PROGRAMMING OF HEPATIC GENE EXPRESSION BY MATERNAL FOLIC ACID AND VITAMIN B12 IMBALANCE

by

Abeer Mohammad Aljaadi

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Abstract

Folate is a B-vitamin required for cell growth and division, and its metabolism is linked to vitamin B12 (B12). Food fortification with folic acid (FA) has improved folate status but approximately 5% of Canadian adults, including pregnant women, are B12 deficient. This is concerning because an association between gestational exposure to high maternal folate and low B12 status and greater adiposity and insulin resistance in children has been reported. My thesis examined the effect of developmental exposure to maternal FA/B12 imbalance on programming of liver gene expression in adult offspring using an animal model.

Female C57BL/6 mice were fed a high FA/adequate B12 (HFA+B12), high FA/no B12 (HFA-B12), or control diet 6 weeks prior to mating and through pregnancy and lactation. At weaning, offspring mice from each maternal diet group were randomly assigned to receive the control diet or a Western diet (45% fat, 35% carbohydrate) for 20 weeks (n=6 male mice/group) or for 40 weeks (n=6 female mice/group). Serum folate and B12 concentrations were quantified by microbiological assays. Relative mRNA expression of key enzymes in methyl metabolism in liver from adult offspring was quantified by real-time PCR.

Male offspring mice from dams fed the HFA-B12 diet had lower *Cbs* and *Mthfr* mRNA expression and this was unaffected by post weaning diet. Male offspring mice fed the Western diet had higher *Mtr* mRNA expression compared to control-fed offspring mice, regardless of maternal diet. Female offspring from dams fed the HFA-B12 diet had lower *Mtr* mRNA expression and this was not affected by post weaning diet. Moreover, female offspring from dams fed the HFA-B12 diet had higher *Mthfr* mRNA expression when they were fed the Western diet. No effect of maternal and post weaning diets was observed for serum folate and B12 concentrations.

In summary, developmental exposure to maternal FA/B12 imbalance was found to program expression of genes involved in folate and methionine metabolism in the liver of adult offspring mice. The functional consequences of this effect requires further investigation in order to consider B12 screening of pregnant women and to inform the debate on whether B12 fortification should be considered.

Preface

This thesis has been prepared in partial fulfillment of the requirement for the degree of Master of Science in Human Nutrition. I have prepared this thesis under the direction and supervision of Dr. Angela Devlin from January 2012 to July 2014. This thesis was reviewed by Dr. Angela Devlin, Dr. Tim Green, Dr. Yvonne Lamers, and Dr. Wendy Robinson.

My work was part of a larger project, which was conducted in collaboration with two other graduate students: Rika Aleliunas (MSc 2013, Pathology and Laboratory Medicine) and Jesse Olson (MSc candidate, Pathology and Laboratory Medicine). I joined the project when the breeding was complete and the offspring were being fed the respective post weaning diets. The mice were housed in the animal unit at Child and Family Research Institute (CFRI) and all procedures were approved by the UBC Animal Care Committee (protocols: A09-0346, A10-0179). I was part of team that prepared the experimental diets, fed the mice, weekly weighing mice, and collection of tissues. All experimental procedures related to liver gene and protein expression, gene-specific methylation and serum vitamin B12 concentrations in male offspring were conducted by me, with the assistance of Dian Sulistyoningrum, at CFRI. I quantified serum folate concentrations in Dr. Green's lab at UBC with the assistance of Yazheng Amy Liu. Serum vitamin B12 concentrations in female offspring were quantified by Dr. Anne Molloy, Trinity College Dublin, Ireland. Liver *S*-adenosylmethionine and *S*-adenosylhomocysteine concentrations were quantified by Dr. Joshua Miller, Rutgers University, New Brunswick, New Jersey. Preliminary results from male offspring were presented as a poster at the 20th International Congress of Nutrition held in Spain, September 2013; the abstract was published in the Annals of Nutrition and Metabolism[1]. None of the text is taken from previously published or collaborative articles.

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List of Abbreviations

AdoHcy	Adenosylhomocysteine
AdoMet	Adenosylmethionine
BER	Base excision repair
BHMT	Betainehomocysteine methyltransferase
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
CBS	Cystathionine beta-synthase
CCAC	Canadian Council on Animal Care
CFRI	Child and Family Research Institute
CHD	Coronary heart disease
CI	Confidence interval
CpG	A cytosine nucleotide next to a guanine nucleotide
ddH2O	Double-distilled water
DEXA	Dual-energy X-ray absorptiometry
DHFR	Dihydrofolate reductase
DMG	Dimethylglycine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOHaD	Developmental origins of health and disease
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate,
EDTA	Ethylenediaminetetraacetic acid
FFA	Free fatty acids
GR	Glucocorticoids receptor
HPLC	High-pressure liquid chromatography
IDT	Integrated DNA technologies
IF	Intrinsic factor
K	Lysine residue
Kcal	Kcalories
kDa	Kilodalton
LSD	Least significant difference
MAT	Methionine adenosyltransferase
MTHFR	Methylenetetrahydrofolate reductase

MTR	Methionine synthase
n.s.	Non significant
NHANES	National Health and Nutrition Examination Survey
Nr3c1	Nuclear receptor subfamily 3, group C, member1
NRC	National Research Council
NTD	Neural tube defect
OR	Odds ratio
PAGE	PolyAcrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
Pparα	Peroxisome proliferator-activated receptor alpha
Psi	Pound-force per square inch
RIPA buffer	Radio immuno precipitation assay buffer
RR	Relative risk
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TAE	Tris-acetate-EDTA
TBS-T	Tris buffer saline tween 20
TG	Triglyceride
THF	Tetrahydrofolate
TS	Thymidylate synthase
US	United States
X-CH3	Methylated product

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I would like to express my special appreciation and thanks to my advisor Dr. Angela Devlin, who has been a tremendous mentor for me through my Master's degree. I thank her for the patient guidance, encouragement and advice she has provided throughout my time as her student. I would also like to thank my committee members, Dr. Tim Green, Dr. Yvonne Lamers, and Dr. Wendy Robinson for their time and support throughout my research. I would especially like to thank my colleagues in the Devlin lab: Rika Aleliunas, Melissa Glier, Jesse Olson, Dian Sulistyoningrum, Kaia Hookenson, Mikaela Barker, Anita Cote, and Daven Tai, all of whom have inspired me to continue my work in this field and supported me throughout the entire process, by helping me put pieces together. I would also like to express my gratitude to the Ministry of Higher Education in Saudi Arabia for the generous funding throughout my study.

I owe particular thanks to my small family. Words cannot express how grateful I am to my beloved husband Kamal, my son Eyad, and my daughter Miral for all of the sacrifices that they have made on my behalf. I would like to express appreciation to my parents who have supported me morally throughout my graduate studies. At the end, I would also like to thank all of my friends who supported me in writing, and incented me to strive towards my goal.

Chapter 1: Literature Review

1.1 Overview

In recent decades, the world has faced a substantial increase in overweight, obesity, and related metabolic disorders among both adults and children. The World Health Organization (WHO) estimates that obesity has doubled since 1980 and over 40 million children aged 5 years and less were overweight or obese in 2012[2]. A comprehensive recent analysis on global and regional prevalence of overweight and obesity between 1980 and 2013 reported that the worldwide prevalence of both overweight and obesity has increased by 28% in adults and by 47% in children[3]. At the same time there has been an increase in risk factors for chronic diseases, such as cardiovascular diseases (CVDs) and Type 2 diabetes (T2D) [4].

There is no clear explanation for the current surge in overweight, obesity, and associated chronic diseases. The increase intake of energy-dense food and physical inactivity are important contributors to this increase. However, there is also growing evidence to support the role of developmental programming, which suggests that maternal and early life environmental stressors play a role in excess weight gain and metabolic dysfunction later in life. Maternal malnutrition during pregnancy is one of these stressors that can predispose, and sometimes lead to, adverse health outcomes in the offspring. Mandatory folic acid fortification of grain products was introduced in North America in 1998 for the prevention of neural tube defects (NTDs) [5,6]. As a result, most Canadians have adequate folate status with an estimated 40% of Canadian adults having high folate status (defined as having red blood cell (RBC) folate concentration >1360 nmol\L) [7].

Folate is metabolically linked to another vitamin, vitamin B12, and both are required for nucleotide synthesis and the generation of methyl groups[8]. Given this metabolic relationship, there

are concerns about metabolic imbalances between vitamin B12 status and folic acid with fortification. Recent reports suggest that 5% of Canadian adults (aged ≥ 20 years) and pregnant women have vitamin B12 deficiency (<148 pmol/L) [9,10]. The consequence of chronic exposure to high folic acid is still not fully understood, especially when accompanied by low vitamin B12 status. However, some concerns have been raised. High serum folate concentration in combination with low vitamin B12 status were reported to be associated with anemia and cognitive impairment in elderly subjects (aged ≥ 60 years) [11]. Furthermore, observational studies have reported an association between gestational exposure to high maternal folate and low vitamin B12 status with adiposity and insulin resistance in children[12-14]. These findings suggest that maternal folate and vitamin B12 status during pregnancy may program adiposity and glucose homeostasis in their offspring and contribute to the development of chronic diseases such as CVD and T2D. However, the strength of these associations and the causative mechanisms remain unknown.

The main goal of my thesis is to explore the underlying mechanisms that may contribute to programming of adiposity and glucose homeostasis in adult offspring exposed during development to maternal high folic acid and low vitamin B12 status. My thesis work is focused on the liver, a key site of methyl nutrient metabolism, and represents a first step in delineating the mechanisms by which maternal folate/vitamin B12 imbalance programs adiposity and glucose homoeostasis.

1.2 Developmental Programming

The Dutch famine, known as the Hunger Winter, lasting from September 1944 to May 1945 during World War II created a study population from which many of the current concepts about developmental programming have been developed[15]. The theory of developmental origins of health and disease (DOHaD) asserts that environmental changes during development, particularly during intrauterine and early postnatal life, can program health outcomes later in life and increases risk for chronic disease in adulthood[16,17]. A few examples of these developmental cues are maternal stress, maternal diet, exposure to chemicals, and prenatal steroid exposure[18-21].

The Dutch famine led to undernourished population where individual rations were 400–800 kcal/day, including pregnant women in their early, mid, and late pregnancy [15,22]. This was an opportunity for researchers to examine the short- and/or long-term consequences of prenatal under-nutrition and stress on health and disease risk at different stages of gestation. Studies in this field started in the early 1970s when a group of investigators assessed the association between mental performance of 19-year-old men and maternal under-nutrition, but found no effect[23]. Another study in 1976 reported associations between famine exposure during different stages of gestation and the likelihood to be obese in men[24]. However, it was not until the early 1990s that David Barker proposed the fetal programming hypothesis when he observed that low birth weight was associated with greater incidence of ischemic heart disease in a British cohort[25]. Subsequent studies on the Dutch famine have supported Barker's hypothesis and added great input to our current understanding of DOHaD[26-30].

1.2.1 Programming of Cardiometabolic Disease

Several observational studies have been conducted to explore developmental programming in relation to cardiovascular and metabolic risk factors. Researchers studying the Dutch famine

have reported poorer glucose tolerance in adults exposed to the famine prenatally[29,31]. This might be attributed to impairments in insulin secretion because the exposure to famine was associated with lower insulin disposition index[27], an estimate of β-cell function calculated based on insulin sensitivity and first-phase insulin response post oral glucose loading[27,32]. In middle-age female adults, prenatal exposure to famine was associated with greater central adiposity as determined by waist circumference[28,33]. Both males and females who were conceived during the Dutch famine had a two times higher rate of coronary heart disease (CHD) before the age of 61 years, higher plasma glucose concentration, and higher ratios of low density lipoprotein (LDL) to high density lipoprotein (HDL) [34]. These people also had an earlier onset of CHD (3 years earlier) compared to those conceived before or after the famine[34]. Whincup et al. conducted a quantitative systematic review investigating published studies on the association between birth weight and T2D in adult population[35]. There were 23 populations that showed an inverse association between birth weight and risk of T2D and the pooled odds ratio (OR) of T2D in a total of 28 studies was 0.75 (95% confidence interval (CI): 0.70, 0.82 per kg). Subsequent analysis of the shape of this association in 3 studies (2 on native North American and 1 from Saskatchewan) revealed a U-shaped association, with increased risk for T2D when birth weight is greater than 4 kg[35]. The review suggests that fetal under-nutrition can be implicated as a risk factor for T2D[35].

Despite the high rate of hunger worldwide, over-nutrition is on the rise, and is becoming an important stressor that may contribute to DOHaD[36]. With the rapid rise in obesity, T2D, and CVD in recent decades, there is increasing evidence that *in utero* and early postnatal environments, such as maternal diet, are important environmental factors that can program metabolic alterations and drive the increase in metabolic disorders[16]. Maternal obesity and gestational weight gain, and weight gain in early childhood are examples of influences that may impact chronic disease risk later

in life. However, little is known about the role of individual dietary factors and poor nutrient status during pregnancy and early development on adulthood health, especially with the limited data from human studies. Formula feeding versus breastfeeding is another example of over-nutrition, and it has been reported that formula milk feeding is associated with increased risk of obesity in childhood and elevated circulating cholesterol concentrations in adulthood compared to breast-fed infants[37,38]. Micronutrient inadequacy or imbalance is possible, even with the over consumption of food, because an energy-dense diet is not necessarily nutrient-dense. Currently, studying vitamin deficiencies in relation to DOHaD is an active area of research.

Obesity during pregnancy has been associated with pregnancy complications such as gestational diabetes mellitus[39]. Gestational diabetes raises the risk of impaired glucose tolerance and excess fat accumulation in the offspring [40,41, & as cited in42]. In a mouse model that naturally develops obesity and T2D (A^{vy}, agouti viable yellow), the offspring have dysfunctional glucose and lipid metabolism and these abnormalities are aggravated to insulin resistance and hepatic steatosis upon exposure to a Western diet, compared to offspring from lean dams with healthy metabolism and the same genetic background[43]. Accordingly, the adverse outcomes of excess weight gain and adiposity not only affect maternal health, but can also influence the well being of the offspring.

During pregnancy maternal smoking habits and weight gain can also have profound effects on the growing fetus. These choices and conditions, consequently, can have long-term effects such as increased risk for obesity. In a meta-analysis of 14 studies conducted in 84,563 children, mothers who smoked during pregnancy had children (at ages 3-33 years) with higher risk of overweight compared to non-smoking mothers (pooled adjusted OR: 1.50; 95% CI: 1.36 -1.65) [44]. Although some studies found this association to be independent of the timing of smoking exposure[45,46], other studies reported that smoking throughout the whole pregnancy was associated with greater risk for

overweight in children in comparison to smoking during early pregnancy[45,47,48]. Moreover, a prospective cohort study of pregnant women and their children (1044 mother-child pairs) found that greater gestational weight gain was associated with increased adiposity in children at 3 years of age as determined by skinfold thickness and BMI z-score, regardless of factors like maternal and paternal BMI, breastfeeding duration, and infant growth[49]. In this study, mothers with excess gestational weight gain based on the 1990 Institute of Medicine guidelines had children who were at four times higher risk to be overweight at age 3 years, compared to children from mothers who gained inadequate weight during pregnancy[49]. The positive association between gestational weight gain and offspring BMI has also been observed in adults at 42 years of age (OR: 1.08; CI: 1.03,1.14 per kg of gestational weight gain), as reported by the Copenhagen Perinatal Cohort study (n = 1540) [50].

Developmental programming has been studied in animal models**,** providing causative and mechanistic insight. For instance, maternal protein restriction during pregnancy and/or lactation in rats, which mimics fetal under-nutrition in humans, was associated with nutritional programming of T2D in offspring during fetal and early postnatal life[51,52]. Programming of T2D in these studies was determined by having smaller pancreatic islets and lower insulin content[51,52]. Offspring from dams fed a low protein diet (8% of energy) during pregnancy, were born smaller and developed a reduction in glucose tolerance as their age increased compared to offspring from dams fed normal chow (20% protein) [51,53,54]. Moreover, moderate protein restriction (8-10% of energy) in rodent dams was reported to have an impact on offspring[55]. These offspring were more susceptible to develop high blood pressure (BP), insulin resistance, or abnormal lipid profile in adulthood, especially when challenged with factors, such as high fat or high salt diet[55]. Petrik et al. reported that a low protein diet during pregnancy and lactation has detrimental effects on β-cell mass of

female offspring at birth and weaning by decreasing the rate of β-cell proliferation and increasing β-cell apoptosis[56].

