and mitral atresia (178).

Additionally, studies in children have reported that HC is an excellent predictor of brain volume in children between 1.7-6 years (177). Results of the Helsinki study, a prospective study conducted in Finland, indicated that early adiposity rebound, which is defined as the second rise in adiposity after the first year of life, is negatively associated with HC (179) and positively associated with a higher risk of obesity later in life (180,181). Together, these findings suggest that neonatal anthropometric measurements may be predictors for obesity later in life.

Recently, evidence from the INTERGROWTH 21th project (182), a multicenter study which included eight countries, and aimed to develop new standards and reference values for feta, newborn and infant growth, reported that not only BW, BL, and HC are critical anthropometric outcomes to predict obesity later in life, but that BW/BL ratio (kg/m) was the best predictor for neonatal fat mass (g), fat-free mass (g) and fat (%) measured by air displacement plethysmography. PI and neonatal BMI were also associated with neonatal fat-free mass and fat mass (183). Furthermore, the relationship between neonatal PI with anthropometric measurements later in life was explored by Pietiläinen et al.(172) in a large longitudinal study (n=4,376) conducted in Finland. The authors found that neonatal PI was positively associated with height, weight and BMI at 16 years of age.

In summary, neonatal anthropometric outcomes have been shown to predict obesity and adiposity later in life. Early stages of pregnancy are a critical period for fetal programming due to the high rate of *de novo* DNA methylation. Whether DNA methylation levels of obesity-related genes such as *RXRA* and *HIF-3A* in the offspring are associated with neonatal anthropometric outcomes is unknown. Thus, exploring the relationship between DNA methylation levels of obesity-related genes in the offspring and neonatal anthropometric outcomes is key to identify potential factors that may predispose for obesity later in life.

1.2.5.2 Betaine and neonatal anthropometric outcomes

Betaine has vital roles as an osmolyte in early embryo development (i.e., <8 weeks of gestation) and as the methyl donor of the "alternative" remethylation pathway of homocysteine to methionine in liver and kidneys (47,48,103). Van Lee et al. (39) found that higher maternal betaine concentration measured between 26-28 weeks of gestation was associated with lower neonatal weight (β =-57.6 g; 95%CI -110, -25.3 g, *P*=0.03), smaller HC (β =-0.20 cm; 95%CI - 0.38, -20.0 cm, *P*=0.03), and decreased mid-upper arm circumference (MUAC) (β =-0.16 cm; 95%CI -0.30, -0.03 cm, *P*=0.02), after adjustment for maternal plasma folate, free choline and B-12 concentrations (Table 1.1). Also, higher maternal betaine concentration was associated with a higher risk for SGA (39). Similar results were found by Du et al. (184) in a prospective study that included Chinese pregnant women in which higher maternal betaine concentration measured at late pregnancy was associated with a lower BW (β =-130 g; 95%CI -245, -15.9 g, *P*=0.03), after adjustment for plasma free choline, DMG, tHcy and methionine concentrations. In contrast, a study including Dutch pregnant women found no association between third-trimester betaine concentration and BW (35).

Potential mechanisms underlying the relationship between maternal betaine concentration and neonatal anthropometric outcomes may be related to the suggested role of betaine as a modulator of the expression of fetal growth-related factors. Findings of an animal study showed that rats supplemented with betaine (10 gr/kg) during pregnancy had significantly reduced weaning weight, insulin growth factor-1 (*IGF-1*) expression and serum IGF-1 concentration in the first litter, while an opposite effect was seen in the second litter, in which betaine supplementation significantly increased weaning weight, *IGF-1* expression and IGF-1 concentration (185). Another mechanism proposed is the potential regulatory effect of betaine in

the lipogenesis pathway. Betaine supplementation (1,000mg of betaine/kg to the diet) significantly increased fatty acid synthase (FAS) expression, a key enzyme of the lipogenesis pathway, in adipose tissue of broilers (186). In contrast, results reported by Huang et al.(187) showed that the addition of 1250 mg of betaine to a pig's diet significantly decreased the expression of FAS and acetyl-CoA carboxylase in subcutaneous adipose tissue compared to pigs receiving the control diet.

Despite previous studies and emerging evidence showing the inverse relationship between plasma betaine concentration and BMI and body fat in newborns and adults (39,188), the role of early-pregnancy betaine concentration during fetal-growth programming and neonatal anthropometric outcomes such as BW and BL is not fully understood. Findings from animal studies indicated that maternal betaine supplementation may modify methylation patterns in the promoter region of fetal growth and fatty acid metabolism-related genes (186,187); however the relationship of maternal betaine concentration and DNA methylation of fetal growth and obesityrelated genes in the offspring needs to be confirmed in human studies (185). Additionally, whether the association of maternal betaine and neonatal and infant anthropometric outcomes depends on other maternal methyl nutrient concentrations such as maternal folate or total B-12 concentrations, needs to be elucidated.

1.2.5.3 Choline and neonatal anthropometric outcomes

Choline is the precursor of betaine, the methyl nutrient donor of the alternative methylation pathway. Pregnancy studies exploring the relationship between maternal free choline concentration measured at late pregnancy (i.e., > 26 weeks of gestation) or at delivery have found no significant association between maternal free choline concentration and BW (35,189).

However, results of the Growing Up in Singapore Towards Healthy Outcomes (GUSTO) study indicated that maternal plasma free choline concentration at late-pregnancy was positively associated with neonatal BMI z-score, triceps and subscapular skinfolds, while findings of the UK Southampton Women's Survey showed that maternal serum free choline concentration measured at 11 weeks of pregnancy was positively associated only with subscapular skinfolds (190).

In animal studies, choline has been shown to play a key role in DNA methylation and fetal programming. Choline-deficient diets (i.e., 0g choline chloride) in rats resulted in DNA damage, as well as hypomethylation of global DNA and specific brain development-related genes (191). In addition, changes in maternal total choline intake in rats was shown to modify DNMT-1 expression and IGF-2 methylation in the offspring. In humans, two pregnancy studies have explored the relationship between maternal total dietary choline intake and DNA methylation of fetal-growth related genes in the offspring. A study conducted by Pauwels et al. (142) in Belgian pregnant women found that maternal second-trimester total dietary choline intake was negatively associated with DNA methylation levels of CpG sites related to DNMT-1 (β =-0.30; 95%CI -0.57, -0.03, P=0.03), while maternal third-trimester total dietary choline intake was positively associated with DNMT-1 DNA methylation levels (β =0.29; 95% CI 0.10,0.84, P=0.02) measured in the cord blood of newborns (23). In a sample of North American pregnant women, periconceptional total dietary choline intake was negatively associated with global DNA methylation in the cord blood of male newborns; however, this relationship was not significant in female newborns (192), which may suggest that the impact of maternal total dietary choline intake on DNA methylation is sex-dependent.

To summarize, our current knowledge on the relationship between maternal free choline concentration and neonatal anthropometric outcomes is limited to a few studies that only determined maternal free choline concentration at late pregnancy. In addition, evidence regarding the relationship between maternal total dietary choline intake and DNA methylation of fetal growth and obesity-related genes in the offspring is only available from one study in Belgium pregnant women. Given that *de novo* DNA methylation occurs at early stages of pregnancy and the potential role of choline on fetal growth programming (23,24), further studies exploring the relationship between early-pregnancy choline status (e.g., maternal free choline concentration), DNA methylation of fetal growth-related genes and neonatal anthropometric outcomes are still needed.

1.2.5.4 Folate and folic acid and neonatal anthropometric outcomes

Folate is the key methyl donor nutrient in all cells and has an important role for methylation reactions and nucleotide synthesis, and subsequently in cellular differentiation and proliferation (41). Pregnancy studies exploring the relationship between maternal folate status and neonatal anthropometric outcomes have shown conflicting results. Current evidence from pregnancy studies conducted in UK (26), Indian (27), and Japanese pregnant women (25), have indicated that maternal RBC folate concentration, an indicator of long-term folate status, measured at early (i.e., <20 weeks of gestation) and late pregnancy (i.e., >20 weeks of gestation) is positively associated with BW z-score and BW, respectively. However, in Italian (30), Dutch (193), and Brazilian pregnant women (194), no relationship was found between RBC folate concentration at early and late pregnancy and BW.

In contrast to pregnancy studies that determined maternal RBC folate concentration, only one study has found a significant association between early-pregnancy plasma folate concentration, an indicator of short-term folate status and BW and BL. Bergen et al. (28) showed that low maternal folate concentration (defined as plasma folate concentration ≤ 9.1 nmol/L) measured at 13 weeks of gestation was associated with a lower BW (β =-113.0 g; 95% CI -160, -66.3 g) and BL (β =-3.3 cm; 95% CI -5.8, -0.8 cm) compared to newborns of pregnant women with high maternal folate concentration (defined as plasma folate concentration ≥ 25.8 nmol/L). Furthermore, pregnancy studies that measured maternal plasma folate or serum folate concentrations at late pregnancy (i.e., >20 weeks of gestation) reported no significant association between maternal folate concentration and neonatal anthropometric outcomes (25,30,32–34,36– 38,194–197).

Despite the positive results of the association between early-pregnancy RBC and plasma folate concentration and neonatal anthropometric outcomes, emerging evidence has suggested that not only low maternal folate, but also high maternal folate concentration, may not be beneficial for the offspring development. A study conducted by van Uitert et al. (198) in Dutch pregnant women reported that low maternal folate concentration, defined as RBC folate concentration <1513 nmol/L measured at 8 weeks of gestation, was inversely associated with embryo growth measured before 13 weeks of gestation (198). Additionally, high RBC folate concentration, defined as RBC folate concentration >1813 nmol/L, was also inversely associated with embryo growth. Furthermore, results of the same study showed that low and high folate concentrations were associated with lower cerebellar growth, compared to those offspring of pregnant women with RBC folate concentration between 1538–1813nmol/L at early pregnancy (199). Findings of a study conducted in North American pregnant women showed a positive

association between early RBC folate concentration (mean 12 weeks of gestation) and BW, however, this relationship was only significant in pregnant women with RBC folate concentration in the second quartile, and not in those with higher RBC folate concentrations (i.e., third and fourth quartiles of RBC folate concentration) (22).

Potential explanations for the relationship between early pregnancy folate status with neonatal anthropometric outcomes and fetal growth may be related to the role of folate, as a methyl donor nutrient, in fetal growth programming by DNA methylation reactions. However, evidence regarding the relationship between early pregnancy folate concentration and DNA methylation of fetal growth-related genes is limited. A meta-analysis including two populationbased birth cohort studies conducted in Northern Europe found that maternal plasma folate concentration (measured at < 22 weeks of gestation) was associated with the DNA methylation levels of more than 48 genes (specific CpG sites) assessed in cord blood (200). Furthermore, some of these genes were involved in the occurrence NTDs and brain development of the offspring during pregnancy, which suggest that maternal folate plays a key role in early stages of zygote and blastocyst differentiation. Additionally, one of the studies included in the metaanalysis, The Generation Study, found that maternal plasma folate concentration measured at early pregnancy (median 13 weeks of gestation) in Dutch pregnant women, was positively associated with fetal HC (201). However, in this study, maternal plasma folate concentration [mean 19.1 ±1 nmol/L] was not significantly associated with fetal growth-related DNA methylation genes (202). Similar findings were reported by Haggarty et al. (203) in pregnant women living in the UK in which maternal RBC folate concentration at 19 weeks of gestation [geometric mean 456 (95%CI 442, 471) nmol/L] was not associated with DNA methylation levels of IGF-2, a well-known gene involved in fetal growth, measured in cord blood (Table 1.2).

In contrast, results from the Newborn Epigenetics Study (NEST) study, a prospective cohort study with pregnant women in the United States (U.S.A) with a mean of RBC folate concentration ~494 nmol/L measured at early pregnancy (mean 12 weeks of gestation) found that only low maternal RBC folate concentration (defined as second quartile folate concentration) was positively associated with the DNA methylation levels of the *IGF-2* gene measured in cord blood (22), which may suggest a potential role of folate in fetal growth programming. Although, further studies in pregnant women are needed to replicate the results reported in European and North American pregnant women, and to explore the potential role of folate in fetal growth programming.

According to international recommendations for the prevention of NTDs (204,205), women who are capable to conceive should consume a daily dose of 400µg folic acid until 12 weeks of gestation. In Canada, the SOGC recommends in their Clinical Practice Guidelines that women with a low risk of NTD and capable to conceive should consume "400 µg folic acid for at least 2 to 3 months before conception throughout the pregnancy, and for 4 to 6 weeks postpartum or as long as breast-feeding continues" (11). The Canadian Maternity Experiences Survey found that 58% of Canadian women took folic acid supplements three months before pregnancy (206), while 84% of pregnant women reported folic acid supplement use throughout pregnancy (207). In Alberta, the APrON study reported that 97%, 95% and 91% of women used folic acid supplements in the first, second and third trimester, respectively (68). In Toronto, the PREFORM study indicated that 90% of the participants consumed folic acid supplements at early pregnancy (i.e., 16 weeks of gestation) (16). Folic acid supplementation has led to high folate status in childbearing-aged and pregnant women (15,16,67). Furthermore, folic acid supplementation and mandatory folic acid fortification have led to the reduction of NTDs occurrences (63). Despite the

WHO (204) and the SOGC (11) recommendation of $400\mu g/day$ of folic acid intake for women with a low risk of NTDs starting before conception, prenatal supplements containing 400-1000 μg of folic acid are readily available in Canada (13,208).

A recent animal study showed that maternal folic acid supplementation (20 mg/kg diet) before and during pregnancy decreases the *DNMT-3A* expression in the brains of the offspring (94). Furthermore, a folic acid supplemented diet (i.e., 20 mg/kg) before and during pregnancy in female mice was shown to significantly decrease embryo and placenta weight compared to those embryos of female mice that received a control diet (i.e., 2 mg/kg) (94).

In humans, periconceptional folic acid intake (i.e., folic acid use from at least 4 weeks before conception until 8 weeks of gestations after conception) was positively associated with DNA methylation levels of the *IGF-2* gene measured in whole blood of 17-month-old toddlers, while results of the same study showed that DNA methylation levels of the *IGF-2* gene was negatively associated with BW (209). Findings of a UK pregnancy study also showed that folic acid supplementation beyond the first 12 weeks of gestation was positively associated with *IGF-2* methylation measured in the cord blood of the newborn (203).

In contrast, a prospective study conducted in North American pregnant women reported that DNA methylation levels of the *IGF-2* gene in newborns of pregnant women who took prenatal folic acid supplement (i.e., during pregnancy) were significantly lower, compared to newborns of pregnant women not using folic acid supplements in the second and third trimester (210). Results from a randomized placebo-controlled trial in Irish pregnant women showed that newborns from women who consumed folic acid supplements (400µg/day) in all trimesters, had significantly lower cord blood DNA methylation levels (%) in *IGF-2* compared to those of mothers who took folic acid supplements only the first trimester (211). Potential explanations for

the discrepancies found between the observational studies may be related to the use of different specimens for the determination of DNA methylation levels (i.e., whole blood vs. cord blood). The time point of specimen collection (i.e., toddlers vs. newborn), and the duration of the use of folic acid supplementation differed between the studies may also contribute to explain the different results between the studies. Furthermore, the replication of the results of the randomized controlled trial is needed to understand the effect of folic acid supplementation on DNA methylation levels of fetal growth-related genes in the offspring.

In summary, current evidence from a limited number of studies in apparently healthy pregnant women and their offspring, the majority of them conducted in non-mandatory folic acid fortified populations, indicated that early and late maternal RBC folate concentrations are positively associated with BW, while only one study reported a positive association between earlypregnancy plasma folate concentration and BW and BL. However, emerging evidence has indicated that increasing folate status is inversely related with embryo and brain growth at early stages of pregnancy. Given the well-known effect of folic acid fortification and extended use of folic acid supplementation during pregnancy on increasing folate status, future studies are needed to understand the relationship between maternal folate status and neonatal anthropometric outcomes under presumably high folate concentrations. Also, whether potentially high (i.e., RBC folate concentration >1360 nmol/L) early and late pregnancy folate concentrations are associated with DNA methylation levels of fetal growth or obesity-related genes in the offspring is currently unknown. Thus, further pregnancy studies in folate-replete populations are needed to understand if DNA methylation is an underlying mechanism linking maternal folate status, fetal growth, obesity programming and neonatal anthropometric outcomes.

1.2.5.5 Homocysteine and neonatal anthropometric outcomes

Folate, in the form of 5-methyl-THF serves as the methyl donor in the folate-B-12 dependent homocysteine remethylation pathway, which emphasizes the role of folate not only as a key nutrient for fetal growth and development, but also for preventing an increase in tHcy concentration (212). Given that homocysteine is a key and central metabolite of the remethylation and transsulfuration pathways (7,42), tHcy concentration is considered as a non-specific functional biomarker for low folate, B-12, B-6 and riboflavin status (213–217). Furthermore, lifestyle factors such as increasing age (218), impaired renal function (219), and smoking (217) have shown a positive association with tHcy concentration in adults.

In early pregnancy, elevated tHcy concentration (defined as tHcy concentration $\geq 8.3 \mu$ mol/L) measured at 13 weeks of gestation was associated with a reduced neonatal HC (β =-1.6 cm; 95%CI -3.1,-0.1 cm, *P*<0.05) and weight (β =-102 g; 95%CI -139,-6.65 g, *P*<0.05) in newborns of Dutch pregnant women (28). Similarly, in the U.S.A, McCullough et al. (29) found that tHcy concentration between 5.1-6.0 µmol/L (i.e., the third quartile of the tHcy concentration) at early pregnancy (mean 12 weeks of gestation) was associated with a lower BW in male (β =-210 g; SE 102 g, *P*=0.04) but not female newborns. In Italian pregnant women, an inverse association was found between early-pregnancy tHcy concentrations and BW, in non-users and users of folic acid supplements (30). In Canada, a study conducted by Wu et al. (36) in pregnant women living in BC found no significant association between maternal tHcy concentration, measured at 16th weeks of gestation, and BW.

Additionally, several pregnancy studies have found an inverse association between late pregnancy maternal tHcy concentration and BW (25,30,32). A recent study led by Jiang et al. (31) found that maternal tHcy concentration at delivery was negatively correlated with BW (*r*=-

0.46, *P*=0.01), BL (*r*=-0.43, *P*=0.03), HC (*r*=-0.40, *P*=0.03) and neonatal BMI (*r*=-0.36, *P*=0.04). Similar results were found in a pregnancy study in Indian women, in which maternal tHcy concentration at 30±2 week of gestation was negatively associated with BW (β =-0.13 g; 95%CI-0.21,-0.05 g, *P*=0.001), MUAC (β =-0.15;95 cm%CI-0.23,-0.07 cm, *P*<0.001), triceps (β =-0.12, 95%CI -0.20, -0.03, *P*=0.006) and subscapular skinfold thickness (β =-0.12, 95%CI -0.20,-0.04, *P*=0.006)(33).

Unlike the findings of the relationship between maternal folate status and newborn anthropometric outcomes, results of the majority of pregnancy studies indicated that tHcy concentration at any time point of pregnancy is negatively associated with neonatal anthropometric outcomes (25,28–30). To date, available evidence from pregnancy studies indicates no significant relationship between maternal tHcy concentration and DNA methylation levels of fetal growth and obesity-related genes in the offspring (29,202). tHcy concentration is an indicator that may reflect an impaired one-carbon metabolism. Further studies are needed to explore potential mechanisms underlying the inverse relationship between maternal tHcy concentration and neonatal anthropometric outcomes.

1.2.5.6 Vitamin B-12, B-6, riboflavin and neonatal anthropometric outcomes

Considering the role of B-12 in one-carbon metabolism and specifically as a cofactor for the enzyme methionine synthase (MS) that catalyzes the folate-B12 dependent homocysteine remethylation, recent studies have suggested that maternal B-12 status may also play a crucial role in fetal growth and programming. To date, conflicting results have been reported about the relationship between maternal B-12 status and neonatal anthropometric outcomes. A meta-analysis conducted by Rogne et al. (220) that included 18 studies in which 9 of the studies were

conducted in Europe, 6 in Asia, 1 study was conducted in North America, 1 in Africa, and 1 in Oceania, reported no significant linear association between maternal serum total B-12 concentration and BW. In contrast a pregnancy study conducted by Jiang et al. (31) found that maternal serum total B-12 measured at delivery was positively correlated with BW, HC, and BMI in newborns of Chinese pregnant women. However, this study included an equal proportion of fetal growth restriction (FGR), appropriate for gestational age (AGA) and large for gestational age (LGA) newborns. Thus, these findings cannot be extrapolated to other populations. In contrast, Frery et al. (195) showed an inverse association between maternal plasma total B-12 concentration at delivery and BW only in pregnant women who were smokers, while in Turkish pregnant women, no significant relationship was found between third-trimester total B-12 concentration and BW (37). Additionally, pregnancy studies conducted in Canadian (36), Asian (34) and Indian pregnant (221) women have found no significant relationship between early and late-pregnancy maternal total B-12 concentration and BW.

Data from animal studies showed that B-12 deficiency induces DNA hypomethylation and inadequate uracil incorporation in the DNA of rat colonic epithelial cells (222,223). The underlying mechanism between maternal B-12 and DNA methylation in the offspring is suggested to be due to the cofactor role of B-12 in the homocysteine remethylation pathway, whereby lower total B-12 concentration may lead to an insufficient supply of methyl groups from folate for DNA methylation. In humans, maternal third-trimester serum total B-12 concentration <148 pmol/L was associated with a lower methylation of the *IGF-2* promoter region in cord blood (224). In contrast, McKay et al. (225) found that total B-12 concentration in the first trimester was not associated with *IGF-2* methylation levels measured in cord blood. Similar results were reported in Asian pregnant women (i.e., Chinese, Malay or Indian) where maternal

serum total B-12 concentration, measured at 26-28 weeks of gestation, was not associated with DNA methylation of fetal-growth related genes measured in cord blood (226).

B-6 participates as a coenzyme in the form of PLP in several reactions of one-carbon metabolism. Results from the NEST study showed that maternal PLP concentration, the biomarker of B-6 status, measured between 4.0–32.5 weeks of gestation (mean 12 weeks of gestation), was positively associated with early life weight gain, defined as the body weight gained in the first 36 months of age (29). Additionally, results of the same study indicated that maternal PLP concentration >7.48 nmol/L was positively associated with *MEG3* DNA methylation levels in cord blood, which is a cell-growth related gene (29).

Riboflavin functions as a coenzyme including for the formation of 5-methyl-THF that serves as methyl donor in the folate-B12 dependent homocysteine remethylation reaction. Dietary riboflavin intake measured in late pregnancy (i.e. 36 weeks of gestation) has been positively associated with BW (227), whereas late-pregnancy dietary riboflavin intake has been associated with BL (β =0.72 cm, *P* =0.002) and BW (β =149 g, *P*=0.001) in Dutch pregnant women (228). However, in another study of Dutch pregnant women, plasma riboflavin concentration measured at 6 and 10 weeks of gestation showed no significant relationship with BW (193). It has been previously reported that dietary riboflavin intake before pregnancy was positively correlated with the DNA methylation index of *ZAC1* DMR, which is a gene involved in fetal growth and metabolism (117). However, whether circulating maternal riboflavin concentration is associated with DNA methylation of fetal growth-related genes needs to be elucidated in future prospective pregnancy studies.

To summarize, previous evidence suggested a link between individual maternal methyl nutrient concentrations and neonatal anthropometric outcomes. However, this evidence is limited

to pregnancy studies conducted in non-mandatory folic acid fortified countries and that have a lower prevalence of prenatal folic acid supplement use and a lower dose of folic acid in prenatal supplements, and which explored the relationship between individual maternal methyl nutrients (e.g., maternal folate concentration) and neonatal anthropometric outcomes. Also, methyl nutrient concentrations were determined at different trimesters of pregnancy (i.e., first trimester or at delivery). Given that *de novo* DNA methylation and fetal programming occur at early stages of pregnancy and the potential role of maternal methyl nutrients in fetal programming through DNA methylation, the relationship between early-pregnancy methyl nutrient concentrations and neonatal anthropometric outcomes need to be explored in future pregnancy studies including folate-replete populations. According to pregnancy studies conducted in Canada, more than 90% of pregnant women consume prenatal supplements containing folic acid supplements across trimesters (16,68) with a median daily intake of 1000µg of folic acid from supplements. The high compliance of folic acid supplementation has led to high folate status (i.e., RBC folate >1360 nmol/L) in Canadian pregnant women (16,67), which represents an ideal opportunity to explore the relationship between early-pregnancy methyl nutrient concentrations and neonatal anthropometric outcomes in a folate-replete population.

Considering that all methyl nutrients participate in interrelated reactions of one-carbon metabolism which is critical for the provision of methyl groups for DNA methylation, as well as the lack of evidence of the potential role of maternal methyl nutrients in fetal growth and obesity programming through DNA methylation, the determination of the relationship of all maternal methyl nutrients and related-metabolites with DNA methylation of fetal growth and obesityrelated genes in the offspring is still needed.

Table 1.1 Longitudinal studies reporting on the relationship between maternal methyl nutrient concentrations and neonatal anthropometric outcomes

		a 1			
Author/	n	Sample	Maternal biomarker concentrations ¹	Neonatal outcomes	Findings
Country		collection (wks)			
(year)					
Du et al.	115	Median 26.3	Plasma free choline, < 35y: 9.1 (7.0,10.6); ≥35 y: 8.4 (7.8,10.4) µmol/L	BW, BL	Plasma betaine was negatively associated with BW
(2019)(184)		(range 22.7-33) ²	Plasma betaine: < 35y: 15.1 (12.3,17.3); \geq 35 y:14.5 (11.1,16.3) µmol/L		ũ .
China			Plasma methionine: $< 35y: 18.7 (15.7, 21.3); \ge 35 y: 18.8 (16.1, 22.9)$		
van Lee et al	SWS 985	SWS 11	SWS_serum total choline: 6.03+0.86 umol/L	BW BL BMLz-score total body	GUSTO: Choline concentration was associated with higher BMI
(2019)(190)	GUSTO, 995	GUSTO, 26-28 ²	GUSTO, plasma free choline: $9.12\pm1.6 \mu$ mol/L	fat, abdominal circumference,	z-score subscapular and tricens skinfold
				Subscapular and triceps skinfolds	SWS: Choline concentration was associated with a higher
SWS: UK					subscapular skinfold at birth
GUSTO: Singapore	498	First prenatal	Plasma tHey: $7.1+2.1 \text{ µmol/}I^{-3}$	BW	No relationshin
(2019)(196)	490	visit	Tashia tricy. 7.1±2.1 µmol/E	DW	No relationship
(=====)(====)					
Ireland					
Bergen et al.	5890	Median 14 $(rom co. 11, 17)^4$	Plasma folate: 15.7 (6.2, 34.3) nmol/L	BW, BL, HC	Folate concentration ≤9.1 nmol/L was associated with lower BW
(2010)(28)		(lange 11-17)	Serum total B-12: 169.0 (83.0.351) $pmol/L$		and BL
			Serum active B-12: 42.0 (20.0,83.0) pmol/L		they concentration ($\geq 8.3 \mu$ mol/L) associated with lower BW and
The Netherlands			· · · · ·		smaller HC
McCullough et al.	496	Mean 12	Plasma total B-12 range: 56.1.3665 ng/L	BW	tHey concentration (>6umol/L) was associated with a lower BW
(2016)(29)		(range 4-33)	[41.5, 2704 pmol/L]		in males
			Plasma PLP range: 0,174 nmol/L		
U.S.A	055	26 282	Plasma tHcy range: 0.3,1.54 µmol/L Plasma bataine: 12.2+2.7 µmol/L	PW PL HC	Detains more discharge size denide DW DL HC and mid
(2016)(39)	955	20-28	Plasma free choline: mean 9.2 μ mol/L	Fat mass, abdominal.	Betaine was negatively associated with BW BL, HC and mid-
()(+,)			Plasma tHcy: mean 5.0 µmol/L	and midupper arm circumferences	upper ann cheunneichee
Singapore					
Jiang et al. $(2016)(21)$	116	At delivery	Serum B-12: FGR, 51.6 \pm 39.2 ng/L, [38.1 \pm 28.9 pmol/L]	BW, BL, HC, BMI, BW/BL	Total B-12 was positively correlated with BW, HC and neonatal
(2010)(31)			nmol/L]		BMI
China			Serum tHcy: FGR, 17.5 ± 28.4 ,		they was negatively correlated with BW, BL, HC, and neonatal
			AGA, 5.86±6.17, LGA, 5.54±6.21 µmol/L		BMI
Chen et al.	999	26-282	Plasma folate: 34.4 (24.5,44.6) nmol/L	BW, BL	No relationship
(2015)(34)			Plasma total B-12: 209 (107,238) $pmol/L$ Plasma PL P: 61 8 (25 9 113) $pmol/I$		
Singapore			1 asina 1 E1 : 01:0 (25:5,115) innov E		
Gadgil et al.	49	36	Plasma folate: 17.8 (15.1, 18.9) ng/ml	BW, BL, HC, abdominal and chest	Folate/B-12 ratio was negatively correlated with BW, BL, HC
(2014)(197)			[40.3 (34.2, 42.8)] nmol/L	circumferences	and chest circumference
India			$[145 (103 \ 193) \text{ pmol/L}]$		
			Plasma tHcy: $08.9 (07.1,10.4) \mu mol/L$		
Hoyo et al.	496	All trimesters	RBC folate, Non-Hispanic Black: 195±83.2 µg/L [442±189 nmol/L]; Non-	BW	BW increased in newborns from women in the second RBC
(2014)(22)		Mean 12	hispanic white: $248\pm66.6 \ \mu g/L$ [562±151 nmol/L]; Hispanic: $222\pm64.1 \ \mu g/L$ (502.145 nmol/L); Others: 204.82 6 $\mu g/L$ [462.180 nmol/L]		folate quartile compared to newborns of pregnant women with
USA			$\mu g/L$ (303±143 mmol/L); Others: 204±83.0 $\mu g/L$ [402±189 mmol/L]		lower RBC folate concentration
0.0.11					

¹Data are presented as median (IQR) or mean \pm SD unless otherwise noted. Folate and B-12 units were converted to [nmol/L] and [pmol/L], respectively. Conversion factor for folate concentration (1µg/L=2.266 nmol/L)(213). ²Fasting samples; ³RBC folate, serum folate, and B-12 were not reported; ⁴Non-fasting samples; AGA, appropriate for gestational age; BMI, body mass index; BW, birth weight; BL, birth length; FGR, fetal growth restriction; GUSTO, Growing Up in Singapore Towards Healthy Outcomes study; HC, head circumference; LGA, large for gestational age; PLP, pyridoxal 5'-phosphate; RBC, red blood cell; SWS, UK Southampton Women's Survey; tHcy, total homocysteine; total B12, total vitamin B-12; UK, United Kingdom; U.S.A, United States; wks, weeks of gestation.
Table 1.1. Longitudinal studies reporting on the relationship between maternal methyl nutrient concentrations and neonatal anthropometric outcomes (con)

Author (year)	n	Sample collection (wks)	Maternal biomarker concentrations ¹	Neonatal outcomes	Findings
Yajnik et al. (2014)(27)	PMNS, 526 Parthenon, 515	PMNS: 29±1 ² Parthenon:29±2 ²	PMNS; Plasma tHcy: 8.6 (6.7,10.8) μmol/L RBC folate: 958 (734, 1261) nmol/L Plasma total B-12: 122 (94, 164) pmol/L Parthenon; Plasma tHcy: 6.0 (5.1,7.1) μmol/L Plasma folate: 34.4 (16.8, 51.2) nmol/L Plasma total B-12: 162 (123, 223) nmol/L	BW	PMNS: RBC folate was positively associated with BW Parthenon: Higher tHcy concentration was negatively associated with BW
Krishnaveni et al. (2014)(33) India	654	30±2	Plasma total B-12: 162 (123, 223) pmol/L Plasma total B-12: 164 (123, 224) pmol/L Plasma tHcy: 6.0 (5.1, 7.0) μmol/L	BW, BL, HC, MUAC, subscapular and triceps skinfold	Higher maternal tHcy was associated with smaller BW, MUAC and triceps skinfold thickness
Hogeveen et al. (2013) (35) The Netherlands	366	30-34	Plasma free choline: 7.0 (6.1, 8.3) μmol/L Plasma betaine: 11.0 (9.2, 12.9) μmol/L	BW	No relationship
Wu et al. (2013)(36)	270	16 th and 32 ^{th2}	Plasma total B-12: 259 (199,351) and 206 (150,284) pmol/L Plasma free choline:6.90 (5.96,8.10); and 9.40 (8.30,11.3) µmol/L Plasma betaine:13.0 (10.6,15.8) and 13.1 (11.3,14.8) µmol/L Plasma tHcy: 4.10 (3.50,4.80) and 4.80 (4.20,5.70) µmol/L Plasma folate: 35.9 (33.3,38.1) and 35.8 (32.8,38.8) µmol/L	BW	No relationship
Halicioglu et al. (37)(2012)	208	37 th	Serum total B-12: 163 pg/ml ³ [120 pmol/L] Serum folate: 8.0 ng/ml ³ [18.1 nmol/L]	BW, BL, HC	No relationship
Hogeveen et al. (2010)(32) The Netherlands	366	30-34	Plasma folate: 9.1 (6.1,16.4) nmol/L Plasma total B-12: 179 (134,219) pmol/L Plasma tHcy: 5.5 (4.5,67) μmol/L	BW	tHcy concentration was negatively correlated with BW
Nilsen et al. (2010) (38) Norway	2934	Median 18 ⁴	Not reported	BW, HC	No relationship
Parazzini et al. (2010)(30)	244	8-12, 16 and 22 ⁴	Non-folic acid users: RBC folate: 39 (19,76), 61 (36,109), 70 (49,109) nmol/L Plasma folate: 11.2 (9.1,18.4), 15.2 (10.6,21.3), 18.4 (12.8,28.8) nmol/L Plasma tHcy: 7.9 (5.0,11.2, 8.1 (5.5,11.2), 8.5 (5.6,12.0) µmol/L Folic acid users: RBC folate: 52 (26,103), 67 (42,106), 85 (58,124) nmol/L Plasma folate: 13.8 (9.2,20.3), 18.0 (11.7,21.7), 20.3 (14,30.1) nmol/L Plasma folate: 13.8 (9.2,20.3), 18.0 (11.7,21.7), 20.3 (14,30.1) nmol/L	BW	Higher tHcy concentration at 8-12 and 22 weeks of gestation was associated with lower BW in both groups

Plasma tHcy: 6.9 (4.7,10.6), 7.3 (5.3,10.4), 7.4 (5.3,11.1) μmol/L ¹Data are presented as median (IQR) or mean± SD unless otherwise noted. Folate and B-12 units were converted to [nmol/L] and [pmol/L], respectively. Conversion factor for folate concentration (1µg/L=2.266 nmol/L)(213). ²Fasting samples; ³median; ⁴Non-fasting. BW, birth weight; BL, birth length; HC, head circumference; MUAC, mid–upper arm circumference; PLP, pyridoxal 5'-phosphate; Parthenon, Parthenon study; PMNS, Pune Maternal Nutrition Study; RBC, red blood cell; tHcy, total homocysteine; total B-12, total vitamin B-12; wks, weeks of gestation.

Table 1.1. Longitudinal studies reporting on the relationship between maternal methyl nutrient concentrations and neonatal anthropometric outcomes (con)

Author (year)	n	Sample collection (wks)	Maternal biomarker concentrations ¹	Neonatal Outcomes	Findings
Takimoto et al. (2007)(25)	94	7–14, 26–29 and 34–36 ²	Plasma tHcy: 5.1±1.5, 5.0±1.3, 5.9±1.4 µmol/L Serum total B-12: 405±146, 301±96, 265±95 pmol/L Serum folate: 23.2±9.7, 19.3±23.8, 23.1±69.3 nmol/L RBC folate: 1317±824, 909±551, 813±475 nmol/l	BW, BL, HC	Second-trimester RBC folate and tHcy concentrations were positively associated with HC Third trimester tHcy concentration was negatively, while RBC folate was positively associated with BW
Japan					
Relton et al. (2005)(26)	683	12±6	RBC folate: $418\pm178 \text{ ng/ml} [947\pm403 \text{ nmol/L}]$ Plasma total B-12: $324\pm132 \text{ pg/ml} [241\pm97.4 \text{ pmol/L}]$	BW z-score	RBC folate concentration was positively associated with BW
UK					
Sram et al. (2005)(229)	766	At delivery	Plasma folate: 24.7±16.3 nmol/L ³	BW	Plasma folate was positively associated with BW (Prague-smoker group)
Czech Republic					
Guerra-Shinohara et al. (2004)(194)	119	At delivery	Serum total B-12: 130 (122, 138) pmol/L ⁴ RBC folate: 643 (591, 701) nmol/L ⁴ Serum folate: 12.9 (12, 14) nmol/L ⁴ Serum tHcy: 6.5 (6.1, 6.9) μmol/L ⁴	BW	No relationship
Brazil			Serum methionine: 16.9 (16.2,17.5) μ mol/L ⁴		
de Weerd et al (2003)(193) The Netherlands	188	6 and 10	Plasma PLP: 47 (40,56), 48 (41,57) nmol/L Plasma riboflavin: 270 (250,300), 280 (250,300) nmol/L Plasma total B-12: 280 (230,330), 250 (180,310) pmol/L Serum folate: 13 (10,17), 14 (11,19) nmol/L PBC folate: 520 (440 650), 550 (440 710) nmol/L	BW	No relationship
Buchman et al. (2001)(189)	25	At delivery	Plasma free choline: 8.4±3.1 nmol/mL	BW and BL	No relationship
U.S.A					
Frery et al. (1992)(195)	188	At delivery	Plasma total B-12: 248 (100,539) pg/mL ⁵ [183 (73.8,398) pmol/L] Plasma folate: not reported	BW	Total B-12 negatively correlated with BW in smokers
France					

¹Data are presented as median (IQR) or mean \pm SD unless otherwise noted. Folate and B-12 units were converted to [nmol/L] and [pmol/L], respectively. Conversion factor for folate concentration (1µg/L=2.266 nmol/L)(213). ²Non-fasting sample; ³mean \pm standard error; ⁴geometric mean (95% CI); ⁵median (5th-95th percentiles). BW, birth weight; BL, birth length; HC, head circumference; PLP, pyridoxal 5'-phosphate; RBC, red blood cell; tHcy, total homocysteine; total B-12, total vitamin B-12; UK, United Kingdom; wks, weeks of gestation.

Table 1.2 Longitudinal studies reporting on the relationship between maternal methyl nutrient concentrations and DNA methylation of fetal growth and obesity related genes in the newborns

Author (year)	n	Sample collection (wks)	Maternal biomarker concentration ¹	Neonatal genes	Tissue	Technique	Findings
Lin et al. (2017)(226)	987	26-28	Not reported	EWAS	СВ	Microarray assay	No relationship
McCullough et al. (2016)(29) U.S.A	496	12 (range 4-32.5)	Plasma total B-12: 56.1,3665 ng/L ² [41.4, 2704 pmol/L] Plasma PLP: 0,174 nmol/L ² Plasma tHcy: 0.3,1.54 µmol/L ²	IGF-2/H19 DMR	СВ	Pyrosequencing	PLP concentration >7.48 nM/L was associated with higher <i>MEG3</i> DNA methylation levels
Hoyo et al. (2014)(22) U.S.A	528	All trimesters Mean 12	RBC folate, Non-Hispanic Black: 1945±83.2 [4407±188 nmol/L]; Non-hispanic white: 248±66.6 [561±151 nmol/L]; Hispanic: 222±64.1 [502±145 nmol/L]; Others: 204±83.6 μg/L [461±189 nmol/L]	IGF2/H19	СВ	Pyrosequencing	Low RBC folate concentration (i.e., second quartile) was positively associated with <i>IGF-2</i> DNA methylation levels
Haggarty et al. (2013)(203) UK	913	19	RBC folate: 456 (442, 471) nmol/L ³	IGF-2	СВ	Pyrosequencing	No relationship
Van Mil et al. (2014)(202) The Netherlands	540	10-17	Plasma folate: 19.1±9.0 nmol/L Plasma tHcy: 7.0 (4.9,10.9) μmol/L ⁴	IGF2 DMR, H19	СВ	MassARRAY EpiTYPER Analyzer	No relationship
McKay et al. (2012)(225) UK	197	11±4	RBC folate: 379 (298,512) ng/ml [859 (675-1160 nmol/L] Serum total B-12: 283 (226,389) pg/ml [209 (167,287 pmol/L]	IGF-2	СВ	Pyrosequencing	No relationship
Ba et al. (2011)(224) China	99	At delivery ⁵	Serum folate: 2.29 (0,11.2) ng/ml ⁶ [5.19 (0, 25.4) nmol/L] Serum total B-12: 175 (57,1120) pg/ml ⁶ [129 (42.1, 826 pmol/L)	<i>IGF-2</i> (P2 and P3 promoter regions)	СВ	Real-time methylation specific PCR	Serum total B-12 concentration was negatively associated with <i>IGF-2</i> DNA (P2) methylation

¹Data are presented as median (IQR) or mean \pm SD unless otherwise noted. Folate, B-12, and tHcy units were converted to [nmol/L], [pmol/L] and [µmol/L], respectively. Conversion factor for folate concentration (1µg/L=2.266 nmol/L)(213).²range; ³geometric mean (95%CI) of log-transformed folate concentration;⁴median (90% range);⁵Fasting samples;⁶mean (range). CB, cord blood, DMR, Differentially methylated regions; EWAS, epigenome-wide association study; IGF-2, insulin growth factor-2; PCR, polymerase chain reaction; PLP, pyridoxal 5'-phosphate; RBC, red blood cell; tHcy, total homocysteine; total B-12, total vitamin B-12; UK, United Kingdom; U.S.A, United States; wks, weeks of gestation.

1.3 Summary and rationale

Methyl nutrients participate in interrelated reactions in one-carbon metabolism, which are crucial for cellular proliferation and DNA methylation reactions (7,41,50). Betaine functions as a methyl donor in the folate-B-12 independent remethylation of homocysteine to methionine (48). High maternal tHcy concentration is associated with adverse pregnancy outcomes such as preeclampsia (88). In Canada, a folate-replete population, B-12, and not folate, has been indicated as the major determinant of tHcy concentration (101). Lower total B-12 concentration has been reported in South Asian compared to European pregnant women in Vancouver, BC (230). The South Asian population is the largest ethnic minority in Canada (99). Thus, the understanding of the relationship between methyl nutrient and tHcy concentrations in pregnant women of a folate-replete population would provide important evidence of the role of other methyl nutrients (i.e., not only folate) in the provision of methyl groups for methylation reactions.

Despite methyl nutrients have interrelated roles in one-carbon metabolism (7,50), evidence from pregnancy studies has revealed conflicting results about the relationship between individual methyl nutrient concentrations during pregnancy (e.g., maternal folate concentration) and neonatal anthropometric outcomes. BW is a prognostic indicator for obesity later in life (172,231), whereas HC and neonatal length are indicators of brain development and adiposity later in childhood, respectively. Additionally, neonatal PI, BMI and BW/BL ratio have been associated with adiposity and changes in BMI later in life (183,232–234). Given the interdependent role of methyl nutrients in one-carbon metabolism, a better understanding of the interrelationship of maternal methyl nutrient concentrations and its relationship with neonatal anthropometric outcomes and indicators is needed.

DNA methylation, histone modification and microRNA expression are well-known epigenetic mechanisms. DNA methylation is one of the most stable epigenetic mechanisms involved in fetal programming (132). Early-pregnancy folate and late maternal B-12 concentrations, respectively, have been associated with DNA methylation of fetal-growth related genes measured in cord blood (22,224). However, these studies have included a limited portfolio of circulating maternal methyl nutrient concentrations involved in methylation reactions, and none of these studies have explored the relationship of combined maternal methyl nutrient concentrations and DNA methylation levels in the offspring. Additionally, the measurement of DNA methylation levels in more homogeneous tissues (e.g., BECs or white blood cells), is recommended to partially avoid variations in DNA methylation levels due to cellular type. Given the occurrence of *de novo* DNA methylation at early stages of pregnancy, exploring the relationship between early-pregnancy methyl nutrient concentrations and DNA methylation levels of obesity and fetal-growth related genes measured in a homogeneous tissue (e.g., BECs) may contribute to the understanding of the role of methyl nutrients on obesity programming.

1.4 Research objectives and hypothesis

The objectives of my dissertation are as follows:

Objective 1: To compare peripheral betaine concentration between European and South Asian pregnant women in their first and second trimester of pregnancy, and to determine the relationship between betaine and tHcy concentrations in early pregnancy (i.e., <20 weeks of gestation), in a group of women living in Metro Vancouver, BC, Canada.

- *Null hypothesis*: Serum betaine concentration does not differ between pregnant women of South Asian and European descent in their first and second trimester of pregnancy, and serum betaine concentration is not associated with tHcy concentration in early pregnancy (i.e., <20 weeks of gestation).
- b. *Research hypothesis*: Serum betaine concentration is higher in pregnant women of South Asian descent compared to pregnant women of European descent in their first and second trimester of pregnancy (P < 0.05), and serum betaine concentration is associated with tHcy concentration in early pregnancy (i.e., <20 weeks of gestation) (P < 0.05).

Objective 2: To explore the interrelationship of maternal methyl nutrient concentrations in the first and second trimester, and to determine the association of first- and second-trimester maternal methyl nutrient patterns with neonatal anthropometric outcomes among Canadian mothernewborn dyads.

- a. *Null hypothesis*: First- and second-trimester maternal methyl nutrient patterns are not characterized by specific maternal methyl nutrient concentrations and are not associated with neonatal anthropometric outcomes among Canadian mother-newborn dyads.
- b. *Research hypothesis*: First- and second-trimester maternal methyl nutrient patterns are characterized by specific maternal methyl nutrient concentrations and are positively associated with neonatal anthropometric outcomes among Canadian mother-newborn dyads (P < 0.05).

Objective 3: To determine DNA methylation level (%) of CpG sites associated with *IGF-2*, *LEP*, leptin receptor (*LEP-R*), *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month old infants of mothers who had high RBC folate concentration (>1360nmol/L) in early pregnancy (i.e., <20 weeks of gestation), compared to infants of mothers with folate status in the reference range.

- a. *Null hypothesis*: DNA methylation level (%) of CpG sites associated with *IGF-2*, *LEP*, *LEP-R*, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month old infants of pregnant women with high RBC folate concentration (>1360nmol/L) in early pregnancy (i.e., <20 weeks of gestation) does not differ from those of infants from pregnant women with folate status in the reference range.
- b. *Research hypothesis*: DNA methylation level (%) of CpG sites related to *IGF-2*, *LEP*, *LEP-R*, *HIF3a*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month old infants of pregnant women with high RBC folate concentration (>1360nmol/L) in early pregnancy (i.e., <20 weeks of gestation) are higher compared with those of 3-monthold infants of pregnant women with folate status in the reference range (P <0.05).

Objective 4: To determine the relationship between maternal methyl nutrient concentrations in early pregnancy (i.e., <20 weeks of gestation) and DNA methylation level (%) of CpG sites associated with *IGF-2*, *LEP*, *LEP-R*, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month-old infants.

- a. *Null hypothesis*: DNA methylation level (%) of CpG sites associated with *IGF-2*, *LEP*,
 LEP-R, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month-old infants are not associated with maternal methyl nutrient patterns in early pregnancy (i.e., <20 gestational weeks).
- b. *Research hypothesis*: DNA methylation level (%) of CpG sites related to *IGF-2*, *LEP*, *LEP-R*, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month-old infants are positively associated with maternal methyl nutrient patterns in early pregnancy (i.e., <20 gestational weeks) (P <0.05).

Chapter 2: MATERNAL SERUM BETAINE CONCENTRATION AND ITS RELATIONSHIP WITH TOTAL HOMOCYSTEINE CONCENTRATION IN EUROPEAN AND SOUTH ASIAN PREGNANT WOMEN

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All inferences, opinions, and conclusions drawn in this chapter are those of the author and do not reflect the opinions or policies of Perinatal Services British Columbia.

2.1 Introduction

Betaine, with the chemical structure of trimethylglycine, is a methyl donor nutrient with a metabolic role in the remethylation of homocysteine to methionine. This one-carbon reaction is critical for DNA methylation, a key epigenetic mechanism involved in fetal programming. Betaine can be obtained from a variety of dietary sources (e.g., wheat, beets, spinach) or by endogenous synthesis through choline oxidation, which is catalyzed by choline dehydrogenase (CHDH) and betaine aldehyde dehydrogenase (48,49). Homocysteine remethylation occurs in two pathways, one is by the action of the ubiquitous enzyme MS using 5-MTHF as the methyl group donor and B-12 as a coenzyme (235). In addition to B-12, the recycling of reduced folate forms depends on B-6 in its coenzyme form PLP and riboflavin. The second pathway uses betaine as the methyl group donor independent of folate and B-12, and is catalyzed by BHMT

with methionine and DMG as products (Figure 1.1). This pathway is responsible for 50% of homocysteine remethylation to methionine in mammalian liver cells (90). High plasma tHcy concentration during pregnancy is associated with adverse pregnancy outcomes such as preeclampsia and LBW (88,91,236). Circulating tHcy concentration is influenced by lifestyle, e.g., smoking (98), and nutritional status (237), and genetic variants (93). In Dutch men and post-menopausal women (50-75 y), folate was a two-fold stronger determinant of tHcy concentration at fasting state compared to plasma betaine and serum total B-12 concentrations (98). Plasma betaine, however, was the strongest determinant of the tHcy increase after a methionine loading test, compared to folate, B-12, and B-6 in healthy men and women aged 34-69 y (93). In folate-replete populations, such as the Canadian population (14), B-12 becomes a major determinant of tHcy concentration (101).

In pregnant women from non-mandatory folic acid fortified populations, plasma folate predicted tHcy across trimesters in women with low folate status (i.e., below median of 27.6 nmol/L) (64). Similarly, a study conducted in the Caribbean with pregnant women of West African descent found that plasma folate was a significant predictor of tHcy only at 9 weeks of gestation and at delivery (65). Plasma betaine was associated with increased tHcy concentration after 20 weeks of gestation (64–66), with the strongest association at low folate status (64,66). Plasma betaine was a stronger predictor of plasma tHcy in the second and third trimester, compared to folate and B-12 (64). In contrast, in Canadian pregnant women with the majority being of European ethnicity, no association between betaine and tHcy concentrations was observed at early- and late-pregnancy (36), which may be related to the high folate status in Canadian pregnant women (16,67).

South Asians are the largest visible ethnic minority in Canada (5% of Canada's total population) (99). Circulating betaine concentrations in South Asian populations in Canada or worldwide have not been described to date. Our group previously reported that South Asian pregnant women in BC, Canada, have significantly lower B-12 status compared to European pregnant women in early pregnancy (230). According to the Canadian Health Measures Survey, B-12 becomes the main determinant of tHcy in the folate-replete Canadian population (14,101). With betaine being a strong determinant of tHcy concentration, we hypothesize that betaine and B-12 are major determinants of tHcy in Canadian pregnant women, with betaine being a stronger determinant than B-12, especially in South Asian pregnant women shown to have lower B-12 status. Thus, the objective of this study was to assess betaine and DMG concentrations, and to determine the association of betaine with tHcy concentration in early pregnancy, among Canadian pregnant women of South Asian and European ethnicity.

2.2 Study design and methods

This was a retrospective cohort study using bio-banked residual samples of 723 healthy pregnant women (19-44 years) who participated in the BC Prenatal Genetic Screening Program. In brief, the Prenatal Genetic Screening Program is a voluntary and free-of-charge program that offers a screening exam for Down's syndrome, Trisomy 18 and open NTDs during early fetal development. During each of the two blood collection visits, with one in the first trimester (between 8 and 13 weeks of gestation) and one in the second trimester (between 14 and 20 weeks of gestation), maternal blood samples along with information on maternal age, ethnicity, and weeks of gestation are collected. Around 50% of pregnant women in the province of BC, Canada, are estimated to participate in the program (238).

The primary goal of this retrospective cohort study was to determine the difference in serum total B-12 concentration between pregnant women of South Asian and European ethnicity, as described earlier (230). Briefly, maternal serum samples collected between November 2014 and May 2016 were retrieved for this study. Samples were excluded if women were current smokers, HIV positive, had a diagnosis of diabetes mellitus Type I or II, had *in vitro* fertilization or intracytoplasmic sperm injection treatment, had twins, were using intravenous or oral steroid medication, and had no ethnicity information. Additionally, pregnant women with an increased risk of chromosome disorders or structural anomalies according to the Prenatal Screening Guidelines for Down's syndrome, Trisomy 13, 18 and open NTDs (238) were not included in this study. The present study was reviewed and approved by The University of British Columbia Children's and Women's Research Ethics board, Vancouver, Canada (H15-00820).

2.2.1 Sample size

The sample size calculation was based on the primary objective of this pregnancy cohort study that was to detect a difference of 40 pmol/L in serum total B-12 concentration between women of European and South Asian ethnicity in both first and second trimesters of pregnancy, with a power of 0.8 and a confidence level (α) of 0.05 (230). In total, available first- and second-trimesters serum samples from up to 750 pregnant women were retrieved for the cohort study (European=350; South Asian=350).

Considering that the objectives of the research presented herein were: 1) to determine the relationship between betaine and homocysteine concentrations in South Asian pregnant women and, by comparison in European pregnant women, across the first and second trimester of pregnancy; and 2) to determine the relationship between maternal betaine, across the first and

second trimester of pregnancy, I conducted a power analysis. For power calculation, I considered a sample size of a total of 700 pregnant women. Using a generalized linear model, I would be able to detect an effect size of 0.15 in maternal tHcy concentration with a level of significance (α) of 0.01 and a power (β) of 0.9.

2.2.2 Maternal demographic, obstetric and neonatal information

Maternal age, ethnicity (self-reported), and weeks of gestation at blood collection (calculated according to the crown-rump length ultrasound) were obtained from the Prenatal Genetic Screening Program. Pre-pregnancy weight and height, smoking status, parity (nulliparous-multiparous) and neonatal sex (male-female) were obtained from the British Columbia Perinatal Data Registry (BCPDR) (239). Briefly, the BCPDR "contains data abstracted from obstetrical and neonatal medical records on nearly 100% of births in the province of BC from over 60 acute care facilities as well as births occurring at home attended by BC registered midwives, including women who had pregnancies ending in a live or still birth of at least 20 weeks of gestation or 500 g BW. The BCPDR also collects data on maternal postpartum readmissions up to 42 days post-delivery and baby transfers and readmissions up to 28 days after birth" (239). Pre-pregnancy BMI [weight in kg by height in m²] of the women was classified as underweight (BMI <18.5 kg/m²), healthy weight (BMI \geq 18.5 to 24.9 kg/m²), overweight (BMI \geq 25 to 29.9 kg/m²) and obese (BMI \geq 30 kg/m²) according to the WHO criteria (240).

2.2.3 Biochemical analyses

2.2.3.1 Blood processing

Maternal non-fasting blood samples were collected using serum separator tubes, following the clinical protocol. Blood samples were left at room temperature to allow for serum separation and, subsequently, were stored at 4°C, until centrifugation (1890 x *g* for 5 min at 4°C) within 24h of sample collection. Serum aliquots were stored at -80° C within 4 days. Details about aliquot preparation and thaw-freeze cycles of the samples analysed in this study have been previously reported by Schroder et al (230).

2.2.3.2 Biomarker quantitation

Maternal serum betaine, DMG, methionine, and tHcy concentrations were determined by isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a single platform (230). The inter-assay coefficient of variation (CV)% for the analytes were estimated based on in-house plasma control samples included in each of the in total 25 batches, and were $\leq 10\%$ for betaine, DMG, tHcy, and methionine. In each batch, two external quality control samples for tHcy and methionine (Clinchek 23082 and IRIS Technologies International) were included; the mean (±SD) tHcy concentration was within the target analytical range for the low (8.0±0.4 µmol/L; target: 9.04; control range 7.23-10.8 µmol/L) and high control samples (23.8±1.0 µmol/L; target: 25.9; control range 20.7-31.0 µmol/L). The mean (±Standard deviation (SD)) methionine concentration was within the target analytical range for the low (30.1±2.1 µmol/L; target: 29.1; control range 23.3-34.9 µmol/L) and high control samples (61.0±5.6 µmol/L; target: 67.0; control range 57.0-77.1 µmol/L).

Serum PLP was quantified by isotope-dilution LC-MS/MS using a method modified by Midttun et al. (241). In each batch, three external quality control samples for PLP concentrations (Clinchek 8873 and IRIS Technologies International) were included. The mean (\pm SD) PLP concentration were within the target analytical range for the low (67.5 \pm 6.1 nmol/L; target: 61.9; control range 49.4-74.5 nmol/L), medium (109 \pm 5.1 nmol/L; target: 97.1; control range 77.1-117 nmol/L) and high control samples (144 \pm 8.6 nmol/L; target: 138; control range 110-165 nmol/L). The intra- and inter-assay CV% for PLP were estimated based on 14 batches. The intra-assay CV% was \leq 5% for the low and medium concentration quality control samples, and \leq 10% for the high concentration quality control samples. The inter-assay CV% was \leq 10% for all control samples.

The quantitation of serum total folate and B-12 biomarker concentrations was previously described (230). In brief, serum total folate concentration was determined by the microbiological assay according to O'Broin and Kelleher (242) and Molloy and Scott (243), using the chloramphenicol-resistant *Lactobacillus rhamnosus* (ATCC 27773) and 5-MTHF [(6S)-5-methyl-5,6,7,8-tetrahydro-pteroyl-L-glutamic acid, sodium salt; Merck Eprova] as calibrator. An in-house serum control sample with folate concentration of 42.8 nmol/L (inter-assay CV%: 10.1%) and an external whole blood control sample (NIBSC 95/528) with a folate content of 28.3 nmol/L (i.e., 12.5 ng/mL; target: 13ng/mL) (inter-assay CV%: 8.8%) were included in each of the twenty-two batches. Serum total B-12 was quantified by a microparticle-enzyme immunoassay (Access 2 by Beckman Coulter) at the BC Children's Hospital Pathology Laboratory (Vancouver, BC, Canada). Results from the inter-assay (CV%) of four manufacturer control samples for serum total B12 (Bio-Rad, mean concentration of 93.6, 245, 335, 407 pmol/L) analysed weekly over 4 months (n=18) ranged from 2.4% to 7.1%.

2.2.4 Statistical analyses

Descriptive analyses. The normality distribution of the data was tested using the Shapiro-Wilk test. All continuous variables had a skewed distribution and data were, thus, expressed as the median and interquartile range (IQR). Maternal age, weeks of gestation and pre-pregnancy BMI were compared between ethnic groups using a Wilcoxon rank-sum test, whereas parity, prepregnancy BMI categories, and neonatal sex were compared using the Chi-square test. In each trimester of pregnancy, maternal biomarker concentrations were compared between ethnic groups using the Wilcoxon rank-sum test. Differences between the first- and second-trimester biomarker concentrations, in the total sample and each ethnic group, were tested using the Wilcoxon signedrank test.

Longitudinal analysis. Generalized linear models (GLM) were used to assess 1) the change in betaine and DMG concentrations across weeks of gestation, and 2) the change in betaine and DMG concentrations between the first and second trimester according to first-trimester betaine and DMG concentrations, respectively. The longitudinal association between betaine concentration, as an independent variable, and tHcy concentration, as the dependent variable, was tested using four multi-level mixed effect models. *Model 1* was adjusted for weeks of gestation, serum folate and total B-12 concentrations. *Model 2* was additionally adjusted for PLP. Considering previous evidence that highlighted the potential influence of methionine concentration in the relationship between maternal betaine and tHcy concentrations (66), maternal serum methionine concentration was included in *Model 3*. In *Model 4*, maternal age, ethnicity, neonatal sex, pre-pregnancy BMI categories and parity were additionally included as confounding variables. All four models were adjusted for repeated measures (by including the

study ID as a random effect to account for the general pattern of change of biomarker concentrations over time). All β -coefficients and 95%CI values were multiplied by ten for interpretability purposes. Results were considered significant at *P*<0.05. All the statistical analyses were conducted using Stata version IC 15 (StataCorp). Graphs were made using R Studio version 3.5.1.

2.3 Results

2.3.1 Maternal characteristics

Maternal and neonatal characteristics are presented in Table 2.1. The median (IQR) age of the women was 31 (28-34) y, and 50% were of South Asian (*n*=365) and 50% of European (*n*=358) ethnicity. The median (IQR) weeks of gestation was 11.4 (10.7-12.3) and 16.1 (15.7-17.1) at the first and second trimester blood collection visit, respectively. Maternal age, weeks of gestation, parity and neonatal sex did not differ by ethnicity. Most women were in the reference range for healthy weight (59.9%). Median pre-pregnancy BMI and the distribution of women by BMI categories did not differ between ethnic groups.

2.3.2 Betaine, DMG, and related metabolite concentrations across weeks of gestation and by ethnicity

Biomarker concentrations are presented in Table 2.2. Median (IQR) betaine concentration decreased from 17.6 (13.7-22.6) μ mol/L to 12.9 (10.6-16.7) μ mol/L in European pregnant women and from 19.8 (16.3-25.0) μ mol/L to 16.1 (12.9-19.8) μ mol/L in South Asian pregnant women between the first and second trimester. Serum concentrations of DMG, total B-12, and tHcy also decreased in both ethnic groups, whereas serum folate increased in South Asian

pregnant women between trimesters. Per week of gestation, betaine concentration decreased by 0.99 μ mol/L in European and by 0.86 μ mol/L in South Asian pregnant women (both *P*<0.001) (Figure 2.1). Maternal DMG concentration decreased by 0.04 μ mol/L and 0.03 μ mol/L per week of gestation in European and South Asian pregnant women, respectively (Figure 2.2). The change in betaine concentration between first and second trimester was negatively associated with first-trimester betaine concentration (β =-0.62; 95% CI -0.68, -0.56), while the change in DMG concentration was positively associated with first-trimester DMG concentration (β =0.36; 95% CI -0.30, 0.42) (both *P*<0.001).

Table 2.1 Maternal and neonatal characteristics of apparently healthy European and South Asian pregnant women and their offspring¹

	All	n	European	n	South Asian	n	P ²
Age (years)	31 (28-34)	723	31 (28-35)	358	31 (28-34)	365	0.87
Weeks of gestation at first trimester	11.4 (10.7-12.3)	707	11.4 (10.7-12.4)	356	11.4 (10.6-12.3)	351	0.27
Weeks of gestation at second trimester	16.1 (15.7-17.1)	707	16.1 (15.6-17.0)	355	16.2 (15.7-17.3)	352	0.32
Parity, %							0.14
Nulliparous	51.1	358	53.9	188	48.3	170	
Multiparous	48.9	343	46.1	161	51.7	182	
Neonatal sex, %							0.32
Male	50.5	357	49.4	174	51.5	183	
Female	49.5	350	50.6	178	48.5	172	
Pre-pregnancy	23.5 (21.2-26.7)	558	23.7 (21.3-27.5)	288	23.4 (20.9-26.2)	270	0.08
BMI (kg/m ²), %							
Underweight	17.7 (16.8-18.0)	22	17.9 (17.7-18.2)	8	17.4 (16.5-17.9)	14	0.17
	3.94%		2.78%		5.19%		
Healthy weight	21.9 (20.7-23.4)	334	21.9 (20.7-23.3)	168	21.9 (20.7-23.5)	166	
	59.9%		58.3%		61.5%		
Overweight	26.8 (26.0-28.2)	132	27.2 (26.0-28.3)	69	26.5 (25.8-27.8)	63	
	23.7%		23.9%		23.3%		
Obesity	33.2 (31.3-36.9)	70	33.6 (31.6-36.9)	43	32.4 (30.8-37.3)	27	
	12.5%		14.9%		10.0%		

¹Data are presented as median (IQR) for continuous variables, and as prevalence (%) for categorical variables; ²Maternal age, weeks of gestation and pre-pregnancy BMI were compared between ethnic groups using a Wilcoxon rank-sum test. Parity, pre pregnancy BMI categories and neonatal sex were compared using a Chi-square test; ³ pre-pregnancy BMI categories; underweight (BMI <18.5 kg/m²), healthy weight (BMI \geq 18.5 to 24.9 kg/m²), overweight (BMI \geq 25 to 29.9 kg/m²) and obese (BMI \geq 30 kg/m²) are expressed as median (IQR) and prevalence (%).



Figure 2.1 Maternal serum betaine concentration across weeks of gestation in European and South Asian pregnant women.

Longitudinal linear regression coefficients (95%CIs) for weeks of gestation (P<0.001 unless otherwise stated) are as follows: European pregnant women (black line) β =-0.99; 95%CI -1.16, -0.82, P<0.001; South Asian pregnant women (grey line) β =-0.86; 95%CI -1.04, -0.68, P<0.001.



Figure 2.2 Maternal serum DMG concentration across weeks of gestation in European and South Asian pregnant women

Longitudinal linear regression coefficients (95% CIs) for weeks of gestation (P<0.001 unless otherwise stated) are as follows: European pregnant women (black line) $\beta=-0.04$; 95% CI -0.06, -0.03, P<0.001; and South Asian pregnant women (grey line) $\beta=-0.03$; 95% CI -0.05, -0.006, P=0.01.

Serum betaine and DMG concentrations were significantly higher in women of South Asian ethnicity compared to those of European ethnicity in the first and second trimester (Figure 2.3 and Figure 2.4). Serum total B-12 concentration was significantly lower in South Asian women, while methionine, PLP, folate, and tHcy concentrations did not differ between ethnicities (Table 2.2).



Figure 2.3 First and second-trimester betaine concentration in European and South Asian pregnant women.

Tukey box plots show median values (solid horizontal line), 50th percentile values (box outline; Q1 and Q3), the upper and lower adjacent values (whiskers; most extreme values within Q3+1.5*(Q3-Q1) and Q1-1.5*(Q3-Q1)), and outlier values (solid circles). Serum betaine concentration without a common letter differ significantly between European (first trimester n=306; second trimester n=286) and South Asian (first trimester n=282; second trimester n=291) pregnant women in each trimester, P<0.05 (Wilcoxon rank-sum test).