High fat diet feeding during pregnancy and lactation is also associated with programming of metabolic dysfunction in the offspring. Dams fed a high fat and high sugar diet during pregnancy and lactation have offspring with greater adiposity, insulin resistance (determined by euglycaemic-hyperinsulinaemic clamps), glucose intolerance, and BP (assessed by radiotelemetry) in adulthood despite the fact that they were weaned on a standard chow diet[21,57]. In sheep, maternal diet-induced obesity during pregnancy is associated with increased expression of fatty acid transporters in the placenta, elevated cholesterol and triglyceride (TG) concentrations in fetal blood, and increased expression of lipogenic genes in the adipose tissue of offspring[58-60]. Although paternal lifestyle and environmental exposures have not been studied to the same extent as maternal factors, there is increasing evidence regarding the role of fathers in programming of offspring metabolic phenotypes[61,62]. For example, in male rats, the consumption of a high fat diet at the time of breeding is associated with impaired glucose-insulin homeostasis in the female offspring[62].

1.2.2 Programming of Liver Disease

The liver is a primary organ in metabolic regulation and is responsible for more than 500 tasks[63]. Although a small portion (10-20%) of functioning liver is enough to keep a person alive, insults to the liver can lead to systemic metabolic dysfunction[63,64]. With the growing interests in the field of DOHaD, several studies have explored the consequences of maternal nutrition on offspring liver. A study in non-human primates reported that females fed a high fat diet before and during pregnancy had offspring with liver TG accumulation, fatty liver phenotype, and higher total body fat and this was independent of maternal obesity and diabetes[65]. Interestingly, when these females

were switched to a control diet for 1-3 months prior to a subsequent pregnancy, the offspring had improvements in hepatic fatty liver phenotype and gene expression[65].

Maternal Western diet during pregnancy and lactation in mice has been found to program susceptibility to non-alcoholic fatty liver disease (NAFLD) in male offspring[66]. Male offspring fed a post weaning Western diet who were exposed prenatally to maternal Western diet had increased weight gain, hepatic TG accumulation, and liver injury at 29 weeks of age, compared to offspring fed a post weaning low fat diet[66]. Another study found similar results in 15 and 30 week-old female offspring and suggested that the onset of NAFLD is attributed to alterations in hepatic mitochondrial metabolism and lipogenic genes expression[67]. It is worth mentioning that 30 week-old female offspring from dams fed a high fat diet during gestation and lactation developed NAFLD (confirmed by histological analysis and Kleiner scoring system) even if they were fed a post weaning control diet[67]. However, offspring who were fed high fat diet post weaning developed a more advanced stage of NAFLD, which is non-alcoholic steatohepatitis (NASH) [67]. All together, these studies support the notion that the liver is susceptible to programming by maternal diet during development.

1.2.3 Epigenetics

The underlying mechanisms of changes or adaptations associated with developmental programming have not yet been well established; however, epigenetic mechanisms may play a role[68,69]. Epigenetics is the study of heritable changes in gene function without a change in DNA sequence[70]. The human genome is estimated to have approximately 23,000 genes required to be expressed in certain cells at specific times, which explains why differentiated cells in the human body express only genes that are essential for their own functions[70]. One of the ways to regulate gene expression and genome stability is through chromatin remodeling[70,71]. In a complex process,

DNA is wrapped around core histone octamers (composed of two H2A, H2B, H3, and H4) in order to form nucleosomes, which are arranged into chromatin[71]. Condensed chromatin (heterochromatin) is associated with limited gene transcription, whereas decondensed chromatin (euchromatin) is associated with active transcription[70,72]. These changes in chromatin structure can affect gene transcription, and are modulated by the following epigenetic processes: DNA methylation, histone modifications, and miRNAs[70,73].

Understanding of epigenetic mechanisms may provide insight into some underlying mechanisms and the pathogenesis of certain diseases, such as cancer, cardiometabolic diseases, mental health conditions, and chromosomal instabilities[70-72]. *In utero* environment, diet, environmental chemicals, and ageing can influence epigenetic mechanisms[71].

DNA methylation involves the addition of a methyl group to the 5' position of cytosines within cytosine and guanine pairs (CpG) in the DNA, resulting in the conversion of cytosine into 5-methylcytosine[72,74]. In addition, methylation of cytosine non-CpG sites has been reported in early development[75]. DNA methyltransferases (DNMTs) are the enzymes responsible for catalyzing the transfer of methyl groups to DNA. Maintenance methylation during mitotic cell division is accomplished by DNMT1, whereas DNMT3a and DNMT3b catalyzes de novo methylation during embryonic development[76].

Histone proteins contain amino-terminal tails, which are exposed on the nucleosome surface and are susceptible to posttranslational modifications[77]. These modifications include: acetylation, methylation, phosphorylation, ubiquitnation, and sumolyation, with the first three types have been widely studied[72,78]. For example, arginine (R) and lysine (K) residues are sites for methylation, whereas serine (S) and threonine (T) are sites for phosphorylation[72,79]. Acetylation of lysine is associated with transcriptional activation, but the effect of lysine methylation on gene expression is

dependent on the specific lysine residues[78]. For example, H3K36 and H3K4 methylation in gene promoter regions is associated with transcriptionally active chromatin, whereas H3K9 and H3K27 methylation are often linked to transcriptional repression[72,77,80]. These reactions are carried out by several enzymes, such as histone deacetylases, histone acetylases, and histone methyltransferases[71,72].

Lastly, miRNAs (20-30 nucleotides) are non-coding RNAs that can function as posttranscriptional regulators of gene expression[73]. Binding of miRNAs to the mRNA mainly at the 3' untranslated region is associated with translational repression[73,81]. It has been also reported that miRNA regulates heterochromatin formation and translational activation[73,81].

Overall, an adverse intrauterine and early postnatal environment can initiate metabolic changes in the offspring, leading to the later development of cardiometabolic disease. Given that epigenetic marks are heritable and govern how genes interact with environment, they may respond to changes in maternal dietary factors and contribute to developmental programming. There are micronutrients required for methylation, such as folate and vitamin B12, and recent observational data have implicated maternal imbalances in these nutrients with adverse outcomes in children[12-14].

1.3 Folate and Vitamin B12

1.3.1 Folic Acid Fortification

Folic acid fortification of grain products was introduced in several countries for the prevention of NTDs. NTDs are a group of developmental anomalies in the central nervous system resulting from incomplete closure of the neural tube during embryonic development[82]. That folic acid taken periconceptionally could prevent NTDs was demonstrated by three trials in the 1990s. The Medical Research Council (MRC) Vitamin Study Group reported that 4 mg/day of folic acid

has a protective effect in reducing NTD recurrence risk[83]. Another two influential studies, one in China[84] and one in Hungary[85], investigated the impact of periconceptional folic acid supplement on minimizing NTDs. The Hungarian trial reported that a vitamin supplement containing 800 μg of folic acid was associated with a significant reduction in the first occurrence of NTD[85]. The Chinese study confirmed that folic acid supplementation (400 μg/day) before pregnancy reduced the risk of NTD and the reduction was of a greater magnitude in the northern region, which had a higher background NTD rate, compared with the southern region, which had a lower NTD rate[84]. Neural tube development and closure normally occurs during the first 28 days post conception in humans[82], which is often before a woman is aware she is pregnant. This led to considering fortification of grain and cereal products with folic acid.

At present, more than 50 countries have mandatory folic acid fortification of at least one widely consumed grain product[86,87]. In 1998, Canada and the United States (US) implemented mandatory folic acid fortification of grains and observed a significant reduction in the incidence of NTDs[5,86]. Women planning for pregnancy are currently recommended to take 400 μg of folic acid from supplement or fortified food in addition to dietary folate[63,88]. Folic acid supplementation, along with food fortification, has been associated with a decline in the prevalence of NTDs[86,89,90]. On the other hand, folic acid fortification has raised blood folate concentrations. The Canadian Health Measures Survey reported that 40% of Canadians (aged 6-79 years) have high folate status as determined by RBC folate concentration >1360 nmol\L[7]. Similarly, data from the US National Health and Nutrition Examination Survey (NHANES) between 1988 and 2004 revealed a dramatic increase in both serum and RBC folate concentration post fortification[91,92]. The implementation of mandatory folic acid fortification for various cereal products has generated concerns and controversial debate regarding the potential metabolic or health impact of chronic exposure to high

folic acid, which is still unknown. The Framingham Offspring Cohort reported a greater prevalence of circulating unmetabolized folic acid in adults post folic acid fortification[93]. Higher intakes of folic acid lead to the release of unmetabolized folic acid into the circulation because the capacity of the dihydrofolate reductase (DHFR) reaction (Figure 1.1) is limited in humans[93-96].

Due to the interrelation between folate and vitamin B12, vitamin B12 deficiency is one of the main concerns during consumption of high amounts of folic acid. A survey from the US found that high serum folate concentrations (> 59 nmol/L) in combination with low vitamin B12 status (serum vitamin B12<148 pmol/L or methylmalonic acid >210 nmol/L) was associated with anemia and cognitive impairment in elderly subjects (aged ≥ 60 years), whereas high serum folate concentrations and adequate vitamin B12 status in this cohort was protective against cognitive impairment[11]. Miller et al. reported that high concentrations of homocysteine and methylmalonic acid, and low concentrations of holo-transcobalamin, which are indicators of vitamin B12 inadequacy, were more pronounced when plasma folate concentration was high (> 45.3 nmol/L) in elderly Latin Americans (n = 1535, aged ≥ 60 years) [97]. Currently, there is no policy for vitamin B12 fortification in Canada. A recent analysis of data from the Canadian Health Measures Survey (2007-2009) suggests that the prevalence of vitamin B12 deficiency (<148 pmol/L) and marginal vitamin B12 deficiency (148-220 pmol/L) among Canadian adults (aged ≥ 20 years) is 5% and 19.7%, respectively[9]. Another study from Ontario conducted on ~ 10,000 women aged 15-46 years reported that 5% of pregnant women were deficient in vitamin B12 (serum vitamin B12 concentration <125 pmol/L) in early pregnancy (28 days or less) and 10% were vitamin B12 deficient after 28 days gestation [10]. A small cross-sectional study conducted in Metro Vancouver on 204 women (19-35 years old) found that 14% had vitamin B12 deficiency (serum vitamin B12 <148 pmol/L) and 20% had marginal deficiency (serum vitamin B12 = 148-220 pmol/L) [98]. To date,

there is no clear understanding of the potential consequences of high folic acid status when accompanied by vitamin B12 deficiency in pregnancy.

1.3.2 Digestion and Absorption of Folate and Vitamin B12

Folate and vitamin B12 are water-soluble B-vitamins. Given that mammals cannot synthesize folate and vitamin B12, these nutrients are essential and must be obtained from dietary sources or supplements[8,63]. Folate is a generic term for a variety of folate compounds that are functionally related. Folate in its natural form can be obtained from dietary sources, such as liver, green leafy vegetables, citrus fruit, legumes, and kidneys[8,63]. Folic acid is the synthetic and more stable form of folate and found in supplement and fortified foods, such as cereal grain products[63,99]. Folic acid is a fully oxidized monoglutamate and it is more bioavailable than naturally occurring folates. Folates from natural foods exist as reduced polyglutamates, which require the hydrolysis of the glutamate residues before being absorbed in the proximal small intestine[94,99]. As such, dietary recommendations are expressed as 'dietary folate equivalents' in order to account for the differences in bioavailability between dietary folate and folic acid[63]. Most dietary folate (from natural food sources) and folic acid is metabolized to 5-methyltetrahydrofolate (5-methylTHF) in the small intestinal enterocytes and enters the blood circulation as 5-methylTHF[94,99,100].

Vitamin B12 is naturally found in animal dietary sources, such as beef, liver, fish, meat, poultry, eggs, milk, and other dairy products[63,101]. In addition to natural food sources, vitamin B12 can also be found in most multivitamin supplements and a variety of fortified foods, such as veggie burgers[63,101]. The ingested natural vitamin B12 is released from food components in the stomach by gastric acid and pepsin and is bound to haptocorrin[102,103]. In the duodenum, haptocorrin is degraded by pancreatic proteases and the freed vitamin B12 is captured by the intrinsic factor (IF), secreted by the gastric parietal cells[63,103]. In the distal ileum, vitamin B12-IF complex is absorbed by

receptor-mediated endocytosis (cubam receptor) and this interaction is calcium dependent[94,102]. The endocytosis process is followed by the degradation of IF inside the enterocyte and the release of vitamin B12 from the enterocyte into plasma by the multidrug resistance protein 1, a member of ABC transporter family[102,103]. In plasma, vitamin B12 binds to the transport proteins haptocorrin (70-80%) or transcobalamin II (20-30%); vitamin B12 bound to transcobalamin II, a complex called holo-transcobalamin, is an active fraction of vitamin B12 being taken up by the body's cells[94,102,103].

1.3.3 One Carbon Metabolism

Folate and vitamin B12 have inter-dependent roles in one carbon metabolism[63,94], a metabolic network that plays a crucial role in the synthesis of purines and pyrimidines and in the generation of methyl groups for the methylation of DNA, RNA, proteins, and phospholipids[94]. Inside the cell, folate has various derivatives that are polyglutamated by folypolyglutamate synthase in order to retain them inside the cell[94,100]. The major circulating form of folate is 5-methylTHF, and this serves as a methyl donor for homocysteine remethylation, a reaction that subsequently forms THF (Figure 2.1). Vitamin B12 with methionine synthase (MTR), present as cbl(I)MTR complex, takes a methyl group from 5-methylTHF and becomes methylcbl(III)MTR. Transferring the methyl group to homocysteine results in methionine and cbl(I)MTR formation. Cbl(I)MTR becomes ready to accept another methyl group from 5-methylTHF and the resulting active form of folate, THF, is available for purine and thymidylate biosynthesis[94,99]. MTR and vitamin B12, by catalyzing this reaction, metabolically link the folate and methionine cycles and vitamin B12 indirectly participates in nucleotide synthesis[94].

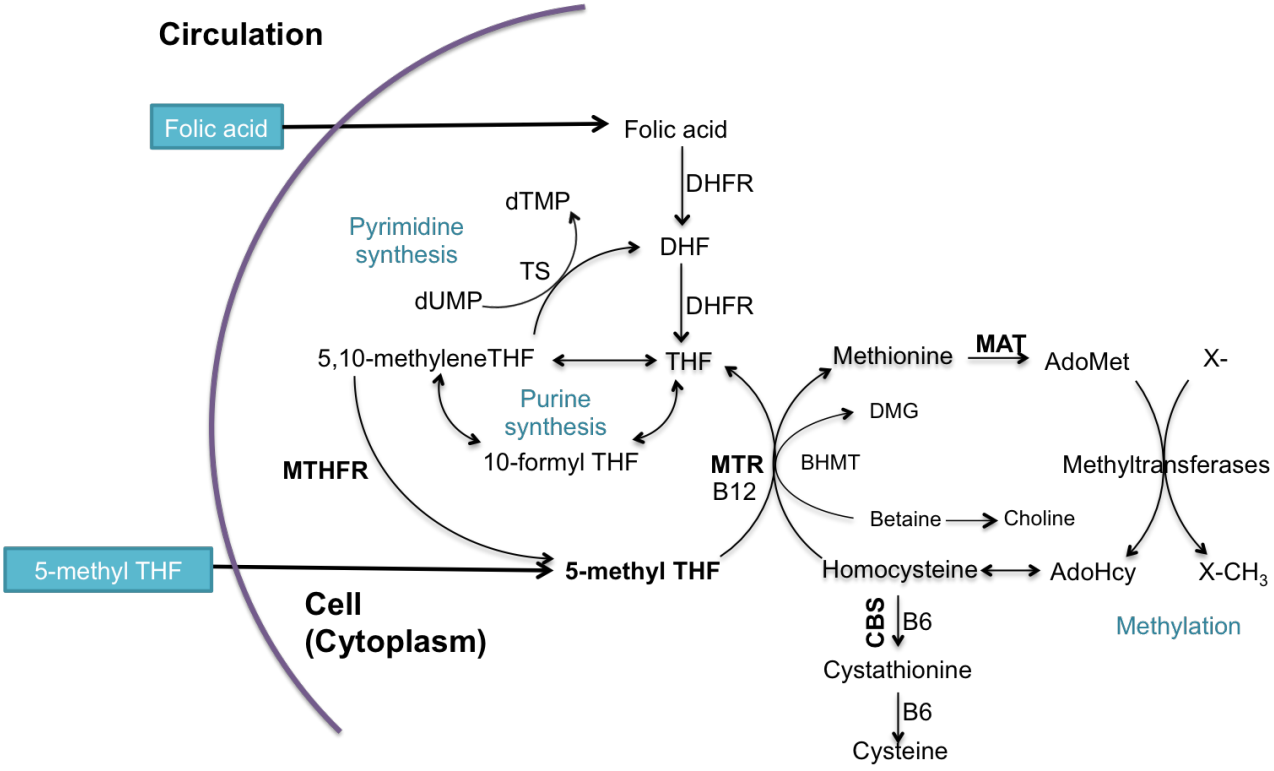

Figure 1.1: Schematic Representation of the Folate and Methionine Cycles
Abbreviations: THF: tetrahydrofolate, MTR: methionine synthase, MTHFR: methylenetetrahydrofolate reductase, DHFR: dihydrofolate reductase, CBS:Cystathionine β-synthase, MAT: methionine adenosyltransferase, BHMT: betainehomocysteine methyltransferase, TS: thymidylate synthase, AdoMet: S-adenosylmethionine, AdoHcy: S-adenosylhomocysteine, DMG: dimethylglycine, dTMP: deoxythymidine monophosphate, dUMP: deoxyuridine monophosphate, X-CH3: methylated product.