Figure 2.4 First and second trimester DMG concentration in European and South Asian pregnant women.

Tukey box plots show median values (solid horizontal line), 50^{th} percentile values (box outline; Q1 and Q3), the upper and lower adjacent values (whiskers; most extreme values within Q3+1.5*(Q3-Q1) and Q1-1.5*(Q3-Q1)), and outlier values (solid circles). Serum DMG concentration without a common letter differ significantly between European (first trimester n=306; second trimester n=286) and South Asian (first trimester n=286; second trimester n=292) pregnant women in each trimester, *P*<0.05 (Wilcoxon rank-sum test).

Serum	All women						European						South Asian				
biomarker	First	n	Second	n	P ²	First	n	Second	n	P ²	First	n	Second	n	P ²	P ³	P ⁴
	trimester		trimester			trimester		trimester			trimester		trimester				
Betaine	18.8 ^a	589	14.5 ^b	577	< 0.001	17.6 ^{a,d}	307	12.9 ^{b,f}	286	< 0.001	19.8 ^{a,c}	282	16.1 ^{b,e}	291	< 0.001	< 0.001	< 0.001
$(\mu mol/L)$	(15.1-23.7)		(11.6-18.1)			(13.7-22.6)		(10.6-16.7)			(16.3-25.0)		(12.9-19.8)				
DMG	1.47 ^a	593	1.29 ^b	578	< 0.001	1.38 ^{a,d}	307	1.19 ^{b,f}	286	< 0.001	1.55 ^{a,c}	286	1.42 ^{b,e}	292	< 0.001	< 0.001	< 0.001
$(\mu mol/L)$	(1.19-1.90)		(1.04-1.68)			(1.12-1.77)		(0.97-1.52)			(1.30-1.96)		(1.14-1.81)				
Total B-12	218 ^a	666	198 ^b	717	< 0.001	247 ^{a,c}	336	224 ^{b,e}	355	< 0.001	191 ^{a,d}	330	170 ^{b,f}	362	< 0.001	< 0.001	< 0.001
(pmol/L)	(157-296)		(146-270)			(189-318)		(165-299)			(137-255)		(126-242)				
Folate	66.8 ^b	656	69.6 ^a	675	0.031	67.9	330	68.7	332	0.148	66.5 ^b	326	70.9 ^a	343	0.006	0.301	0.564
(nmol/L)	(53.0-81.3)		(55.3-87.8)			(54.2-82.5)		(53.8-88.2)			(52.0-78.8)		(56.8-86.8)				
tHcy	5.01ª	610	4.37 ^b	578	< 0.001	4.99 ^a	324	4.38 ^b	286	< 0.001	5.03ª	286	4.36 ^b	292	< 0.001	0.432	0.707
(µmol/L)	(4.47-5.69)		(3.78-5.10)			(4.37-5.65)		(3.67-5.10)			(4.51-5.75)		(3.85-5.13)				
PLP	90.1ª	575	66.3 ^b	496	< 0.001	95.4ª	287	66.9 ^b	254	< 0.001	82.1ª	288	63.3 ^b	242	< 0.001	0.184	0.381
(nmol/L)	(50.7-176)		(43.1-121)			(51.4-191)		(44.8-129)			(50.3-164)		(41.5-116)				
Methionine	22 1ª	610	21 2 ^b	578	0.002	22.4	324	21.4	286	0.0529	21 7ª	286	21 1 ^b	292	0.012	0 314	0 445
(µmol/L)	(19.3-25.4)	010	(18.4-24.7)	570	0.002	(19.4-25.6)	521	(18.3-25.2)	200	5.0527	(19.2-25.1)	200	(18.5-24.0)	272	0.012	0.017	0.115

Table 2.2 Serum biomarker concentrations in the first and second trimester of pregnancy by ethnicity¹

¹Data are presented as median (IQR). DMG, dimethylglycine; PLP, pyridoxal 5'-phosphate; tHcy, total homocysteine. ²Biomarker concentrations without a common letter (a,b) differ significantly by trimester (*P*<0.05, Wilcoxon signed-rank test).

³First-trimester biomarker concentrations without a common letter (c,d) differ significantly by ethnicity (P<0.05, Wilcoxon rank-sum test).

⁴Second-trimester biomarker concentrations without a common letter (e,f) differ significantly by ethnicity (*P*<0.05, Wilcoxon rank-sum test).

2.3.3 Association between maternal betaine and total homocysteine concentrations in early pregnancy

Serum tHcy concentration decreased by 0.19 μ mol/L for each ten-unit increase in serum betaine concentration across weeks of gestation after including B-12 and folate concentrations into the model (Table 2.3). We did not observe substantial changes in the relationship between betaine and tHcy concentrations after inclusion of methionine, PLP, maternal age, ethnicity, neonatal sex, parity, and pre-pregnancy BMI categories into the models. Also, serum tHcy concentration decreased by 0.03 μ mol/L for each ten-unit increase in serum total B-12 concentration across weeks of gestation, after adjusting for confounding factors.

Independent variables	Model 1	P^2	Model 2	P ³	Model 3	P ⁴	Model 4	P ⁵
P	β (95%CI)	-	β (95%CI)	-	β (95%CI)	-	β (95%CI)	-
Serum betaine, µmol/L	-0.19	0.004	-0.19	0.005	-0.19	0.004	-0.21	0.002
	(-0.32, -0.06)		(-0.32, -0.06)		(-0.3, -0.63)		(-0.34, -0.07)	
Weeks of gestation	-1.36	< 0.001	-1.38	< 0.001	-1.37	< 0.001	-1.38	< 0.001
	(-1.62, -1.10)		(-1.65, -1.11)		(-1.64, -1.11)		(-1.65, -1.11)	
Serum total B-12,	-0.03	< 0.001	-0.03	< 0.001	-0.03	< 0.001	-0.03	< 0.001
pmol/L	(-0.04, -0.02)		(-0.04, -0.02)		(-0.04, -0.02)		(-0.04, -0.02)	
Serum folate, nmol/L	-0.01	0.33	-0.14	0.31	-0.01	0.34	-0.01	0.29
	(-0.04, 0.01)		(-0.04, 0.01)		(-0.04, 0.01)		(-0.04, 0.01)	
Serum PLP, nmol/L			-0.003	0.45	-0.004	0.35	-0.003	0.38
			(-0.01, 0.005)		(-0.01, 0.004)		(-0.01, 0.004)	
Serum methionine,					0.13	0.10	0.11	0.18
µmol/L					(0.02, 0.28)		(-0.05, 0.26)	
Maternal age							0.14	0.19
							(-0.07, 0.35)	
Ethnicity							-1.72	0.07
South Asian							(-3.58, 0.14)	
Neonatal sex							-0.68	0.45
Female							(-2.43, 1.07)	
Parity							-3.20	0.001
Multiparous							(-5.03, -1.38)	
Pre-pregnancy BMI								
Healthy weight							Reference	
Underweight							3.34	0.15
							(-1.24, 7.92)	
Overweight							-1.02	0.35
<u>.</u>							(-3.16, 1.12)	0.50
Obesity							-0.48	0.73
							(-3.21, 2.25)	

Table 2.3 Association of maternal serum betaine and total homocysteine concentrations across weeks of gestation in 495 apparently healthy European and South Asian pregnant using longitudinal regression models¹

¹Data presented as coefficients with 95%CI; coefficients and 95%CI values were multiplied by ten for interpretability purposes. PLP, pyridoxal 5'-phosphate; BMI, body mass index; underweight (BMI <18.5 kg/m²), healthy weight (BMI \geq 18.5 to 24.9 kg/m²), overweight (BMI \geq 25 to 29.9 kg/m²) and obese (BMI \geq 30 kg/m²);²Model 1 was adjusted for repeated measures, weeks of gestation, total B-12, and folate concentrations; ³Model 2 was additionally adjusted for PLP concentration; ⁴Model 3 was additionally adjusted for methionine concentration; ⁵Model 4 was additionally adjusted for maternal age, ethnicity, neonatal sex, parity, and pre-pregnancy BMI.

2.4 Discussion

Betaine is the methyl donor nutrient required for the folate-B-12 independent remethylation of homocysteine to methionine (48). There is a higher requirement for methyl nutrients during rapid stages of growth including pregnancy. South Asian pregnant women are at risk for B-12 deficiency during pregnancy. To the best of my knowledge, this is the first study to determine betaine concentration in South Asian pregnant women, and the first to explore the association between maternal betaine and tHcy concentration in this ethnicity.

In this retrospective cohort study with samples of 723 pregnant women, serum betaine and DMG concentrations declined across weeks of gestation. Betaine concentration decreased from 18.8 µmol/ to 14.5 µmol/L between the first and second trimester, and DMG concentration from 1.47µmol/L to 1.29 µmol/L, respectively. Similarly, Velzing et al. (65) reported a decrease in plasma betaine concentration from 16.3 µmol/L to 11.5 µmol/L, and in DMG concentration from 1.96 µmol/L to 1.54 µmol/L, between 9 and 16 weeks of gestation, respectively, in 50 West African pregnant women. A significant decrease in betaine and DMG concentrations in early pregnancy (i.e., <20 weeks of gestation) was also reported in a prospective cohort study with 522 Spanish pregnant women (64). The observed decrease in these metabolites may be partially related to the preferential transport of betaine and DMG from the mother to the fetus. Betaine and DMG concentrations at delivery were significantly higher in umbilical cord blood (21.1 µmol/L and 2.45 µmol/L, respectively) compared to maternal blood (9.52 µmol/L and 1.81 µmol/L, respectively) in Irish pregnant women (84). Similarly, cord blood betaine and DMG concentrations were two-fold higher than maternal betaine and DMG concentrations in Spanish pregnant women (64). Hemodilution may be a contributing factor in the decline of betaine and DMG during pregnancy, however, maternal betaine concentration was not associated with

hematocrit concentration in West African pregnant women (65). Other pregnancy-related physiologic changes may lead to the decline, such as the increase in the glomerular filtration rate (244) and hormonal changes (245,246). Betaine and DMG are excreted by urine and changes in renal clearance may promote betaine excretion. The decreased osmolarity in kidney during pregnancy (247) may also promote a lower resorption of betaine by the sodium- and chloridecoupled betaine/GABA transporter (248), thereby increasing the excretion of betaine. Hormonal changes such as the increase in estradiol can stimulate BHMT gene expression (245,246) and thereby the use of betaine as methyl donor, which may contribute to the decrease in maternal plasma betaine concentration during the first and second trimester of pregnancy. Additionally, it has been previously reported that the homocysteine remethylation rate is significantly higher in the third compared to the first trimester (249) likely due to an increased demand of cellular proliferation and differentiation that may also explain the decreasing betaine concentrations that we found between the first and second trimester. Thus, we hypothesize that the use of betaine in the remethylation pathway depends not only on the availability of betaine in circulation but also on methylation rates in each trimester of pregnancy.

South Asian pregnant women had significantly higher serum betaine and DMG concentrations compared to those of European ethnicity in this study. South Asians are the largest ethnic minority in Canada, and the second largest in Metro Vancouver, BC (99) The main dietary source of betaine are vegetables and roots such as spinach and beets, respectively. Given the retrospective study design, it was not possible to retrieve dietary intake data. However, in recent cross-sectional studies conducted in Metro Vancouver BC, it was observed an up to two-fold higher prevalence of vegetarianism in South Asian pregnant (100) and non-pregnant (250) young adult women, while the use of supplement was not significantly different between South Asian and other ethnicities included in these studies (100,250). South Asian adults in Vancouver were

reported to have increased their vegetable and fruit intake since their immigration to Canada (251). Also, higher betaine concentration might be related with a total dietary choline intake and subsequent choline oxidation, forming betaine. It has been shown that the administration of intravenous choline, in the form of choline chloride, in pigs significantly increased plasma, liver and kidney concentration of betaine (252). However, the intake of eggs, the main dietary source of choline, was 50% lower in South Asian pregnant women compared to other ethnicities which may suggest that dietary choline intake is not immediately oxidized to betaine after consumption (100).

Serum betaine concentration responds acutely to betaine intake in healthy adults (253). In rat liver tissue, betaine concentration and BHMT activity doubled within 3 days of a betaineenriched versus a betaine-choline-deficient diet (i.e., 0mg/kg) (254). A higher dietary betaine intake, and thereby increased BHMT activity, may lead to higher betaine and DMG concentrations, respectively. Additionally, an accumulation of serum betaine concentration may have also promoted higher serum DMG concentration, due to its inhibitory role on BHMT activity (255), as it was found in South Asian pregnant women. Current research is underway to investigate whether the differing metabolite concentrations, such as higher betaine, in South Asian women are related to dietary factors or genetic variants such as the common variant BHMT 742G>A (256). Although the BHMT 742G>A genetic variant has not been associated with differences in plasma concentrations of folate, free choline, betaine and tHcy through pregnancy and at delivery, pregnant women carrying the heterozygous BHMT GA or the homozygous BHMT AA genotypes had significantly lower DMG concentration in early pregnancy (<15 weeks of gestation) compared to those carrying the common BHMT GG genotype (97). Further pregnancy studies are needed to confirm the potential differences on BHMT by ethnicity and its influence on betaine and DMG concentration.

Maternal tHcy concentration was associated with serum betaine and total B-12 but not by folate concentrations across weeks of gestation in this cohort. In contrast, plasma betaine concentration was not associated with tHcy concentration in early pregnancy in Spanish or West African pregnant women (64,65), but was a strong predictor in late pregnancy (64–66) and under low methionine concentrations (66). In the Reus-Tarragona Birth Cohort Study, fasting plasma folate concentration was negatively associated with tHcy concentration across weeks of gestation and at labour in women with low folate status, defined as having plasma folate concentration below the median of 27.6 nmol/L (64), and in late pregnancy in women with high folate status. Betaine was associated with tHcy in late pregnancy and labour in women with low folate status, but only at labor in women with high folate status. Similarly, in a study conducted in 50 West-African pregnant women from a non-mandatory folic acid fortified population, fasting plasma folate concentration (geom. mean of 16.4 nmol/L) was a strong predictor of maternal tHcy concentration at 9 weeks of gestation, while betaine concentration was the strongest predictor only after 20 weeks of gestation (65).

Potential explanations for the discrepancies found between the results of this study (i.e., a significant association between betaine and tHcy concentrations) and the lack of association between betaine and tHcy concentrations at early pregnancy reported by previous studies, might be related to an interaction between the folate and betaine dependent methylation pathways (257,258). Rats fed a diet deficient in choline (i.e., 0.006% choline), the main precursor of betaine, had 31% lower hepatic folate concentration (258), whereas the folate deficient diet caused hepatic choline depletion after 2 weeks (259). In healthy adults, who had fasting median folate concentration ~ 12 nmol/L at baseline, folic acid supplementation ($< 800 \mu g/day$) increased plasma betaine concentration by $\sim 15\%$ after 12 weeks (98), which may suggest a sparing effect of betaine as methyl donor and promoting the use of folate for homocysteine remethylation. In

contrast, offspring of mice receiving a high folic acid diet (20 mg folic acid/kg diet) during pregnancy and lactation had 3-fold lower liver 5,10-methylenetetrahydrofolate reductase (MTHFR) protein levels (94). 5,10-MTHFR is the enzyme catalyzing the generation of 5-MTHF, the folate form required in homocysteine remethylation (260). In maternal liver of mice, messenger ribonucleic acid levels of MS, the enzyme catalyzing homocysteine remethylation using 5-MTHF, were decreased as a consequence of the high folic acid diet (94). In this study, a high non-fasting serum folate concentration of ~67 nmol/L was reported in both trimesters, which reflects a high folate status and/or folic acid intake, and may impair the folate-dependent methylation pathway, while diminishing the use of folate as methyl donor nutrient and promoting the use of betaine for the remethylation of homocysteine. High serum folate concentration reported in the current study may be explained by the high prevalence of prenatal supplement use in Canadian pregnant women and high dose of folic acid in commercially available products. Due to the retrospective design of this cohort study, we were unable to collect data on supplement use. Results from the Canadian Maternal Experiences Survey showed that 94% of pregnant women consume folic acid-containing prenatal supplements in the first three months of pregnancy, with >90% continuing until the end of pregnancy and/or lactation (206). The long duration of supplement use and high folic acid content, most commonly 1mg/d, throughout pregnancy (206), likely explains the high circulating folate concentrations in Canadian pregnant women (16).

This is the first study to assess betaine and DMG concentrations as well as the relationship between betaine and tHcy concentrations in South Asian pregnant women. Strengths of this study include the longitudinal approach, the large sample size, and the lack of consent bias. Due to the retrospective study design, the current study however is limited by the absence of maternal dietary intake information, and the lack of data on supplement use. Choline is the endogenous precursor of betaine; however, the biobanked specimen available was serum and given the

instability of free choline concentration in serum samples (261), we did not include maternal free choline data in our analyses. Also, the determination of tHcy concentration in serum samples requires coagulation time at room temperature. Plasma tHcy concentration increases by about 10% per hour at room temperature which may affect the interpretation of serum tHcy (262). However, tHcy concentration determined in the current study is comparable to previous pregnancy studies in Canadian pregnant women using plasma tHcy concentration (36), and also were lower than pregnancy studies using serum tHcy concentration in a non-mandatory folic acid fortified population (66).

In summary, South Asian pregnant women had significantly higher betaine and DMG concentration compared to European pregnant women. For first time, this study is showing that maternal betaine concentration is an important predictor of tHcy concentration at early pregnancy in a folate-replete population. Results of this study emphasized the key role of betaine at pregnancy, and may be used to potentially decrease the risk of adverse pregnancy outcomes associated with elevated tHcy concentrations. Further pregnancy studies in folate-replete populations including clinical outcomes are still needed. Additionally, further studies are needed to explain the ethnic differences and should include dietary intake and supplement use data, as well as determine genetic variants related to homocysteine and betaine metabolism, e.g., for BHMT, to understand potential underlying mechanisms.

Chapter 3: FIRST- AND SECOND-TRIMESTER MATERNAL METHYL NUTRIENT PATTERNS AND THEIR ASSOCIATION WITH NEONATAL ANTHROPOMETRIC OUTCOMES IN CANADIAN MOTHER-NEWBORN DYADS

Acknowledgments: All inferences, opinions, and conclusions drawn in this chapter are those of the author and do not reflect the opinions or policies of Perinatal Services British Columbia.

3.1 Introduction

Maternal adequacy in folate, riboflavin, vitamins B-12 and B-6, methionine, choline, and betaine – also commonly defined as methyl nutrients – is crucial for optimal fetal growth and development given the nutrients' interdependent roles in one-carbon metabolism (41,42) (Figure 1.1). Folate, in the form of 5-MTHF with B-12 acting as a cofactor, and betaine serve as methyl donors in the remethylation of homocysteine to methionine (41,48,59). Methionine is the precursor of the universal methyl donor, S-adenosylmethionine, which is vital for DNA methylation and cell proliferation.

DNA methylation is one of the most stable epigenetic mechanisms involved in fetal programming. Demethylation and remethylation, also known as *de novo* DNA methylation, of embryonic DNA strands occur in the first ten weeks of gestation – also referred to as the plasticity window (130,132) – and are vital for early growth and development, as well as for potential anatomical, endocrine and metabolic adaptions which may impact health conditions later in life (22,139). BW, BL and HC are key indicators of fetal growth (177). In the U.S.A, the results of the NEST study showed a negative association between low maternal RBC folate

concentration in early pregnancy (i.e., first RBC folate quartile at ~12 weeks of gestation) and methylation levels of the *IGF-2/H19* gene in cord blood, a well-known gene involved in fetal growth, which may suggest a critical role of maternal methyl nutrient concentrations in fetal growth programming by DNA methylation modifications.

Despite that findings from animal and human studies have indicated that the utilization of methyl donors (i.e., folate and betaine) for remethylation reactions depend on the availability of methyl donors as well as on coenzyme concentrations (i.e., B-12 for the folate-dependent reaction) (64,94,263), evidence of the interrelationship of maternal methyl nutrients in early pregnancy in human studies is lacking. Additionally, pregnancy studies have only investigated the association of individual methyl nutrients with neonatal anthropometric outcomes, which have revealed conflicting results. For example, in European women, low maternal plasma folate concentration (defined as folate concentration $\leq 9.1 \text{ nmol/L}$) at <15 weeks of gestation was negatively associated with BW and BL (28), while in Asian pregnant women, only late-pregnancy betaine, and not folate or B-12, concentration was negatively associated with BW, HC, BL, and subcutaneous adipose tissue in newborns (34,39).

Given the interdependent role of methyl nutrients in one-carbon metabolism, I sought, to my knowledge for the first time, to explore the interrelationship of maternal methyl nutrient and tHcy concentrations in the first and second trimester of pregnancy using a principal component analysis (PCA) approach. Also, in this study I explored the association between the derived maternal methyl nutrient pattern scores with neonatal anthropometric outcomes in Canadian mother-newborn pairs.

3.2 Study design and methods

This was a retrospective pregnancy cohort study for which bio-banked, non-fasting serum samples of 729 apparently healthy pregnant women, aged between 19-44 years who participated in the BC Prenatal Genetic Screening Program, Canada, between November 2014 and May 2016 were retrieved. Information about maternal samples collection, exclusion criteria, ethical approval and BC Prenatal Genetic Screening Program was provided in Chapter 2.2.

3.2.1 Maternal and neonatal information

3.2.1.1 Maternal data

Information on maternal age, weeks of gestation at sample collection and self-reported ethnicity (European or South Asian) was obtained from the BC Prenatal Genetic Screening Program. Data on pre-pregnancy maternal weight (kg), height (cm), parity (nulliparous/multiparous), delivery type (vaginal birth/caesarean section), hypertensive disorder of pregnancy (HTN) (yes/no) and GD (yes/no) were obtained from the BCPDR, Canada (239). Briefly, the BCPDR "contains data abstracted from obstetrical and neonatal medical records on nearly 100% of births in the province of BC from over 60 acute care facilities as well as births occurring at home attended by BC registered midwives, including women who had pregnancies ending in a live or still birth of at least 20 weeks of gestation or 500g BW".

Neighborhood income was estimated using the median family income of each pregnant woman's residential Forward Sortation Area (FSA) reported in the Canadian Census 2016 (22). The FSA of each pregnant woman was retrieved from the BCPDR. According to Statistics Canada, the FSA is defined as "a way to designate a geographical unit based on the first three characters in a Canadian postal code" (264). The FSA-median income was adjusted for the

average family size in each FSA, i.e., FSA median household income/√average number of FSA household members. The median neighborhood income adjusted for the average family size for all FSAs in BC were divided into quintiles and each pregnant woman was allocated to the corresponding quintile (99). Pre-pregnancy BMI [weight (kg)/height (m)²] was calculated using the pre-pregnancy body weight, or body weight collected before 11 weeks of gestation, and height. Nutritional status was classified using BMI according to the WHO criteria (240).

3.2.1.2 Neonatal data

Gestational age (GA) at birth (weeks), BW (kg), BL (cm), HC (cm), and neonatal sex were obtained from the BCPDR. Missing data on gestational age at birth, BW and sex from the BCPDR (n=8) was replaced by data available from the BC Newborn Screening Program, Canada. LBW was classified as BW <2,500 g (265). SGA, AGA and LGA were defined as BW <10th percentile, BW between 10th-90th percentile and BW >90th percentile, respectively, according to the Perinatal Services BC (PSBC) growth chart (266). The PSBC growth charts for BW are based on BC data registered by the Vital Statistics Agency between 1981-2000, whereas PSBC growth charts for BL and HC are based on BC data registered by the Vital Statistics Agency between 1995-2000. The Vital Statistics Agency registers all births that occur in BC. BW, BL and HC z-scores were derived from the PSBC growth charts (266). Furthermore, neonatal PI, BMI and BW/BL ratio were included as proxy indicators of neonatal body composition (183), which were calculated as follows: PI was calculated as BW (kg) divided by BL (m^3), whereas BW/BL was calculated as BW (kg) by BL (m). Neonatal BMI was calculated as BW (kg) divided by BL (m^2). In addition to the PSBC growth chart, BW z-scores using

Kramer's growth charts (267), which were developed based on Canadian population born between 1994-1996 (except for Ontario) was calculated.

3.2.2 Biochemical analyses

Non-fasting serum samples were collected using serum separator tubes. Details about sample collection, processing, aliquot preparation and freeze-thaw cycles have been described earlier (230).

3.2.2.1 Biomarker quantitation

Analytical methods used for serum tHcy, methionine, PLP, betaine, folate and total B-12 concentrations were described in Chapter 2.2.3. Riboflavin was quantified by isotope-dilution LC-MS/MS using a method modified by Midttun et al. (241). The intra- and inter-assay CVs% for riboflavin were estimated based on 14 batches and were <10%. Maternal serum holotranscobalamin (holoTC) and total B-12 were quantified by automated immunoassays (Architect by Abbott Laboratories and Access 2 by Beckman Coulter, respectively) at the pathology laboratories at St. Paul's Hospital and BC Children's Hospital, respectively. The mean (SD) concentration for the holoTC control samples run once per batch for three batches of study samples was 46 (1.5) pmol/l (CV: 3.3 %) for high and 6 (2.0) pmol/l (CV: 12 %) for low control samples, respectively.

3.2.3 Statistical analyses

Because of skewed distributions, descriptive statistics for biomarker and demographic variables are reported as median and IQR for continuous variables and as percentages (%) for categorical variables. A Wilcoxon signed-rank test was used to compare biomarker

concentrations between trimesters, while a Wilcoxon rank-sum test was used to compare biomarker concentration between pregnant women included in the PCA in the first and second trimester. Additionally, a Wilcoxon rank-sum test was also used to compare neonatal anthropometric outcomes by neonatal sex, maternal ethnicity, HTN, GD, and parity. A Kruskal-Wallis test and a Dunn-Bonferroni test as a post hoc test were used to compare neonatal anthropometric outcomes by neighborhood income quintile and pre-pregnancy BMI categories. Additionally, a chi-square test was used to compare birth status (preterm/term), delivery type, and the prevalence of SGA, AGA, and LGA by neonatal sex and maternal ethnicity.

3.2.3.1 Principal components analysis

Considering the importance of methyl nutrients for the provision of methyl groups for DNA methylation at specific time windows of pregnancy, e.g., for *de novo* DNA methylation reactions through interrelated reactions of the one-carbon metabolism, uncorrelated maternal methyl nutrient principal components for first- and second-trimester biomarker concentrations were derived separately, by conducting two PCAs. The derivation of the first (PCA 1) and second (PCA 2) trimester maternal methyl nutrient principal components involved several steps which are detailed as follows. First, maternal biomarkers were selected to be included in the PCAs. The selection of the maternal biomarkers was based on their role in related methylation reactions of one-carbon metabolism: folate, betaine and methionine, serve as methyl group donors, whereas B-12 participates as a coenzyme for the folate-dependent remethylation reaction, and PLP participates as a coenzyme in the catabolism of homocysteine, an intermediate metabolite of the remethylation reaction, as well as in the provision of one carbon units for the formation of 5-MTHF in one-carbon metabolism. Also, riboflavin concentration was included due to its role as a
precursor of FMN and FAD, both coenzymes in one-carbon metabolism, while holoTC was included because this is the B-12 form taken up by cells, also referred to as 'active B-12'. Given the role of homocysteine as an intermediate metabolite of methylation reactions in one-carbon metabolism, tHcy concentration was included in the PCAs. The PCAs were restricted to pregnant women with complete data for maternal biomarker concentrations, i.e., without missing observations. The sample size of complete data for first- and second-trimester biomarker concentrations was n=505 and n=393, respectively. The next step included the log-transformation of the maternal serum PLP, tHcy, total B-12, folate, betaine, methionine, holoTC, and riboflavin concentrations to improve the normality of the data, and the subsequent exploration of the correlation between the maternal biomarker concentrations at each trimester using a Pearson correlation test.

After this, the PCAs were conducted. The number of maternal methyl nutrient principal components derived equaled the number of variables included in each PCA (i.e., 8 maternal biomarker concentrations). The PCA enabled us to summarize 8 correlated maternal biomarker variables to uncorrelated maternal methyl nutrient principal components in each trimester, which were subjected to orthogonal rotation. After the derivation of the 8 maternal methyl nutrient principal components, the number of them to be retained was based on the examination of 1) Eigenvalues, which represents the variance of the data explained by each maternal methyl nutrient principal component using Kayser's rule (i.e., >1) (268), 2) the exploration of scree plots of the maternal methyl nutrient principal components for each trimester, which are the graphical representation of the percentages of variation that each maternal methyl nutrient principal component accounts for (Figure 3.1 and Figure 3.2) and 3) explained cumulative variance of the biomarker data). Retained maternal principal methyl nutrient principal components (i.e., >50% of the variance of the biomarker data). Retained maternal principal methyl nutrient components, henceforth referred to

maternal methyl nutrient patterns, were labeled according to the factor loadings, also known as the contribution or proportion of each biomarker to the retained component (i.e., |factor loadings| \geq 0.5 were considered to contribute significantly to each component) and overall interpretability. Finally, scores of the retained maternal methyl nutrients patterns were predicted for each pregnant woman.



Figure 3.1 Scree plot of eigenvalues of derived first-trimester principal components



Figure 3.2 Scree plot of eigenvalues of derived second-trimester principal components

Characteristics of mother-newborn pairs included in PCA 1 versus those included in PCA 2 were compared using Wilcoxon rank-sum test for continuous variables (i.e., maternal age, weeks of gestation, median of neighborhood income, GA at birth, BW, BL, HC, PI, neonatal BMI and BW/BL) and chi-square test for categorical variables (i.e., pre-pregnancy BMI categories, parity, ethnicity, neonatal sex, BW categories, mode of delivery, and prevalence of GD, HTN, preterm birth, and LBW).

Linear regression models were used to explore the association between the predicted scores of each retained maternal methyl nutrient pattern in each trimester, considered as the exposure variable, and neonatal anthropometric variables as outcome variables. Specifically, the models included as outcome variables: BW (Model 1), BL (Model 2), HC (Model 3), PI (Model 4), BW/BL (Model 5), and neonatal BMI (Model 6), BW z-score (Model 7), BL z-score (Model 8), and HC z-score (Model 9). For significant associations between retained maternal methyl nutrient patterns and neonatal anthropometric outcomes, additional regression models were used in each trimester to explore the association between log-transformed individual maternal methyl nutrient concentrations with the highest contribution (i.e., log-transformed individual methyl nutrient concentrations with |factor loadings| \geq 0.5 to each pattern) as the exposure variable, and neonatal anthropometric outcomes.

Based on results from previous studies (183,269–274), all models were adjusted, except Models 7-9, for maternal age, pre-pregnancy BMI, maternal height, GA at birth, and neighborhood income quintiles, parity, GD, HTN, maternal ethnicity and neonatal sex. Models 7-9 were not adjusted for GA at birth and neonatal sex, because the derivation of BW, BL and HC z-scores considers both variables. Because previous studies reported body composition and neonatal anthropometric outcomes to differ between preterm and term newborns (183), a sensitivity analysis was conducted which restricted the regression analysis to term newborns, excluding preterm newborns.

In the first trimester analysis (n=505), missing data for maternal variables (i.e., weeks of gestation at blood collection (n=6), GD (n=16), HTN (n=16), parity (n=16), and neighborhood median household income per year (n=17)), and neonatal variables (i.e., sex (n=15), BW (n=15), BW z-score (n=15), BL (n=24), BL z-score (n=24), HC (n=23), HC z-score (n=23), BW/BL (n=24), BMI (n=24), PI (n=24), GA at birth (n=15), preterm (n=15), and delivery type (n=16), represented <5% of the total sample size. In the second trimester analysis (n=393); maternal variables (i.e., GD (n=5), HTN (n=5), parity (n=5), and neighborhood median household income per year (n=6)), and neonatal variables (i.e., sex (n=6), BW (n=6), BW z-score (n=6), BL (n=13), BL z-score (n=13), HC (n=12), HC z-score (n=12), BW/BL (n=13), BMI (n=13), PI (n=13), GA at birth (n=6), preterm (n=6) and delivery type (n=5)) represented <4% of the total sample size. Given that in the first and second-trimester analysis, missing data for the aforementioned variables represented <5% of the data, missing observations were omitted, and no missing data categories were created.

For pre-pregnancy BMI, 23% (n=115) and 22% (n=87) of data were missing for women included in the first- and second trimester PCAs, respectively. Thus, an additional BMI category for missing data was created, coded as 999, to account for the missing BMI data in the regression analysis. Data for maternal height were missing for 16% (n=81) and 15% (n=57) of women included in PCA 1 and 2, respectively. Missing data for height were coded as 999, and an indicator variable with two categories (missing height data=0; height data available=1) was created. Regression models were run including maternal height as a continuous variable, as well as the indicator variable. Results were considered significant at P<0.05. All the statistical analyses were conducted using STATA version IC 15 (StataCorp). Graphs were made using R Studio version 3.5.1.

3.3 Results

3.3.1 Maternal characteristics

Median (IQR) maternal age was (28-34) y. Of all pregnant women, 49.5% of them were of European and 50.5% of South Asian ethnicity. First- and second-trimester blood sample collection visits occurred at 11.4 (10.7-12.3) and 16.1 (15.7-17.1) weeks of gestation, respectively. Fifty-one percent of women were nulliparous and 49% multiparous. The women's pre-pregnancy BMI indicated that 3.9% (n=22), 59.9% (n=334), 23.7% (n=132) and 12.5% (n=70) were underweight, healthy weight, overweight and obese, respectively, before entering pregnancy or at <11 weeks of gestation. During pregnancy, 15.8% were diagnosed with GD and 4.14% with HTN. The prevalence of GD was significantly higher in South Asian pregnant women (23.3%) compared to European pregnant women (8.3 %) (P<0.001).

3.3.1 Neonatal characteristics

Neonatal characteristics are presented in (Table 3.1). Most of the newborns were born at term and were AGA. Female newborns had a lower BW and BL, and had a smaller HC compared to males (P<0.05 for all). Newborns from South Asian pregnant women had lower BW, BW *z*-score, BL, BL *z*-score, and smaller HC and HC *z*-score (P<0.05), while newborns from mothers of European ethnicity had higher PI and BW/BL ratio compared to newborns of South Asian women (P<0.001 for all). Additionally, the prevalence of SGA was higher in newborns of South Asian women, while LGA prevalence was higher in newborns of European women (P<0.001 for all). BW of newborns from pregnant women with GD [3239 (3024-3575) g] or HTN [3130 (3005-3620) g] was lower compared to those newborns of pregnant women without GD [3403 (3102-3703) g] or HTN [3386 (3100-3700) g] (both P<0.05). Newborns of pregnant women with GD had lower BMI [12.6 (11.9-13.5) vs. 12.8 (12.0-13.8) kg/m³] and BW/BL ratio [6.41 (6.02-7.0) vs. 6.60 (6.17-7.14) kg/m] compared to newborns of non-GD pregnant women. Additionally,

within European or South Asian pregnant women, BW of newborns from GD-pregnant women did not significantly differ, compared to those newborns from non-GD-pregnant women (P>0.05).

Pregnant women with HTN had shorter newborns [50 (49-52) vs. 51 (50-53) cm] compared to those not reported to have HTN (P<0.05). Newborns from mothers with prepregnancy obesity had higher BW z-score, HC and HC z-score compared to newborns of women healthy weight or underweight before pregnancy (P<0.05). Newborns of pregnant women who lived in a neighborhood with a median neighborhood income in the lowest quintile had a lower BW, BW z-score, BL, BL z-score, BMI, BW/BL ratio and PI compared to newborns of pregnant women who lived in a neighborhood with a median neighborhood income in the 4th or 5th quintiles (P<0.05).

3.3.2 Maternal methyl nutrient concentrations

The sample sizes for maternal methyl nutrient data are shown in Figure 3.3. Missing methyl nutrient data resulted from insufficient serum volume to quantify the metabolite. Serum PLP, betaine, methionine, riboflavin, total B-12, holoTC and tHcy concentrations significantly decreased from the first to the second trimester (P<0.05). In contrast, serum folate concentration increased (P<0.05) (Table 3.2).

Neonatal	n	All	n	Female	n	Male	n	European	n	South Asian	P ²	P ³
characteristics												
Birth status, %	699		348		351		350		349		0.38	0.22
Term		93.4		94.3		92.6		94.6		92.3		
Preterm		6.58		5.75		7.40		5.43		7.74		
Delivery type, %	701		343		349		349		352		0.08	0.003
Vaginal birth		61.3		64.1		57.6		66.8		56.0		
Caesarean section		38.7		35.9		42.4		33.2		44.0		
GA at birth, weeks	699	39.0	348	39.0	351	39.0	350	39.0	349	39.0	0.69	0.0003
		(38.0-40.0)		(38.0-40.0)		(38.0-40.0)		(38.0-40.0)		(38.0-40.0)		
BW, g	700	3380	349	3322	351	3424	350	3540	350	3244	0.006	< 0.001
		(3086-3691)		(3070-3652)		(3115-3735)		(3240-3790)		(3005-3525)		
LBW, %	20	2.86	10	2.87	10	2.85	<5	≤1.43	18	5.14	0.99	< 0.001
BW z-score	699	0.05	348	0.09	351	0.005	350	0.32	349	-0.17	0.52	< 0.001
		(-0.58-0.68)		(-0.54-0.66)		(-0.61-0.71)		(-0.31-0.96)		(-0.77-0.41)		
BL, cm	688	51.0	341	51.0	347	52.0	346	52.0	342	51.0	< 0.001	< 0.001
		(50.0-53.0)		(50.0-52.0)		(50.0-53.0)		(50.0-53.0)		(50.0-52.0)		
BL z-score	688	0.10	341	0.04	347	0.10	346	0.15	342	0.003	0.80	0.003
		(-0.31-0.50)		(-0.27-0.54)		(-0.31-0.50)		(-0.27-0.61)		(-0.37-0.42)		
HC, cm	689	35.0	342	35.0	347	35.0	346	35.0	343	35.0	< 0.001	< 0.001
		(34.0-36.0)		(34.0-35.0)		(34.0-36.0)		(34.0-36.0)		(34.0-36.0)		
HC z-score	689	0.23	342	0.23	347	0.25	346	0.25	343	0.17	0.92	0.04
		(-0.19-0.61)		(-0.06-0.54)		(-0.21-0.67)		(-0.15-0.71)		(-0.21-0.54)		
PI, kg/m ³	688	24.9	341	25.1	347	24.8	346	25.4	342	24.5	0.26	< 0.001
		(23.3-26.8)		(23.3-27.4)		(23.3-26.6)		(23.8-27.5)		(22.9-26.1)		
BW/BL ratio, kg/m	688	6.56	341	6.52	347	6.61	346	6.82	342	6.37	0.08	< 0.001
		(6.14-7.09)		(6.06-7.04)		(6.19-7.18)		(6.36-7.25)		(5.96-6.85)		
BMI, kg/m^2	688	12.8	341	12.8	347	12.8	346	13.1	342	12.6	0.66	< 0.001
		(11.9-13.7)		(11.9-13.8)		(12.1-13.7)		(12.3-14.0)		(11.7-13.3)		
SGA, %	699	5.29	348	5.46	351	5.13	350	3.43	349	7.16	0.94	< 0.001
AGA, %		83.7		83.9		83.4		81.7		85.7		
LGA, %		11.0		10.6		11.4		14.9		7.16		

Table 3.1 Newborn characteristics¹

¹Values are median (IQR) for continuous variables or prevalence (%) for categorical variables; *P*² are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for categorical variables by neonatal sex; *P*³ are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for categorical variables by maternal ethnicity. AGA, appropriate for gestational age; BL, birth length; BMI, body mass index; BW, birth weight; BW/BL, weight-to-length ratio; GA, gestational age; HC, head circumference; LGA, large for gestational age; LBW, low birth weight; PI, ponderal index; SGA, small for gestational age.



Figure 3.3. Flowchart showing serum samples analyzed and sequence of biomarkers determined for each trimester of pregnancy HoloTC, holotranscobalamin; tHcy, total homocysteine; PLP, pyridoxal 5'-phosphate; NSQ, no sufficient quantity.

	All						РСА				
Serum concentrations	n	First trimester	n	Second trimester	P ²	n	First trimester	n	Second trimester	P ³	
Folate, <i>nmol/L</i>	658	66.8 (53.2-81.3)	680	69.4 (55.3-87.6)	0.03	505	66.7 (51.9-81.4)	393	67.9 (53.4-86.8)	0.19	
PLP, nmol/L	577	90.1 (50.8-176.0)	499	66.4 (43.2-121.0)	< 0.001	505	93.4 (51.8-181)	393	66.4 (42.7-124)	< 0.001	
Riboflavin, nmol/L	579	22.0 (16.6-32.4)	499	21.4 (15.5-32.7)	0.02	505	22.0 (16.6-32.6)	393	21.2 (15.5-33.2)	0.11	
Betaine, µ <i>mol/L</i>	591	18.8 (15.0-23.9)	580	14.5 (11.5-18.1)	< 0.001	505	18.9 (15.0-24.1)	393	14.5 (11.4-18.3)	< 0.001	
Methionine, nmol/L	612	22.1 (19.3-25.4)	581	21.2 (18.4-24.7)	0.002	505	22.1 (19.2-25.4)	393	21.2 (18.4-25.0)	0.03	
Total B-12, pmol/L	668	218 (157-297)	723	199 (146-271)	< 0.001	505	218 (159-297)	393	209 (151-279)	0.04	
HoloTC, pmol/L	656	83.6 (60.3-115.2)	634	77.9 (54.9-107.7)	< 0.001	505	84 (60.4-117)	393	81.0 (55.5-109)	0.07	
tHcy, µmol/l	612	5.0 (4.5-5.9)	581	4.4 (3.8-5.1)	< 0.001	505	5.0 (4.5-5.7)	393	4.38 (3.76-5.03)	< 0.001	

Table 3.2 First and second-trimester methyl nutrient and related metabolite concentrations of apparently healthy Canadian pregnant women in the total sample and in those women included in the first or second-trimester PCA¹

¹Values are median (IQR). *P*²values are from Wilcoxon signed-rank test; *P*³ values are from Wilcoxon rank-sum test. B-12, vitamin B-12; HoloTC, holotranscobalamin; PCA, principal component analysis; PLP, pyridoxal-5'-phosphate; tHcy, total homocysteine.

3.3.3 Maternal methyl nutrient patterns

After conducting the PCAs, 3 maternal methyl nutrient patterns were retained for each trimester of pregnancy (Table 3.3) (Figure 3.4 and Figure 3.5). First-trimester maternal methyl nutrient patterns explained 57% of the variation of the biomarker data, whereas second-trimester maternal methyl nutrient patterns explained 59% of the variation of the biomarker data. In the first trimester, maternal methyl nutrient patterns were labeled according the highest biomarker contributor of each retained component as follows 1) B-12 pattern (in this case, total B-12 and holoTC concentrations), 2) methyl donor/riboflavin pattern (including folate, betaine as well as riboflavin concentrations), and 3) methionine pattern (i.e., methionine concentration). The B-12 pattern score tended to increase with increasing log-transformed total B-12 and holoTC, which were the highest contributors for the B-12 pattern. Similarly, the methyl donor/riboflavin pattern score showed an increasing trend with increasing log-transformed folate, betaine and riboflavin that were the most important contributors for methyl donor/riboflavin pattern. The methionine pattern score increased with increasing log-transformed metholone.

In the second trimester, maternal methyl nutrient patterns were characterized primarily by 1) B-12 pattern (in this case, total B-12, holoTC and tHcy concentrations), 2) B-6/riboflavin pattern (i.e., PLP and riboflavin concentrations), and 3) methyl donor pattern (including betaine and methionine concentrations). Similar to the results of the first trimester, the B-12 pattern score showed an increasing trend as log total B-12 and log holoTC increased, and a decreasing trend with increasing log tHcy concentration. The B-6/riboflavin pattern score showed also an increasing trend with increasing log-transformed B-6 and riboflavin, whereas the methyl donor pattern score tended to increase with increasing log-transformed betaine and methionine.

Maternal age, pre-pregnancy BMI, prevalence of GD and HTN, ethnicity, parity, neighborhood median income, prevalence of preterm birth, delivery type, GA at birth, neonatal sex, and neonatal anthropometric outcomes did not differ between mother-newborn pairs included in the first-trimester versus the second-trimester PCA (Table 3.4 and Table 3.5).

First trimester	B-12 pattern ¹	Methyl donor/riboflavin	Methionine pattern ³
		pattern ²	
Variance explained	25.7%	16.7%	14.5%
Folate	-0.0096	0.6163	-0.1337
PLP	0.2333	0.1865	0.3594
Total B-12	0.5913	0.0052	0.0657
Riboflavin	0.0573	0.5411	0.2844
Methionine	0.0013	-0.0734	0.7624
tHcy	-0.4697	0.0556	0.3833
HoloTC	0.6048	-0.0056	0.0384
Betaine	-0.0790	0.5330	-0.1952
Second trimester	B-12 pattern ⁴	B6/riboflavin pattern⁵	Methyl donor pattern ⁶
Variance explained	25.1%	18.2%	15.5%
Folate	0.0909	0.4323	-0.1669
PLP	0.1102	0.5140	0.1268
Total B-12	0.5772	0.0736	-0.0138
Riboflavin	-0.0715	0.7129	0.0012
Methionine	0.0335	-0.0036	0.6953
tHcy	-0.5107	0.1866	-0.0187
HoloTC	0.6150	0.0140	0.0001

Table 3.3. Loadings of methyl nutrients for the first and second-trimester maternal methyl nutrient patterns

¹First trimester B-12 pattern refers to maternal total B-12 and holoTC loadings;²Methyl donor/riboflavin pattern refers to maternal folate, riboflavin and betaine loadings;³Methionine patters refers to maternal methionine loadings; ⁴Second trimester B-12 pattern refers to maternal total B-12 and holoTC, and tHcy loadings;⁵B6-riboflavin pattern refers to maternal PLP and riboflavin loadings;⁶Methyl donor pattern refers to maternal methionine and betaine loadings. B-12, vitamin B-12; HoloTC, holotranscobalamin; PLP, pyridoxal-5'-phosphate; tHcy, total homocysteine.



Figure 3.4. Retained maternal methyl nutrient patterns in the first trimester B-2, riboflavin; HoloTC, holotranscobalamin; PLP, pyridoxal 5' phosphate; tHcy, total homocysteine.



Figure 3.5. Retained maternal methyl nutrient patterns in the second trimester B-2, riboflavin; HoloTC, holotranscobalamin; PLP, pyridoxal 5' phosphate; tHcy, total homocysteine.

]	First trimester PCA	S	econd trimester PCA		
Maternal characteristics	n		n		P	
Age, y	505	31.0 (28.0-34.0)	393	31.0 (28.0-35.0)	0.45	
Weeks of gestation	499	11.4 (10.7-12.3)	393	16.3 (15.7-17.1)	< 0.001	
Pre-pregnancy BMI, kg/m ²	390	23.5 (21.2-26.9)	306	23.4 (21.2-26.4)	0.55	
BMI categories,					0.94	
Underweight, % (BMI)	14	3.59 (17.8; 16.8-18.0)	12	3.92 (17.7; 17.1-17.9)		
Healthy weight, % (BMI)	231	59.2 (21.8; 20.6-23.4)	189	61.8 (21.9; 20.7-23.4)		
Overweight, % (BMI)	95	24.4 (26.9; 26.0-28.3)	71	23.2 (26.7; 26.0-28.3)		
Obesity, % (BMI)	50	12.8 (33.3; 31.4-36.4)	34	11.1 (33.1; 31.1-36.3)		
GD, %	72	14.7	63	16.2	0.54	
HTN, %	22	4.50	18	4.60	0.92	
Ethnicity, %	505		393		0.67	
European	263	52.1	199	50.6		
South Asian	242	47.9	194	49.4		
Parity, %	488		388		0.73	
Nulliparous	254	51.9	197	50.8		
Multiparous	235	48.1	191	49.2		
Median neighborhood income per year, CAD	488	51,217 (45,066-55,370)	387	51,439 (43,778-55,370)	0.99	

Table 3.4. Characteristics of apparently healthy Canadian pregnant women included in first or second trimester PCA^1

¹Values are medians (IQR) for continuous variables or prevalence (%) for categorical variables. *P* values are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for categorical variables between pregnant women included in first and second trimester PCA analysis. Pre-pregnancy BMI categories; underweight (BMI <18.5 kg/m²), healthy weight (BMI ≥18.5 to 24.9 kg/m²), overweight (BMI ≥25 to 29.9 kg/m²) and obese (BMI ≥30 kg/m²). BMI, body mass index; GDM; gestational diabetes; HTN, hypertensive disorder of pregnancy; PCA, principal component analysis.

	First trimester PCA			Second trimester PCA	
Neonatal characteristics	n		n		P
Ethnicity, %					
European	263	52.1	199	50.6	0.67
South Asian	242	47.9	194	49.4	
Neonatal sex, %					
Female	245	50.0	181	46.8	0.34
Male	245	50.0	206	53.2	
Birth status, %	490		387		0.71
Term		92.7		93.3	
Preterm		7.4		6.72	
Delivery type, %	489		388		0.91
Vaginal birth		62.0		38.4	
Caesarean section		38.0		61.6	
GA at birth, weeks	490	39.0 (38.0-40.0)	387	39.0 (38.0-40.0)	0.62
BW, g	490	3380 (3075-3682)	387	3390 (3111-3715)	0.26
LBW, %	14	2.86	11	2.84	0.99
BW, z-score	490	0.06 (-0.53-0.66)	387	0.16 (-0.52-0.71)	0.38
BL, cm	481	51.0 (50.0-53.0)	380	51.0 (50.0-53.0)	0.22
BL, z-score	481	0.05 (-0.32-0.49)	380	0.10 (-0.28-0.54)	0.31
HC, cm	482	35.0 (34.0-36.0)	381	35.0 (34.0-36.0)	0.11
HC z-score	482	0.23 (-0.21-0.60)	381	0.25 (-0.15-0.61)	0.29
PI, kg/m ³	481	25.0 (23.3-26.8)	380	25.0 (23.2-26.6)	0.99
BW/BL, kg/m	481	6.57 (6.12-7.08)	380	6.57 (6.19-7.14)	0.36
BMI, kg/m ²	481	12.8 (11.9-13.7)	380	12.9 (12.0-13.7)	0.69
SGA, %	27	5.51	17	4.40	0.54
AGA, %	415	84.7	325	83.9	
LGA, %	48	9.80	45	11.6	

Table 3.5 Characteristics of newborns of pregnant women included in the first or second trimester PCA $^{\rm 1}$

¹Values are median (IQR) for continuous variables or prevalence (%) for categorical variables. *P* values are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for categorical variables between newborns included in first and second trimester PCA analysis. AGA, appropriate for gestational age; BL, birth length; BMI, body mass index; BW, birth weight; BW/BL, birth weight-to-length ratio; GA, gestational age; HC, head circumference; LGA, large for gestational age; LBW, low birth weight; PCA, principal component analysis; PI, ponderal index; SGA, small for gestational age.

3.3.4 Association between first-trimester maternal methyl nutrient pattern scores and neonatal anthropometric outcomes

The first-trimester maternal methyl nutrient pattern scores were not significantly associated with neonatal anthropometric outcomes in this sample of term and preterm newborns (Table 3.6). After the exclusion of preterm newborns (n=36), however, a 1-unit increase in score of the methyl donor/riboflavin pattern, which mainly reflects increasing maternal betaine, riboflavin and folate concentrations, was associated with a decrease of 0.18 cm and 0.06 in BL and BL z-score, respectively (P < 0.05 for all). Among the biomarkers with the highest contribution for the methyl donor/riboflavin pattern (i.e., factor loadings $\geq |0.5|$), only maternal log-transformed betaine was negatively associated with BL [(β =-0.53; 95%CI -1.04, -0.02, P=0.04). Additionally, maternal log-transformed betaine was weakly associated with BL z-score $(\beta = -0.17, 95\%$ CI -0.33, -0.01; P = 0.03) after adjustment for confounding factors. No significant association was found between log folate or log riboflavin and BL or BL z-score, after controlling for confounding variables. Consistent with findings obtained using BW z-score calculated from PSBC growth charts, BW z-score calculated using Kramer's growth chart was not associated with maternal methyl nutrient patterns (P < 0.05) before or after the exclusion of preterm newborns (Table 3.7)

3.3.5 Association between second-trimester maternal methyl nutrient pattern scores and neonatal anthropometric outcomes

In the second trimester, only the B-12 pattern score, but not methyl nutrient donor and B-6/ riboflavin pattern scores, was associated with HC and HC z-score in the complete sample of newborns, including term and preterm newborns. The 1-unit increase in score of the B-12 pattern, which mainly reflects decreasing tHcy and increasing holoTC and B-12 concentrations, was associated with a decrease of 0.13 cm in HC and 0.05 in HC z-score, after adjustment for confounding factors ($P \le 0.05$ for all) (Table 3.6) Among the biomarkers with a higher contribution to the B-12 pattern, only log total B-12 was negatively associated with HC (β =-0.43; 95%CI -0.77, -0.08, P=0.02) and HC z-score (β =-0.16; 95%CI -0.31, -0.02, P=0.03). The exclusion of mother-newborn pairs with pretern birth (n=26) did not significantly modify the association between B-12, methyl donor and riboflavin/B-6 patterns and neonatal anthropometric outcomes. Additionally, BW z-score calculated using Kramer's growth chart was not associated with second-trimester maternal methyl nutrient patterns before or after the exclusion of preterm (Table 3.7)

	BW BW z-score		e	BL		BL z-score		НС		HC z-score		
First Trimester	β (95%CI)	P ¹	β (95%CI)	P^2	β (95%CI)	P^1	β (95%CI)	P^2	β (95%CI)	P^1	β (95%CI)	P^2
B-12 pattern ³	-22.7 (-47.5,1.98)	0.07	-0.05 (-0.11,0.005)	0.07	-0.12 (-0.26,0.01)	0.07	-0.04 (-0.08,0.005)	0.09	-0.03 (-0.12,0.07)	0.58	-0.002 (-0.04,0.04)	0.93
Methyl donor/ riboflavin	-23.4 (-53.7,6.89)	0.13	-0.06 (-0.13,0.009)	0.09	-0.13 (-0.30,0.03)	0.11	-0.05 (-0.09,0.003)	0.07	0.05 (-0.06,0.17)	0.38	0.02 (-0.03,0.07)	0.34
Methionine pattern ⁵	-9.26 (-23,4.16)	0.57	0.01 (-0.06,0.09)	0.71	0.13 (-0.05,0.31)	0.16	0.03 (-0.02,0.09)	0.23	0.02 (-0.10,0.14)	0.73	-0.0002 (-0.05,0.05)	0.99
Second Trimester	β (95%CI)	P ¹	β (95%CI)	P^2	β (95%CI)	P ¹	β (95%CI)	P^2	β (95%CI)	P^1	β (95%CI)	P^2
B-12 pattern ⁶	-17.8 (-47.1,11.5)	0.23	-0.04 (-0.11,0.02)	0.21	-0.13 (-0.28,0.03)	0.11	-0.04 (-0.08,0.01)	0.15	-0.13 (-0.24, -0.03)	0.019	-0.05 (-0.09, -0.009)	0.02^{10}
B6/ riboflavin pattern ⁷	9.55 (-24.2,43.3)	0.58	0.02 (-0.06,0.09)	0.67	-0.09 (-0.27,0.09)	0.35	-0.03 (-0.09,0.02)	0.24	0.05 (-0.08,0.17)	0.46	0.01 (-0.04,0.06)	0.62
Methyl donor pattern ⁸	8.13 (-28.9,45.1)	0.67	0.001 (-0.08,0.08)	0.99	-0.11 (-0.31,0.08)	0.26	-0.04 (-0.09,0.02)	0.20	0.04 (-0.09,0.18)	0.54	0.03 (-0.03,0.08)	0.34

Table 3.6 Associations of maternal methyl nutrient patterns in the first and second trimester of apparently healthy Canadian pregnant women with neonatal anthropometric outcomes

 P^1 value is from linear regression model for the corresponding coefficient. Models adjusted for maternal age, gestational age at birth, pre-pregnancy BMI, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal ethnicity, neonatal sex and parity. P^2 value is from linear regression model for the corresponding coefficient. Models adjusted for maternal age, pre-pregnancy BMI, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal ethnicity and parity.³B-12 pattern refers to maternal B-12 and holoTC loadings; ⁴Methyl donor/riboflavin pattern refers to maternal folate, riboflavin and betaine loadings; ⁵Methyl donor pattern refers to maternal methionine loadings; ⁶B-12 pattern refers to maternal B-12, holoTC and tHcy loadings; ⁷B-6/riboflavin pattern refers to maternal PLP and riboflavin loadings; ⁸Methyl donor pattern refers to maternal betaine and methionine loadings.⁹Adjusted R²=0.25; ¹⁰Adjusted R²=0.07. B-6, vitamin B-6; B-12, vitamin B-12; BL, birth length; BMI, body mass index; BW, birth weight; BW/BL, birth weight-to-length ratio; CI, confidence interval; HC, head circumference; PI, ponderal index. Median (IQR) neighborhood income, 1st q

	BW/BL		B	MI	Ι	PI		
First Trimester	β (95%CI)	P^1	β (95%CI)	P^1	β (95%CI)	<i>P</i> ¹		
B-12 pattern ²								
	-0.03	0.19	-0.03	0.49	-0.009	0.92		
	(-0.07,0.01)		(-0.12,0.06)		(-0.20,0.18)			
Methyl donor/riboflavin								
pattern ³	-0.03	0.31	-0.02	0.71	0.02	0.86		
	(-0.08,0.03)		(-0.12,0.09)		(-0.22,0.26)			
Methionine pattern ⁴	0.006	0.82	-0.01	0.83	-0.08	0.56		
	(-0.05,0.06)		(-0.12,0.09)		(-0.33,0.18)			
Second Trimester	β (95%CI)	P^1	β (95%CI)	P^1	β (95%CI)	P^1		
B-12 pattern ⁵	-0.02	0.34	-0.02	0.67	0.01	0.90		
	(-0.08,0.03)		(-0.12,0.07)		(-0.20,0.23)			
B6/riboflavin pattern ⁶	0.03	0.27	0.09	0.12	0.22	0.08		
	(-0.03,0.09)		(-0.02,0.20)		(-0.03,0.47)			
Methyl donor pattern ⁷	0.03	0.39	0.07	0.25	0.19	0.18		
	(-0.04,0.09)		(-0.05,0.20)		(-0.08,0.46)			

Table 3.6 Associations of maternal methyl nutrient patterns in the first and second trimester of apparently healthy Canadian pregnant women with neonatal anthropometric outcomes (con)

 P^{1} value is from linear regression model for the corresponding coefficient. Models adjusted for maternal age, gestational age at birth, pre-pregnancy BMI, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal ethnicity, neonatal sex and parity. BMI, body mass index..²B-12 pattern refers to maternal B-12 and holoTC loadings; ³Methyl donor/riboflavin pattern refers to maternal folate, riboflavin and betaine loadings;⁴Methionine pattern refers to maternal methionine loadings;⁵B-12 refers to maternal B-12, holoTC and tHcy loadings;⁶B-6/riboflavin refers to maternal PLP and riboflavin loadings;⁷Methyl donor refers to maternal betaine and methionine loadings. B-6, vitamin B-6; B-12, vitamin B-12; BL, birth length; BMI, body mass index; BW, birth weight; BW/BL, weight-to-length ratio; CI, confidence interval; HC, head circumference; PI, ponderal index. Median (IQR) neighborhood income, 1st quintile: \$ 41,116 (\$ 40,386-\$ 42,062) CAD; 2nd quintile: \$ 49,294 (\$ 47,641-\$ 50,586) CAD; 3rd quintile: \$ 53,218 (\$ 52,143-\$ 54,713) CAD; 4th quintile: \$ 57,107 (\$ 56,456-\$ 59,251) CAD; 5th quintile: \$ 65,716 (\$ 63,478-\$ 68,554) CAD.

Table 3.7 Associations of maternal methyl nutrient patterns in the first and second trimester of apparently healthy Canadian pregnant women with neonatal anthropometric outcomes using Kramer's growth charts

	BW z-score		BW z-score	
First trimester	β (95%CI)	P^1	β (95%CI)	P^2
B-12 pattern ³	-0.05 (-0.11,0.003)	0.07	-0.05 (-0.11,0.009)	0.10
Methyl donor/riboflavin pattern ⁴	-0.06 (-0.13,0.007)	0.08	-0.07 (-0.14,0.003)	0.06
Methionine pattern ⁵	0.02 (-0.06,0.09)	0.70	0.02 (-0.06,0.09)	0.65
Second trimester	β (95%CI)	P ¹	β (95%CI)	P^2
B-12 pattern ⁶	-0.04 (-0.11,0.02)	0.20	-0.03 (-0.09,0.04)	0.40
B6/riboflavin pattern ⁷	0.02 (-0.06,0.09)	0.67	0.03 (-0.05,0.11)	0.49
Methyl donor pattern ⁸	-0.002 (-0.09,0.08)	0.95	0.003 (-0.08,0.09)	0.95

 P^{1} value is from linear regression model for the corresponding coefficient. ¹z-score calculated using Kramer's growth chart; ²z-scores calculated using Kramer's growth chart including only full-term newborns. Models adjusted for maternal age, pre-pregnancy BMI, maternal height, gestational diabetes, hypertensive disorder of pregnancy, neighborhood income quintiles, maternal ethnicity and parity. ³B-12 pattern refers to maternal total B-12 and holoTC loadings, ⁴Methyl donor/riboflavin pattern refers to maternal folate, betaine, and riboflavin loadings; ⁵Methionine pattern refers to maternal methionine loadings; ⁶B-12 pattern refers to maternal total B-12, holoTC and Hcy loadings; ⁷B6/riboflavin pattern refers to maternal PLP and riboflavin loadings; ⁸Methyl donor pattern refers to maternal methionine and betaine loadings; BW, birth weight; CI, confidence interval; Median (IQR) neighborhood income, 1st quintile: \$ 41,116 (\$ 40,386-\$ 42,062) CAD; 2nd quintile: \$ 49,294 (\$ 47,641-\$ 50,586) CAD; 3rd quintile: \$ 53,218 (\$ 52,143-\$ 54,713) CAD; 4th quintile: \$ 57,107 (\$ 56,456-\$ 59,251) CAD; 5th quintile: \$ 65,716 (\$ 63,478-\$ 68,554) CAD.

3.4 Discussion

To the best of my knowledge, this is the first study to describe first and second-trimester maternal methyl nutrient patterns using a PCA approach, and to explore the association between maternal methyl nutrient patterns and neonatal anthropometric outcomes.

Maternal methyl nutrient concentrations. First- and second-trimester serum betaine concentrations (18.8 and 14.5 μ mol/L) in this sample of 723 pregnant women were similar to fasting plasma betaine concentrations reported in Spanish (~21 and ~15 μ mol/L at <12 and 15 weeks of gestation, respectively) (64,275) and Canadian pregnant women (13 μ mol/L at 16 weeks of gestation) (36). First- and second-trimester serum holoTC concentrations (83.6 and 77.9 pmol/L) were also comparable to those reported in Spanish pregnant women (86.0 and 72.4 pmol/L at <12 and 15 weeks of gestation, respectively) (64,275). Serum total B-12 concentration was similar to findings of other pregnancy cohorts in Canada, especially when comparing total B-12 concentration of European pregnant women in this study (247 and 224 pmol/L in first and second trimester, respectively) with cohorts that mostly included European women in Vancouver (259 pmol/L at 16 weeks of gestation) (36) and Toronto (219 pmol/L at 12-16 weeks of gestation) (72).

Serum tHcy concentrations in both trimesters (5.0 and 4.4 μ mol/L) were also comparable to plasma tHcy concentration reported for Vancouver pregnant women at 16 weeks of gestation (4.1 μ mol/L) (36). In contrast, serum PLP concentration in this current study (90.1 and 69.4 nmol/L) was lower compared to pregnant women from Toronto (107 nmol/L at 12-16 weeks of gestation) (71), while serum folate concentration in both trimesters (~68 nmol/L) was higher compared to those reported in previous pregnancy studies conducted in European and Asian countries (28,34,39,65). Serum riboflavin concentration (~22 nmol/L) was almost two-fold

higher than that in non-fasting plasma samples at 18 gestational weeks (11.8 ± 0.26 nmol/L) of pregnant women in the Norwegian Mother and Child Cohort Study (MoBa) (276). Samples analyzed in the current study were collected under non-fasting conditions, which may partially explain the discrepancies found in biomarker concentrations. Recent dietary intake of folate and riboflavin rich foods or supplements acutely raise circulating plasma concentrations of these metabolites (16,277–279). Information on supplement use was not available in this retrospective study. Previous pregnancy cohort studies in Canada reported about 90% of pregnant women to consume a folic acid containing multivitamin-multimineral prenatal supplement in the first and second trimesters (68,71). A major contributor to the higher serum folate concentration in the current study is likely the fact that most prenatal supplements contain folic acid doses of \geq 1000µg (206) as well as the extended use of folic acid containing supplements throughout pregnancy until post-partum (11) in Canada. Results of the MoBa study showed 36% of women consumed folic acid supplements in the first 4 months of pregnancy, whereas 31% consumed multivitamin-minerals supplements (280). The higher prevalence of prenatal supplement use and the mandatory flour fortification with riboflavin in Canada, may also explain the higher serum riboflavin concentration observed in this study. Additionally, differences in analytical methods used to determine PLP concentration (i.e., LC-MS/MS and nonradioactive enzymatic assay) may also explain the differences that we found between PLP concentration of the current study and PLP concentration reported in the PREFORM study (281).