In vitamin B12 deficiency, folate is trapped as 5-methylTHF leading to impaired nucleotide synthesis[94]. However, it is important to note that folic acid can serve as a substrate for DHFR, which enables folic acid to enter the folate cycle to produce THF[94,95]. Accordingly, THF (from excess folic acid) can participate in nucleotide synthesis, bypassing the vitamin B12-dependent step. As such, excess folic acid can ameliorate and mask the hematological signs of vitamin B12 deficiency, but the irreversible neurological damage associated with vitamin B12 deficiency can progress[94,95]. Vitamin B12 deficiency can be distinguished from folate deficiency by quantifying

serum vitamin B12 and circulating methylmalonic acid concentrations because vitamin B12 but not folate is a cofactor in the conversion of methylmalonyl-CoA to succinyl-CoA (Figure 1.2). In vitamin B12 deficiency, methylmalonic acid, a by-product of methylmalonyl-CoA, accumulates in blood and urine[95,102].

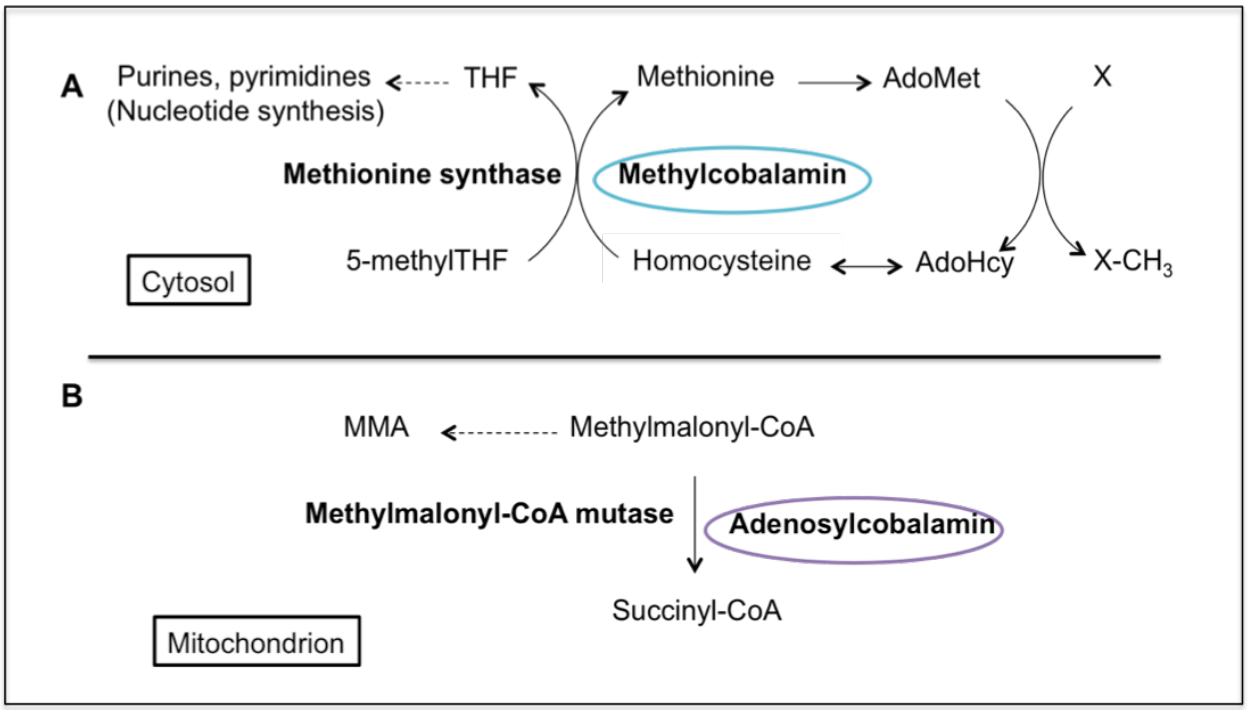

Figure 1.2: Vitamin B12 Coenzyme Functions
A. Vitamin B12 in the form of methylcobalamin serves as a coenzyme for cytosolic methionine synthase.
B. Vitamin B12 in the form of adenosylcobalamin serves as a coenzyme for mitochondrial methylmalonyl-CoA mutase.

MethylTHF can be formed by the irreversible reduction of 5,10-methyleneTHF in the folate cycle. This reaction is catalyzed by 5,10-methylene tetrahydrofolate reductase (MTHFR), an enzyme that regulates the availability of 5-methylTHF for homocysteine remethylation[94,99]. Homocysteine can also be remethylated to methionine via betainehomocysteine methyltransferase (BHMT) that uses a methyl group from betaine. Unlike MTR that is ubiquitously expressed, BHMT is tissue-specific enzyme expressed mainly in the liver and kidneys[99]. Both remethylation reactions that are catalyzed by MTR and BHMT form methionine. Methionine is the precursor of S-adenosylmethionine (AdoMet), which is the principal methyl donor for methylation reactions.

The biosynthesis of AdoMet is catalyzed by methionine adenosyltransferase (MAT). MAT has different isozymes encoded by two different genes in mammals: *MAT1A* encodes MAT I and MAT III and it is mainly expressed in healthy adult liver, whereas *MAT2A* encodes MAT II and is expressed in other tissues and fetal liver[104]. Decreased *MAT1A* expression in the liver is associated with increased hepatic *MAT2A* expression and it is implicated liver cirrhosis and hepatocellular carcinoma[104,105]. *S*-adenosylhomocysteine (AdoHcy) is formed following methyl donation by AdoMet. Homocysteine is formed by the reversible liberation of adenosine from AdoHcy by AdoHcy hydrolase. There are two vitamin B6-dependant enzymes contributing to the irreversible degradation of homocysteine: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). CBS functions to condense homocysteine and serine to cystathionine, which is consequently hydrolyzed to cysteine and α-ketobutyrate by CTH[94,99]. Disturbances in homocysteine metabolism can result in hyperhomocysteinemia, a condition that is associated with several adverse health outcomes including CVDs, dementia-type disorders, and osteoporosis[106-109].

1.3.4 Folate and Vitamin B12 in Pregnancy

Many factors during pregnancy can affect fetal growth and development. Among the most important modifiable factors is the nutritional status of pregnant women. Given the role of folate and vitamin B12 in DNA methylation, these vitamins may influence adult phenotype through epigenetic processes. Supplementation with methyl nutrients in rodents during pregnancy is associated with alterations in gene expression and DNA methylation in the offspring[110-113]. For example, maternal folate depletion (0.4 mg folic acid/kg of diet) prior to mating and during pregnancy in C57BL/6J was associated with increased fetal body weight and lower methylation of *Slc39a4* gene in fetal gut[112]; *Slc39a4* encodes one of the zinc/iron-regulated transporter-like proteins (ZIP) and its expression has been associated with some types of cancers, such as pancreatic and liver cancers[114-116]. Further, maternal diet completely depleted in folate or vitamin B12 during pregnancy and lactation was associated with lower birth weight in rat offspring, but this effect was demolished 7 days after birth in offspring exposed prenatally to a folate-deficient diet[113]. The same investigators reported lower hepatic expression of *Ppara* and *Pparg* mRNA in offspring exposed to vitamin B12-deficient diet, but not in those developmentally exposed to folate-deficiency[113]; *Ppara* and *Pparg* encode peroxisome proliferator-activated receptors (PPARs) α and γ, respectively, which are transcription factors that play different roles in glucose and fat metabolism[117-119]. Langie et al. reported that maternal folate depletion (0.4 mg folic acid/kg of diet) was associated with elevated base excision repair (BER) activity (a primary DNA repair pathway) in 4 brain regions of male offspring at weaning, and with hypermethylation of BER-related gene *(Ogg1)* at 6 months of age[120]. Moreover, maternal serum vitamin B12 concentration in a group of women (n=121) from the North Cumbria Community Genetics Project was reported to be inversely associated with infant

global DNA methylation in cord blood (r = 0.18, p = 0.04) as determined by the luminometric methylation assay[121].

Given the increased folate status of the Canadian population following folic acid fortification[7] and the high prevalence of vitamin B12 deficiency[9,10,98], a potential imbalance of high folic acid with low vitamin B12 is of concern particularly among pregnant women. Women in the lowest tertile of serum vitamin B12 concentration during the first, second, or third trimester of pregnancy were at greater risk for infants with intrauterine growth retardation (IUGR) [adjusted ORs (95%CI): 5.98 (1.72, 20.74), 9.28 (2.90, 29.68), and 2.81 (1.01, 7.87), respectively] compared to women with serum vitamin B12 concentrations in the highest tertile (n = 136) [122]; the medians in the lowest and highest tertiles slightly differed across the three trimesters, but they were on average ~113 and ~205 pmol/L, respectively. However, no association between RBC folate and IUGR was found in this study[122]. A study conducted in mice reported that female mice fed a vitamin B12-deficient diet during pregnancy and lactation had offspring with less body weight (8 ± 0.9 vs 13 ± 1.4 g) and lower serum vitamin B12 concentrations (218.5 ± 23.6 vs 507.7 ± 39.9 pmol/L) at weaning compared to offspring from control-fed dams [123]. Another study reported that feeding female mice a diet high in folic acid (40 mg/kg of diet) was associated with increased weight gain and insulin resistance, and decreased serum adiponectin concentrations in male offspring after 8 weeks on a high fat diet[124]. The reduction in serum adiponectin concentrations was associated with decreased adiponectin mRNA expression in white adipose tissue of male offspring before and after the introduction of the high fat diet[124]. Adiponectin is an adipokine mainly produced by adipocytes and is involved in regulating glucose metabolism[125-127]. Deficiency of B-vitamins (folate, vitamin B12, and vitamin B2) and choline during pregnancy and lactation resulted in decreased brain AdoMet:AdoHcy, suggesting a methyl group shortage, of male and female offspring at birth, and

lower total glutathione concentrations as well as higher plasma total homocysteine 28 days after birth[128].

The intake of high folate and low vitamin B12 during the second trimester of pregnancy was also reported to be associated with higher risk for small-for-gestational-age infants (adjusted relative risk (RR): 2.73; 95% CI: 1.17, 6.37) in a cohort of South Asian women (n = 315) [129]. Researchers in another study from South Asia reported that low plasma vitamin B12 concentrations (<150 pmol/L) are associated with a higher prevalence of gestational diabetes compared to non-deficient women and the prevalence increased as plasma folate concentrations increased from lowest to highest tertile[130]. A follow up study found that maternal gestational diabetes in the same population was a major predictor of adiposity and insulin resistance in children at 9.5 years of age[131]; adiposity was determined by measuring skinfold thickness, while insulin resistance was estimated based on the homeostatic model assessment of insulin resistance (HOMA-IR).

The Pune Maternal Study reported greater adiposity and insulin resistance in children aged 6 years (n = 653) who were exposed prenatally to high maternal RBC folate at 28 weeks of gestation[12]. In the same study, low maternal plasma vitamin B12 status was also associated with insulin resistance in the children, but the most insulin resistant children were from mothers with both low plasma vitamin B12 and high folate concentrations[12]. Adiposity in those children was determined by dual-energy X-ray absorptiometry (DEXA) scan, and insulin resistance was estimated via HOMA-IR. Stewart et al. conducted a randomized control trial of antenatal multivitamin supplementation in rural Nepal and reported that only maternal vitamin B12 deficiency was associated with higher risk of insulin resistance in the children (aged 6-8 years, n = 598) [13]. This study, however, used plasma folate as an indicator of folate status and not RBC folate, which is a better indicator of the long-term folate status[13]. In contrast, a recent observational study

conducted in India found no association between maternal plasma vitamin B12 concentrations and offspring adiposity (measured by bioelectrical impedance method) or insulin resistance (estimated by HOMA-IR). However, this study did find that high maternal plasma folate concentrations were positively associated with insulin resistance (estimated by HOMA-IR) in children at 9.5 and 13.5 years of age[14].

1.4 Overweight and Obesity

Environmental stressors during development have the potential to affect offspring metabolic health as discussed in section 2.1. Exposures to maternal influences during development, such as obesity, and to obesogenic environment postnatally, such as high fat diet, have been associated with metabolic complications[66,132-135]. Worldwide obesity, defined as a Body Mass Index (BMI) ≥ 30 kg/m^2, has increased dramatically since 1980, and is not limited to high-income countries[2,3]. The WHO reported that 500 million adults were obese in 2008[2]. According to Public Health Agency of Canada, data from 2007- 2009 revealed that one in four Canadian adults is obese while 8.6% of Canadian children (aged 6 to 17 years) are obese[136]. BMI is a surrogate measure of adiposity and is used to define overweight and obesity[137]. A healthy adult BMI ranges from 18.5 to 24.9 kg/m^2, whereas overweight is defined as a BMI of 25.0-29.9 kg/m^2 , and obesity as a BMI of ≥ 30 kg/m^2[63,137].

1.4.1 Abdominal Obesity

Regional distribution of body fat is an important factor to consider when assessing obesity-related health issues. There are two major types of fat deposition: android “apple-shape” and gynoid “pear-shape”. The android obesity is characterized by excess subcutaneous fat on the trunk/abdominal region and it is more common in men, while the gynoid fat distribution is

characterized by excess fat in the thighs and buttocks and it is more common in women[63,138]. The android type of obesity is associated with metabolic disturbances, such as glucose intolerance, hypertension, and hyperlipidemia more than the gynoid type[63,139,140]. The accuracy of BMI in assessing adiposity and risk for disease is questionable. BMI does not take into consideration differences in body composition, age, sex, and ethnicity[141,142]. It is calculated from total body weight without distinction between fat mass and lean (muscle) mass[142]. For example, an athlete may be classified as overweight based on BMI but the elevated BMI may due to an increased muscle mass and not adiposity. In addition, age and different sexes and ethnicities have been reported to have different rates of abdominal (visceral) fat accumulation[63,141,143,144].

Simple methods are available to estimate body fat and body fat distribution such as waist circumference, skinfold thickness, and calculation of waist-to-hip circumference ratios[8,145]; waist circumference is the most practical surrogate indicator of abdominal fat and one of the best anthropometric predictors of CVD risk[146,147] and healthy ranges based on sex and ethnicity have been established[8,146-148]. However, the use of waist circumference to predict cardiometabolic dysfunction has some limitations, as it cannot distinguish between visceral and subcutaneous adipose tissue. Visceral adipose tissue is the fat that surrounds abdominal organs such as liver, kidneys, and pancreas and is associated with greater cardiometabolic risk[149-151], whereas subcutaneous adipose tissue is the fat located between the skin and muscles. There are more accurate techniques to assess body composition and body fat distribution, such as magnetic resonance imaging, DEXA, and computerized tomography[145,152].

There are individuals with a BMI in the healthy range who are considered metabolically obese because of their metabolic complications, such as reduced insulin sensitivity, elevated circulating TG, and excess liver fat, which may be related to their body fat distribution[63,153]. On the

other hand, not all individuals with obesity (BMI ≥ 30 kg/m^2) have metabolic complications, which is partially attributed to lower visceral fat deposition[153]. However, this 'healthy obesity' phenotype may change over time and eventually lead to adverse health outcomes. A recent study reported that metabolically healthy subjects with obesity had a higher risk for cardiovascular events and all-cause mortality in the long-term (≥ 10 years), compared to subjects with healthy BMIs[154,155].

1.4.2 Causes of Obesity

The etiology of obesity is still an active area of research and multiple factors are believed to contribute to obesity, making it a complex phenomenon. Genetic, environmental, and physiological influences and interactions play a causative role in obesity[8,63]. Evidence from genome-wide association studies, family studies, and twin studies suggests that a significant portion of variation in weight gain patterns can be attributed to genetic variants. Such variants affect feeding behaviours, such as an increase in hunger and overeating, or metabolism, such as tendency to store body fat rather than using it as fuel[63,156]. Congenital or monogenic obesity, such as that caused by variants in the leptin receptor gene, is rare[8,157]. Bardet-Biedl and Prader-Willi syndromes are two rare congenital disorders associated with obesity[158,159]. Most obesity is polygenic with genetic factors interacting with several environmental factors[156,160].

Genetic factors can influence vulnerability to obesity, but environmental factors are also key determinants for obesity in susceptible individuals. Both dietary and physical activity patterns play crucial roles in weight gain and energy balance. By increasing energy input without compensating with increasing energy output, the body becomes in a state of positive energy balance, leading to storage of excess energy as fat[8,63]. It is estimated that for each extra 3500 kcalories, one pound of fat is stored[8]. Even though macronutrient intake is the main component considered in energy input and weight gain, the nutritional qualities of the food is gaining attention. The term "Western diet"

has been used to describe current unhealthy dietary patterns characterized by high consumption of refined grains, refined sugars, saturated and trans- fats, and processed food as well as low consumption of fiber[161]. Although the term was originated in Westernized populations, it is a common dietary practice in developed and developing countries[161]. Furthermore, our current environment fosters physical inactivity, placing an additional burden on the individual's ability to maintain healthy body weight. The WHO reported that 31% of the world's population, 1 in 3 people, is not physically active[162]. Urbanization, screen time, sedentary nature of several occupations, and modern modes of transportation are some of the factors that led to the current decline in physical activity level[2,8,163].

1.4.3 Obesity-related Complications

Obesity is associated with risk for chronic disease. Aside from the economical and psychological concerns of obesity, several adverse health outcomes and increased mortality have been linked to overweight and obesity. According to the WHO, the fifth leading risk for deaths worldwide is overweight and obesity[2] and it is estimated that 300,000 people die annually from obesity-related diseases[164]. Body weight and adiposity have been widely studied in relation to cardiovascular risk factors and diseases. A meta-analysis of 21 prospective cohort studies reported that, compared to healthy-weight participants, overweight was associated with 32% higher risk for developing CHD and this percentage went to 81% in obese participants[165].

Adipose tissue in the abdominal area is believed to be metabolically active and is able to induce metabolic changes that lead to abnormalities like insulin resistance[166]. In the Nurses' Health Study and the Health Professionals Follow-up Study, weight gain and increased BMI and waist circumference in adulthood were associated with higher risk for T2D[167,168]. The term "cardiometabolic risk" covers a wider range of risk factors for the development of T2D as well as

CVD[169]. Important elements of increased cardiometabolic risk include: abdominal obesity, smoking, insulin resistance, high BP, high LDL cholesterol, low HDL cholesterol, high TG, high fasting blood glucose, and disturbed inflammatory profile[169].

As the liver plays a central role in glucose and lipid metabolism, it is one of the primary organs that can be adversely affected by obesity and an unhealthy diet. Products of carbohydrate digestion, such as galactose and fructose, are converted into glucose in the hepatocyte, and glucose can be stored in the liver as glycogen (glycogenesis) [8,63]. Glycogen can be broken down into glucose that is released into the blood when glucose is required for energy (glycogenolysis) [8,63]. In addition, the liver produces glucose from non-carbohydrate compounds (gluconeogenesis), such as glucogenic amino acids, lactic acid, and glycerol[8,63]. Fatty acids can be synthesized by the liver (lipogenesis) from acetyl CoA, produced during the metabolism of glucose and amino acids. Circulating free fatty acids (FFAs) are transported to the liver to be oxidized to produce energy, re-esterified to TG and stored in adipose cells, or packed into lipoproteins to be transported to other tissues via blood[63,170]. Normally, excess dietary fat is stored as TG in the adipocytes as an energy reserve to be used in fasting or other catabolic states (e.g prolonged exercise) [63]. However, with the chronic ingestion of high fat diet, especially when accompanied with lack of physical activity, the body starts to store TG ectopically in other organs, such as heart, liver, kidneys, and skeletal muscle, which can lead to lipotoxicity and impairment of several metabolic processes in these tissues[64].

One of the most common liver disorders in obesity is NAFLD[171]. NAFLD is considered an umbrella term for several related hepatic diseases and is characterized by excessive fat accumulation in the liver (> 5% by weight) that is not the result of alcohol consumption[170,172]. Non-alcoholic steatohepatitis (NASH) is different from simple steatosis as it is characterized by the

presence of inflammation, fibrosis, and hepatocyte injury and it is likely to progress to cirrhosis[170,171]. It is estimated that up to 34% of the US adults have hepatic steatosis and it was more prevalent in European men compared to European women and in Hispanic Americans compared to European Americans and African Americans, respectively[173]. A study from Italy found that the prevalence of NAFLD is much higher in obese individuals compared to non-obese participants, 76% and 16%, respectively[174]. Although the prevalence of NAFLD is relatively low in children (2.6%), it has been reported to be as high as 53% in obese children[175,176].

Chapter 2: Rational and Hypothesis

Folate and vitamin B12 are crucial for DNA methylation through the generation of AdoMet. Changing maternal methyl nutrient supply during pregnancy and lactation has been reported to affect gene expression and DNA methylation patterns in mice and sheep[110,177,178]. In addition, alterations in the periconceptional supply of methyl nutrients, including folic acid and vitamin B12, are associated with metabolic disturbances in the offspring[123,124,128,177]. Accordingly, alterations in adiposity and insulin resistance observed in children exposed prenatally to maternal high folate and low vitamin B12 status[12-14] may be due to disturbances in methyl metabolism. Human studies in this area of research are mainly observational and provide no data on causality, mainly because it is unethical to restrict pregnant women from attaining adequate nutrition and it is difficult to collect human tissue. Therefore, animal studies are ideal to study a more mechanistic approach in a controlled setting. Based on this background**, I hypothesize that maternal high folic acid and low vitamin B12 intakes during pregnancy and lactation program liver gene expression in adult offspring and contribute to cardiometabolic dysfunction (excess adiposity and insulin resistance).** My hypothesis was addressed by determining the effect of developmental exposure to maternal dietary folic acid/vitamin B12 imbalance during pregnancy and lactation on the following parameters in male and female adult offspring mice:

1. Serum folate and vitamin B12 concentrations in adult offspring.
2. Expression of key enzymes in methyl metabolism and *Nr3c1* and *Ppara* in liver from adult offspring.
3. The relationship between changes in gene expression to changes in gene-specific DNA methylation and relationship to methyl metabolites in the liver.

I conducted my research in C57BL/6J mice, which are susceptible to diet-induced obesity and insulin resistance[179]. Given that programming events can be exacerbated upon exposure to an obesogenic environment[43,66,135], a group of offspring was challenged with an obesogeneic diet (Western diet). The liver is a key target for studying the impact of developmental exposure to folate and vitamin B12 imbalance on genes expression and chronic disease development for different reasons. In addition to its role in macronutrients metabolism, 85% of the body's methylation reactions occur in the liver and up to half of dietary methionine is converted to AdoMet in mammalian hepatocytes[180,181]. The link between insulin resistance and fatty liver disease has been established, but it is still unclear which one comes first. Some studies suggest that insulin resistance can lead to fatty liver disease [182,183]. On the other hand, some linked fatty liver disease to insulin resistance and atherosclerosis[184].

I examined gene expression changes in liver of adult offspring who were exposed to maternal folic acid and vitamin B12 imbalance prenatally (during gestation) and during early postnatal life (suckling period). In Chapter 3, I describe my research design and methods. My research findings are presented in Chapter 4 and discussed in Chapter 5.

Chapter 3: Materials and Methods

3.1 Experimental Design

This project was conducted in C57BL/6J mice purchased from the UBC Centre for Disease Modeling (males) and Charles River, Montreal (females). These mice were acclimatized to the animal unit at CFRI for one week before mating. Dams were fed standard laboratory chow (PicoLab® Mouse Diet 20 (code 5058), LabDiet, PMI Nutrition International, St. Louis, MO) and were bred at 6 weeks of age to males of the same age. After their first litter, the dams were randomly assigned to one of three diets: control, high folic acid with no vitamin B12 (HFA-B12), or high folic acid with adequate vitamin B12 (HFA+B12) for 6 weeks prior to mating and through pregnancy and lactation. Both HFA-B12 and HFA+B12 diets contained 10 mg folic acid (Sigma, Sigma-Aldrich)/kg of diet, which is five times higher than the control group (2 mg folic acid/kg of diet) and the recommended nutritional requirements for mice[185]. Control and HFA+B12 groups received adequate amounts of vitamin B12 (50 μg/kg cyanocobalamin, Sigma), whereas HFA-B12 group had no vitamin B12 in their diet (Table1). Pectin was added (50 g/kg, Sigma) to enhance vitamin B12 depletion[186].

At weaning (3 weeks of age), offspring mice were randomly assigned to receive a control diet or a Western diet [high in fat and simple carbohydrate (sucrose)] for 20 weeks (male offspring) and for 40 weeks (female offspring) (Figure 3.1). Male mice are more susceptible to diet-induced obesity and glucose intolerance and develop these conditions earlier than female mice when fed a Western diet[57,187,188]. At the end of feeding period, mice were anaesthetized using isofluorane, blood was collected by cardiac puncture, and tissue were harvested after cervical dislocation. Upon harvest, liver tissue was flash frozen in liquid nitrogen, then stored at -80°C. Blood was left at room

temperature to coagulate for 15 min, then centrifuged at 8000 rpm at 4°C for 15 minutes to obtain serum, which was consequently stored at -80°C until further analyses.

Mice were housed in the Animal Care Unit at CFRI, which is regularly inspected by the Canadian Council on Animal Care. Mice were housed in groups (3-5 mice/cage) under a standard 12-hour light/12-hour dark cycle and had unlimited access to food and water.

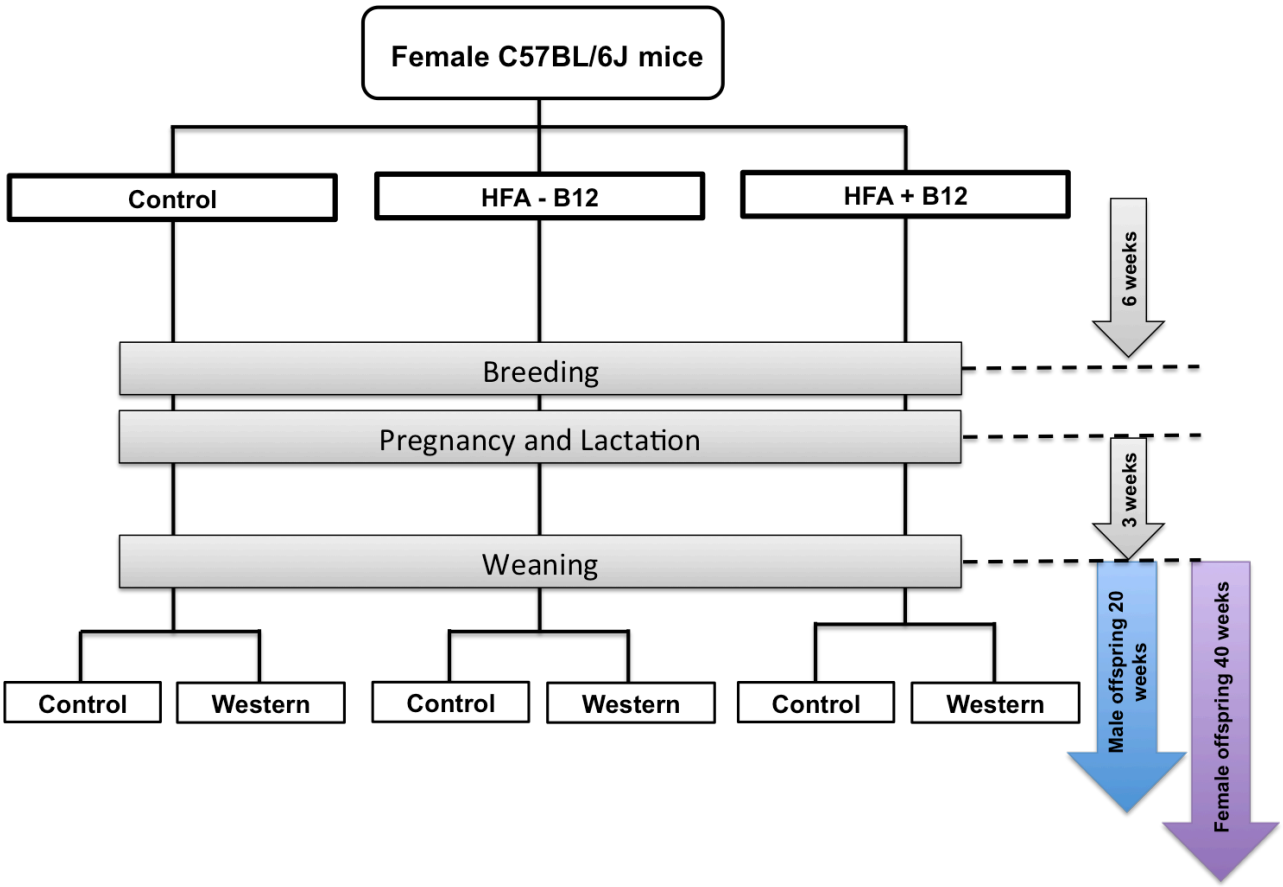

Figure 3.1: Research Design Overview
Overall, 6 groups of offspring mice were studied (maternal diet/post weaning diet): control/control; HFA-B12/ control; HFA+B12/control; control/Western; HFA-B12/Western; HFA+B12/Western. Each group had 6 mice/sex/diet group.

Table 3.1 Maternal and Offspring Diet Composition Based on National Research Council (NRC) of Canada.

	Control	Western	HFA-B12	HFA+B12
Total Energy (kcal/kg)	3948	4700	3948	3948
Fat (% of total energy)	16	45	16	16
Carbohydrate (% of total energy)	64 (100%Cornstarch)	35 (70%Sucrose 30%Cornstarch)	64 (100%Cornstarch)	64 (100%Cornstarch)
Protein (% of total energy)	20	20	20	20
Folic Acid (mg/kg)	2.0	2.0	10.0	10.0
Vitamin B12 (µg/kg)	50.0	50.0	0	50.0
Pectin (g/kg)	50.0	50.0	50.0	50.0

Note: Amounts are per kg of diet. All other micronutrients are consistent between groups
The primary source of fat was soybean oil in all diets except the Western diet, which composed of a mixture of soybean oil, butter, lard, and vegetable shortening.