First and second-trimester maternal methyl nutrient patterns. Of the maternal methyl nutrient patterns, the B-12 patterns explained the highest variation of the first (26.7%) and second trimester (25.1%) biomarker data, with log total B-12 and log holoTC as the main positive contributors to the first-trimester B-12 pattern. The derivation of the B-12 pattern is likely explained by the stronger correlation between first-trimester log B-12 and holoTC (r=0.70,

P<0.001), compared to correlations among other methyl nutrients included in PCA 1. Similar results between the B-12 biomarkers was reported in first-trimester Spanish pregnant women (r=0.62, P<0.001) (38). Despite that the loading of log tHcy to the B-12 pattern was slightly below the cut-off used to characterize the pattern (i.e., |0.5|), this biomarker still had a meaningful and negative loading to the B-12 pattern (factor loading: -0.47), and it was correlated with total B-12 (r=-0.30) and holoTC (r=-0.35) (P<0.001 for both), which is expected because serum tHcy is a functional B-12 indicator, and a specific biomarker of B-12 status in folate-replete populations such as the Canadian population (47).

Similarly, in the second trimester, log B-12 and log holoTC were positive contributors, while log tHcy was a negative contributor to the B-12 pattern. Also, these results are explained by the stronger correlation of the B-12 biomarkers (total B-12 and holoTC: r=0.68; tHcy and B-12: r=-0.30; tHcy and holoTC: r=-0.39) (*P*<0.001 for all). The negative contribution of log tHcy to the pattern is in line with the inverse relationship of B-12 with tHcy (47). Given that the derivation of the patterns is partly based on the correlation of the data, we speculate that under folate-replete conditions such as in the Canadian population, B-12, becomes a critical nutrient for the remethylation of Hcy to methionine, which results in the strongest correlation between B-12 biomarkers (i.e., B-12, holoTC and tHcy), and subsequently, the derivation of the B-12 pattern in both trimesters.

After the B-12 pattern, the second highest variation of the methyl nutrient concentrations was explained by the first-trimester methyl-donor/riboflavin pattern with log folate, betaine and riboflavin being the main contributors, and the second-trimester B-6/riboflavin pattern with log riboflavin and B-6 as the main contributors. The positive correlation between first-trimester log folate and riboflavin (r=0.25, P<0.001) likely explained the derivation of methyl-donor/riboflavin pattern, which may be related to the coenzyme role of riboflavin in the form of FAD for the

formation of 5-MTHF (48). Riboflavin, in the form of FMN, functions also as a coenzyme in the formation of the B-6 coenzyme form PLP by the action of pyridoxine-phosphate oxidase (49), which may explain the positive correlation between second-trimester log PLP and riboflavin (r=0.35, P < 0.001), and the subsequent derivation the B-6/riboflavin pattern. Lastly, first-trimester methionine pattern and the second-trimester methyl donor pattern explained 15% and 16% of the maternal methyl nutrients variation, respectively. Only log methionine was a positive contributor for the methionine pattern, which can be due to the lack of a strong correlation between log methionine and other methyl nutrients, and the combined regulation of methionine concentration by nutritional (e.g., folate, dietary protein intake) and hormonal factors (i.e., insulin concentration) (1,50). In the second trimester, not only log methionine but also log betaine were positive contributors to the methyl donor pattern, which is likely explained by the significant correlation between these methyl nutrients (r=0.23, P=0.001). We speculate that a higher remethylation rate in the second compared to the first trimester (51), in addition to an impaired use of folate as a methyl donor for the remethylation of homocysteine to methionine, due to a high folic acid intake which acts as folate antagonist (10), promote the use of betaine as a methyl donor, resulting in a stronger correlation between log betaine and methionine.

First-trimester maternal methyl donor/riboflavin pattern and neonatal anthropometric outcomes. First-trimester methyl donor/riboflavin pattern was negatively associated with BL and BL z-score, only in full-term newborns. We speculate that differences in median (IQR) folate concentrations (i.e., mothers of term babies: ~67 (51-81) nmol/L, mothers of preterm babies: ~79 (60-89) nmol/L) may modify the pattern score, and subsequently, the association with BL and BL z-score. Among the main contributors to this pattern, only log betaine was negatively associated with BL and BL z-score. Our results are similar to those reported in the GUSTO study, in which a 1-unit increase in plasma betaine concentration at late-pregnancy was significantly associated

with a decrease of 0.29 cm in BL (39). These findings can be partially explained by the previously reported critical role of betaine, as a methyl donor, in fetal programming and *de novo* DNA methylation (47,102,263). Results from animal studies have indicated that maternal supplementation with betaine decreased the expression of genes involved in adipose tissue metabolism (186), increased serum growth-hormone concentration (282) and regulated the expression of *IGF-1* and *IGF-2* genes in the offspring, which may affect fetal growth, and subsequently, anthropometric outcomes of the offspring (185). However, the relationship between maternal betaine concentration and DNA methylation levels of fetal growth-related genes needs to be confirmed in human studies.

Second-trimester maternal B-12 pattern and neonatal anthropometric outcomes. In the second trimester, an inverse association between the B-12 pattern scores with HC and HC z-score was found. Among the main contributors to this pattern, only log total B-12 was negatively associated with HC and HC-z-score. We hypothesized that the relationship between maternal B-12 concentration and HC and HC z-score became significant at the second trimester due to the occurrence of the "ontogenesis window" of brain development. The formation of the frontal and occipital sulci, and the neural cellular migration peak occurs mainly between 8-24 weeks of gestation(283,284). Given that DNA methylation is key for cellular and organ differentiation (e.g., neural cell), we speculate that B-12 may become critical for the provision of methyl groups for DNA methylation reactions in this folate-replete population. Despite that available evidence from pregnancy studies have reported no significant linear associations between maternal B-12 and neonatal anthropometric outcomes (28,220), results of a pregnancy study in China showed that maternal serum total B-12 concentration at delivery was negatively associated with DNA

methylation levels of *IGF-2* in cord blood, which highlight the potential role of second and third trimester B-12 in fetal growth and brain programming (224).

Strengths and limitations. To my knowledge, this is the first observational study in a large sample size of pregnant women that explored the interrelationship of maternal methyl nutrient concentrations by using PCA. PCA allowed us to derive non-correlated maternal methyl nutrient patterns, which prevented collinearity between maternal methyl nutrient concentrations in the regression models. PSBC growth charts were used to calculate BW, BL, and HC-z-scores, which are based on the BC population and portray neonatal anthropometric outcomes in the context of the BC health care system. Limitations of the current study include the equal proportion of apparently healthy South Asian and European pregnant women, which limits the generalization of our results to other populations. Additionally, this study lacks other maternal methyl nutrients in the PCAs such as free choline concentration. Due to the instability of maternal free choline in serum samples (261), data for maternal free choline concentration was not included in the PCA analysis.

In conclusion, this study shows that first and second-trimester maternal B-12 patterns explained the highest variation of the maternal methyl nutrient concentrations in both trimesters in 700 pregnant women residing in BC. Second-trimester maternal B-12 pattern was negatively associated with HC and HC z-score which may suggest a critical role of B-12 on fetal brain programming. However, further pregnancy studies exploring the interrelationship of maternal methyl nutrients using PCA are needed to assess the reproducibility of the derivation of the maternal methyl nutrients patterns and their relationship with neonatal anthropometric outcomes in other populations.

Chapter 4: COMPARISON BETWEEN DNA METHYLATION LEVELS IN CPG SITES OF FETAL GROWTH AND OBESITY-RELATED GENES IN 3-MONTH OLD INFANTS OF MOTHERS WITH RBC FOLATE CONCENTRATION WITHIN AND ABOVE THE REFERENCE RANGE IN EARLY PREGNANCY

4.1 Introduction

Folate is a methyl donor nutrient that has a critical role in nucleotide synthesis and methylation reactions, such as DNA methylation, through one-carbon metabolism (7). Findings from two randomized controlled trials conducted in pregnant women with high risk for NTDs and low risk for NTDs showed that supplementation with $4000\mu g/day$ (8) and $0.8\mu g/day$ (9), respectively, of folic acid, the synthetic form of folate, before and during the first trimester of pregnancy reduced the incidence of NTDs by >60%. Currently, the SOGC recommends a daily dose of $400\mu g$ of folic acid, starting at least 3 months before pregnant to lactation, for low risk women to prevent the occurrence of NTD (11). However, most of the available prenatal supplements on the Canadian market contain 1000 μg of folic acid (12,13).

According to the Canadian Maternity Experiences Survey, 58% of Canadian women took multivitamin supplements containing folic acid during the three months before conception, and 90% took folic acid supplements at some point during the first trimester (206). Prospective cohort studies conducted in Canadian pregnant women (i.e., the PREFORM and APrON studies) have reported that >90% of the participants consume folic acid containing prenatal supplements by the end of the first trimester (16,68). Findings of from the PREFORM and the APrON studies, both Canadian pregnancy studies, indicated that >50% of pregnant women had high RBC folate concentration (>1360 nmol/L) in early and late pregnancy (16,67).

Early pregnancy is considered a critical window for fetal growth programming mainly due to the occurrence of *de novo* DNA methylation in the offspring (18). Evidence from previous pregnancy studies have shown that maternal factors such as pre-pregnancy BMI may influence fetal growth and obesity programming of the offspring through modifications in fetal DNA methylation levels (285,286). However, evidence of the relationship between maternal folate status and DNA methylation levels of fetal growth or obesity related genes in the offspring is limited and with conflicting results. Results of a previous study conducted in North American pregnant women (n=496 mother-newborn dyads) with mean RBC folate concentration of ~494 nmol/L, indicated that DNA methylation levels of *IGF-2* measured in cord blood, were higher in offspring of pregnant women with low maternal RBC folate concentration (i.e., second quartile) at ~12 weeks of gestation, compared to offspring of pregnant women with RBC folate concentration in the third and fourth quartiles (22). In contrast, findings from two pregnancy studies conducted in the UK, where women had RBC folate concentration <500 nmol/L, showed no significant association between maternal RBC folate concentration at early pregnancy (i.e., <20 weeks of gestation) and DNA methylation levels of *IGF-2* measured in cord blood (203,225). Whether early pregnancy folate status is associated with infant DNA methylation levels of CpG sites associated with genes involved in fetal growth and obesity programming in folate-replete populations is currently unknown. Given the high maternal folate status reported in Canadian pregnant women, the current study sought to compare DNA methylation levels of CpG sites associated with fetal growth and obesity programming between infants of pregnant women with high folate status (i.e., \geq 1360 nmol/L) and folate status in the reference range (i.e., <1360 nmol/) measured before 22 weeks of gestation, in Canadian mother-infant dyads.

4.2 Study design and methods

The current study is nested within the APrON study. The APrON study is a prospective cohort study of pregnant women and their children with follow-up visits until 3 years postpartum, which was conducted in the central and southern regions of Alberta, Canada. Details of the APrON study have been previously described (287). Briefly, during the study, 2192 pregnant women, living in Edmonton, Calgary, or nearby areas, were enrolled between May 2009 and July 2012 if they were >16 years, at <27 weeks of gestation, were able to speak in English, and who had planned to not move out of the province during the time of the study. A study visit was conducted in the first trimester in women recruited between 1-13 weeks of gestation, while for those recruited after, a study visit was conducted at the second (14-26 weeks of gestation) and third (27-42 weeks of gestation) trimester. All study visits were conducted by trained research assistants. Non-fasting maternal blood samples were collected at each study visit. Blood or cheek swab samples were collected from infants at 3 months of age.

The APrON study was approved by the University of Calgary Health Research Ethics Board and the University of Alberta Health Research Ethics Biomedical Panel (REB14-1702, Pro00002954). The present study was reviewed and approved by The University of British Columbia / Children's and Women's Health Centre of British Columbia Research Ethics Board, Vancouver, Canada (H17-03141).

Inclusion criteria

The present study included mother-infant dyads that have available maternal samples collected at <22 weeks of gestation, and information of DNA methylation levels measured in infants at 3 months of age, from women who reported to not smoke (i.e., non-smokers or smoked <100 cigarettes in her life) or consume alcohol (i.e., never consumed alcohol) or drug users (i.e., never used recreational drugs) or had type 1 or type 2 diabetes.

4.2.1 Maternal lifestyle, demographic and anthropometric information

Data about maternal age (years), ethnicity [self-reported; Black (i.e., African, African North American), Caucasian/white (i.e., English, French, German, Greek, Irish, Polish, Russian, Scottish, Ukrainian) Chinese, Filipino, Japanese, Korean, Latin American, Native/Aboriginal Peoples of North America (i.e., First Nations, North American Indian, Metis, Inuit) South Asian, South East Asian, Arab, West Asian], education (less than high school, completed high school diploma, completed university undergraduate degree diploma, completed post-graduate degree, completed technical, vocational school, or trade, business/community college), total family income per year (<\$20,0000, \$20,000-\$39,999, \$40,000-\$69,999, \$70,000-\$99,999, ≥\$100,000), marital status (single, common-law, married, widowed, divorced and separated), parity (nulliparous/multiparous), GD, type 1 and type 2 diabetes, primary hypertension, gestational hypertension, preeclampsia, smoking history, drug use, and alcohol consumption was collected at the first study visit (287). Additionally, maternal weight (kg) and height (cm) were measured at each study visit by trained research assistants. Weight was measured using a professional scale with 200 kg capacity and variation of 0.01 kg Healthometer Professional 752KL, Pelstar LLC, Bridgeview, IL, USA. Height (m) was measured using a digital stadiometer (HM200P Portstad Portable Stadiometer, Charder, Seattle, WA, USA) with a variation of 0.1 cm.

4.2.2 Maternal supplement intake

A supplement intake questionnaire (SIQ) developed by the APrON study team was used to estimate the daily supplement intake of vitamins and minerals (e.g., prenatal multi-vitamins) at each trimester, as described in details by Gomez et al (68). Briefly, the SIQ contained three sections. The first section included a list of 20 commonly used multivitamins/minerals; the second section contained a list of 23 single-nutrient supplements, and the third section contained

a list of 43 herbal products, homeopathic remedies, probiotic, traditional medicines and animalderived products.

4.2.3 Infant demographic and anthropometric information

BW, BL, sex, birth status (term/preterm), and gestational age at birth were obtained from medical records. Infant age (weeks) and infant weight (kg) information was collected by the APrON study's research team at the ~12-week post-partum visit. Infant weight was measured by calculating the difference in body weight between the mother holding her infant (undressed except for a dry diaper) and the mother alone using a scale Healthometer Professional 752KL, Pelstar LLC, IL, U.S.A with a precision of 0.01 kg (288). LBW was classified as BW <2,500 g (265). Infant length (cm) was obtained from medical records from a pediatrician appointment one week before the study visit, whereas infant age was provided by the mother.

4.2.4 Infant DNA methylation information

Infant DNA methylation data were available for 246 infants. Briefly, a blood (n=109) or cheek swab (n=137) sample was collected from infants ~12 weeks post-partum. Blood samples were collected by a certified phlebotomist using either a butterfly needle or a 25G^{3/4} standard infant needle and EDTA tubes. Blood samples were processed within 6 hours after collection and were centrifuged at 3000 r/min for 15 min to separate the plasma, buffy coat (leukocytes and platelets), and erythrocytes. The buffy coat was separated, and processed leukocytes were stored in microcentrifuge tubes at -80°C for DNA extraction. For those infants who were not able to provide a blood sample, BEC samples were collected. BECs were collected by rolling a sterile cytology brush up and down 10 times along each cheek of the infant (duplicate). Both BECs samples were dried for 10–15 min in a drinking glass at room temperature and were placed and

sealed in a labeled plastic bag and then stored at -80°C until DNA extraction. DNA was extracted from leukocytes and BECs by cellular lysis and then it was purified using Gentra Buccal DNA purification kits. Approximately 750 ng of genomic DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). After, ~160 ng of bisulfite converted DNA was processed using the 450K array, according to manufacturer's instructions (Illumina) (287) at the Kobor Laboratory at the University of British Columbia. The selection of CpG sites to include in the current study was based on the highest variability of DNA methylation levels at each CpG site associated with *LEP*, *LEP-R*, *HIF3a*, *IGF-2*, *RXRA*, *DNMT-1*, *DNMT-3A* and *DNMT-3B* genes according to the information provided in the BEcon platform (289). The detailed list of CpG sites is provided in Table 4.1.

LEP	LEP	HIF3a	IGF-2	RXRA	DNMT-1	DNMT-3A	DNMT-3B
	receptor						
cg00666422	cg03050981	cg01552731	cg00570518	cg02127980	cg07627628	cg00277048	cg00300969
cg00840332	cg11228758	cg07684068	cg02045936	cg13487983	cg21063296	cg00912598	cg09835408
cg03084214	cg11807188	cg12068280	cg02807948	cg13510651	cg21892967	cg05544807	cg13788819
cg07464571	cg23688719	cg14153927	cg07096953	cg13595495	cg01347596	cg09986894	cg14224313
cg12782180		cg16672562	cg05384664	cg13677043		cg10749994	cg17475857
cg13381984		cg20667364	cg11717189	cg13689699		cg11354105	cg17482740
cg25435800		cg22891070	cg12528452	cg13847322		cg15150970	cg21235334
cg19594666		cg23548163	cg13165070	cg13931640		cg15843262	cg22052056
cg26814075		cg25196389	cg26401390	cg13941235		cg19489797	cg22605822
		cg25460031		cg14051662		cg20303441	cg24403338
		cg26749414		cg14121282		cg20669908	
		cg27146050		cg14236758		cg26544247	
				cg14472716		cg27369452	
				cg14570632		cg00856404	
				cg21201924			

Table 4.1 CpG sites associated with fetal growth and obesity-related genes included in this study

4.2.5 Biochemical analyses

4.2.5.1 Blood processing

Non-fasting maternal samples were collected by a certified phlebotomist. Blood samples were drawn using vacutainer tubes with EDTA or serum separator gel and processed immediately after collection. RBC samples were aliquoted and stored at -80 °C (67,287).

4.2.5.2 Biomarker quantification

RBC folate concentration was determined in a sub-sample of pregnant women (n=154) using an on-capture method. Details of the determination of RBC folate concentration have been described previously (67,287). Briefly, a hemolysate (1:10) was prepared directly after blood sampling. The hemolysate was prepared using 0.1 mL of blood (with EDTA) and 0.9 mL ascorbic acid (1%). Then, the hemolysate was incubated at 37°C for 30±5 minutes and underwent a 1:2 dilution with AxSYM Folate RBC Protein Diluent. Samples were analysed using the AXSYM® analyzer (Abbott, Mississauga, ON, Canada) according to the manufacturer's instructions. RBC folate concentration was classified into two groups; 1) RBC folate concentration \leq 1360 nmol/L, and 2) RBC folate concentration >1360 nmol/L, the latter indicated the cut-off for high folate status in the adult population (290).

4.2.6 Statistical analysis

Descriptive analysis

The normality of the data was tested using a Shapiro-Wilk test. Maternal age is normally distributed and is presented as mean (SD). All other continuous variables had skewed distributions and, thus, are presented as median (IQR). Categorical maternal and infant variables

are presented as prevalence (%). Differences in the percentage of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration >1360 nmol/L between tissues (i.e., BECs and buffy coat) were compared using a chi-square test. An independent Student's *t*-test was used to compare maternal age between pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration >1360 nmol/L. A Wilcoxon rank-sum test was used to compare weeks of gestation at blood collection, gestational age at birth, BW, BL, infant age, infant weight and infant length between infants of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \leq 1360 nmol/L a

>1360 nmol/L, while parity, birth status (term/preterm), infant sex, and LBW were compared using a chi-square test. Because the sample size for at least one cell was <5, pre-pregnancy BMI categories (i.e., underweight, overweight, healthy weight and obesity) and maternal ethnicity between pregnant women with RBC folate concentration \leq 1360 nmol/L and pregnant women with RBC folate >1360 nmol/L were compared using a Fisher's exact test.

Furthermore, an independent Student's *t*-test was used to compare maternal age between the total sample of pregnant women (n=246) and the subset of pregnant women with available maternal RBC folate concentration (n=154). A Wilcoxon rank-sum test was used to compare weeks of gestation, gestational age at birth, BW, BL, infant age, infant weight and infant length between the total sample of pregnant women and the subset of pregnant women with available maternal RBC folate concentration. Additionally, a chi-square test was used to compare prepregnancy BMI categories (i.e., underweight, healthy weight, overweight, and obesity), parity, maternal ethnicity, infant sex, birth status (preterm/term) and LBW.

DNA methylation analysis

A Wilcoxon-rank sum test was used to compare DNA methylation levels (i.e., β -values) of each CpG site measured in infants of pregnant women with RBC folate concentration \leq 1360 nmol/L and infants of pregnant women with RBC folate concentration >1360 nmol/L, separately by tissue type (291). The β -value is calculated as a ratio between the intensities of methylated probes divided by the sum of unmethylated + methylated probes

 $(\beta = \frac{Methylated intensity probes}{Unmethylated intensity probes}) (292). Results were corrected for multiple comparisons using the Benjamini-Hochberg test. All the statistical analyses were conducted using Stata version IC 15 (StataCorp).$

4.3 Results

4.3.1 Maternal characteristics

Maternal characteristics are presented in Table 4.2. For the sample of 246 women mean \pm SD maternal age was 30.9 \pm 3.8 y. In the present study, 78% identified as Caucasian, and 70% had a completed university degree or postgraduate level degree, while only 2% had an uncompleted high school diploma. Furthermore, >70% of pregnant women had a total annual family income \geq \$ 70,000, whereas only 2% had a total annual family income \leq \$ 20,000. Most of pregnant women (87.5%) were married, 9.8% were living with their partners, and only 2.5% were single. Regarding the BMI classification, 38% of pregnant women had overweight or obesity, whereas 59% had a healthy weight. Only 1.2% (*n*=3) and 0.8% (*n*=2) had GD and preeclampsia, respectively, while no pregnant women had primary hypertension, gestational hypertension or type 1 or type 2 diabetes.

In total, 97.5% of pregnant women consumed a daily folic acid supplement in early pregnancy. The median daily folic acid dose used was $1000\mu g$ (IQR: 862-1000 μg ; minimummaximum 14.3-6000 μg), and 23.8% consumed >1000 μg folic acid/day. Of the total sample of pregnant women (*n*=246), 154 had available RBC folate concentration data. Median (IQR) RBC folate concentration was 1334 (1111-1668) nmol/L. No significant differences in maternal and infant characteristics were found comparing the sample size of 246 pregnant women and those with available RBC folate data.

Of pregnant women with available RBC folate concentration, 51.3% (n=79) had RBC folate concentration \leq 1360 nmol/L with a median (IQR) RBC concentration of 1116 (996-1231) nmol/L, and 48.7% (n=75) had RBC folate concentration >1360 nmol/L with a median (IQR) RBC concentration of 1702 (1516-1887) nmol/L. Weeks of gestation were significantly higher in pregnant women with RBC folate concentration >1360 nmol/L compared to those with RBC
folate concentration ≤1360 nmol/L. No further significant differences were found in maternal

characteristics comparing pregnant women with RBC folate concentration ≤1360 nmol/L and

those with RBC folate concentration >1360 nmol/L (Table 4.2).

Maternal	n	All	n	RBC folate	n	RBC folate	Р
characteristics				≤1360 nmol/L		>1360 nmol/L	
Maternal age, y	238	30.9±3.8	75	31.0±4.2	75	31.3±3.5	0.61
Weeks of gestation at	246	15.4	79	15.0	75	15.7	0.03
blood sample collection		(13.6-17.4)		(12.6-16.6)		(13.4-17.4)	
Pre-pregnancy BMI, % (kg/m ²)	241	23.7 (21.5-26.8)	76	23.0 (21.6-25.4)	74	24.8 (22.5-29.4)	
Underweight	7	2.90	2	2.63	0	-	0.11
		(18.3;17.8-18.4)		(18.1;17.8-18.5)		52.7	
Healthy weight	144	59.8	50	65.8	39	(22.6; 20.8-24.2)	
		(22.0;20.9-23.5)		(22.0; 21.2-23.1)		24.3	
Overweight	52	21.6	15	19.7	18	(26.6; 25.9-28.3)	
		(26.7;25.6-28.0)		(26.4; 25.2-27.7)		23.0	
Obesity	38	15.8	9	11.8	17	(33.1; 31.9-36.2)	
		(34.0;31.9-37.2)		(36.1; 33.2-38.1)			
Parity, %	240		77		75		0.77
Nulliparous	91	37.9	30	39.0	31	41.3	
Multiparous	149	62.1	47	61.0	44	58.7	
Ethnicity, %	238		75		75		0.24
European	190	80	57	76.0	69	92.0	
Chinese	8	3.4	3	4.0	3	4.0	
Latin American	9	3.8	6	8.0	1	1.3	
South East Asian	8	3.4	2	2.7	1	1.3	
Other	23	9.4	7	9.3	1	1.3	

Table 4.2 Maternal characteristics of the total sample of pregnant women and those with RBC folate concentration ≤1360 nmol/L and RBC folate concentration >1360 nmol

¹Values are median (IQR) or mean (SD) for continuous variables and prevalence (%) for categorical variables. *P* values are from Wilcoxon ranksum test or independent Student's *t*-test for comparison of continuous variables and chi-square test or Fisher's exact test for categorical variables between pregnant women with RBC folate \leq 1360 nmol/L and those with RBC folate >1360 nmol/L. Pre-pregnancy BMI categories; underweight (BMI <18.5 kg/m²), healthy weight (BMI \geq 18.5 to 24.9 kg/m²), overweight (BMI \geq 25 to 29.9 kg/m²) and obese (BMI \geq 30 kg/m²). BMI, body mass index; RBC, red blood cells.

4.3.1 Infant characteristics

Most of the infants included in the current study were full-term newborns (94%) (Table 4.3), and only 5.7% of them were classified as LBW. Of the total sample of infants, 52% of were male (n=128) and 48% were female (n=118). No significant differences in infant characteristics

were found comparing infants of the total sample size of pregnant women with infants of pregnant women with available RBC folate concentration data. Also, the characteristics of infants of pregnant women with RBC folate concentration \leq 1360 nmol/L did not significantly differ from infants of pregnant women with RBC folate concentration >1360 nmol/L.

Infant characteristics	n	All	n	RBC folate ≤1360 nmol/L	n	RBC folate >1360 nmol/L	Р
Birth status, % Term Preterm	246 231 15	93.9 6.10	79 76 3	96.2 3.80	75 67 8	89.3 10.7	0.09
Gestational age at birth, wks	246	39.7 (38.4-40.1)	79 70	39.7 (39.0-40.0)	75	39.9 (38.3-40.4)	0.88
BW, g LBW, % BL.cm	240 14 243	5.69 51 1 (49 5-53 3)	79 6 79	7.59 51.0 (50.0-53.3)	75 4 74	5.33 51 8 (49 5-53 3)	0.56 0.57 0.62
Infant age, wks	209	12.0 (11-13)	70	12.0 (12.0-13.0)	65	12.0 (11.0-13.0)	0.16
Infant weight, g	236	6095 (5600-6600)	77	6110 (5620-6570)	74	5925 (5450-6550)	0.18
Infant length, cm	181	59.0 (57.7-61.0)	59	59.5 (58.0-61.0)	59	59.0 (56.0-61.0)	0.30

Table 4.3 Infant characteristics of the total sample of infants and those of pregnant women with RBC folate concentration ≤1360 nmol/L and RBC folate concentration >1360 nmol

¹Values are median (IQR) or mean (SD) for continuous variables and prevalence (%) for categorical variables. *P* values are from Wilcoxon ranksum test or independent Student's *t*-test for comparison of continuous variables and chi-square test for categorical variables between infants of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration >1360 nmol/L. BW, birth weight; BL, birth length; LBW, low birth weight; RBC, red blood cell.

4.3.2 Comparison of DNA methylation levels of CpG sites associated with fetal growth and obesity-related and DNA methyltransferase genes in offspring by maternal RBC folate concentration

The proportion of pregnant women with folate status in the reference range and high folate status did not significantly differ by tissue sample (i.e., buffy coat or BECs) (P> 0.05). DNA methylation levels in specific CpG sites associated with *DNMT-3A/3B*, *HIF-3a*, *LEP*, *LEP*-*R* and *RXRA*, sites, measured in BECs, and CpG sites associated with genes, measured in the buffy coat, differed comparing folate status in pregnant women (Table 4.4). However, these results were no longer significant after Benjamini-Hochberg correction.

Table 4.4 Comparison of DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes in infants of mothers with RBC folate \leq 1360 nmol/L and RBC folate >1360 nmol/L at early pregnancy¹

CpG sites	BECs		Bu			
•	RBC folate	RBC folate	RBC folate	RBC folate	_	
	≤1360 nmol/L	>1360 nmol/L	≤1360 nmol/L	>1360 nmol/L		
	(n=46)	(n=37)	(n=33)	(n=38)	P^2	P ³
DNMT-1						
cg07627628	0.28 (0.25-0.30)	0.29 (0.25-0.33)	0.85 (0.83-0.86)	0.84 (0.83-0.86)	0.33	0.86
cg21063296	0.04 (0.04-0.05)	0.05 (0.04-0.05)	0.46 (0.45-0.47)	0.46 (0.45-0.47)	0.90	0.84
cg21892967	0.19 (0.17-0.19)	0.19 (0.17-0.20)	0.18 (0.18-0.19)	0.19 (0.18-0.20)	0.67	0.34
cg01347596	0.81 (0.76-0.84)	0.82 (0.79-0.84)	0.94 (0.94-0.95)	0.94 (0.94-0.95)	0.40	0.36
DNMT-3A						
cg00277048	0.96 (0.95-0.97)	0.96 (0.95-0.96)	0.96 (0.96-0.97)	0.96 (0.96-0.97)	0.85	0.51
cg00856404	0.75 (0.74-0.76)	0.77 (0.74-0.77)	0.30 (0.29-0.31)	0.30 (0.29-0.30)	0.03	0.11
cg00912598	0.06 (0.06-0.07)	0.06 (0.06-0.07)	0.06 (0.06-0.07)	0.06 (0.06-0.07)	0.58	0.95
cg05544807	0.23 (0.22-0.24)	0.23 (0.22-0.25)	0.72 (0.70-0.73)	0.71 (0.69-0.72)	0.96	0.05
cg09986894	0.93 (0.92-0.94)	0.93 (0.93-0.93)	0.94 (0.94-0.95)	0.95 (0.94-0.95)	0.99	0.36
cg10749994	0.09 (0.08-0.11)	0.09 (0.08-0.11)	0.03 (0.02-0.03)	0.02 (0.02-0.03)	0.99	0.34
cg11354105	0.16 (0.15-0.18)	0.17 (0.16-0.19)	0.81 (0.80-0.82)	0.81 (0.80-0.82)	0.11	0.84
cg15150970	0.51 (0.49-0.52)	0.51 (0.50-0.52)	0.20 (0.19-0.21)	0.20 (0.19-0.21)	0.51	0.64
cg15843262	0.46 (0.42-0.49)	0.46 (0.43-0.48)	0.02 (0.02-0.02)	0.02 (0.02-0.03)	0.99	0.09
cg19489797	0.07 (0.06-0.08)	0.06 (0.06-0.08)	0.07 (0.06-0.07)	0.07 (0.06-0.08)	0.70	0.96
cg20303441	0.42 (0.39-0.43)	0.40 (0.38-0.43)	0.01 (0.008-0.01)	0.01 (0.01-0.02)	0.45	0.03
cg20669908	0.80 (0.79-0.81)	0.80 (0.79-0.81)	0.33 (0.33-0.34)	0.33 (0.32-0.34)	0.22	0.51
cg26544247	0.43 (0.40-0.44)	0.43 (0.40-0.45)	0.04 (0.04-0.05)	0.05 (0.04-0.05)	0.67	0.11
cg27369452	0.03 (0.03-0.04)	0.04 (0.03-0.04)	0.04 (0.04-0.05)	0.04 (0.04-0.05)	0.16	0.78
DNMT-3B						
cg00300969	0.88 (0.87-0.89)	0.88 (0.87-0.89)	0.58 (0.56-0.60)	0.58 (0.56-060)	0.79	0.64
cg09835408	0.76 (0.74-0.77)	0.75 (0.74-0.76)	0.42 (0.39-0.45)	0.41 (0.40-0.44)	0.04	0.91
cg13788819	0.88 (0.86-0.89)	0.88 (0.86-0.89)	0.31 (0.30-0.33)	0.32 (0.30-0.34)	0.97	0.35
cg14224313	0.89 (0.88-0.90)	0.89 (0.88-0.90)	0.92 (0.92-0.93)	0.92 (0.92-0.93)	0.63	0.58
cg17475857	0.86 (0.84-0.88)	0.86 (0.84-0.87)	0.58 (0.56-0.60)	0.58 (0.57-0.60)	0.65	0.56
cg17482740	0.47 (0.47-0.49)	0.47 (0.46-0.49)	0.59 (0.58-0.60)	0.59 (0.57-0.60)	0.45	0.52
cg21235334	0.09 (0.08-0.10)	0.08 (0.07-0.09)	0.24 (0.22-0.25)	0.23 (0.21-0.25)	0.89	0.92
cg22052056	0.95 (0.94-0.95)	0.95 (0.94-0.95)	0.82 (0.81-0.83)	0.82 (0.80-0.83)	0.29	0.78
cg22605822	0.86 (0.85-0.86)	0.86 (0.85-0.87)	0.87 (0.87-0.88)	0.88 (0.87-0.88)	0.04	0.49
cg24403338	0.82 (0.81-0.83)	0.82 (0.81-0.83)	0.67 (0.64-0.69)	0.66 (.065-0.68)	0.71	0.95
IGF-2						
cg00570518	0.40 (0.39-0.41)	0.41 (0.39-0.42)	0.59 (0.57-0.60)	0.58 (0.57-0.59)	0.07	0.30
cg02045936	0.84 (0.83-0.86)	0.84 (0.83-0.86)	0.91 (0.91-0.92)	0.91 (0.91-0.92)	0.85	0.64
cg02807948	0.30 (0.29-0.32)	0.30 (0.29-0.31)	0.51 (0.50-0.54)	0.52 (0.51-0.54)	0.93	0.73
cg05384664	0.44 (0.43-0.45)	0.45 (0.43-0.46)	0.62 (0.60-0.63)	0.62 (0.60-0.64)	0.15	0.84
cg07096953	0.11 (0.10-0.12)	0.11 (0.10-0.13)	0.34 (0.32-0.38)	0.35 (0.32-0.38)	0.93	0.94
cg11717189	0.32 (0.30-0.34)	0.31 (0.30-0.35)	0.38 (0.35-0.41)	0.39 (0.37-0.42)	0.80	0.26
cg12528452	0.50 (0.46-0.53)	0.50 (0.46-0.54)	0.61 (0.57-0.63)	0.62 (0.58-0.64)	0.88	0.57
cg13165070	0.33 (0.32-0.35)	0.33 (0.31-0.35)	0.36 (0.34-0.38)	0.35 (0.33-0.37)	0.21	0.08
cg26401390	0.84 (0.83-0.86)	0.84 (0.83-0.86)	0.91 (0.91-0.92)	0.91 (0.91-0.92)	0.84	0.60
LEP						
cg00666422	0.41 (0.38-0.44)	0.40 (0.36-0.42)	0.47 (0.46-0.49)	0.46 (0.44-0.49)	0.24	0.28
cg00840332	0.04 (0.04-0.05)	0.04 (0.03-0.05)	0.16 (0.14-0.18)	0.14 (0.13-0.16)	0.62	0.03
cg03084214	0.79 (0.77-0.81)	0.79 (0.76-0.81)	0.90 (0.89-0.91)	0.90 (0.89-0.91)	0.91	0.13
cg07464571	0.10 (0.08-0.12)	0.09 (0.08-0.11)	0.37 (0.35-0.38)	0.35 (0.34-0.37)	0.51	0.08
cg13381984	0.16 (0.14-0.18)	0.15 (0.13-0.18)	0.36 (0.34-0.37)	0.34 (0.33-0.37)	0.93	0.23
cg19594666	0.09 (0.06-0.10)	0.08 (0.07-0.11)	0.40 (0.37-0.43)	0.38 (0.35-0.41)	0.64	0.03
cg26814075	0.14 (0.11-0.16)	0.14 (0.12-0.17)	0.40 (0.37-0.42)	0.38 (0.36-0.40)	0.48	0.10
cg12782180	0.11 (0.08-0.13)	0.10 (0.08-0.12)	0.40 (0.38-0.42)	0.39 (0.37-0.41)	0.07	0.44
cg25435800	0.07 (0.05-0.09)	0.07 (0.06-0.09)	0.94 (0.93-0.95)	0.94 (0.93-0.95)	0.48	0.65

¹Values are median (IQR) for beta values. P^2 values are from Wilcoxon rank-sum test between DNA methylation levels of CpG sites measured in BECs of infants of pregnant women with RBC folate concentration \geq 1360 nmol/L and those with RBC folate concentration \geq 1360 nmol/L. With RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration \geq 1360 nmol/L. BECs, buccal epithelial cells; RBC, red blood cell.