3.2 Biochemical Analyses

3.2.1 Quantification of Serum Folate Concentrations

Serum folate concentrations were quantified by a microbiological assay using 96-microtitre plates and the chloramphenicol resistant strain of *Lactobacillus casei* (Strain NCIB 10463; obtained from Cedarlane, Ontario, Canada) as outlined by O'Broin et al. [189]. The assay was conducted at UBC in collaboration with Dr. Tim Green (UBC Food, Nutrition and Health). Glycerol-cryopreserved cultures were used as described by Grossowitz et al. [190] and modified by Wilson and Horne[191,192]. A stock solution of folic acid standard was made in advance and stored in aliquots at -80°C. From this stock solution, working folic acid standard solution was made fresh to make the standard curve. Serum samples were diluted with 0.5% sodium ascorbate based on the expected folate concentrations in C57BL/6 mice. For each sample 400 µl of cryo-preserved *Lactobacillus casei* bacteria was added to each 100ml of assay medium. Assay medium was made fresh and consisted of (5.3 g Difco™ Folic Acid Casei Medium (BD: Becton Dickinson), 3 mg chloramphenicol (Sigma), 30 µl Tween 80 (Sigma), and 75 g ascorbic acid (Sigma) dissolved in 100 ml distilled water. The assay medium contained all necessary nutrients required to grow the bacteria except folate, therefore the amount of bacterial growth was proportional to the folate concentration of the serum sample analyzed. After 40-hour incubation at 37°C, turbidity was measured using a multiscan spectrophotometer set at 585 nm (Thermo Labsystems, Helsinki, Finland). Serum folate concentrations were determined by polynomial equations derived from the standard curve.

The concentration of folic acid standard was confirmed by a spectrophotometer. All samples were run at the same time in five plates and folic acid standards were used in the 1st, 3rd,and 5th plate. On each plate, I ran blanks, which were assay broth without the bacteria, and a control

sample, which was a mouse serum obtained from the same project. Blanks and each folic acid standard were run in quadruplicate. Serum samples and the control were run in 2 dilutions, 1/600 and 1/1200; each dilution was run in duplicates.

3.2.2 Quantification of Serum Vitamin B12 Concentrations

Serum vitamin B12 concentrations were quantified in dams and male offspring by a commercial microbiological kit using *Lactobacillus delbrueckii subsp. lactis* coated microtitre plate (ID-Vit® Vitamin B12, Immundiagnostik) and following the manufacturer's protocol. Standards, controls, and samples were run in duplicate. In order to obtain values that fall within the range of the standard curve (110.4 - 993.4 pmol/L), a serial dilution experiment was conducted with the serum samples in order to determine the best dilution for detection. Samples were diluted 1 in 40 with water (provided by the kit).

Serum vitamin B12 concentrations from female offspring were analyzed by Dr. Anne Molloy (Trinity College Dublin, Ireland). The method used was a microbiological assay using colistin sulphate (colomycin) resistant *Lactobacillus delbrueckii* (NCIMB 12519, ATCC 43787) as described by Kelleher and O'Broin[193]. These vitamin B12-dependant bacteria were inoculated into the growth media and added to wells, which contained the samples at the appropriate dilution. Vitamin B12 was extracted from serum samples by diluting each sample 1 in 10 with an extraction buffer [sodium hydroxide (8.3 mmol/L), acetic acid (20.7 mmol/L), and sodium cyanide (0.45 mmol/L), pH 4.5]. Samples were mixed with the extraction buffer, then autoclaved for 10 minutes at 121°C at 15 psi, and centrifuged at 3000 rpm for 15 minutes. The supernatant was diluted as required with the extraction buffer. Before adding the assay medium, the total volume in all wells had to be 100 µl, so compensating volumes of vitamin B12 extraction buffer were added as needed. The assay medium consisted of 6.2 g of Difco™ B12 Assay Medium (BD), 150 µl Tween 80, and

11 mg colistin sulphate dissolved in 100 ml ddH2O. A 100 µl of the cryopreserved bacteria was added to each 100 ml of media and then 200 µl of this mixture was added to each well. Vitamin B12 standards were run in quadruplicates and each sample were run in 2 dilutions and each dilution was run in quadruplicates.

3.3 Quantification of Liver AdoMet/AdoHcy Concentrations

To quantify hepatic AdoMet and AdoHcy concentrations, 0.15 g of liver was homogenized with 750 µl of 0.4 M perchloric acid using the Bullet Blender® tissue homogenizer for 3 minutes at level 9 or until tissue was fully lysed. The tissue homogenate was spun down at 4°C for 5 minutes at 14,000 rpm and supernatant was stored at -80˚C until time of analysis. Concentrations of AdoMet and AdoHcy were quantified in Dr. Joshua Miller's lab by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection as described by Fell et al. [194] and modified by Miller et al. [195]. A 3 µm ODS Hypersil, 150 mm x 2 mm (Keystone Scientific, Bellafonte, PA) column was used with a 0.3 mL/min flow rate. The mobile phase consisted of a gradient over 20 minutes from 100% solvent A to a mixture of 75% solvent A+ 25% solvent B. Solvent A contained 0.01 mmol/L ammonium formate with 4 mmol/L heptanesulfonic acid at pH 4.0; solvent B contained 50% solvent A plus 50% acetonitrile at pH 4.0. The 75% solvent A and 25% solvent B mixure was maintained from 20 to 25 minutes, followed by 100% solvent A for 15 minutes (equilibration). Peaks of AdoMet and AdoHcy were detected at 254 nm by UV absorption, and then external standards were used to obtain AdoMet and AdoHcy concentrations. AdoMet:AdoHcy ratio were calculated by dividing AdoMet concentration by AdoHcy concentrations.

3.4 Quantification of mRNA

Total RNA was extracted from liver using the RNeasy Mini Kit (Qiagen). Samples were treated with RNase-Free DNase (Qiagen) to remove contaminating genomic DNA. RNA integrity was confirmed by the presence of 18s and 28s ribosomal RNA (rRNA) on 1.5% agarose gels. RNA concentrations were calculated based on absorbance at 260 nm (NanoDrop spectrophotometer) and the 1.9-2.1 260 nm/280 nm absorbance ratio was considered as good quality RNA. RNA (500 ng) was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems). Real time PCR was used to quantify mRNA levels using the ΔΔCt method of relative quantification[196,197]. The amount of target gene was normalized to an endogenous reference (18s rRNA) in the ΔΔCt assay and was calculated relative to findings obtained from offspring from the maternal control diet group and fed the post weaning control diet. TaqMan® Gene Expression Master Mix and and the following mouse-specific gene expression primers (Applied Biosystems®) were used: *Mtr* (Mm 01340053_m1), *Cbs* (Mm 00460654_m1), *Mat1a* (Mm00522563_m1), *Mthfr* (Mm 01255752_m1), *Ppara* (Mm 00440939_m1), and *Nr3c1* (Mm00433832_m1). Data were analyzed using 7500 System Sequence Detection software (Applied Biosystems®). Each sample was run in duplicate to examine intra-assay variability and the experiment were repeated two separate times to determine inter-assay variation.

3.5 Cbs Immunoblot

Expression of Cbs was quantified in liver by immunoblot. Frozen liver tissue (50 mg) was homogenized in 500 µl lysis buffer [1x RIPA buffer (Cell Signalling: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate , 2.5 mM

sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin) and protease inhibitor cocktail (Roche Diagnostics) in distilled water]. Each was sonicated on ice for 30 seconds or until tissue was fully homogenized. Samples were then centrifuged at 8000 rpm at 4°C for 10 min and the resulting supernatant was stored in -20°C for immunoblot analyses. The Bradford assay was performed to determine protein concentrations in homogenates[198].

Liver homogenates (30μg of protein) were mixed with 5x Laemmli sample buffer (250mM Tris-HCl, pH 6.8, 10% SDS (Invitrogen), 50% glycerol (Fisher Scientific), 0.02% bromophenol blue (Fisher Scientific), and 10% β-mercaptoethanol (Sigma)) and boiled for 15 minutes to denature the proteins. Samples were resolved by electrophoresis on 10% SDS-polyacrylamide gels with a 4% stacking polyacrylamide gel for ~100 minutes at 110V. Samples were then electrotransferred to nitrocellulose membranes (Bio-Rad) for 90 minutes at 110V, using 1x transfer buffer (25 mM Tris base, 190 mM glycine, and 20% methanol). Membranes were then immersed in Ponceau S staining solution (Sigma) to confirm successful transfer and to visualize protein bands, and then de-stained by washing in ddH2O.

For immunodetection of proteins, membranes were blocked in 5% milk solution, which consists of milk powder dissolved in Tris Buffer Saline Tween 20 (TBS-T) buffer (0.2M Tris base, 2.5M NaCl, and 0.05% Tween 20), for 1 hour at room temperature. The blocking step is to prevent non-specific binding of the antibodies to the membrane in the subsequent steps. Membranes were incubated with the following primary antibodies overnight at 4°C on a shaker: rabbit Cbs polyclonal IgG (sc-67154, Santa Cruz Biotechnology) at 1:5000 dilution in 5% bovine serum albumin (BSA), and rabbit anti mouse β-actin polyclonal IgG as a loading control (sc-1616-R, Santa Cruz Biotechnology) at 1:1000 dilution in 5% BSA. After incubation with primary antibodies, membranes were washed in TBS-T for 30 minutes, and then incubated at room

temperature with goat anti-rabbit IgG-conjugate to alkaline phosphatase (sc-2007, Santa Cruz Biotechnology) at 1:2000 dilution in 5% milk solution (milk powder dissolved in TBS-T) for 1.5 hour. Membranes were washed with TBS-T for 30 minutes and then incubated with Lumi-Phos WB Substrate (ThermoScientific), a chemiluminescence reagent, for 5 minutes. Chemiluminescence from the membranes was detected by ChemiGenius gel imaging system (Perkin Elmer) by exposing the membranes for 10 minutes; 5 images were captured. Relative protein densities (Cbs expression relative to actin expression) were quantified by GeneTools software (Perkin Elmer). Each sample was run two times (in two different blots) and the average value was used in the analysis.

3.6 Quantitative Analysis of *Mthfr* DNA Methylation

The methylation status of the *Mthfr* promoter was quantified by bisulfite pyrosequencing[199]. The mouse *Mthfr* gene is located in distal chromosome 4[200]. *Mthfr* has a complex genomic structure and two major promoters have been identified[201,202]. *Mthfr* methylation has not been studied before in mice. *Mthfr* promoter CpG-rich regions were identified by *in silico* analysis using MethPrimer and the UCSC genome browser[203,204]. Genomic DNA was extracted from liver tissue (25mg) using the DNeasy Blood & Tissue Kit (Qiagen) and included RNase I treatment. DNA concentrations were quantified by NanoDrop spectrophotometer. DNA (500 ng) was bisulfite-treated using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH) and stored at -20°C until further analysis.

PCR and sequencing primers were designed using PyroMark Assay Design Software (Qiagen). A 235 bp region of the *Mthfr* downstream promoter between -312bp and -77bp, relative to the translational start site, was amplified by PCR using HotStarTaq DNA Polymerase (Qiagen) and the following primers: MmMthfr-P2-F, 5'- GGGGATGTGGGTTTTAGAG -3' and MmMthfr-P2-RB, 5'- CCCATACACACCCAACAC -3' (IDT). This region has been reported to be required

for *Mthfr* promoter activity in Neuro-2a (derived from mouse neuroblastoma cells) and RAW 264.7 (derived from mouse macrophage cell line)[202] and it is adjacent to exon1 (Figure 4.2). For each sample, PCR master mix consisted of 2.5 µl of 10x PCR buffer, 5 µl of Q-solution, 10mM of dNTP, 0.25µl of HotStarTaq DNA polymerase, 0.5 µl of 10µM forward primer (IDT, Coralville, IA) and 0.5 µl of 5'biotin-labelled reverse primer (IDT) and 1µl of Bisulfite-treated DNA. The cycling conditions were as follow: 95°C for 15 minutes followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds and a final extension at 72°C for 10 minutes. A water blank was used as a negative control and agarose electrophoresis (1.2% agarose) was performed to visualize the PCR products.

PCR products were sequenced using the PyroMark MD System (Biotage, Qiagen) following the manufacturer's suggested protocol. Two sequencing primers were used to sequence the PCR product: MmMthfr-P2-Seq2, 5'- TTTATTAGGTAGTTGT -3' and MmMthfr-P2-Seq3B, 5'- TTTTGGGTAGGAGTTGTAAG -3' (IDT). The former sequencing primer was used to sequence the first 6 CpG sites while the latter was used to sequence the last 3 CpG sites (Figure 3.2). The percent methylation at each CpG site was quantified by Pyro Q-CpG software (Biotage, version 1.0.9).

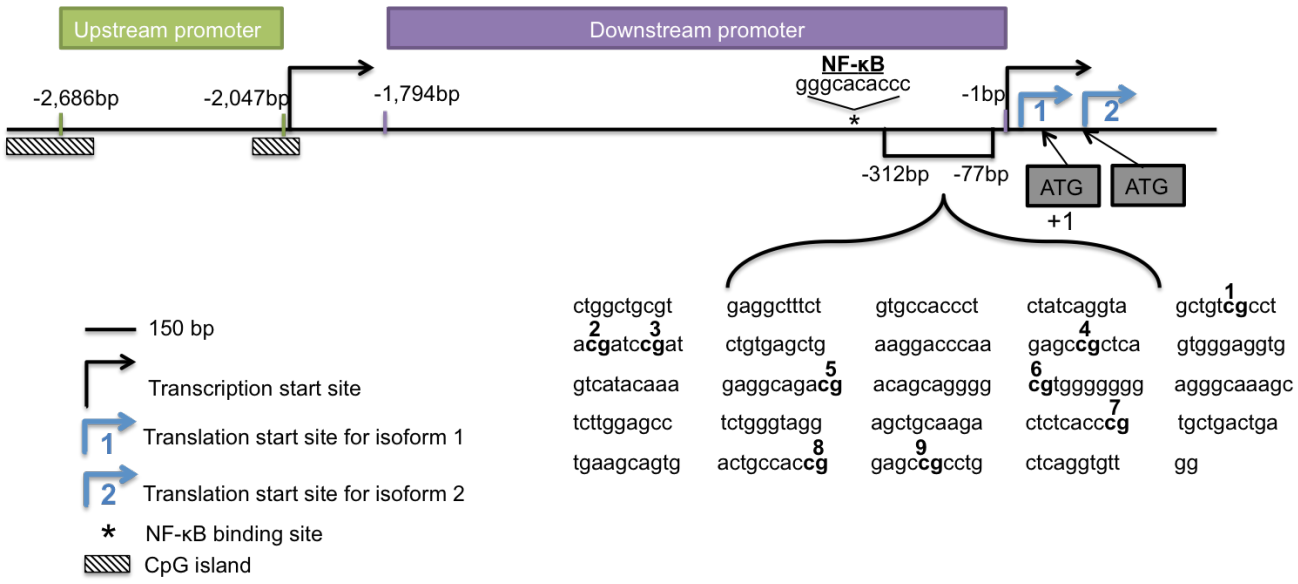

Figure 3.2: Schematic Representation of the 5' Region of Mouse *Mthfr* Gene in Chromosome 4 Illustrating Two Promoters Upstream of the First Exon
Isoform 1 = long isoform of MTHFR (77 kDa), Isoform 2 = short isoform of MTHFR (70 kDa)
The figure is adapted from Pickell et al. [205].

3.7 Statistical Analyses

Data were analyzed by two-way analysis of variance (ANOVA). The independent variables were maternal diet and post weaning offspring diet. If an interaction was found, one-way ANOVA was used to assess the effects of maternal diet on post weaning control-fed and Western-fed mice separately followed by the least significant difference (LSD) test for multiple comparisons. Male and female offspring were analyzed separately. Simple linear regression analyses were performed to explore the relationship between gene expression and other variables, such as *Mthfr* promoter methylation and AdoMet and AdoHcy concentrations. Analyses were conducted using SPSS version 22 (IBM, US) and p-value < 0.05 was considered statistically significant. All results are presented as means ± standard error of the mean (SEM).

3.7.1 Folate Microbiological Assay Intra- and Inter-assay Variability

For the folate microbiological assay, the inter-assay and intra-assay coefficient of variability (CV) were calculated as follow: %CV = [(standard deviation (SD) between plates or between duplicates/ average value) x100]. Inter-assay CV was calculated for the concentration as well as the absorbance reading of a control sample that was measured in two dilutions (1/600 and 1/1200). They are represented in Table 3.2 as control 1 (1/1200) and control 2 (1/600). I calculated the intra-assay CV for the absorbance reading of control 1 and 2 in each plate as shown in Table 3.3.