Table 4.4 Comparison of DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes in infants of mothers with RBC folate \leq 1360 nmol/L and RBC folate >1360 nmol/L at early pregnancy (con)¹

CpG sites	BECs		Buffy	coat	_	
	RBC folate	RBC folate	RBC folate ≤1360	RBC folate		
	\leq 1360 nmol/L	>1360 nmol/L	nmol/L	>1360 nmol/L		
	(n=46)	(n=37)	(n=33)	(n=38)	P^2	P^3
HIF3a.						
cg14153927	0.40 (0.39-0.41)	0.40 (0.38-0.42)	0.28 (0.27-0.29)	0.28 (0.27-0.29)	0.62	0.10
cg16672562	0.10 (0.09-0.12)	0.11 (0.10-0.13)	0.97 (0.96-0.98)	0.97 (0.96-0.97)	0.34	0.67
cg20667364	0.11 (0.10-0.13)	0.13 (0.12-0.15)	0.27 (0.24-0.33)	0.27 (0.24-0.33)	0.008	0.59
cg22891070	0.23 (0.22-0.26)	0.24 (0.24-0.26)	0.91 (0.89-0.92)	0.90 (0.90-0.92)	0.85	0.63
cg23548163	0.21 (0.19-0.22)	0.22 (0.20-0.23)	0.55 (0.50-0.62)	0.55 (0.49-0.62)	0.10	0.82
cg25196389	0.57 (0.53-0.58)	0.56 (0.55-0.59)	0.83 (0.80-0.85)	0.83 (0.80-0.84)	0.65	0.48
cg25460031	0.87 (0.86-0.89)	0.86 (0.87-0.89)	0.92 (0.92-0.93)	0.93 (0.92-0.94)	0.35	0.61
cg26749414	0.39 (0.36-0.42)	0.41 (0.39-0.43)	0.78 (0.71-0.84)	0.76 (0.71-0.81)	0.05	0.91
cg27146050	0.17 (0.16-0.19)	0.18 (0.15-0.19)	0.66 (0.64-0.68)	0.66 (0.64-0.67)	0.65	0.50
cg01552731	0.64 (0.60-0.67)	0.66 (0.63-0.68)	0.89 (0.87-0.90)	0.90 (0.83-0.91)	0.09	0.64
cg07684068	0.84 (0.81-0.86)	0.84 (0.82-0.86)	0.93 (0.91-0.94)	0.93 (0.91-0.94)	0.52	0.79
cg12068280	0.37 (0.36-0.39)	0.37 (0.36-0.40)	0.69 (0.68-0.71)	0.69 (0.68-0.70)	0.95	0.53
RXRA						
cg13941235	0.06 (0.05-0.07)	0.07 (0.06-0.08)	0.73 (0.70-0.75)	0.72 (0.69-0.75)	0.06	0.50
cg14051662	0.18 (0.17-0.19)	0.18 (0.17-0.19)	0.92 (0.91-0.92)	0.91 (0.91-0.92)	0.81	0.53
cg14121282	0.05 (0.04-0.05)	0.05 (0.04-0.05)	0.22 (0.21-0.23)	0.24 (0.22-0.26)	0.68	0.008
cg14236758	0.13 (0.12-0.14)	0.13 (0.11-0.14)	0.68 (0.65-0.72)	0.68 (0.63-0.71)	0.69	0.94
cg14472716	0.29 (0.28-0.31)	0.29 (0.28-0.30)	0.39 (0.37-0.42)	0.39 (0.38-0.42)	0.50	0.53
cg14570632	0.66 (0.61-0.72)	0.65 (0.60-0.72)	0.97 (0.96-0.98)	0.97 (0.96-0.97)	0.87	0.70
cg21201924	0.29 (0.27-0.31)	0.28 (0.25-0.31)	0.78 (0.77-0.80)	0.78 (0.77-0.79)	0.61	0.71
cg02127980	0.07 (0.06-0.07)	0.06 (0.05-0.07)	0.56 (0.54-0.60)	0.56 (0.54-0.59)	0.18	0.95
cg13487983	0.13 (0.13-0.14)	0.13 (0.12-0.14)	0.31 (0.30-0.32)	0.32 (0.31-0.33)	0.49	0.29
cg13510651	0.72 (0.70-0.73)	0.72 (0.70-0.73)	0.90 (0.89-0.90)	0.90 (0.90-0.91)	0.87	0.53
cg13595495	0.18 (0.17-0.19)	0.18 (0.17-0.19)	0.94 (0.94-0.95)	0.95 (0.94-0.95)	0.97	0.85
cg13677043	0.08 (0.07-0.10)	0.09 (0.07-0.10)	0.92 (0.91-0.92)	0.92 (0.91-0.92)	0.65	0.46
cg13689699	0.40 (0.38-0.43)	0.42 (0.37-0.44)	0.94 (0.94-0.95)	0.94 (0.94-0.95)	0.99	0.44
cg13847322	0.24 (0.22-0.25)	0.23 (0.22-0.25)	0.64 (0.62-0.66)	0.63 (0.61-0.65)	0.93	0.67
cg13931640	0.98 (0.97-0.98)	0.97 (0.96-0.98)	0.62 (0.60-0.64)	0.62 (0.59-0.64)	0.58	0.88
LEP-R						
cg03050981	0.98 (0.97-0.99)	0.98 (0.97-0.99)	0.96 (0.95-0.97)	0.97 (0.96-0.97)	0.37	0.61
cg11228758	0.71 (0.69-0.73)	0.70 (0.69-0.72)	0.92 (0.91-0.92)	0.92 (0.91-0.92)	0.45	0.28
cg11807188	0.54 (0.51-0.56)	0.53 (0.52-0.55)	0.89 (0.89-0.90)	0.88 (0.88-0.89)	0.71	0.003
cg23688719	0.34 (0.32-0.35)	0.33 (0.31-0.36)	0.96 (0.95-0.96)	0.96 (0.95-0.96)	0.45	0.80

¹Values are median (IQR) for beta values. P^2 values are from Wilcoxon rank-sum test between DNA methylation levels of CpG sites measured in BECs of infants of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration >1360 nmol/L. P^3 values are from Wilcoxon rank-sum test between DNA methylation levels of CpG sites measured in buffy coat of infants of pregnant women with RBC folate concentration \leq 1360 nmol/L and those with RBC folate concentration >1360 nmol/L. BECs, buccal epithelial cells; RBC, red blood cell.

4.4 Discussion

Folate is the most important methyl donor for remethylation reactions. Findings from the current study indicate there are no significant differences in DNA methylation levels of CpG sites associated with *LEP*, *LEP-R*, *RXRA*, *HIF3α*, *DNMT-1*, *IGF-2*, *DNMT-3A* and *DNMT-3B* genes measured in infants of mothers with high folate concentration compared to those with folate concentration in the current reference range.

Maternal RBC folate concentration. The median (IQR) maternal RBC folate concentration in the current study was 1334 (1111-1688) nmol/L which was higher than maternal RBC folate concentration previously reported in European pregnancy studies. Cavalle-Busquets et al. (293) showed that the geometric mean (95%CI) of RBC folate concentration was 901 (859-945) nmol/L at 12 weeks of gestation in Spanish pregnant women. Similarly, a study conducted by O'Malley et al.(294) in Irish pregnant women showed that first-trimester RBC folate concentration was 1137.5 (SD 443.0) nmol/L (294), while in the UK, a geometric mean of 456 nmol/L for RBC folate concentration was reported in pregnant women at 19 weeks of gestation (203). In contrast, the PREFORM study (16), a pregnancy study in Canadian pregnant women living in Toronto, showed that maternal RBC folate concentration measured at 12 weeks of gestation was higher (~2400 nmol/L) compared to the results of the current study. Discrepancies in folate concentration between the pregnancy studies may be partially explained by differences in folic acid supplement use at early pregnancy (i.e., < 20 weeks of gestation). In the current study, 98% of pregnant women in the first trimester (i.e., <14 weeks of gestation) consumed folic acid supplement with at least 14µg/day, 42% using a daily dose of folic acid of 1000µg, and 24% using a folic acid dose of greater than 1000µg/day. In comparison, 94%, 98%, and 83% of Spanish (64,293), Irish (294) and UK pregnant women (203), respectively, consumed a daily dose of 400µg of folic acid in their first trimester of pregnancy (i.e., <14 weeks of gestation),

which likely explains the higher RBC folate concentration in the current study. Additionally, 83% of pregnant women that participated in the PREFROM study consumed a daily dose of folic acid $\geq 1000 \mu$ g, which may result in higher RBC folate concentration, compared to the results of the present study (16).

Furthermore, differences in the analytical methods used to measure folate concentration may also contribute to the discrepancies found among the aforementioned pregnancy studies. In the pregnancy studies conducted in Spain, Ireland, and the UK, RBC folate concentration was measured by microbiological assay, while in the present study RBC folate concentration was measured by the ion-capture method (67), and in the PREFORM study, it was measured by folate-binding protein with chemiluminescence detection. It has been previously suggested that the latter method may overestimate RBC folate concentration resulting in higher RBC folate concentration as it was reported in the PREFORM study, compared to RBC folate measured by microbiological assay (295).

Differences in DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes by maternal folate status. In contrast to my hypothesis, DNA methylation levels of CpG sites associated with *IGF-2*, *LEP*, *RXRA*, *HIF-3a*, and *LEP-R* genes did not differ between infants of pregnant women with RBC folate concentration \leq 1360 nmol/L, and those of pregnant women with RBC folate concentration >1360 nmol/L. Similar to our findings, Hoyo et al. (22) reported that higher maternal RBC folate concentration (i.e., third and fourth quartiles) at early pregnancy was not associated with DNA methylation levels of CpG sites associated with *IGF-2* gene measured in cord blood.

To the best of my knowledge, there is no available evidence in the relationship between maternal RBC folate concentration and DNA methylation levels of CpG sites associated with *LEP*, *LEP-R*, *RXRA*, and *HIF-3A* in the offspring. Offspring of Belgium pregnant women that

used folic acid supplementation 6 months before conception, had higher DNA methylation levels in CpG sites associated with *LEP*, measured in cord blood, compared to offspring of women that did not take folic acid supplements (23). Folic acid supplementation is the most important positive predictor of RBC folate concentration in Canadian childbearing-aged women (15). Additionally, findings of the same study showed that offspring of Belgium pregnant women that used folic acid supplementation across all trimesters had higher DNA methylation levels in CpG sites associated with *RXRA*, compared to offspring of pregnant women that stopped consumption in the second trimester (23). Overall, previous studies suggest that maternal folate status and folic acid supplementation before and during pregnancy may have a role in fetal growth and obesity programming, however, the current evidence is limited.

Comparison between studies is challenging and it should be made with caution. It has been previously reported that DNA methylation levels measured in blood vary by age. For example, findings from a recent study indicated that DNA methylation levels of specific CpG sites (i.e., CpG 11) associated with the *LEP* gene increased by 22% from birth to 12 months (296). Furthermore, genetic variants of obesity-related genes such as *LEP* have been associated with DNA methylation of CpG sites associated with the *LEP* gene (296). Results of a study conducted by Mansell et al. (166) showed that DNA methylation levels measured in cord blood significantly differed by genotypic variants of *HIF* (i.e., rs112087991 TT/CT/CC). Additionally, differences between methods to measure DNA methylation (i.e., microarray vs. pyrosequencing) may also contribute to the differences found between studies (297). For example, pyrosequencing detects small differences of DNA methylation levels (i.e., <5%) (297), which may also contribute to the discrepancies between studies.

Given these and other factors may influence DNA methylation levels of CpG sites associated with obesity-related genes, further prospective pregnancy studies measuring DNA methylation levels in the same specimen (e.g., BECs) between studies, as well as, the determination of genetic variant information, are needed to replicate the results, and understand the potential role of maternal folate concentration at early pregnancy in obesity programming.

Findings of the present study also showed that DNA methylation levels of CpG sites associated with *DNMT-1*, *DNMT-3A* and *DNMT-3B* genes did not differ comparing offspring of pregnant women with RBC folate concentration \leq 1360 nmol/L and those of pregnant women with RBC folate concentration >1360 nmol/L. Results from animal studies reported that male mice that received a postnatal methyl donor-supplemented diet (i.e., folic acid, betaine, choline chloride, methionine and B-12) during 3 weeks after weaning, increased the expression of *DNMT-3A* by 6% in brain tissue (298). In contrast, Bahous et al. (94) showed that the offspring of dams fed with a folic acid supplemented diet during pregnancy showed a significantly lower expression of the *DNMT-3A* gene in the cortex and hippocampus, which, we speculate, may potentially be mediated by modifications in DNA methylation. However, further animal studies are needed to replicate and confirm whether the association between methyl nutrient intake and *DNMT-3A* expression is underlaid by modifications in DNA methylation levels of CpG sites associated with *DNMT-3A*.

Potential mechanisms underlying the lack of differences in DNA methylation levels in CpG sites associated with fetal growth and obesity related genes

We hypothesize that several and complex factors may explain the lack of significant differences. First, findings of the current study indicate that most of pregnant women consumed folic acid supplements and 24% used a supplemental folic acid dose >1,000µg/day. High folic acid consumption, and the subsequent production of DHF (299), has been shown to lead to a pseudo-MTHFR deficiency, characterized by a decrease in MTHFR expression and protein levels, which we speculate, may lead to a decreased availability of 5-MTHF for the remethylation

of homocysteine to methionine and further decreased synthesis of SAM, the universal methyl donor for DNA methylation (300). Second, the median RBC folate concentration was ~1300 nmol/L, which may promote an adequate synthesis of SAM for the provision of methyl groups for methylation reactions; however, a potential negative feedback may occur under RBC folate concentration >1360 nmol/L that results in no changes in DNA methylation levels (200). The provision of methyl groups through one-carbon metabolism is regulated. High SAM concentration has an inhibitory effect on the enzyme MTHFR which catalyzes the formation of 5-MTHF for homocysteine remethylation to methionine. Inhibition of MTHFR may thereby decrease the synthesis of methionine, and subsequently, of SAM (7), that may partially also explain the lack of differences in DNA methylation levels between pregnant women with RBC folate ≤ 1360 nmol/L and those with RBC folate ≥ 1360 nmol/L. Further pregnancy studies including a larger sample size of pregnant women with early-pregnancy folate status data and DNA methylation levels of CpG sites associated with obesity-related genes measured only in one type of specimen (i.e., BECs) collected from their offspring are needed to confirm the reproducibility of the current findings.

Strengths and limitations. The strengths of the current study include the prospective design as well as the availability of early-pregnancy RBC samples collected at a critical developmental window for fetal programming. Furthermore, the determination of DNA methylation levels in cord blood has been disputed due to the heterogeneity of the specimen (i.e., varied cell composition) which may challenge the interpretation of DNA methylation. The determination of DNA methylation levels in a more homogeneous specimen such as BECs or buffy coat (i.e., leucocytes) is recommended, which were used in this study. However, the current study also has limitations. The present study included apparently healthy pregnant women with high educational levels and high household income, which limits the generalizability of the

findings. The determination of DNA methylation levels in two tissues (i.e., BECs and leucocytes) resulted in a smaller sample size as results cannot be combined. The small sample size may have not allowed detect differences in DNA methylation levels between pregnant women with RBC folate \leq 1360 nmol/L and those with RBC folate >1360 nmol/L. Additionally, the current study did not include the determination of genetic variants, which may influence DNA methylation levels of the genes examined.

In conclusion, early-pregnancy RBC folate concentration was not significantly associated with DNA methylation levels of CpG sites associated with DNA methyltransferase coding genes and fetal growth and obesity related genes, in these 154 mother-infant dyads. Further pregnancy studies with a larger sample size, data on early pregnancy folate status, and data on genetic variants of interrogated genes in folate-replete and non-folate-replete populations are needed to expand the findings of the current investigation.

Chapter 5: RELATIONSHIP BETWEEN MATERNAL METHYL NUTRIENT PATTERNS IN EARLY PREGNANCY AND INFANT DNA METHYLATION LEVELS OF FETAL GROWTH, OBESITY-RELATED GENES AND *METHYLTRANSFERASE* CODING GENES.

5.1 Introduction

Maternal nutrition is critical for adequate fetal growth and development and possibly even health in later life. Methyl nutrients such as folate, betaine, choline, B-6, B-12 and riboflavin have an interrelated role the one-carbon metabolism. Vitamins B-6, in the form of PLP, riboflavin, in the form of FMN and FAD, and B-12 participate as coenzymes for the synthesis of SAM (7,50,59), the universal methyl donor, whereas folate and betaine donate methyl groups for SAM formation (48), which is critical for the provision of methyl donors for methylation reactions such as DNA methylation.

DNA methylation is a well-recognized stable epigenetic mechanism, which plays a key role in the regulation of gene expression (139). In early pregnancy, *de novo* DNA methylation occurs, which is characterized by the removal of the methyl groups in embryonic DNA, and the subsequent re-methylation of the DNA strand (18,138,139). This physiological process makes early pregnancy a critical plasticity window for fetal programing and development (20). Current evidence from animal and human studies support the notion that modifications of DNA methylation levels of fetal growth and obesity-related genes in the offspring may predispose the offspring to obesity later in life (151,301). DNA methylation levels of *LEP* and *HIF3a* genes, which participate in appetite control and hypoxic cellular conditions such as obesity, have shown an inverse and positive association, respectively, with BMI in both children (302) and adults (164). DNA methylation levels of *IGF-2* and *RXRA* genes, both genes involved in fetal growth

and cellular proliferation, showed an inverse and positive association with subcutaneous adipose tissue in young adults (303) and fat mass in children, respectively (151).

Despite findings from previous pregnancy studies that indicated pre-pregnancy dietary folate and betaine intake was associated with DNA methylation levels of *IGF-2* and *LEP* genes measured in cord blood (23) and BECs of the offspring (24), evidence of pregnancy studies exploring the relationship between individual maternal methyl nutrient concentrations and DNA methylation levels of *IGF-2* in the offspring has shown conflicting results. Haggarty et al. (203) found no relationship between maternal RBC folate concentration measured at 19 weeks of gestation in British pregnant women (*n*=913) and DNA methylation levels of *IGF-2* in cord blood. Whereas, third-trimester serum total B-12 concentration measured in 99 Chinese pregnant women (224), was negatively associated with DNA methylation levels of *IGF-2* measured in cord blood, which may suggest a potential role of maternal B-12 status in concentrations in obesity programming.

However, the aforementioned studies included only a single maternal biomarker (e.g., maternal RBC folate concentration) as the exposure variable and without adjustment for other circulating methyl nutrients, which, from a metabolic perspective, is challenging to interpret due to the interrelated role of these methyl nutrients in one-carbon metabolism and in the provision of methyl groups. Furthermore, the use of cord blood for the determination of DNA methylation levels has been debated mainly due to the presence of different types of cells. Given the crucial role of *de novo* DNA methylation in fetal programming and the interrelated functions of methyl nutrients in one-carbon metabolism and thus potentially also DNA methylation, we aimed to determine the relationship between maternal methyl nutrient patterns in early pregnancy, derived using PCA, and DNA methylation levels of CpG sites associated with *LEP*, *HIF3a*, *RXRA*, *IGF-2*, *LEP-R*, and DNA methyltransferase genes (i.e., *DNMT-1* and *DNMT-3*) in their infants.

5.2 Study design and participants

Information on the study design, exclusion criteria, ethical approval, maternal, and infant characteristics and infant DNA methylation data is provided in Chapter 4.2. Briefly, the present study is nested within the APrON study, and includes maternal lifestyle and anthropometric information, in addition to infant DNA methylation and anthropometric data collected as part of the APrON study. The APrON study is a prospective cohort study that included pregnant women and their children, which were followed until 3 years postpartum, and was conducted in Alberta, Canada.

5.2.1 Biochemical analyses

5.2.1.1 Blood processing

As detailed in Chapter 4.2.5.1, non-fasting maternal blood samples were collected using vacutainer tubes with EDTA or serum separator gel by a certified phlebotomist and processed immediately after collection. Serum, plasma and buffy coat were aliquoted and stored at -80° C (67,287).

5.2.1.2 Biomarker quantification

Plasma free choline, betaine, methionine, and tHcy concentrations were determined by LC-MS/MS using a single platform (304). We used in-house plasma control samples in each of the in total 4 batches to estimate the inter-assay CV% for the analytes. The inter-assays CVs were $\leq 10\%$ for free choline, $\leq 10\%$ for betaine, $\leq 5\%$ for tHcy, and $\leq 7\%$ for methionine concentrations. Two external quality control samples for tHcy and methionine concentrations (ClinChek 23082,

RECIPE Chemicals and Instruments; IRIS Technologies International) were included in each batch; the mean (\pm SD) tHcy concentration was within the target analytical range for the low (8.7 \pm 0.5 μ mol/L; target: 9.04; control range 7.23-10.8 μ mol/L) and high control samples (24.9 \pm 2.3 μ mol/L; target: 25.9; control range 20.7-31.0 μ mol/L). The mean (\pm SD) methionine concentration was within the target analytical range for the low (28.4 \pm 0.4 μ mol/L; target: 29.1; control range 23.3-34.9 μ mol/L) and high control samples (61.9 \pm 3.5 μ mol/L; target: 67.0; control range 57.0-77.1 μ mol/L).

Plasma PLP, riboflavin, FMN and FAD concentrations were determined by isotopedilution LC-MS/MS using a method modified by Midttun et al. (23). In each batch, three external quality control samples for PLP concentration (ClinChek 8873, RECIPE Chemicals and Instruments; IRIS Technologies International) were included. The mean (±SD) PLP concentration was within the target analytical range for the low (62.7±5.0 nmol/L; target: 61.9; control range 49.4-74.5 nmol/L), medium (100±1.9 nmol/L; target: 97.1; control range 77.1-117 nmol/L) and high (128±5.0 nmol/L; target: 138; control range 110-165 nmol/L) control samples. The intra- and inter-assay CV% for PLP concentration were estimated based on 3 batches. The intra-assay CV% was \leq 7% for the low concentration quality control samples, and \leq 5% for the medium and high concentration quality control samples. The inter-assay CV% was \leq 5% for all control samples. For riboflavin, FMN and FAD in-house plasma control samples were included in each of the three batches to estimate the inter-assay CV% of these metabolites, and were $\leq 13\%$ for riboflavin, $\leq 10\%$ for FAD and $\leq 13\%$ for FMN concentrations. Serum total B-12 was quantified by a microparticle-enzyme immunoassay (Access 2 by Beckman Coulter) at the BC Children's Hospital Pathology Laboratory (Vancouver, BC, Canada). RBC folate concentration was determined using a by ion-capture method. Details of the determination of RBC folate concentration were described in Chapter 4.2.5.2.

5.2.2 Statistical analysis

A Shapiro-Wilk test was used to test the normality of the data. Maternal age is normally distributed and is presented as mean (±SD). Because of skewed distributions, weeks of gestation at maternal blood collection, gestational age at birth, pre-pregnancy BMI, maternal biomarker concentrations, BW, BL, infant age, infant weight, and infant length are presented as median (IQR). Categorical variables are presented as percentages (%). Student's *t*-test was used to compare maternal age, while a Wilcoxon rank-sum test was used to compare weeks of gestation and maternal biomarker concentrations, and chi-square test was used to compare parity, between tissue type for DNA methylation data (i.e., BECs or buffy coat) and between pregnant women included in the PCA and the total sample of pregnant women. Given the expected values for at least one cell were <5, pre-pregnancy BMI categories (i.e., underweight, healthy weight, overweight and obesity) and maternal ethnicity categories were compared between tissue type, and between PCA sample and the total sample using Fisher's exact test.

For infant characteristics, a Wilcoxon rank-sum test was used to compare gestational age at birth, BW, BL, infant age, infant weight and infant length by tissue, sex and by infant of pregnant women included in the PCA with the total sample of pregnant women. A chi-square test was used to compare birth status (preterm/term) and LBW by tissue, sex and by infants of pregnant women included in the PCA with the total sample of pregnant women.

5.2.2.1 Principal component analysis

Given the interrelated role of methyl nutrients in one-carbon metabolism, and the critical role that they play in the provision of methyl groups for DNA methylation, especially for *de novo* DNA methylation at early stages of development, PCA was used to derive uncorrelated maternal

methyl nutrient principal components at early pregnancy. The derivation of maternal methyl nutrient patterns was conducted through several steps. Based on their role as methyl donors (i.e., folate, betaine, choline), coenzymes (i.e., total B-12, PLP, FMN and FAD) or intermediate metabolites of one-carbon metabolism (i.e., methionine, tHcy), the aforementioned biomarkers were included in the PCA. PCA was restricted to pregnant women with complete data for maternal biomarker concentrations, i.e., without missing observations. Of the total sample size (n=246), 92 pregnant women had missing RBC folate data. Of the remaining sample (n=154) 12 had missing total-B12 data and 3 had missing PLP/FMN/FAD data. In total, 139 pregnant women with complete biomarker data were included in the PCA (n=139).

After the selection of maternal biomarkers to include in the PCA, maternal biomarker concentrations were log-transformed to improve the normality of the data, and the correlation between the maternal biomarker concentrations was explored, using a Pearson correlation test.

The PCA was conducted. The number of maternal methyl nutrient principal components derived indicated the number of variables included in the PCA (i.e., 9 maternal biomarker concentrations). The maternal methyl nutrient principal components were subjected to orthogonal rotation. Of the total derived maternal methyl nutrient components (n=9), retained maternal methyl nutrient components were selected using the following criteria; 1) the examination of Eigenvalues, which represents the variance of the data explained by each maternal methyl nutrient principal component using Kayser's rule (i.e., >1) (233), 2) the exploration of scree plots of the maternal methyl nutrient principal components, which are the graphical representation of the percentages of variation that each maternal methyl nutrient principal components accounts for (Figure 5.1), and 3) the explained cumulative variance of the biomarker data).

Retained maternal methyl nutrient principal components, henceforth referred to as maternal methyl nutrient patterns, were labeled according to the factor loadings, which indicates the contribution of each biomarker of the retained component (i.e., |factor loadings| \geq 0.45 were considered to contribute significantly to each component) and overall interpretability. Finally, scores of the retained maternal methyl nutrient patterns were predicted for each pregnant woman.



Figure 5.1 Scree plot of eigenvalues of derived early-pregnancy principal components
Longitudinal analysis. Linear regression models were used to explore the association

between the predicted scores of each retained maternal methyl nutrient pattern at early
pregnancy, as the exposure variable, and 1) DNA methylation levels of each of the 77 CpG sites
(i.e. M values) (292) associated with *IGF-2*, *RXRA*, *HIF3a*, *DNMT-1*, *DNMT-3*, *LEP* and *LEP*receptor genes measured in offspring buffy coat, as the outcome variables; and 2) DNA
methylation levels of each of the 77 CpG sites associated with *IGF-2*, *RXRA*, *HIF3a*, *DNMT-1*, *DNMT-3*, *LEP* and *LEP-R* genes measured in offspring BECs, as the outcome variables. Given
previous evidence has shown that infant age, sex, maternal ethnicity (Caucasian/Non-Caucasian)

and pre-pregnancy BMI may influence DNA methylation levels of CpG sites (166,296,305,306), all models were adjusted for these variables. Benjamini-Hochberg procedure was used to control for false-positive rate. Because missing data for maternal variables [i.e., maternal age (n=4), prepregnancy BMI (n=1), parity (n=2), ethnicity (n=4), type 1 diabetes (n=2), type 2 diabetes (n=2), gestational diabetes (n=2), primary hypertension (n=2), gestational hypertension (n=2), preeclampsia (n=2)] represented <5% of the database, no missing categories were created. Results were considered significant at P<0.05. Descriptive analyses and PCA were conducted using Stata version IC 15 (StataCorp). Longitudinal regression analyses were conducted using R studio version 3.5.1.

5.3 Results

5.3.1 Maternal characteristics

Maternal characteristics are presented in Table 5.1. Mean (SD) age of pregnant women at their first study visit was 30.9 (3.8) y, and 78% reported being Caucasian. Only 2% of women had an incomplete high school diploma, whereas 9% had a high school diploma, 19% had a technical degree, 48% had completed university and 22% had a post-graduate level degree. Also, >50% of women had a total family income/year \geq \$100,000, while less than 10% had a total family income/year <\$40,000. Most of the women had a healthy pre-pregnancy BMI, while 16% of women had a BMI that classified them as obese. None of the participants had primary hypertension, gestational hypertension, type 1 or type 2 diabetes. Only 1.2% (*n*=3) of the participants had GD, whereas 0.8% (*n*=2) developed preeclampsia during pregnancy. Maternal characteristics and methyl nutrient concentrations did not differ between pregnant women who had infants in which DNA methylation levels were measured in BECs compared to those in which DNA methylation levels were measured in buffy coat (Table 5.1 and Table 5.2).