Table 3.2: Folate Microbiological Assay Inter-Assay CV

Control	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Average	SD	%CV
Concentration	56.81	52.12	53.22	62.66	62.26	57.41	4.92	8.6
Abs (control 1)	0.198	0.165	0.180	0.203	0.176	0.184	0.01	7.6
Abs (control 2)	0.320	0.265	0.269	0.320	0.303	0.295	0.02	8.1

Table 3.3: Folate Microbiological Assay Intra-Assay CV

Control		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Total %CV
Control 1	Abs 1	0.207	0.173	0.181	0.201	0.188	5.2
	Abs 2	0.188	0.156	0.178	0.204	0.164	
	Average	0.198	0.165	0.180	0.203	0.176	
	SD	0.01	0.01	0.00	0.00	0.02	
	%CV	6.80	7.31	1.18	1.05	9.64	
Control 2	Abs 1	0.335	0.282	0.280	0.326	0.303	5.0
	Abs 2	0.304	0.248	0.257	0.314	0.302	
	Average	0.320	0.265	0.269	0.320	0.303	
	SD	0.02	0.02	0.02	0.01	0.00	
	%CV	6.86	9.07	6.06	2.65	0.23	

Overall, the inter-assay CV for the folate microbiological assay ranged from 7.6 to 8.6%. Further, the average intra-assay CV for the folate microbiological assay was 5.1%.

3.7.2 Vitamin B12 Microbiological Assay Intra- and Inter-assay Variability

The inter- and intra-assay CV for the vitamin B12 microbiological assay is shown in Tables 3.4 and 3.5, respectively. Inter-assay % CV was calculated for the absorbance reading and the concentration of a control sample came with the kit. Intra-assay CV is shown for the control and the first standard (Std 1).

Table 3.4: Vitamin B12 Microbiological Assay Inter-Assay CV

Control	Plate 1	Plate 2	Plate 3	Average	SD	%CV
Concentration (ng/L)	580	600	575	585	13.23	2.3
Abs	0.250	0.254	0.283	0.262	0.02	6.9

Table 3.5: Vitamin B12 Microbiological Assay Intra-Assay CV

Control		Plate 1	Plate 2	Plate 3	Total %CV
Control	Abs 1	0.243	0.241	0.281	4.1
	Abs 2	0.257	0.267	0.285	
	Average	0.250	0.254	0.283	
	SD	0.01	0.02	0.00	
	%CV	3.96	7.26	1.02	
Std 1	Abs 1	0.108	0.104	0.115	1.8
	Abs 2	0.108	0.101	0.109	
	Average	0.108	0.265	0.112	
	SD	0.00	0.02	0.004	
	%CV	0.17	1.53	3.79	

Vitamin B12 microbiological assay inter-assay CV for the concentration is 2.3% and for the absorbance is 6.9%, which are both acceptable (less than 15%). Intra-assay CV for the control and standard 1 are 4.1% and 1.8%, respectively. Both values are less than 10% of variation.

3.7.3 Real-time PCR Intra- and Inter-assay Variability

For real-time PCR experiments, the SD between Ct of duplicates was ≤ 0.200; values over this cutoff were rejected and samples were repeated. Inter-assay CV between plates was calculated based on the relative quantification (RQ) values as suggested by Livak et al. [196]. The RQ results presented in the following tables are from samples of offspring fed the control diet during prenatal development and post weaning.

Table 3.6: Real-time PCR Inter-assay CV for Male Offspring

Mtr	**RQ1**	**RQ2**	**SD**	**Average RQ**	**CV (%)**	**Average CV (%)**
1	1.053	0.993	0.04	1.023	4.15	5.9
2	0.835	0.776	0.04	0.806	5.18	
3	0.824	0.966	0.10	0.895	11.22	
4	0.946	0.966	0.01	0.956	1.48	
5	0.771	0.826	0.04	0.799	4.87	
6	0.629	0.557	0.05	0.593	8.59	
Cbs	RQ1	RQ2	SD	Average RQ	CV (%)	Average CV (%)
1	0.880	1.007	0.09	0.944	9.52	6.6
2	0.886	0.914	0.02	0.900	2.21	
3	0.618	0.595	0.02	0.606	2.70	
4	1.079	0.943	0.10	1.011	9.54	
5	1.129	0.997	0.09	1.063	8.81	
Mthfr	**RQ1**	**RQ2**	**SD**	**Average RQ**	**CV (%)**	**Average CV (%)**
1	0.475	0.530	0.04	0.530	7.32	6.7
2	2.295	2.052	0.17	2.174	7.91	
3	0.587	0.694	0.08	0.641	11.81	
4	0.841	0.749	0.07	0.795	8.18	
5	0.912	0.906	0.00	0.909	0.47	
6	0.401	0.429	0.02	0.415	4.77	

Table 3.7: Real-time PCR Inter-assay CV for Female Offspring

Cbs	RQ1	RQ2	SD	Average RQ	CV (%)	Average CV (%)
1	1.454	1.609	0.11	1.531	7.15	6.0
2	1.296	1.229	0.05	1.262	3.72	
3	0.630	0.696	0.05	0.663	7.03	
4	0.509	0.554	0.03	0.532	6.03	
Mthfr	**RQ1**	**RQ2**	**SD**	**Average RQ**	**CV (%)**	**Average CV (%)**
1	1.543	1.427	0.08	1.485	5.50	4.1
2	1.714	1.636	0.05	1.675	3.28	
3	0.795	0.767	0.02	0.781	2.51	
4	0.766	0.782	0.01	0.774	1.49	
5	1.090	0.976	0.08	1.033	7.81	
Mat1a	**RQ1**	**RQ2**	**SD**	**Average RQ**	**CV (%)**	**Average CV (%)**
1	1.126	0.999	0.09	1.063	8.50	9.0
2	0.975	0.763	0.15	0.869	17.20 *	
3	0.864	0.786	0.05	0.825	6.66	
4	1.129	1.070	0.04	1.099	3.81	

The inter-assay CV for real-time PCR experiments ranged from 4.1 to 9.0%. * this sample showed high CV between plates although the SD was less than 0.2.

Chapter 4: Results

4.1 Offspring Body and Liver Weights

Offspring weights were measured at weaning and then on a weekly basis. Liver weights were recorded immediately during tissue collection. Liver weight is expressed in Figure 4.1C and 4.2C as a percentage of body weight, calculated as follows: liver weight (g)/body weight (g) x100. As shown in Figure 4.1A, there was no effect of maternal diet on male offspring body weight at weaning. As predicted, after 20 weeks on diet (Figure 4.1B), male offspring who were weaned onto the Western diet weighed more (48.45 ± 1.06 vs 38.29 ± 1.32 g; $p < 0.001$) than those weaned onto the control diet. Although there were no statistically significant effects of the maternal diets, male mice from dams fed the HFA-B12 diet were smaller at 20 weeks post weaning than those from control-fed dams (41.22 ± 2.58 vs 45.29 ± 1.77 g; $p = 0.063$). Moreover, liver mass was greater ($p < 0.001$) in male offspring fed the Western diet post weaning (5.23 ± 0.16) compared to those fed the control diet (4.22 ± 0.16) (Figure 4.1C).

There was no effect of maternal diet on female offspring body weight at weaning (Figure 4.2A). After 40 weeks on diet (Figure 4.2B), there was a significant interaction ($p = 0.049$) between maternal diet and post weaning diet on body weight. In female offspring mice fed the post weaning Western diets, those from dams fed the HFA-B12 weighed less compared to female offspring from dams fed the control diet (50.36 ± 3.33 vs 58.29 ± 1.60; $p < 0.05$). No effect of maternal diet was observed in female offspring fed the post weaning control diet. Liver weight was greater ($p < 0.01$) in female offspring mice fed the post weaning Western diet (4.36 ± 0.23) compared to control-fed female offspring (3.66 ± 0.09). This effect was independent of maternal diet.

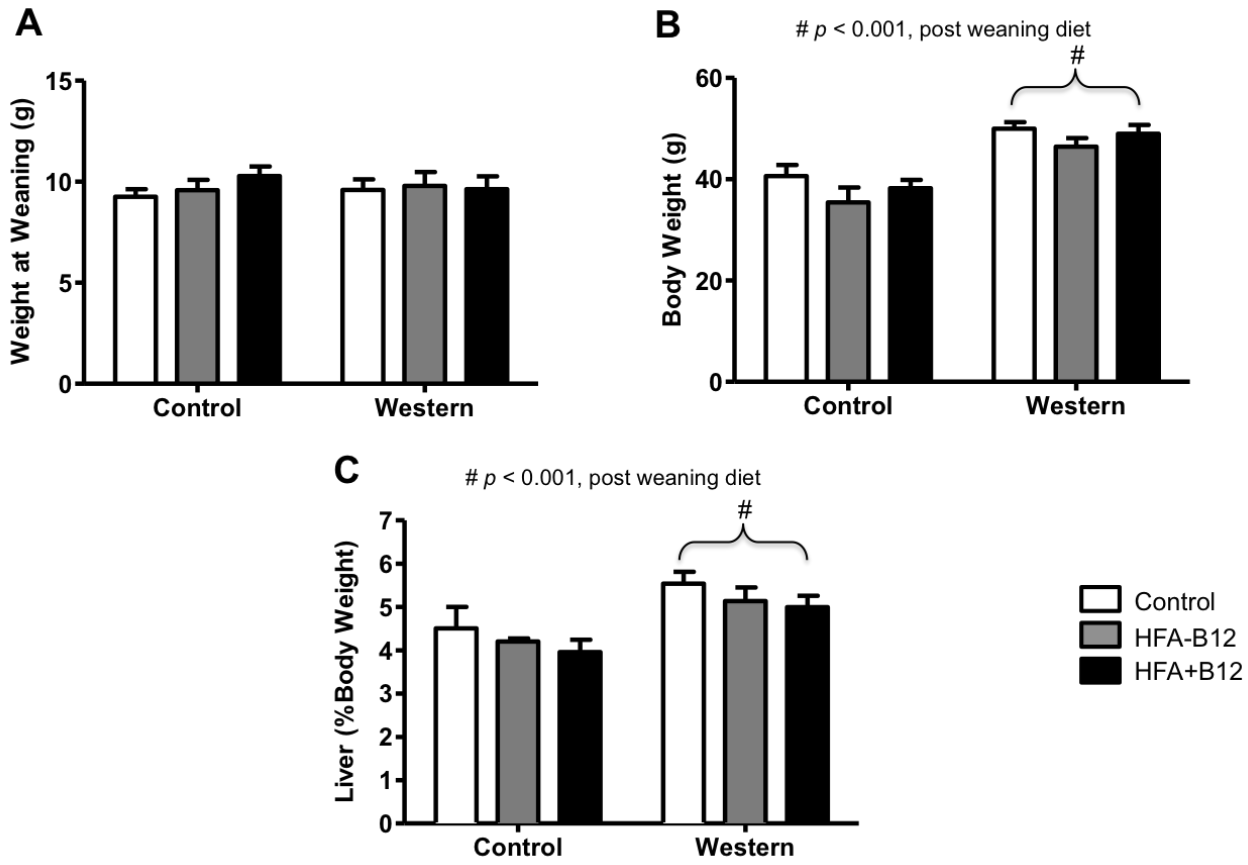

Figure 4.1: Body Weight and Liver weight of Male Offspring
A. Body weight at weaning (3 weeks old). **B.** Body weight at 20 weeks post weaning. **C.** Liver Weight at 20 weeks post weaning.
Data are presented as mean ± SEM (n=6-7/group). ANOVA was conducted to analyze differences between groups.

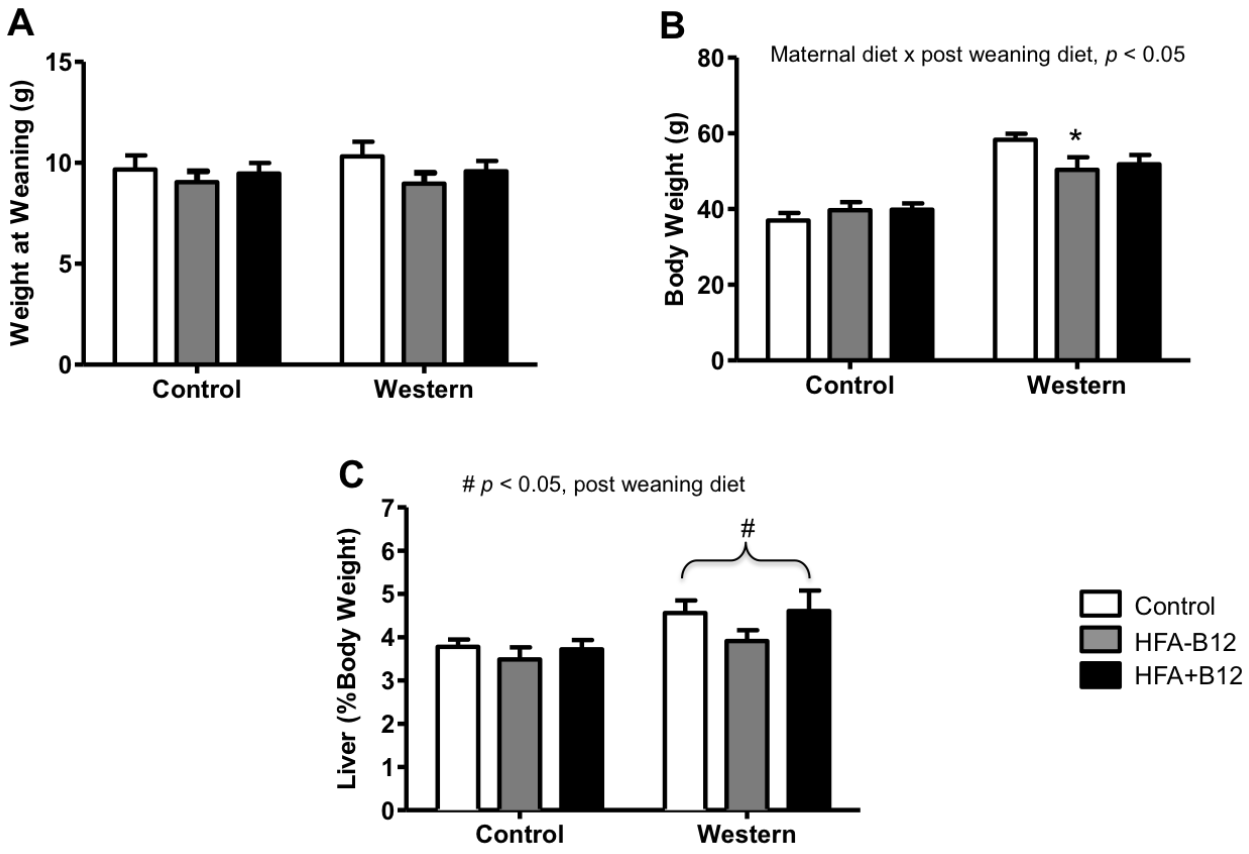

Figure 4.2: Body Weight and Liver Weight of Female Offspring
A. Body weight at weaning (3 weeks old). **B.** Body weight at 40 weeks post weaning. **C.** Liver Weight at 40 weeks post weaning.
Data are presented as mean ± SEM (n=6-7/group). ANOVA was conducted to analyze differences between groups. *$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

4.2 Serum Folate Concentrations

I quantified serum folate concentrations in male and female offspring to determine the effect of maternal dietary folate intakes during pregnancy and lactation on folate status in the offspring. I found no effect of maternal diet or post weaning offspring diet on serum folate concentrations in male and female offspring (Figure 4.3).