Maternal	All women		Specimen DNA methylation			Women included in the PCA				
characteristics	n		n	Buffy coat	n	BECs	P^2	n	PCA	P ³
Maternal age, y	238	30.9±3.8	107	31.1±4.2	131	30.8±3.5	0.69	135	31.2±3.9	0.64
Weeks of gestation at blood sample collection	246	15.4 (13.6-17.4)	109	15.4 (13.1-17.4)	137	15.3 (13.7-17.1)	0.08	139	15.1 (12.7-16.7)	0.74
Pre-pregnancy BMI, % (kg/m ²)	241	23.8 (21.7-26.9)	109	23.7 (21.5-23.4)	132	23.7 (21.6-26.7)	0.88	135	24.1 (21.8-26.8)	0.75
Underweight	7	2.90 (18.3;17.8-18.4)	2	1.83 (17.9;17.8-18)	5	3.8 (18.3;18.3-18.4)		2	2.5 (18.1; 17.8-18.5)	
Healthy weight	144	59.8 (22.0;20.9-23.5)	64	58.7 (21.8;20.7 22.9)	80	50.6 (22.7;21.0-23.7)		80	56.1 (20.1; 21.1-23.5)	
Overweight	52	21.6 (26.7;25.6-28.0)	26	23.9 (26.8;25.9-28.0)	26	19.7 (26.4;25.6-28.1)		33	22.3 (26.5; 25.5-28.0)	
Obesity	38	15.8 (34.0;31.9-37.2)	17	15.6 (35.2;31.9-37.0)	21	15.9 (33.2;32.1-37.2)		20	19.1 (32.9; 31.6-36.1)	
Parity, %	240		107		133		0.98	137		0.88
Nulliparous	91	37.9	40	37.4	51	38.4		53	38.7	
Multiparous	149	62.1	67	62.6	82	61.7		84	61.3	
Ethnicity, %	238		107		131		0.97	135		0.17
European	190	80	92	86.0	98	74.8		114	84.4	
Chinese	8	3.4	2	1.9	6	4.58		4	2.94	
Latin American	9	3.8	3	2.8	6	4.58		6	4.44	
South East Asian	8	3.4	1	0.93	7	5.34		3	2.22	
Other	23	9.7	9	8.4	14	10.7		8	6	

Table 5.1 Maternal characteristics of all women with infant DNA methylation data by tissue and those included in the PCA¹

¹Values are mean (SD) or median (IQR) for continuous variables or prevalence (%) for categorical variables. P^2 values are from Wilcoxon rank-sum test or independent Student's *t*-test for comparison of continuous variables and chi-square test or Fisher's exact test for categorical variables between pregnant women who had infant DNA methylation data measured in buffy coat or BECs. P^3 values are from Wilcoxon rank-sum test or independent Student's *t*-test, and chi-square test for categorical variables between the total sample and those pregnant women that were included in the PCA. or Fisher's exact test for categorical variables between the total sample and those pregnant women that were included in the PCA. or Fisher's exact test for categorical variables between the total sample and those pregnancy BMI categories; underweight (BMI <18.5 kg/m²), healthy weight (BMI ≥18.5 to 24.9 kg/m²), overweight (BMI ≥25 to 29.9 kg/m²) and obese (BMI ≥30 kg/m²). BMI, body mass index; BECs, buccal epithelial cells, PCA, principal component analysis; Wks, weeks.

5.3.2 Maternal methyl nutrient patterns

No significant differences were found in maternal characteristics or maternal biomarker concentrations between pregnant women included in the PCA compared to the total sample of pregnant women (Table 5.2). After conducting the PCA, 5 maternal methyl nutrient patterns were retained which explained 72.3% of the variation of maternal methyl nutrient data (Table 5.3). Maternal methyl nutrient patterns were labeled according to loading of each maternal methyl nutrient to the pattern: 1) B-12/methyl donor pattern (including total B-12, betaine and free choline concentrations), 2) Homocysteine pattern (tHcy concentration), 3) Riboflavin pattern (including FAD and FMN concentrations), 4) Folate pattern (RBC folate concentration) and 5) PLP/methionine pattern (including PLP and methionine concentrations). Maternal B-12/methyl donor pattern score tended to increase with increasing log-B-12, log- free choline and log-betaine concentrations, the main contributors to the pattern. Similarly, maternal homocysteine and riboflavin pattern scores tended to increase with increasing log-tHcy, and log-FMN and log-FAD concentrations, respectively. Maternal folate pattern scores also increased with increasing log-RBC folate, and the PLP/methionine pattern scores increased with increasing log-PLP and logmethionine concentrations.

Biomarker concentrations		All women		Specimen DNA methylation					Women included in	the PCA
	n		n	Buffy coat	n	BECs	n	P^2	PCA	P^3
Plasma betaine, µmol/L	238	18.1 (14.5-22.8)	107	18.0 (15.3-22.9)	131	18.1 (14.3-22.3)	139	0.73	18.4 (14.3-24.1)	0.98
Plasma free choline, µmol/L	238	11.3 (9.35-13.5)	107	11.5 (9.45-13.5)	131	11.2 (9.27-13.4)	139	0.40	11.4 (9.45-13.6)	0.99
Plasma tHcy, µmol/L	238	4.47 (3.72-5.43)	107	4.19 (3.40-5.37)	131	4.63 (4.05-5.51)	139	0.07	4.52 (3.76-5.46)	0.90
Plasma methionine, μ mol/L	238	20.6 (17.5-24.2)	107	20.0 (17.1-23.4)	131	21.0 (17.8-25.1)	139	0.26	20.5 (17.0-24.6)	0.55
Serum total B-12, pmol/L	223	246 (184-322)	98	247 (190-322)	125	240 (169-315)	139	0.36	255 (188-338)	0.74
Plasma PLP, nmol/L	239	72.8 (46.8-128)	107	64.4 (45.8-119)	132	74.5 (49.2-134)	139	0.33	74.0 (51.8-133)	0.64
Plasma FMN, nmol/L	239	18.8 (16.1-21.6)	107	18.7 (15.8-21.1)	132	18.8 (16.2-22.1)	139	0.24	19.3 (16.2-22.1)	0.60
Plasma FAD, nmol/L	238	78.0 (65.8-93.1)	107	77.7 (64.4-92.9)	131	78.6 (66.8-93.7)	139	0.55	76.0 (64.3-93.1)	0.49
RBC folate, nmol/L	154	1334 (1111-1688)	71	1427 (1130-1688)	83	1306 (1095-1762)	139	0.53	1337 (1111-1702)	0.95

Table 5.2 Maternal biomarker concentrations of all women with infant DNA methylation data by tissue and those included in the PCA¹

¹Values are median (IQR). *P*² values are from Wilcoxon rank-sum test for comparison of continuous variables between pregnant women who had infant DNA methylation data measured in buffy or BECs. *P*³ values are from Wilcoxon rank-sum test for comparison between the total sample and those pregnant women that were included in the PCA. BECs, buccal epithelial cells; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PCA, principal component analysis; PLP, pyridoxal-5'-phosphate; tHcy, total homocysteine.

Maternal methyl nutrient	B-12/methyl	Homocysteine	Riboflavin	Folate	PLP/methionine
patterns	donor pattern ¹	pattern ²	pattern ³	pattern ⁴	pattern ⁵
Variance explained	17.4%	14.6%	14.4%	13.3%	12.6%
Total B-12	0.4700	-0.3251	-0.0397	0.4030	0.0870
FAD	-0.2485	0.3355	0.5816	0.2860	0.0276
FMN	0.1091	-0.2086	0.7845	-0.1554	0.0101
tHcy	0.0352	0.7500	-0.1370	-0.0670	0.0743
PLP	0.1364	0.1819	0.0311	0.2284	0.7060
RBC folate	-0.084	-0.0432	-0.0632	0.7539	0.0050
Betaine	0.6717	0.0424	0.0091	-0.1709	0.0758
Free choline	0.4618	0.3426	0.1448	0.0329	-0.231
Methionine	0.1189	0.1393	-0.0012	0.2763	-0.6546

Table 5.3 Loadings of methyl nutrients for early-pregnancy methyl nutrient patterns

¹B-12/methyl donor pattern refers to maternal total B-12, free choline and betaine loadings;²Homocysteine pattern refers to maternal tHcy loading; ³Riboflavin pattern refers to maternal FAD and FMN loadings; ⁴Folate pattern refers to maternal RBC folate loading; ⁵PLP/methionine pattern refers to maternal PLP and methionine loadings. FAD,flavin adenine dinucleotide; FMN, flavin mononucleotide; PLP, pyridoxal-5'-phosphate; RBC, red blood cell; tHcy, total homocysteine.

5.3.1 Infant characteristics

Neonatal and infant characteristics are presented in Table 5.4. Of the total sample, 48% of infants were female (n=118) and 52% were male (n=128). Most of the infants were born at term. Females were lighter [3353 (2970-3545) g vs. 3494 (3143-3833) g] and shorter [52.0 (50.0-53.3) cm vs. 50.8 (49-53.3)] cm than male newborns (P<0.05 for both). No significant differences were found comparing neonatal and infant characteristics by tissue type in the total sample of pregnant women (P>0.05). Furthermore, no significant differences were found comparing infant characteristics of infants of pregnant women that were included in the PCA compared to the total sample of pregnant women.

5.3.1 Association of maternal methyl nutrient patterns and DNA methylation levels of fetal growth and obesity-related genes in the offspring

Derived maternal methyl nutrient patterns were not significantly associated with DNA methylation levels of CpG sites associated with *IGF-2*, *RXRA*, *HIF3a*, *DNMT-1*, *DNMT-3*, *LEP* and *LEP-R*, measured in BECs or buffy coat, before or after the Benjamini-Hochberg test adjustment (P>0.05). Additionally, maternal pre-pregnancy BMI, maternal ethnicity, infant sex and, infant age were not significantly associated with DNA methylation levels of CpG sites associated with *IGF-2*, *RXRA*, *HIF3a*, *DNMT-1*, *DNMT-3*, *LEP*, and *LEP-R*.

Infant		All infant	ts		Specime	n DNA methylatior	W	Women included in the PCA		
characteristics	n		n	Buffy coat	n	BECs	P^2	n	PCA	P^3
Birth status, %	246		109	-	137		0.05	139		0.61
Term	231	93.9	106	97.3	125	91.2		129	92.8	
Preterm	15	6.10	3	2.75	12	8.76		10	7.19	
Gestational age	246	39.7	109	39.7	137	39.7	0.45	139	39.9	0.27
at birth, wks		(38.4-40.1)		(38.9-40.1)		(38.0-40.1)			(38.9-40.3)	
BW, g	246	3380	109	3410	137	3343	0.33	139	3400	0.39
-		(3060-3731)		(3120-3731)		(3020-3710)			(3100-3785)	
LBW, %	14	5.69	4	3.67	10	6.30	0.22	8	5.79	0.97
BL, cm	243	51.1	106	51.0	137	51.5	0.68	139	51.5	0.64
		(49.5-53.3)		(49.5-53.3)		(50.0-53.3)			(50-53.3)	
Infant age, wks	209	12 (11-13)	96	12 (12-13)	113	12 (11-13)	0.97	123	12 (11-13)	0.92
Infant weight, g	236	6095 (5600-6600)	108	6100 (5645-6745)	128	6000 (5500-6590)	0.35	136	6045 (5520-6555)	0.73
Infant length,	181	59.1 (57.1-61.0)	85	60.0 (58.0-61.0)	96	58.7 (57.0-61)	0.08	103	59.0 (57.0-70.0)	0.59

Table 5.4 Infants characteristics of offspring with DNA methylation data by tissue and those included in the PCA¹

¹Values are median (IQR) for continuous variables or prevalence (%) for categorical variables. P^2 values are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for comparison of categorical variables between tissues. P^3 values are from Wilcoxon rank-sum test for comparison of continuous variables and chi-square test for the infants of pregnant women of the total sample and infants of pregnant women that were included in the PCA. BECs, buccal epithelial cells; BW, birth weight; BL, birth length; LBW, low birth weight; PCA, principal component analysis; wks, weeks

5.4 Discussion

DNA methylation is the most stable epigenetic mechanism, and it has been suggested that it might be involved in fetal growth and obesity programming. Methyl nutrients such as folate, riboflavin, vitamins B-6 and B-12, choline, and betaine participate in interrelated pathways of one-carbon metabolism, which critical for the provision of methyl groups for DNA methylation. The findings of the present study showed no significant relationship between maternal methyl nutrients patterns at early pregnancy and DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes (i.e., *IGF-2, LEP, LEP-R, RXRA,* and *HIF3a* genes) and methyltransferase coding genes in the offspring.

Maternal methyl nutrient concentrations. Early-pregnancy betaine (median 18.1 nmol/L), tHcy (median 4.47 μ mol/L), total B-12 (median 246 pmol/L) and methionine (median 20.6 μ mol/L) concentrations reported in the current study are similar to maternal betaine, tHcy, total B-12 and methionine concentrations reported by Wu et al. (36) (betaine: median 13.0 μ mol/L; Hcy: median 4.10 μ mol/L; total B-12: median 259 pmol/L; methionine: median 20.6 μ mol/L) in pregnant women living in Vancouver, BC, at 16 weeks of gestation, and in Spanish pregnant women (betaine: geometric mean ~18 μ mol/L between 12-15 weeks of gestation) (64). However, maternal non-fasting free choline concentration was almost two-fold higher (11.3 (9.35-13.5) μ mol/L) compared to early fasting maternal free choline concentration reported by Wu et al. (36) (median: 6.9 μ mol/L at 16 weeks of gestation). Differences in maternal free choline concentration might be partially explained by non-fasting conditions in the samples collected in the present study which may increase free choline concentration compared to those collected under fasting conditions (36,261,307). However, Visentin et al. (308) reported that Canadian pregnant who were not asked to fast had a free choline concentration of 7.2 μ mol/L at <50 km/s (10.2 km/s) (10.2 km/s) (20.2 k

of gestation. Additionally, maternal free choline concentration increases across weeks of gestation, showing a more pronounced physiological increase after 15-16 weeks of gestation (64,65), mainly due to *de novo* choline synthesis by the PEMT pathway, and the subsequent generation of free choline (87). In the present study, 40% of pregnant women were at >16 weeks of gestation during sample collection. Therefore, different time points of maternal sample collection (i.e., <22 weeks of gestation vs. <16 weeks of gestation) may also explain the differences found with the maternal free choline concentration reported in the PREFORM study (308).

Furthermore, maternal RBC folate concentration (median: 1334 nmol/L) was lower compared to results reported in Canadian pregnant women who participated in the PREFORM study (mean RBC folate 2417 nmol/L) at 16 weeks of gestation. The difference may be related to the folic acid dose in prenatal supplements consumed. In the PREFORM study, 93% of the participants consumed folic acid supplementation at early pregnancy with 83% of them using a dose $\geq 1000 \mu g/d$ (16). While, in the AprON study, 97% of the participants consumed folic acid supplements in the first trimester, however, only 22% used a dose $>1000 \mu g/day$ (68). Also, PLP concentration (median: 73 nmol/L) was lower in the current study compared to the PREFORM study (geometric mean: 107 nmol/L; 95% CI 98, 116 nmol/L). We speculate that differences in analytical methods used to determine PLP concentration (non-radioactive enzymatic assay vs. LC-MS-/MS) may partially explain the differences (71).

To my knowledge, only one previous study conducted in the Netherlands has reported FMN and FAD concentrations during pregnancy. The study was an intervention trial that included women who had a previous NTD affected pregnancy and were planning to become pregnant. Findings of the study showed that FMN (~36 nmol/L at 6 and 9 weeks of gestation) and FAD (~250 nmol/L at 6 and 9 weeks of gestation) concentrations were at least two-fold higher than the FMN (median:18.8 nmol/L) and FAD (median: 78 nmol/L) in the present study (309). We speculate that differences on FMN and FAD concentrations between studies may be partially related to the analytical methods used in each study (HPLC vs. LC-MS/MS), however further pregnancy studies are needed to understand potential pregnancy-related metabolic adaptations that may influence FAD and FMN concentrations.

Maternal methyl nutrient patterns. Maternal B-12/methyl donor pattern explained the highest variation of the biomarker data (17.4%), followed by homocysteine (14.6%), riboflavin (14.4%), folate (13.3%) and PLP/methionine (12.6%) patterns, which explained similar variation of the biomarker data. The derivation of the maternal B-12/methyl donor pattern is likely attributed to the significant and stronger correlation between log-betaine and log-free choline (r=0.45, P<0.001) and log-betaine and log-total B-12 (r=0.23, P=0.007), compared to the correlation among other methyl nutrients included in the PCA. A positive correlation between maternal free choline and betaine was previously reported by Wu et al. (36) in Canadian pregnant women ($\rho=0.60$, P<0.01). The strong correlation can be related to the role of choline as the precursor of betaine due to the action of the enzyme CHDH (49). Furthermore, choline supplementation (i.e., choline chloride), the precursor of betaine, resulted in higher serum holoTC and holoTC/total B-12 ratio in North American pregnant women (310), which may contribute to explain the significant correlation between plasma betaine, of which choline is precursor, and B-12 found in the current study.

Given the interrelationship between the folate-B-12-dependent and the alternative methylation pathways, the provision of choline may decrease the demand for the folate-B-12 dependent pathways, which may result in higher B-12 biomarker concentrations. Despite that a higher proportion of choline is used for PC during pregnancy, results of a randomized controlled trial in pregnant women showed that the provision of supplemented choline diets (i.e., 380 mg of

total dietary choline plus 100 mg of choline chloride or 550 mg choline chloride) enhanced the production of betaine by the choline oxidation pathway (87). Results of a prospective pregnancy study conducted in BC indicated that third-trimester plasma betaine concentration was lower in those pregnant women with deficient (<148 pmol/L) or marginal (148-221 pmol/L) B-12 status, compared to those classified with a sufficient B-12 (>221 pmol/L) status (36), which may indicate an interaction between betaine and B-12, especially in folate-replete populations.

Considering that homocysteine is a central intermediary metabolite in the one-carbon metabolism and is remethylated through either the folate-B-12 dependent or the alternativebetaine dependent pathways, tHcy concentration may be influenced by total B-12, folate, betaine, PLP, and riboflavin related coenzymes. Therefore, the derivation of the maternal tHcy pattern with the only significant loading of log-tHcy (factor loading> |0.45|) was unexpected. It has been previously reported that total B-12 concentration is significantly associated with tHcy concentration in Canadian population (101). Additionally, as reported in Chapter 2, maternal betaine concentration was negatively associated with tHcy concentration across the first and second trimester. We speculate that the potential small sample size of the current study may not have allowed the detection of a stronger correlation of log-tHcy with the other methyl nutrients included in the PCA. The derivation of the riboflavin pattern is likely explained by the strong correlation of log-FMN and log-FAD (r=0.26, P=0.001) which is expected because both metabolites are coenzyme forms and potential biomarkers of riboflavin status.

The derivation of the folate pattern, in which only log-RBC folate had a significant loading to the pattern (factor loading> |0.45|), and the subsequent lack of a strong correlation with the other methyl nutrients and related metabolites included in the PCA, such as tHcy concentration is partially expected in pregnant women from folate-replete populations. For example, in pregnant women living in Vancouver, plasma folate was not associated with the tHcy concentration at early or late pregnancy (36). However, the lack of correlation between RBC folate and the other biomarkers such as total B-12, free choline, betaine, FMN, and FAD needs to be confirmed in further pregnancy studies. Among the retained patterns, the PLP/methionine pattern explained the lowest variation of biomarker data. PLP has a ubiquitous role as a coenzyme in the provision of one-carbon groups for the formation of 5-MTHF, the methyl donor form of folate for the remethylation of homocysteine to methionine, which may partially explain the contributions of PLP and methionine to the pattern. Overall, the derivation of early-pregnancy methyl patterns was driven mainly by maternal total-B12, betaine, and homocysteine concentrations, which is similar to the results reported in Chapter 2.3. However, the explained biomarker variation for each pattern tended to be equal (i.e., ~17-14%), which may be related to the small sample size, and the lack of strong correlations among biomarker concentrations potentially due to 'replete methyl nutrient conditions'.

Association between maternal methyl nutrient patterns and DNA methylation levels of *fetal growth and obesity-related genes in the offspring*. The findings of this study indicated that there is no significant association between early pregnancy methyl nutrient patternsand DNA methylation levels of CpG sites associated with the *IGF-2*, *RXRA*, *HIF3a*, *LEP*, *LEP-R*, *DNMT-1* and *DNMT-3* genes in the offspring. To my knowledge, evidence on the association between early-pregnancy methyl nutrient status and DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes in the offspring is limited. In the U.S.A, Hoyo et al. (22) found that in a sample of 496 pregnant women, low maternal RBC folate concentration (i.e., second quartile, folate concentration not reported) was associated with increased DNA methylation compared to 3rd and 4th quartiles.

Different factors may be involved in the discrepancies between the results of the current study and the findings reported by Hoyo et al. (22) First, Hoyo et al. (22) explored the association

between an individual maternal biomarker concentration (RBC folate concentration) and DNA methylation levels of *IGF-2* in the offspring, while in the current study, I explored the relationship of combined early-pregnancy methyl nutrient concentrations and DNA methylation levels of *IGF-2* in the offspring. Second, Hoyo et al. (22) measured DNA methylation levels in cord blood, while in our study DNA methylation levels were measured in infant buffy coat (i.e., leucocytes) and BECs. It has been previously reported that DNA methylation levels vary by cell type (311). For example, DNA methylation levels of *IGF-2* were consistently lower in BECs compared to DNA methylation levels of *IGF-2* measured in cord blood (311) which may attenuate the potential relationship between maternal methyl nutrient patterns and DNA methylation levels of *IGF-2*, measured in BECs, in the offspring.

The methods used to measure DNA methylation in the aforementioned studies were pyrosequencing or mass spectrometer, whereas in the present study DNA methylation was measured using a microarray assay (Illumina 450k). Pyrosequencing measures specific genes of interest (target genes), while the microarray assay examines DMRs, covering more than 450,000 CpG sites of the genome. It has been reported that pyrosequencing can detect small differences of DNA methylation levels (<5%) compared to other technologies used (297), including the Illumina 450K, which may partially explain the lack of association in the present study, and the significant association reported in North American pregnant women (22). The current study included a relatively small sample size (n < 100) which may have not allowed the detection of potential associations with small effect sizes between maternal methyl nutrient patterns and DNA methylation levels of CpG sites associated with *IGF-2, LEP, LEP-R, RXRA, HIF3a, DNMT-1* and *DNMT-3* genes in the offspring.

Strengths and limitations. Strengths of the present study include a comprehensive portfolio of maternal methyl nutrient concentrations at early pregnancy, and DNA methylation

levels of CpG sites associated with fetal growth and obesity-related genes in the offspring which allowed to test the association of maternal methyl nutrient status and DNA methylation levels in the offspring in a critical developmental window of pregnancy. Additionally, as it was described in Chapter 3.4, the statistical approach used (i.e., PCA) allowed us to explore the interrelationship between maternal methyl nutrients by the derivation of non-correlated maternal methyl nutrient patterns, which prevented potential multicollinearity among the maternal biomarkers included in the regression models. However, this prospective cohort study was limited by its small sample size as well as by the determination of DNA methylation levels in two different tissues (i.e., BECs and buffy coat), which potentially affected the capacity to detect small effect sizes in the association between maternal methyl nutrient patters and infant DNA methylation levels. Furthermore, the small sample size did not allow the inclusion of weeks of gestation at collection of maternal blood samples in the regression model. We prioritized the inclusion of maternal ethnicity, infant age and pre-pregnancy BMI because previous evidence has shown that DNA methylation levels vary according to ethnic background and age (312). Also, pre-pregnancy BMI has been associated with obesity and overweight in the offspring, which may be related to DNA methylation (312). Of the total maternal samples that we included in the present study, 30% of them were collected at the first trimester (i.e., 14 weeks of gestation) and 70% were collected between 14 and <22 weeks of gestation. We acknowledge that maternal methyl nutrient concentrations change across weeks of gestation during early pregnancy, for example, maternal betaine (i.e., $\sim 0.5 \,\mu$ mol/L/week at < 20 weeks of gestation) (65), total B-12 (i.e., $\sim 9 \,\mu$ mol/L/week at <20 weeks of gestation) (65) and PLP concentrations (i.e., ~2 nmol/L/week between 12-16 weeks of gestation and delivery) (71). Given that most of the samples included in this study were collected in the second trimester of pregnancy, it is unlikely that the inclusion of weeks of gestation in the regression model would have modified our findings. Additionally, as shown in

Chapter 33.3, the derivation of the maternal methyl nutrient patterns was characterized by maternal B-12 status in both trimesters. Therefore, we speculate that the potential derivation of maternal methyl nutrient patterns at the first and second trimester separately would likely provide similar retained patterns in both trimesters.

Additionally, mother-infants dyads included in this study were apparently healthy, predominantly of Caucasian ethnicity, with a high household income and maternal education level, limiting the generalizability of our results. Also, the current study did not include other factors such as genetic variants, which were suggested as important contributors to the variation of DNA methylation levels of CpG sites associated with obesity-related genes (e.g., *LEP*) (313,314). In conclusion, the current study showed that early-pregnancy B-12/methyl donor pattern explained the highest variation of the biomarker data. Additionally, no significant associations between early pregnancy methyl nutrient patterns and DNA methylation levels of CpG sites associated with *IGF-2*, *LEP*, *LEP-R*, *RXRA*, *HIF3a*, *DNMT-1* and *DNMT-3* genes in the offspring were found. Further pregnancy studies including larger sample size or pooled data including other pregnancy cohort studies are needed to explore the relationship between maternal methyl nutrients at early pregnancy and DNA methylation of CpG sites associated with fetal growth and obesity-related genes in the offspring.

Chapter 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

6.1 General discussion of the key findings

In Chapter 6, a summary of the key findings of my Ph.D. research work is presented, followed by overall limitations of the present research. Lastly, future research directions and the novelty, significance and contribution of my work to the current knowledge of the relationship between maternal methyl nutrient concentrations and fetal growth and obesity programming, and neonatal anthropometric measurements are presented.

6.1.1 Summary of my Ph.D. research work

Folate, betaine, choline, methionine, riboflavin and vitamin B-12 and B-6, - also known as methyl nutrients, have a combined role in the one-carbon metabolism, which is key for nucleotide synthesis, cellular proliferation and the provision of methyl groups for methylation reactions, such as DNA methylation. DNA methylation is a well-recognized stable epigenetic mechanism that is involved in fetal growth and obesity programming (Chapter 1.2). My Ph.D. research is focused on exploring the interrelationship of maternal methyl nutrient concentrations in early pregnancy, and its relationship with infant DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes, and neonatal anthropometric outcomes in Canadian mother-newborn dyads.

 The first objective of this research was to compare circulating betaine concentration between European and South Asian pregnant women in their first and second trimester of pregnancy, and to determine the relationship between betaine and tHcy concentrations in early pregnancy (i.e., <20 weeks of gestation), in a group of women living in Metro

Vancouver, BC, Canada. For this objective, we hypothesized that serum betaine concentration is higher in pregnant women of South Asian descent compared to pregnant women of European descent in their first and second trimester of pregnancy (P < 0.05), and that serum betaine concentration is associated with tHcy concentration in early pregnancy (P < 0.05). This hypothesis was supported by results presented in Chapter 2. We described that maternal betaine and DMG concentrations (µmol/L) in South Asian [first trimester, betaine 19.8 (16.3-25.0), DMG 1.55 (1.30-1.96); second trimester, betaine 16.1 (12.9-19.8), DMG 1.42 (1.14-1.81)] were significantly higher compared to European [first trimester, betaine 17.6 (13.7-22.6), DMG 1.38 (1.12-1.77); second trimester, betaine 12.9 (10.6-16.7), DMG 1.19 (0.97-1.52)] pregnant women living in Metro Vancouver. Furthermore, in this sample of Canadian pregnant women, early-pregnancy serum betaine concentration was negatively associated with tHcy concentration (β = -0.21; 95%CI -0.34, -0.07, P=0.002) across weeks of gestation after adjusting for serum folate, total B-12, methionine, PLP, maternal age, ethnicity, neonatal sex, parity, and pre-pregnancy BMI categories. Additionally, serum total B-12 concentration was negatively associated with serum tHcy concentration (β =-0.031; 95%CI -0.04, -0.02, P<0.001), after adjustment for confounding factors.

2. The second objective of this research was to explore the interrelationship of maternal methyl nutrient concentrations in the first- and second trimester, and to determine the association of first- and second-trimester methyl nutrient patterns with neonatal anthropometric outcomes among Canadian mother-newborn dyads. We hypothesized that first- and second-trimester maternal methyl nutrient patterns were characterized by specific maternal methyl nutrient concentrations and are positively associated with

neonatal anthropometric outcomes among Canadian mother-newborn dyads (P<0.05). The results presented in Chapter 3 partly supported this hypothesis. First and second trimester maternal methyl nutrient patterns were characterized by B-12 status (i.e., serum total B-12 and holoTC concentrations). First and second trimester maternal B-12 status explained the highest variation of the maternal biomarker data (i.e., 26% and 25%, respectively) in a subset of pregnant women of European and South Asian ethnicity living in BC, Canada, using trimester-specific PCA. In contrast to our hypothesis, second-trimester maternal B-12 pattern was negatively associated with HC (β =-0.13 cm; 95%CI - 0.24, -0.03) and HC-z-score (β =-0.05; 95%CI -0.09, -0.009), after adjustment for confounding factors (P≤0.05 for all).

3. The third objective of this thesis was to determine DNA methylation level (%) of CpG sites associated with *IGF-2*, *LEP*, *LEP-R*, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A*, *DNMT-3B* genes in 3-month old infants of mothers who had high RBC folate concentration (>1360 nmol/L) in early pregnancy (i.e., <20 weeks of gestation), compared to infants of mothers who had RBC folate concentration ≤1360 nmol/L. For this objective, we hypothesized DNA methylation level (%) of CpG sites related to *IGF-2*, *LEP*, *LEP-R*, *HIF3α*, *RXRA*, *DNMT-1*, *DNMT-3A* and *DNMT-3B* genes in 3-month old infants of pregnant women with high RBC folate concentration (>1360 nmol/L) in early pregnancy are higher compared to those of 3-month-old infants of pregnant women who had RBC folate concentration ≤1360 nmol/L) in early pregnancy are higher concentration ≤1360 nmol/L (*P* <0.05).</p>

The results presented in Chapter 4 did not support this hypothesis. Median (IQR) RBC folate concentration was 1334 (1111-1668) nmol/L in a subset of pregnant women (*n*=154) that participated in the APrON study, conducted in Alberta, Canada. Of the total

subset of pregnant women, 51% (*n*=79) had folate concentration \leq 1360 nmol/L [median (IQR) RBC folate concentration of 1116 (996-1231) nmol/L], whereas 49% (*n*=75) had high folate status defined as RBC folate concentration >1360 nmol/L [median (IQR) RBC folate concentration of 1702 (1516-1887) nmol/L]. DNA methylation levels of *DNMT-3B*, *LEP*, *LEP-R DNMT-3A*, *HIF-3a*, *RXRA*, *IGF-2*, and *DNMT-1* in the infants did not significantly differ by maternal folate status.

4. The fourth objective was to investigate the relationship between maternal methyl nutrient patterns in early pregnancy (i.e., <20 weeks of gestation) and DNA methylation level (%) of CpG sites associated with *DNMT-3B*, *LEP*, *LEP-R DNMT-3A*, *HIF-3α*, *RXRA*, *IGF-2*, and *DNMT-1* genes in 3-month-old infants. We hypothesized that DNA methylation level (%) of CpG sites related to *DNMT-3B*, *LEP*, *LEP-R DNMT-3A*, *HIF-3α*, *RXRA*, *IGF-2*, and *DNMT-1* genes in 3-month-old infants are positively associated with maternal methyl nutrient patterns in early pregnancy (*P*<0.05). The results presented in Chapter 5 did not support this hypothesis. Maternal methyl nutrient patterns were characterized by B-12 and methyl donor concentrations (i.e., serum total B-12, betaine and free choline). The maternal B-12/methyl donor pattern explained the highest variation of the variation of biomarker concentrations at early pregnancy. No significant association was found between early-pregnancy maternal methyl nutrient patterns and infant DNA methylation of CpG sites associated with *LEP*, *RXRA*, *HIF-3α*, *IGF-2*, *LEP-R*, *DNMT-1*, *DNTM-3A* and *DNTM-3B* genes.

In conclusion, the findings of my Ph.D. research emphasize the role of betaine, in addition to B-12, for homocysteine remethylation in pregnant women from a folatereplete population. The derivation of maternal methyl nutrients patterns at early
pregnancy was mainly driven by total B-12, holoTC, tHcy, betaine and riboflavin related metabolite concentrations which indicate that other methyl nutrients such as B-12 and not folate, may have a stronger relationship with the other methyl nutrients in folate-replete pregnant women. I only found a significant relationship between early-pregnancy methyl nutrient patterns and HC, as well as, no significant relationship between early-pregnancy methyl nutrient patterns or maternal folate status and DNA methylation levels of CpG sites associated with the investigated genes.

6.2 Discussion of key findings

6.2.1 Maternal betaine concentration and its relationship with tHcy concentration in a folate-replete population

Folate and betaine have been indicated as methyl donors of the B-12 dependent and alternative remethylation pathways, respectively, in one-carbon metabolism in which both nutrients provide a methyl group for the remethylation of homocysteine to methionine. Methionine is then converted to SAM, the universal methyl donor, which is critical for methylation reactions such as DNA methylation (Chapter 1.2). In my Ph.D. research, I found that early-pregnancy betaine concentration, but not folate, was negatively associated with tHcy concentration (β =-0.21; 95%CI -0.34, -0.07 µmol/L) across early pregnancy (i.e., <20 weeks of gestation) in folate replete Canadian pregnant women. Additionally, total B-12 but not folate, was also negatively associated with tHcy concentration (β =-0.031; 95%CI -0.040, -0.022) (Chapter 2.3).

In the last years, betaine has become a nutrient of interest mainly due to its protective role as an intracellular osmolyte, as well as its role as a methyl donor in the alternative remethylation pathway in the liver and kidneys (47,48,103). An interaction between the folate-B12 dependent and alternative remethylation pathways has been described in human studies (93,95,98). In Dutch men [plasma folate concentration: 11.8 (7.8–17.7) nmol/L] and postmenopausal women [plasma folate concentration: 13.1 (8.7–19.3) nmol/L], betaine concentration was a negative predictor of tHcy concentration (standardized β =-0.20) (98). Furthermore, results of a study in Spanish pregnant women (64) showed that betaine became a negative predictor of tHcy concentration at 24-27 weeks of gestation but only in women with low folate status (plasma folate concentration: geometric mean 7.2 nmol/L), which may indicate lower intracellular availability of folate, with subsequent use of betaine as a methyl donor. Meanwhile, a daily dose of folic acid supplementation (50, 100, 200, 400, 600, or 800 µg) showed a dose-dependent effect, increasing betaine concentration which suggested a sparing effect of folate as a methyl donor (98).

Although folate has been indicated as the most important methyl donor in pregnancy studies conducted in non-folate-replete populations (64–66), in folate-replete populations, such as the Canadian population (67,207), betaine, in addition to B-12 (101), are critical for the remethylation of homocysteine to methionine. As discussed in Chapter 2.4, findings from pregnancy studies conducted in Canada indicated that most of the pregnant women (>90%) use folic acid supplements before pregnancy and across all trimesters, with most supplements containing more than two-fold (i.e., 1000 μ g of folic acid) of the recommended dose for women with a low risk of NTDs (i.e., 400 μ g of folic acid), which has led to high folate status (i.e., RBC folate concentration >1360 nmol/L). Evidence from a recent animal study showed that high doses of folic acid supplementation (i.e., 20 mg of folic acid/kg) before and during pregnancy, and lactation caused a pseudo-MTHFR deficiency in the offspring, characterized by a decreased enzymatic activity and expression of MTHFR in the liver (94). Additionally, offspring of dams that received folic acid supplementation, had significantly lower expression of the *DNMT-3A* gene in the brain, compared to offspring of dams that received the control diet (94). While no

effect was seen in the expression of the *DNMT-1* gene. These findings suggest that under folatereplete conditions not only a pseudo-intracellular folate deficiency may occur with the subsequent use of betaine as a methyl donor, but also, potential critical adverse consequences in *de novo* DNA methylation.

De novo DNA methylation, which occurs at early stages of pregnancy, is vital for cellular differentiation and offspring growth. Findings of the present study indicate that betaine is negatively associated with tHcy concentration in early pregnancy (i.e., <20 weeks of gestation), compared to previous pregnancy studies that showed an inverse association only after 20 weeks of gestation (64–66). These findings suggest that in pregnant women from folate-replete populations and characterized by extended use of high dose folic acid supplements from early pregnancy to lactation, the use of betaine, as methyl donor, is promoted earlier during pregnancy which may be crucial for fetal growth programming. A recent study in mice indicated that the offspring of female mice who received betaine supplementation (1% betaine anhydrous) showed a significantly lower expression of *IGF-2* gene, which is a key gene involved in fetal growth and placental development (315), and may suggest a key role of betaine in fetal growth programming. Additionally, betaine has also shown a regulatory role in the expression of enzymes involved in lipid metabolism and body composition in pigs (187), which we speculate, might be critical for obesity programming at the early stages of pregnancy.

In humans, results of the GUSTO study (65) showed that late-pregnancy betaine concentration was negatively associated with a lower neonatal total body fat and newborn size. Similarly, I reported in Chapter 3.3.4, that first-trimester betaine concentration of those pregnant women included in first-trimester PCA was negatively associated with BL and BL z-score only in full-term babies, which may suggest that early-pregnancy betaine concentration has not only a key role for the remethylation of homocysteine to methionine in this folate-replete population,

but also may have a crucial role in fetal growth and body composition programming through DNA methylation.

Given the lack of national data in Canadian pregnant women, the present study contributes to the knowledge of maternal methyl nutrient concentrations in a sample of South Asian and European pregnant women in early pregnancy, which is known as a critical plasticity window for fetal development. Additionally, this research provides key information about the role of betaine in the provision of methyl groups in the remethylation pathway of one-carbon metabolism, which are further use for DNA methylation and potentially for fetal programming.

6.2.2 Early-pregnancy methyl nutrient patterns are characterized by betaine andB-12 and riboflavin related metabolites in Canadian pregnant women

Early stages of pregnancy are a critical developmental and plasticity window in which the fate of pluripotent stem cells and offspring organs is programmed. One of the mechanisms suggested to be involved in fetal programming is DNA methylation. In this context, the interrelationship of maternal methyl nutrients is crucial in providing methyl groups as well as for the synthesis of DNA, and subsequently, for cellular proliferation (Figure 1.2).

In the present research, early-pregnancy methyl nutrient patterns were derived in two subsets of pregnant women living in BC and Alberta, Canada, using a PCA approach. Overall, they were comparable as is detailed in in Chapter 3.3.3 and Chapter 5.3.2. In both subsets of pregnant women, maternal B-12 patterns explained the highest variation of the maternal biomarker data. Additionally, maternal B-12 biomarker and betaine concentrations were the most important drivers of the maternal methyl nutrient patterns. The derivation of the early pregnancy methyl nutrient patterns indicated that in these two subsets of Canadian pregnant women from a folate-replete population, direct biomarkers of B-12 status (i.e., holoTC, total B-12) explained the

highest variation of the maternal biomarker data. As discussed in Chapter 3.3, a strong correlation seen between total B-12 and holoTC, likely explains the subsequent derivation of the B-12 pattern in pregnant women of BC, while in pregnant women of the APrON study, B-12 also showed a significant contribution to the B-12/methyl donor pattern. Furthermore, tHcy concentration, a functional biomarker of B-12 status, was a significant contributor to the B-12 pattern only in BC pregnant women, and it showed a significant and inverse relationship with total B-12. Regardless of the lack of a significant relationship between total B-12 and tHcy concentration in the subset of pregnant women from the APrON study, and the subsequent lack of contribution of tHcy to the B-12/methyl donor pattern, we speculate that this is likely due to the lower sample size of the subset of APrON participants included in the PCA. The correlation of tHcy and B-12 concentration in the total sample size (n=253) was weak, but significant (r=-0.17, P=0.02) compared to the results obtained in the subset of pregnant women included in the PCA (r=-0.12, P=0.03).

Furthermore, 3 patterns were retained in pregnant women of BC, while 5 patterns were retained in the subset of participants of the APrON study. Differences in the amount of retained patterns may be explained by the different biomarkers included in each PCA, which we speculate may contribute to a partial redistribution of the relationship between the biomarkers and the subsequent retention of more patterns in the subset of pregnant women of the APrON study. For example, first trimester serum folate showed a significant correlation with riboflavin (r=0.25, P<0.001) in BC pregnant women, whereas RBC folate concentration was not significantly correlated with any of the biomarkers determined in pregnant women of the APrON study, which may contribute to the differences in the retained patterns.

Additionally, the subset of pregnant women included in each PCA (i.e., BC pregnant women: n=505 at first trimester and n=392 at the second trimester; APrON study n=139: <22

weeks of gestation) was different. For example, 50% of BC pregnant women were European and 50% were South Asian, while 80% of the pregnant women of the APrON study were Caucasian and 6% were South Asian. In the BC study, South Asian pregnant women had significantly lower total B-12 and higher betaine concentration compared to European pregnant women, which may have also influenced the strength of the relationship among the biomarkers and the subsequent derivation of maternal methyl nutrients patterns.

Furthermore, differences in the time point of maternal blood samples collection between BC pregnant women and the subset of the participants of the APrON study may also explained the derivation of different maternal methyl nutrients patterns. Two PCAs were conducted in the BC pregnancy study; the first PCA was conducted using biomarker concentrations measured in samples collected at ~11 weeks of gestation. The second PCA was conducted using biomarker concentrations measured in collected at ~16 weeks of gestation. While in the subset of participants of the APrON study only, one PCA was conducted using biomarker concentrations of maternal samples collected ~15 weeks of gestation. It has been previously reported that maternal methyl nutrients may decrease (i.e., PLP concentration ~2 pmol/L per week) (71) or increase (i.e., free choline concentration ~0.2 μ mol/L per week) (65) across weeks of gestation due to pregnancy-related factors, such as hemodilution or increased estrogen concentration.

Given the folate-replete status of Canadian pregnant women, as well as the extended use of high dose of folic acid (16,67), we speculate that B-12, in addition to betaine, may become a critical nutrient for the remethylation of homocysteine to methionine. High doses of folic acid intake have shown to inhibit the expression of *MTHFR* and *MS* genes, and MTHFR and MS are both critical enzymes for the folate-B-12 dependent methylation pathway (94). In contrast, evidence from an *in vitro* study indicated that B-12 modulates the translation of MS (316,317). The inhibition of the expression of *MTHFR* and *MS*, and the subsequent decreased activity of MTHFR and MS enzymes may cause pseudo-folate deficiency conditions. In this context, B-12 may become a key nutrient attenuating the inhibitory effect of folic acid on MS activity and promoting the remethylation of homocysteine to methionine.

In addition to the role of B-12 in the remethylation pathway of one-carbon metabolism, evidence from animal studies has indicated that B-12 has an important role at early stages of pregnancy. A study conducted by Kumar et al. (318) showed that offspring of dams that received a B-12 deficient diet (i.e., without B-12) had a decreased lean body mass (%) and fat-free mass (%), and had higher fasting plasma glucose and insulin concentrations at 12 months. Additionally, findings of the same study indicated that the B-12 deficient diet led to alterations in DNA methylation levels of genes involved in fatty acid metabolism and energy expenditure in the offspring (319). Offspring of dams that received a B-12 deficient diet had higher DNA methylation levels of the Adipor2 gene that encodes adiponectin receptor 2, and it was expressed in adipose tissue and liver (319). Adiponectin receptor 2 is involved in glucose and insulin metabolism (320). These results suggest that B-12 may have an important role not only in the remethylation of homocysteine to methionine but also in fetal programming through DNA methylation reactions. A potential role of B-12 in early stages of pregnancy has also been suggested in human studies. In Canada, low maternal B-12 concentration was associated with a higher risk of NTDs in the era of folic acid fortification (321). Neural tube closure is part of the primary neurulation process that occurs in the first 4 weeks of gestation (322). Neural tube closure involves cellular differentiation (i.e., neural stem cells) and migration; therefore, these findings suggest that B-12 may potentially play a crucial role, in addition to folate, in the cellular fate and early formation of the nervous system in the offspring (321).

In addition to the direct and functional biomarkers of B-12 status, findings of the current study indicate that betaine and riboflavin related metabolites had a significant contribution to

maternal methyl donor patterns (Chapters 3.3.3 and 5.3.2). We speculate that under pseudo-folate deficiency conditions, the use of the alternative remethylation pathway may be promoted (48). It has been previously suggested that folate deficiency induces choline depletion, which is the precursor of betaine, in the liver (259). Limited evidence is available regarding the potential role of betaine in early stages of pregnancy. Findings of animal studies have shown that both, betaine and folate, are important for the provision of methyl groups during embryogenesis stage (263). Additionally, specific betaine transporters have been identified in mice embryos which may indicate that betaine is needed for early stages of development and growth (102,103). Furthermore, in humans, late-pregnancy betaine concentration was inversely associated with neonatal anthropometric outcomes such as BW, BL and adiposity measurements (39). However, whether betaine is involved in the programming of fetal growth and neonatal anthropometric outcomes needs to be explored in future pregnancy studies.

In addition to betaine and B-12, plasma riboflavin was an important contributor to the patterns which is likely explained by the role of riboflavin in the form of FAD as a coenzyme for the formation of 5-MTHF (7), the form of folate involved in homocysteine remethylation. In cells, riboflavin is the precursor of FMN which is a coenzyme for the formation of PLP. PLP functions as a coenzyme in the reaction providing one-carbon groups, e.g., the reaction catalyzed by the enzyme cytosolic serine hydroxymethyltransferase (SHMT1), and in the irreversible catabolism to cysteine in the transsulfuration pathway (79).

Findings from studies conducted in healthy adults indicated that plasma riboflavin concentration showed a negative association with tHcy concentration in adults carrying the CT/TT (323) and the CC/TT genotype of the *MTHFR C677T* polymorphism (324), which may suggest an important role of riboflavin in the remethylation pathway of homocysteine to methionine. It has been reported that individuals carrying the TT genotype have 30% MTHFR

activity (325). Results of the Framingham Offspring Cohort study in adults showed that the inverse relationship between plasma riboflavin and tHcy concentration was restricted to those with low folate status (defined as plasma folate <12.5 nmol/L) and TT genotype (326). We speculate that under pseudo-folate deficiency due to impaired activity of MTHFR (i.e., under potentially excessive folic acid consumption), riboflavin becomes an important nutrient for the remethylation of homocysteine to methionine, which may potentially explain the contribution of riboflavin to the derived patterns in this sub-sample of Canadian pregnant women of the APrON study. In addition, findings from animal studies have indicated that riboflavin deficiency in dams was associated with impaired embryo growth and cardiac development in the offspring (110), which may also suggest that riboflavin participates in early stages of development and cellular proliferation and differentiation. However, the relationship between maternal riboflavin status and offspring development needs to be confirmed in human studies.

Taken together, the derivation of maternal methyl nutrient patterns in early pregnancy indicated that under folate-replete conditions, B-12, betaine and riboflavin and related metabolites are the main drivers of maternal methyl nutrient patters in folate-replete Canadian pregnant women. These findings suggest that in pregnant women with high folate status and/or folic acid intake, B-12, betaine and riboflavin may have a predominant role in the interrelationship of methyl nutrients in early pregnancy, which is vital for provision of methyl groups for DNA methylation, and potentially in fetal growth programming. 6.2.3 Early-pregnancy methyl nutrient patterns were associated with HC, while DNA methylation levels of fetal growth and obesity-related genes in the infants were not associated with maternal methyl nutrient patterns or maternal folate status at early pregnancy

Given the interrelated role of the methyl nutrients in one-carbon metabolism for the provision of methyl groups for DNA methylation, we hypothesized that early-pregnancy methyl nutrient patterns are associated with neonatal anthropometric outcomes. Additionally, we speculated that this relationship is mediated by DNA methylation of CpG sites associated with fetal growth and obesity-related genes in infants. However, the findings of the current research did not support our hypothesis. To my knowledge, there is no available evidence on the relationship between maternal methyl nutrient patterns and neonatal anthropometric outcomes. Previous pregnancy studies that have explored the relationship between individual early-pregnancy methyl nutrient concentration and neonatal anthropometric outcomes have shown conflicting results. A pregnancy study conducted in Dutch pregnant women showed that low maternal folate concentration (i.e., folate concentration ≤ 9.1 nmol/L) measured at 14 weeks of gestation was associated with a lower BW (28), while studies including Italian and Canadian pregnant women have reported that early-pregnancy concentrations of folate, total B-12, free choline, and betaine were not significantly associated with BW (30,36).

Although maternal methyl nutrient patterns were not associated with neonatal anthropometric outcomes, which is similar to the findings of previous pregnancy studies that explored the relationship of individual maternal methyl nutrients and neonatal anthropometric outcomes, the derivation of maternal methyl nutrient patterns provides useful information about the interrelationship of maternal methyl nutrients, taking into consideration the contribution of all maternal methyl nutrients. As described in Chapter 1.2.1, methyl nutrients participate in

interdependent reactions of the one-carbon metabolism, thus, restricting the analysis to individual maternal methyl nutrient concentration (i.e., including only maternal folate concentration as exposure variable) may limit the understanding of the role of methyl nutrients in early growth and development.

Consistent with the results of the association between maternal methyl nutrient patterns and neonatal anthropometric outcomes, my findings showed no significant associations between maternal methyl nutrient patters and DNA methylation of CpG sites associated with fetal growth and obesity-related genes measured in the infants. Additionally, findings of the present study showed no significant differences in DNA methylation levels of CpG sites associated with the interrogated genes comparing RBC folate \leq 1360 nmol/L and high maternal folate status (i.e., RBC folate >1360 nmol/L).

Evidence of the relationship between individual methyl nutrient concentrations at early pregnancy and DNA methylation levels of CpG sites associated with fetal growth genes is limited. Results of a prospective pregnancy study conducted in North American pregnant women (22) indicated that low RBC folate concentration (i.e., second quartile), measured at ~12 weeks of gestation, was positively associated with DNA methylation levels of CpG sites associated with *IGF-2*, determined in cord blood. In contrast, McKay et al. (225) found no significant association between maternal RBC folate concentration measured at ~11 weeks of gestation and DNA methylation levels of CpG sites associated with *IGF-2* in cord blood.

Given the differences between methods for the determination of DNA methylation levels (i.e., microarray assay vs. pyrosequencing) and tissue and cell types used (i.e., cord blood vs. infant blood and BECs), comparisons between studies is challenging. It has been previously reported that pyrosequencing is able to detect smaller differences on DNA methylation levels (<5%), compared to other methods (297). In addition, a study conducted by Murphy et al. (311)

showed that DNA methylation levels of *IGF-2* DMR measured in 9 pairs of matched cord blood and BECs samples collected at birth, were slightly lower in BECs compared to DNA methylation levels measured in cord blood, which may contribute to partially explain the discrepancies found. Furthermore, in the current study, we included the measurement of single CpG sites, while, the prospective study conducted by Hoyo et al. (22) in North American pregnant women, measured DNA methylation levels in *IGF-2* DMR. *DMR* consists of a group of CpG sites associated with *IGF-2*. We speculate that differences in the results of the relationship between RBC folate concentration and DNA methylation of CpG sites associated with *IGF-2* may be also related to differences in DNA methylation levels obtained from measuring DMR or single CpG sites.

Differences in RBC folate concentration between the studies may also contribute to explain the discrepancies found between the studies. In the current study, the median RBC folate concentration was ~1300 nmol/L, while in the study conducted by Hoyo et al. (22) the mean RBC folate concentration was ~494 nmol/L. We speculate that *IGF-2* DMR may have a more sensitive response to 'low folate status' compared to 'high folate status'. However, further prospective pregnancy cohort studies including pregnant women from populations with different folate status, as well as the measurement of DNA methylation levels in CpGs and DMRs associated with *IGF-2* measured in the offspring, are needed to understand the potential differences in DNA methylation levels comparing individual CpG sites and DMRs.

6.3 **Overall strengths and limitations**

Specific strengths and limitations of each study i.e., the BC retrospective pregnancy cohort study and the prospective APrON study, are discussed in each chapter. This section summarizes the overall strengths and limitations of the current Ph.D. research work. The strengths of this research included the longitudinal design of pregnancy studies with available maternal blood samples collected at early pregnancy which allowed us to explore the relationship of early-pregnancy methyl nutrients patterns with neonatal anthropometric outcomes and DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes in motheroffspring dyads in a critical developmental window of pregnancy. Additionally, the present research includes the determination of a comprehensive portfolio of maternal methyl nutrients and related metabolite concentrations. Furthermore, the statistical approach used (i.e., PCA) allowed us to explore the interrelationship between maternal methyl nutrients by the derivation of non-correlated maternal methyl nutrient patterns, which prevented potential multicollinearity among the maternal biomarkers included in the regression models.

We acknowledge some limitations. The blood samples retrieved for the BC retrospective pregnancy cohort study were from pregnant women of the BC Prenatal Genetic Screening Program, while pregnant women of the APrON study were recruited by convenience sampling, limiting the representativeness of the study. Given that the primary objective of the BC retrospective pregnancy cohort study was to compare serum total B-12 concentration between South Asian and European pregnant women in the first and second trimester, the research described in Chapters 2.2 and 3.2 included biospecimen and data from an equal distribution of European and South Asian pregnant women, which also limits the generalizability of my findings. Furthermore, according to their estimated income, pregnant women included in both studies had a higher income or they reside in higher income neighbourhoods compared to the average national individual income provided by Statistics of Canada in 2017 (327). Additionally, most pregnant women included in the APrON study as described in Chapter 4 and 5 had high education level and were non-smokers. Thus, it is possible that pregnant women included in the current research had better health care access, a greater willingness to participate in a study about nutrition, more knowledge of nutrition, and were more health conscious which may influence

their consumption of folic acid, multivitamin or mineral supplements, and subsequently affecting their folate status (i.e., higher RBC folate concentration), compared to pregnant women of the general population. However, despite the limitations regarding the generalizability of the findings, the present research provides valuable information on maternal characteristics and Bvitamin status of Canadian pregnant women of which there is no available national data. Thus, longitudinal pregnancy studies become an important source of information of health and nutritional status of this specific life stage and provides a key opportunity to study maternal health and fetal development at early stages of life. Additionally, the current research provides maternal and neonatal data on pregnant women of South Asian ethnicity, the largest ethnic minority in Canada and BC and thus, this research represented an opportunity to study this specific population group.

The inclusion of different biomarkers resulted in the derivation of different maternal methyl nutrient patterns; therefore, the comparison of maternal methyl patterns needs to be done with caution. Because of the blood collection protocol for the biobanked maternal serum samples retrieved for the retrospective pregnancy cohort study (Chapters 2 and 3), data on serum free choline concentration were not included in the data analysis. Choline participates in one-carbon metabolism, e.g., as a precursor for betaine, the methyl donor of the alternative homocysteine remethylation pathway. Although, betaine and total B-12 have been identified as significant predictors of tHcy, the inclusion of free choline could have offered a more comprehensive understanding of the interrelationship of methyl nutrients and the derivation of maternal methyl nutrient patterns in the BC study. A recent study conducted by van Lee et al. (190) which included maternal free choline, total choline and neonatal anthropometric data from two pregnancy cohort studies (i.e., GUSTO and SWS studies) showed that early-pregnancy total

choline and free choline concentrations were positively associated with total body fat (SWS study) and neonatal BMI (GUSTO study), respectively.

The authors indicated that potential mechanisms underlying the relationship between maternal choline concentration (i.e., free choline and total choline concentrations) with neonatal anthropometric outcomes and body fat are related to epigenetic mechanisms, such as DNA methylation. Specifically, the authors suggested that maternal choline may have a key role as a methyl donor in the control of gene expression of body composition-related genes through DNA methylation in the offspring (190). Additionally, it suggested that acetylcholine, a neurotransmitter synthesized from choline, may be involved in the regulation of glucose and lipids in adipose tissue. Thus, including maternal free choline in the derivation of maternal patterns could have also provided a better understanding of the relationship between maternal methyl nutrient patterns and neonatal anthropometric outcomes. Furthermore, because of the lack of availability of specimen (e.g., RBC samples in the BC study), it was not possible to derive maternal methyl nutrient patterns using the same maternal biomarker portfolio in the two subsets of pregnant women included in my Ph.D. research, which would have allowed for an accurate comparison of the patterns and an assessment of reproducibility.

The determination of DNA methylation was conducted using two different types of tissues (i.e., BECs and buffy coat), therefore comparison and interpretation of the DNA methylation levels of the targeted CpG sites included in my Ph.D. research work should be made with caution. Despite that several patterns of DNA methylation are shared by different types of cells, it has been widely reported that DNA methylation may differ by cell type or tissue (311). Tissue and cell types have been indicated as the most important sources of variation in DNA methylation levels (313). For example, DNA methylation levels have greater variability for genome wide and specific CpG sites when measured in BECs compared to peripheral blood

mononuclear cells (328). Given the lack of an independent subset of mother-infants dyads data with available infant DNA methylation data and maternal folate status, I was unable to replicate the results of Chapters 4 and 5. Furthermore, the age of the offspring at the time of sample collection for DNA methylation analyses needs to be taken under consideration. DNA methylation levels of CpG sites associated with the *LEP* gene have been shown to significantly differ between newborns (i.e., measured in cord blood) and 1-year-old infants (i.e., measured in whole blood) (296). Therefore, discrepancies between our findings and results of previous studies may also be related to differences in the time point of sample collection for DNA methylation analyses.

6.4 Future research directions

6.4.1 Periconceptional methyl nutrient status in women and DNA methylation levels of fetal growth and obesity-related genes in the offspring

Most of the current studies have explored the relationship between maternal methyl nutrient concentrations measured after 10 weeks of gestation and DNA methylation levels in the offspring. *De novo* DNA methylation occurs at the very early stage of pregnancy (i.e., <10 weeks of gestation), a period when most women are unaware of being pregnant. The determination of periconceptional methyl nutrient concentrations (i.e., one month before conception to two months after conception) would provide an ideal opportunity to explore the relationship of early maternal methyl nutrients concentrations, and DNA methylation levels of fetal growth and obesity-related genes, in this critical plasticity window.

Therefore, a prospective study including the determination of methyl nutrient concentrations before conception, and the measurement of DNA methylation levels of obesityrelated genes in the offspring is needed to explore whether pre-conceptional maternal methyl

nutrient status is involved in obesity growth programming. Furthermore, maternal genetic variants have shown to contribute to the variation of DNA methylation levels of genes included in the present research (i.e., *LEP* gene) (296). Thus, the measurement of genetic variants of obesity-related genes in the offspring would provide a better understanding of maternal factors potentially involved in DNA methylation variation.

Additionally, evidence from pregnancy studies in Canada has shown that >90% of pregnant women use folic acid supplements in the first trimester of pregnancy; however, results of the Canadian Maternity Experience Survey indicated that only 60% of women consumed folic acid supplements before pregnancy (206). Critical milestones in the embryonic development (e.g., closure of the neural tube or the differentiation of germ layers) occur before 28 days of gestation. This is a gestational period in which most women are unaware of being pregnant. Furthermore, findings of a recent study in Canadian pregnant women indicated that women had a lower knowledge related to the DOHaD theory, compared to their knowledge of pregnancy guidelines (329). These findings can be considered as a lack of knowledge translation about the DOHaD-related evidence. However, these findings can also be considered as an opportunity to conduct studies that contribute novel evidence and knowledge of the critical role of preconceptional and early-pregnancy nutrition and health for later stages of life, and to develop strategies to improve the DOHaD-related knowledge translation.

6.4.2 Obesity programming and its consequences later in life

DNA methylation is a stable and critical epigenetic mechanism involved in fetal programming. However, whether these modifications in DNA methylation levels of fetal growth or obesity-related genes in the offspring result in modifications in the expression of the target molecules (i.e., serum IGF-2 or insulin concentrations) is not fully understood. Prospective

cohort studies including the measurement of DNA methylation levels of CpG sites associated with fetal growth and obesity related-genes (i.e., *LEP*, *HIF-3A*, *RXRA*) at birth or early childhood (i.e., infants), the determination of biochemical parameters potentially involved in obesity such as insulin or leptin concentrations, and the occurrence of obesity during adolescence are needed to understand the metabolic pathway behind the relationship of DNA methylation levels of obesity-related genes and the risk or occurrence of obesity later in life.

Furthermore, evidence from observational studies that followed newborns until early adolescence indicated that the trajectory of weight gain as well as the timing of the adiposity rebound are important predictors of obesity later in life (330). Thus, exploring the relationship between DNA methylation levels of fetal growth and obesity-related genes in the offspring and weight gain in childhood and adiposity rebound is key to understand the potential role of DNA methylation levels in the offspring in obesity programming and its consequences later in life.

6.4.3 Paternal methyl nutrient concentrations and DNA methylation in the offspring

Maternal nutrition and health are fundamental for adequate fetal growth and development. The majority of pregnancy studies to date have focused on studying the association or effect of maternal factors on offspring outcomes. In addition to maternal nutrition, evidence from animal and human studies have reported that paternal lifestyle factors such as obesity and paternal dietary intake before conception are also involved in fetal programming (331–333). Recently, Sahara et al. (334) indicated that the offspring of male mice who received a methyl donor deficient diet showed a decrease in anxiety-like behavior which was mediated by modifications of DNA methylation levels of cognitive-related genes. While in humans, paternal dietary betaine and methionine intakes were positively associated with global DNA methylation levels and DNA methylation levels of *IGF-2* in the newborn, respectively (333). Therefore, future studies including the determination of paternal methyl nutrient concentrations before conception and its association with DNA methylation levels of fetal growth and obesity-related genes in the offspring are needed to understand the potential paternal role in fetal growth and obesity programming.

6.4.4 Derivation of maternal methyl nutrient patterns in non-folate-replete populations

The derivation of maternal methyl nutrient patterns is highly influenced by biomarker concentrations and the relationship among them. Despite the central role of folate as methyl donor, folate was not a significant driver of maternal methyl nutrient patterns in the present research. Pregnant women included in this research were from a folate-replete population, and with a primarily high SES. Thus, maternal methyl nutrient patterns derived in this research may not be replicable in other populations with different folate status. Whether the interrelationship of maternal methyl nutrients and the subsequent characteristics of the derived maternal methyl patterns are different in non-folate-replete populations is unknown. Therefore, further prospective studies conducted across all trimesters including pregnant women from other populations are needed to explore potential differences in the relationship between maternal methyl nutrients, as well as to identify other drivers of maternal methyl nutrient patterns under non-folate-replete conditions. Given that critical methylation reactions occur during pregnancy and that the relationship of methyl nutrients varies according to methyl nutrient status (i.e., low or high folate status), the understanding of the relationship of methyl donors and homocysteine, as a central metabolite of the remethylation pathway, is needed in non-folate-replete populations.

6.5 Significance and contribution of research

The current research work contributed three new findings about the interrelationship of methyl nutrient concentrations during early pregnancy and its relationship with DNA methylation of fetal growth and obesity-related genes and neonatal anthropometric outcomes. Findings of the present study showed that maternal betaine concentration was negatively associated with tHcy concentration in early pregnancy, while folate concentration did not show a significant association with tHcy in this sample of Canadian pregnant women from a folate-replete population. Given the higher daily folic acid intake in pregnant women (335) (i.e., 1000 μ g) compared to the recommended dose of 400 μ g/day for NTD prevention, these results are particularly important because they might provide a novel perspective about the homocysteine remethylation reaction of one carbon metabolism, in particular the key role, of betaine as a methyl donor of the alternative remethylation pathway under folate-replete conditions.

Second, the derivation of maternal methyl nutrient patterns in the present study allowed to explore the interrelationship of maternal methyl nutrient concentrations in early pregnancy. Maternal methyl nutrient patterns were mainly driven by the relationship of direct and functional biomarkers of B-12 of status (i.e., total B-12 and holoTC and tHcy concentrations). Additionally, betaine and riboflavin related metabolites (i.e., free riboflavin, FMN and FAD) showed significant contributions to the patterns. These results suggest that in folate-replete pregnant women, total B-12, betaine, and riboflavin concentrations may play critical roles in homocysteine remethylation, for the formation of methionine, the precursor of SAM. These findings are particularly relevant because they may suggest that not only folate, as it has been reported in non-folate-replete populations, but also betaine and B-12 may become critical for the provision of methyl groups for DNA methylation reactions that are central to *de novo* DNA methylation and fetal programming.

Third, early-pregnancy methyl nutrient patterns showed only a significant association with HC at birth, while no significant association was seen between maternal methyl nutrient patterns or maternal folate status, and DNA methylation levels of CpG sites associated with *LEP*, *LEP-R*, *IGF-2*, *RXRA*, *HIF-3a*, *DNMT-1*, *DNMT-3A* and *DNMT-3B* in infants. These findings suggest that in apparently healthy mother-offspring dyads, the interrelationship of maternal methyl nutrient concentrations and maternal folate status alone during early pregnancy may not be significantly related to neonatal anthropometric outcomes and DNA methylation levels of CpG sites associated with fetal growth and obesity-related genes.

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Appendices

A 1. Maternal and neonatal information obtained from Prenatal Genetic Screening Program and British Columbia Perinatal Data Registry

Maternal variables	Description	Source of information
Age	-	Prenatal Genetic Screening Program
Ethnicity	Self-reported: European or South Asian	Prenatal Genetic Screening Program
Weeks of gestation at blood collection (weeks)	Crown-rump length by ultrasound	Prenatal Genetic Screening Program
Pre-pregnancy weight (kg)	Defined as weight before pregnancy or ≤ 11 weeks	British Columbia Perinatal Data Registry
Pre-pregnancy height (m)	Defined as height before pregnancy	British Columbia Perinatal Data Registry
Parity	Number of pregnancies that reached 20 weeks gestation or 500 grams birth weight (nulliparous/multiparous)	British Columbia Perinatal Data Registry
Delivery type	Vaginal birth/caesarean section	British Columbia Perinatal Data Registry
Hypertensive disorder of pregnancy	Yes/No	British Columbia Perinatal Data Registry
Gestational diabetes	Yes/No	British Columbia Perinatal Data Registry
Neighborhood income	Median family income of each pregnant woman's residential Forward Sortation Area (FSA)	Canadian Census 2016
Neonatal variables		
Gestational age (GA) at birth (weeks)	Gestational age at birth, in completed weeks – calculated by algorithm incorporating LNMP ¹ , first ultrasound, newborn examination, and maternal	British Columbia Perinatal Data Registry
Birth weight (kg)	-	British Columbia Perinatal Data Registry
Birth length (cm)	-	British Columbia Perinatal Data Registry
Head circumference (cm)	-	British Columbia Perinatal Data Registry
Neonatal sex	Biological sex of the newborn	British Columbia Perinatal Data Registry

¹Lastmenstrual period