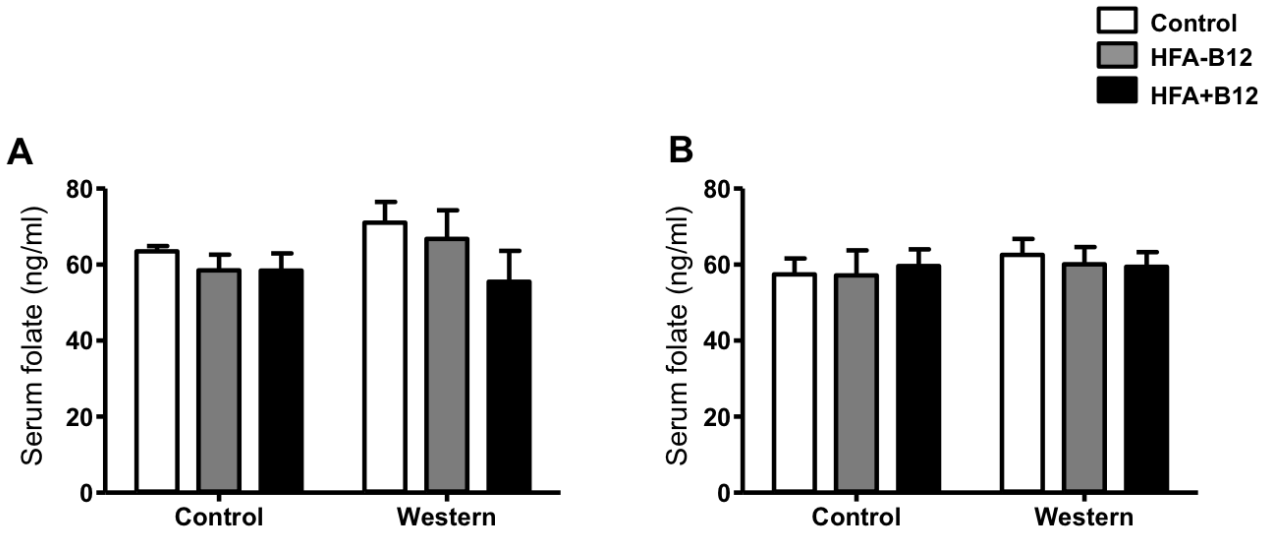

Figure 4.3: Offspring Serum Folate Concentrations
A. Male offspring. **B.** Female Offspring.
Data are presented as mean ± SEM (n=4-7/group). ANOVA was conducted to analyze differences between groups.

4.3 Serum Vitamin B12 Concentrations

I quantified serum vitamin B12 concentrations in dams to confirm vitamin B12 depletion and in offspring to determine the effect of maternal dietary vitamin B12 intake during pregnancy and lactation on offspring vitamin B12 status. Although there are several direct and functional biomarkers (e.g. holo-transcobalamin and methylmalonic acid) to determine vitamin B12 status in humans, serum vitamin B12 is commonly used in animal studies[206,207]. Dams were fed the different

diets for 13 weeks and serum samples were collected at end-point (1 week after offspring's weaning). As shown in Figure 4.4, dams fed the HFA-B12 diet have significantly lower ($p < 0.01$) serum vitamin B12 concentrations (563.79 ± 31.53 pmol/L) compared to dams fed the control and HFA+B12 diets (12,344 ± 3,866.44 and 19,050.46 ± 1942.98 pmol/L, respectively). Control-fed dams had relatively lower serum vitamin B12 compared to dams fed HFA+B12, however this was not significantly different (12,344 ± 3,866.44 vs 19,050.46 ± 1942.98 pmol/L; $p = 0.06$).

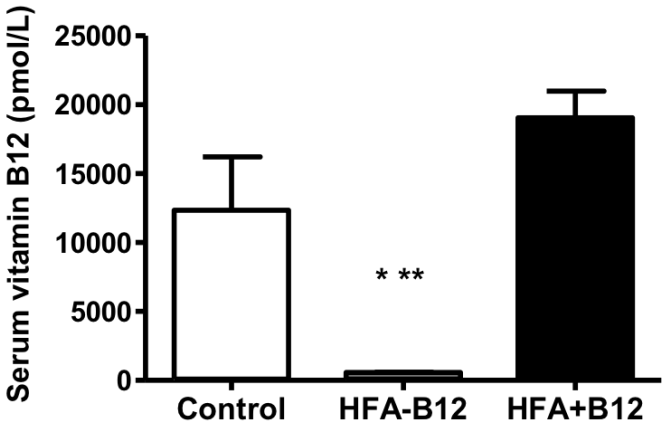

Figure 4.4: Maternal Serum Vitamin B12 Concentrations
Data are presented as mean ± SEM (n=4-6/group).
ANOVA was conducted to analyze differences between groups.
*$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

4.3.1 Male Offspring Serum Vitamin B12 Concentrations

Despite differences in maternal vitamin B12 concentrations, there was no significant effect of maternal diet on male offspring serum vitamin B12 concentrations (Figure 4.5A). Control-fed offspring have lower serum vitamin B12 concentrations compared to offspring fed the post weaning Western diet, however this was not statistically significant (14,101.10 ± 2,382.24 vs 18,199.46 ± 695.91 pmol/L; $p = 0.06$).

4.3.2 Female Offspring Vitamin B12 Concentrations

There were no effects of maternal diet or post weaning diet on serum vitamin B12 concentrations in female offspring (Figure 4.5B). However, female offspring fed the post weaning control diet tended to have lower vitamin B12 concentrations compared to offspring fed the post weaning Western diet (17,974.06 ± 439.57 vs 19,889.72 ± 1,099.20 pmol/L; $p = 0.12$).

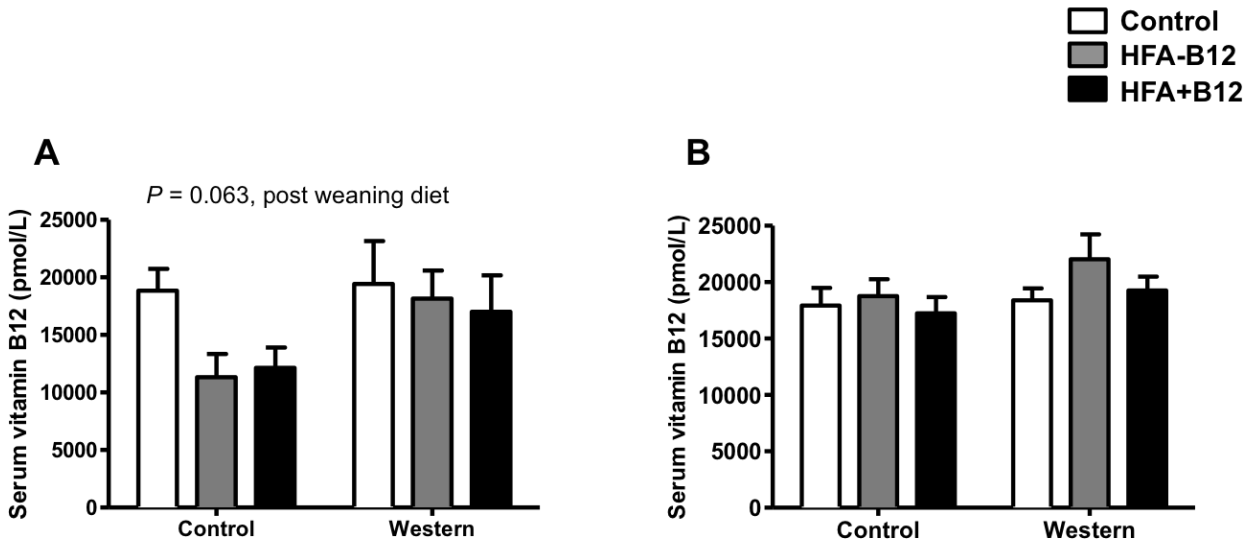

Figure 4.5: Offspring Serum Vitamin B12 Concentrations
A. Male offspring. **B.** Female offspring.
Data are presented as mean ± SEM (n=4-6/group). ANOVA was conducted to analyze differences between groups.

4.4 Hepatic AdoMet and AdoHcy

Alterations in hepatic AdoMet and AdoHcy concentrations have been associated with changes in activity and expression of methyl metabolism enzymes[104,208,209]. AdoMet is the principal methyl donor in mammalian cells. AdoHcy is considered an inhibitor of several methyltransferases[210,211].

Figure 4.6 illustrates the hepatic concentrations of AdoMet and AdoHcy in male offspring. No significant effect of maternal diet or post weaning diet was found for hepatic AdoMet concentrations. However, AdoHcy was significantly lower in male offspring fed the post weaning Western diet (13.81 ± 1.66 vs 20.48 ± 1.793 nmol/g; $p < 0.05$). There was a significant effect of maternal diet and post weaning diet on AdoMet/AdoHcy ratio. Offspring from dams fed the HFA-B12 diet had a lower AdoMet/AdoHcy ratio (2.24 ± 0.23) than offspring from dams fed the control (3.02 ± 0.23) or HFA+B12 (2.96 ± 0.22) diets. AdoMet/AdoHcy ratio was higher in offspring fed the Western diet compared to those fed the control diet (3.08 ± 0.40 vs 2.41± 0.16; $p < 0.05$).

Hepatic AdoMet and AdoHcy concentrations in female offspring are shown in Figure 4.7. There was a trend toward an effect of maternal diet on AdoMet concentrations ($p = 0.068$, n.s.). Offspring from dams fed the HFA-B12 diet had lower hepatic AdoMet concentrations compared to offspring from control-fed dams (33.37 ± 2.24 vs 40.78 ± 2.35 nmol/g; $p <0.05$) and from dams fed HFA+B12 (33.37 ± 2.24 vs 39.18 ± 2.24 nmol/g; $p = 0.08$). There was no significant effect of maternal diet on hepatic AdoHcy concentrations or AdoMet/AdoHcy ratio and no significant effect of post weaning diet on offspring hepatic AdoMet and AdoHcy concentrations or AdoMet/AdoHcy ratio.

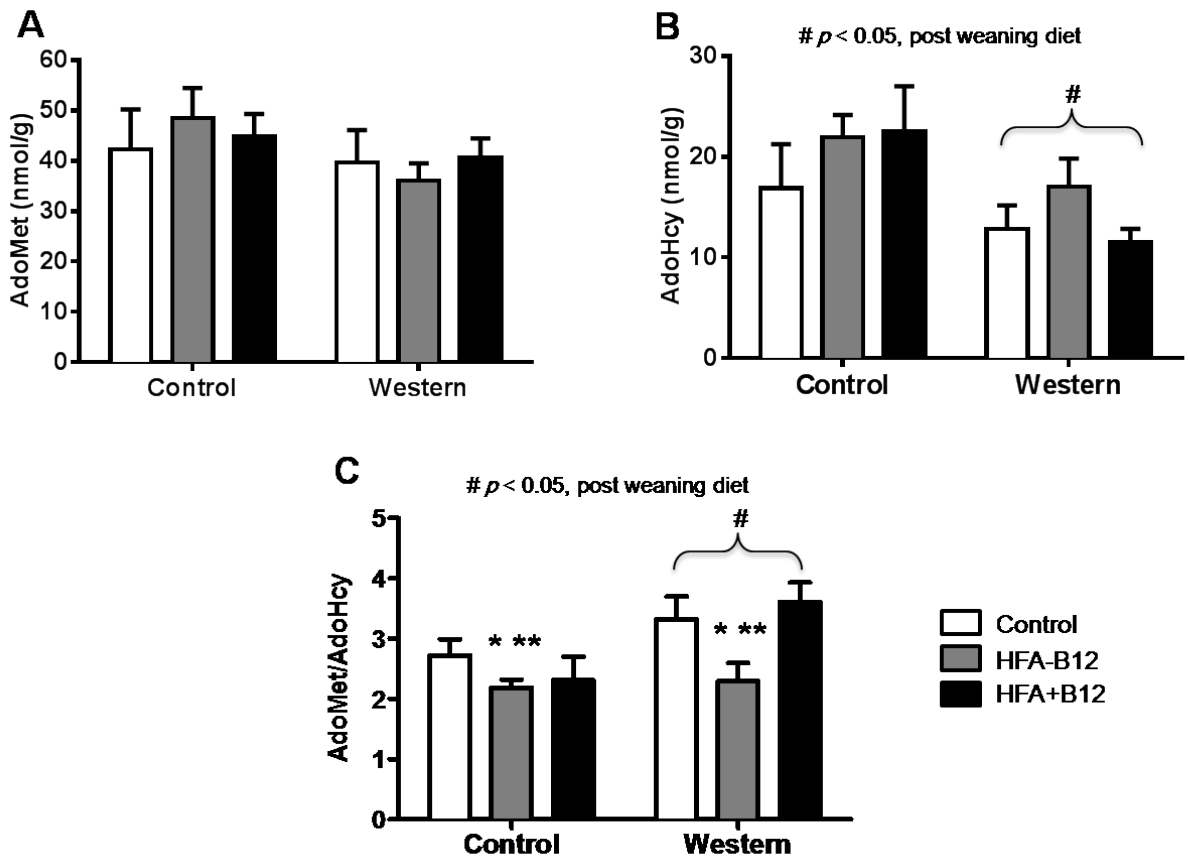

Figure 4.6: Hepatic AdoMet and AdoHcy Concentrations in Male Offspring
A. AdoMet Concentrations. **B.** AdoHcy Concentrations. **C.** AdoMet:AdoHcy Ratio.
Data are presented as mean ± SEM (n=5-6/group). ANOVA was conducted to analyze differences between groups. *$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

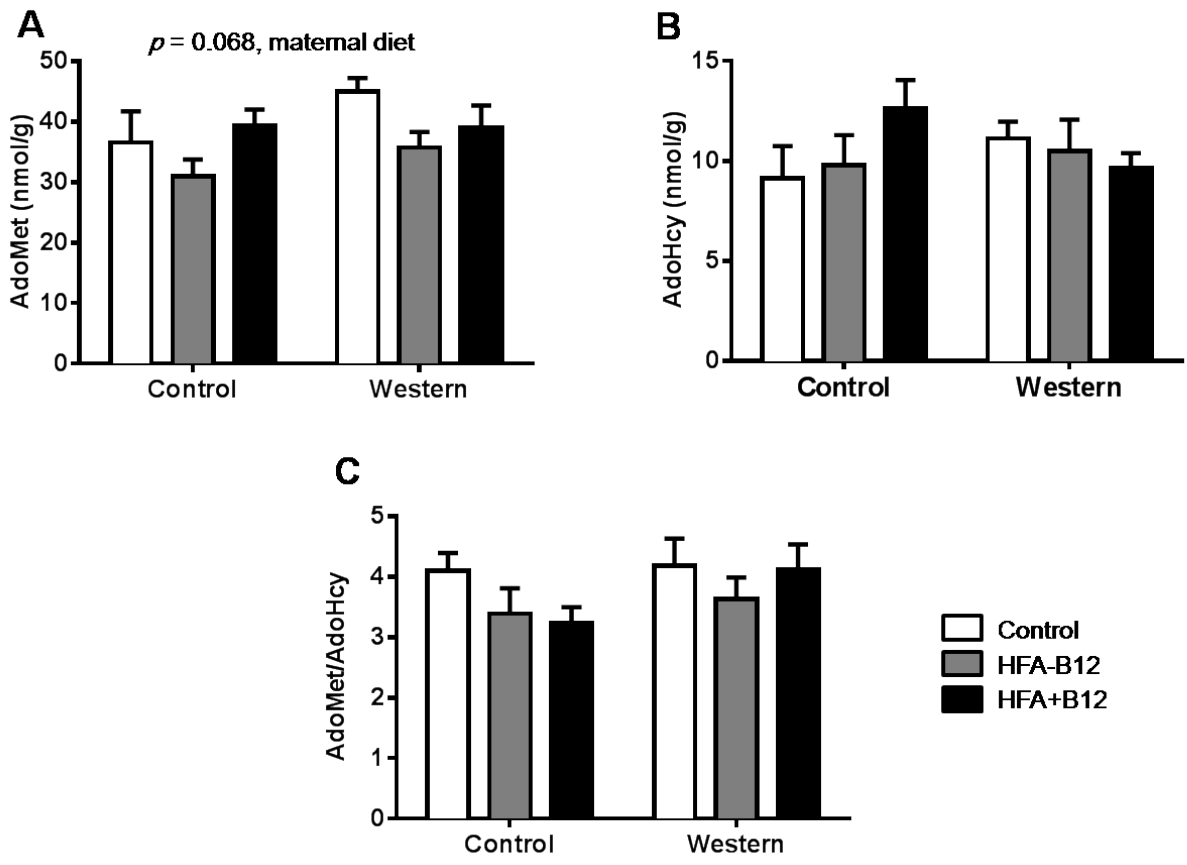

Figure 4.7: Hepatic AdoMet and AdoHcy Concentrations in Female Offspring
A. AdoMet Concentrations. **B.** AdoHcy Concentrations. **C.** AdoMet:AdoHcy Ratio.
Data are presented as mean ± SEM (n=5-6/group). ANOVA was conducted to analyze differences between groups.

4.5 Hepatic mRNA Expression of Methyl Metabolism Enzymes

I quantified mRNA expression of genes encoding key enzymes in methyl metabolism in the liver to explore whether maternal folate and vitamin B12 imbalance programs gene expression. Disturbances in one carbon metabolism have been implicated in CVD, oxidative stress, cancer, and liver disease[94,104,212,213]. *Mthfr* encodes MTHFR, which catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF. *Mtr* encodes MTR, which is one of only two vitamin B12-dependant enzymes, and it metabolically links the folate cycle with the methionine cycle by catalyzing the remethylation of homocysteine to methionine. *Mat1a* is expressed in adult liver and encodes MAT. *Cbs* encodes CBS, which catalyzes the first step in the transsulfuration pathway that irreversibly degrades homocysteine to cysteine.

4.5.1 Male Offspring

Male offspring from dams fed the HFA-B12 diet had lower ($p < 0.05$) *Cbs* mRNA expression compared to offspring from dams fed the control diet. Offspring from dams fed the HFA-B12 diet also had lower *Mthfr* mRNA expression compared to those from dams fed the HFA+B12 (Figure 4.8A). Offspring fed the post weaning Western diet had higher ($p < 0.05$) *Mtr* mRNA expression than offspring fed the post weaning control diet (Figure 4.8C). No effect of maternal and post weaning diets was observed for *Mat1a* mRNA expression (Figure 4.8D).

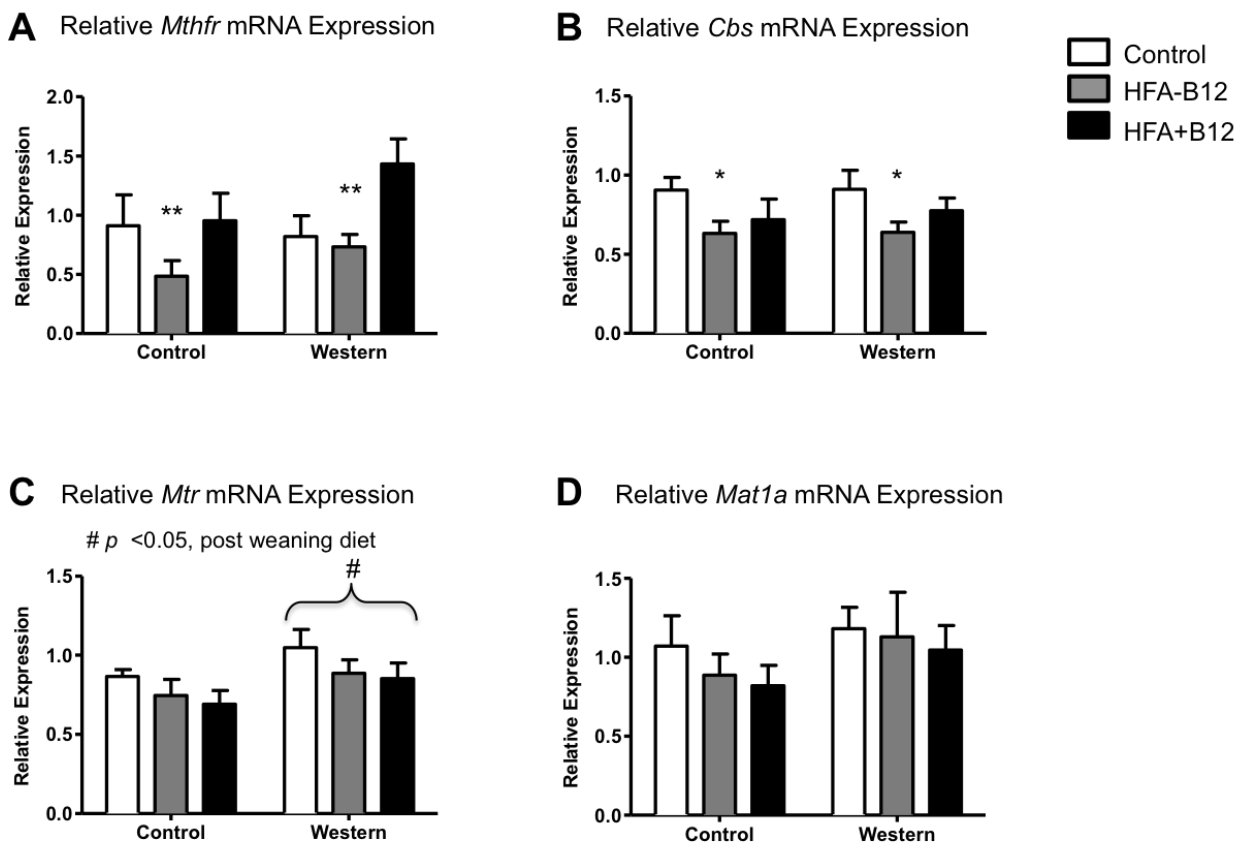

Figure 4.8: Hepatic Gene Expression of Methyl Metabolism Enzymes in Male Offspring
A. Relative *Mthfr* mRNA expression. **B.** Relative *Cbs* mRNA expression. **C.** Relative *Mtr* mRNA expression. **D.** Relative *Mat1a* mRNA expression.
Data are presented as mean ± SEM (n=5-6/group). ANOVA was conducted to analyze differences between groups. *$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

4.5.2 Female Offspring

There was an interaction ($p < 0.05$) between maternal diet and post weaning offspring diet for *Mthfr* mRNA expression. In female offspring fed the post weaning Western diet, those from dams fed the HFA-B12 diet had higher ($p < 0.05$) *Mthfr* mRNA expression compared to those from dams fed the HFA+B12 diet and from dams fed the control diet (Figure 4.10A). In contrast, female offspring fed the post weaning control diet responded differently; offspring from HFA+B12-fed

dams have higher *Mthfr* mRNA expression compared to those from control-fed and HFA-B12-fed dams ($p = 0.052$ and $p = 0.055$, respectively).

Moreover, *Mtr* mRNA expression was affected by maternal diet with female offspring from dams fed the HFA-B12 diet had lower *Mtr* mRNA expression compared to those from control-fed dams; no effect of post weaning diet on *Mtr* mRNA expression was observed. Lastly, there was no statistically significant effect of maternal diet or offspring post weaning diet on *Cbs* and *Mat1a* mRNA expressions in female offspring.

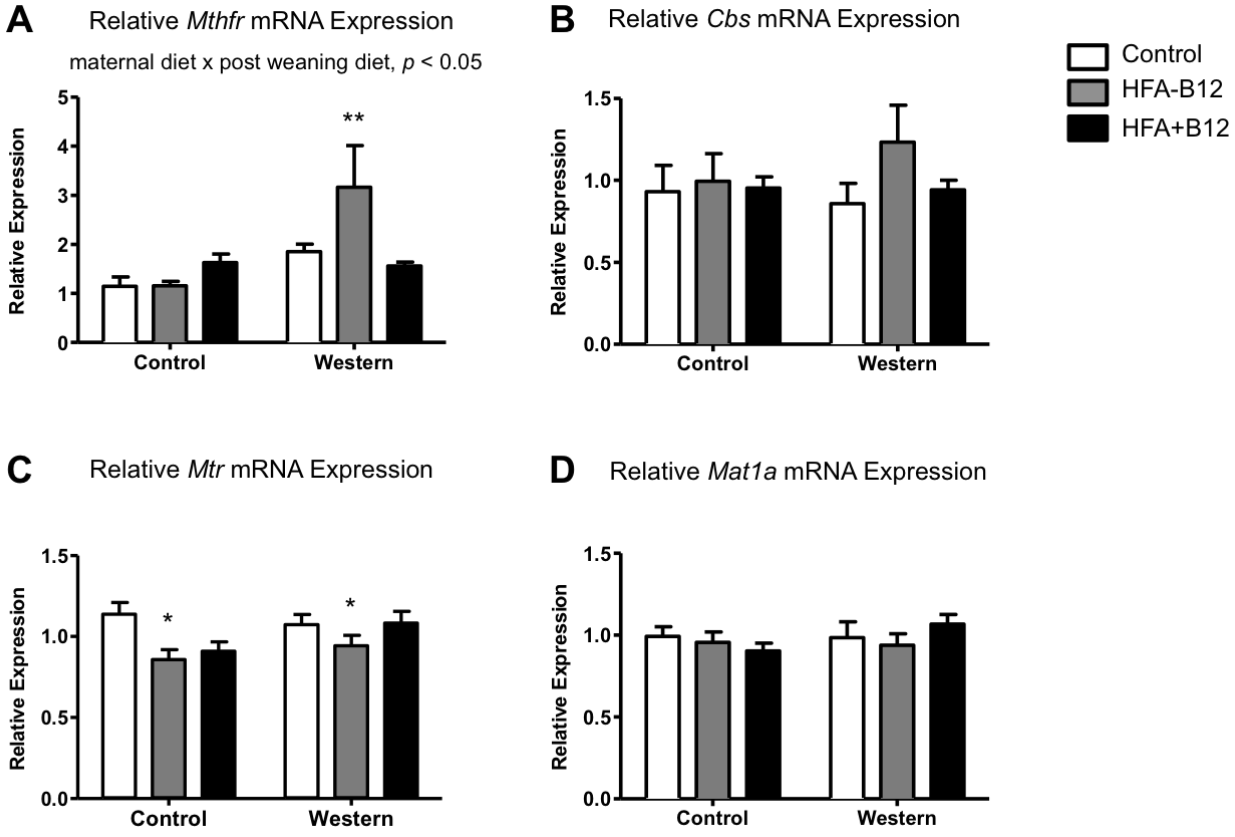

Figure 4.9: Hepatic Gene Expression of Methyl Metabolism Enzymes in Female Offspring
A. Relative *Mthfr* mRNA expression. **B.** Relative *Cbs* mRNA expression. **C.** Relative *Mtr* mRNA expression. **D.** Relative *Mat1a* mRNA expression.
Data are presented as mean ± SEM (n=5-6/group). ANOVA was conducted to analyze differences between groups. *$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

I further investigated the relationship between the mRNA expression of methyl metabolism genes and AdoMet and AdoHcy concentrations by linear regression. *Mtr* mRNA expression was negatively associated (r = 0.45, $p = 0.011$) with AdoMet concentrations in male offspring (Figure 4.10). Expression levels of *Mthfr*, *Cbs*, and *Mat1a* were not significantly associated with AdoMet and AdoHcy concentrations in male offspring or female offspring.

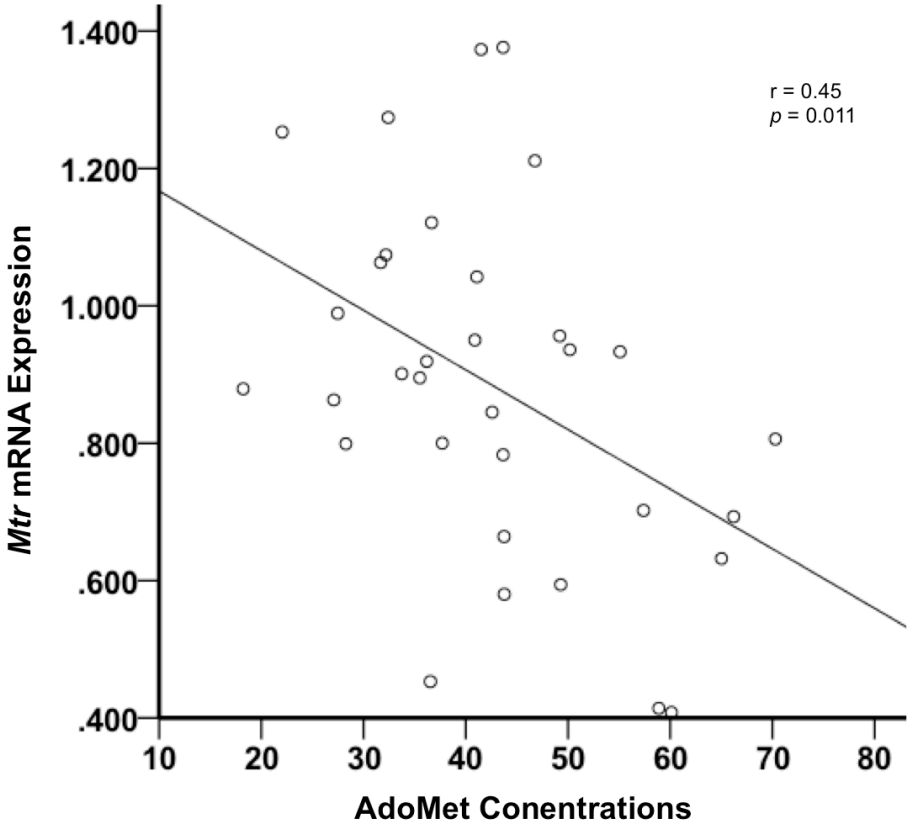

Figure 4.10: Relationship between *Mtr* gene expression and AdoMet Concentrations in Liver from Male Offspring
Simple linear regression was used (n = 33).

4.6 Hepatic Gene Expression of *Nr3c1* and *Ppara*

I further quantified *Nr3c1* and *Ppara* mRNA expression in the liver, given these genes have been shown to be developmentally programmed[19,214-216] and their expression is influenced by DNA methylation[119,217]. *Nr3c1* encodes the glucocorticoid receptor (GR). Glucocorticoids are a class of steroid hormones that bind GR to activate several immunological and metabolic responses, such as the regulation of carbohydrate, protein and fat metabolism[214]. *Ppara* encodes PPARα, a nuclear receptor that plays a role in regulating hepatic expression of genes required for fatty acid synthesis and oxidation, and glucose homeostasis[118,218,219]. In male offspring, *Nr3c1* and *Ppara* mRNA expression levels were not affected by maternal diet or offspring post weaning diet (Figure 4.11).

In female offspring, *Nr3c1* mRNA expression was not affected by maternal diet or post weaning diet (Figure 4.12A). There was a trend towards an interaction ($p = 0.075$) between maternal diet and post weaning diet on hepatic *Ppara* mRNA (Figure 4.12B). When I analyzed the data separately for post weaning control-fed and Western-fed female offspring by one-way ANOVA, Western-fed female offspring mice from dams fed the HFA-B12 diet had lower ($p < 0.05$) *Ppara* mRNA expression compared to offspring from dams fed the HFA+B12 diet. This effect was not observed in offspring fed the post weaning control diet.

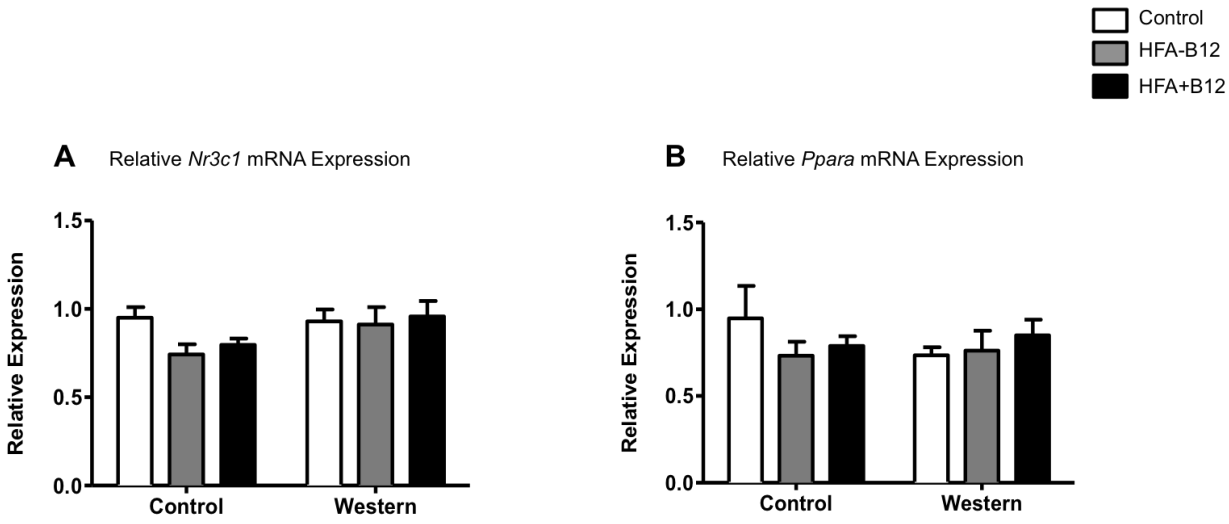

Figure 4.11: Hepatic *Nr3c1* (A) and *Ppara* (B) Expressions in Male Offspring
Data are presented as mean ± SEM (n=5-6/group).
ANOVA was conducted to analyze differences between groups.

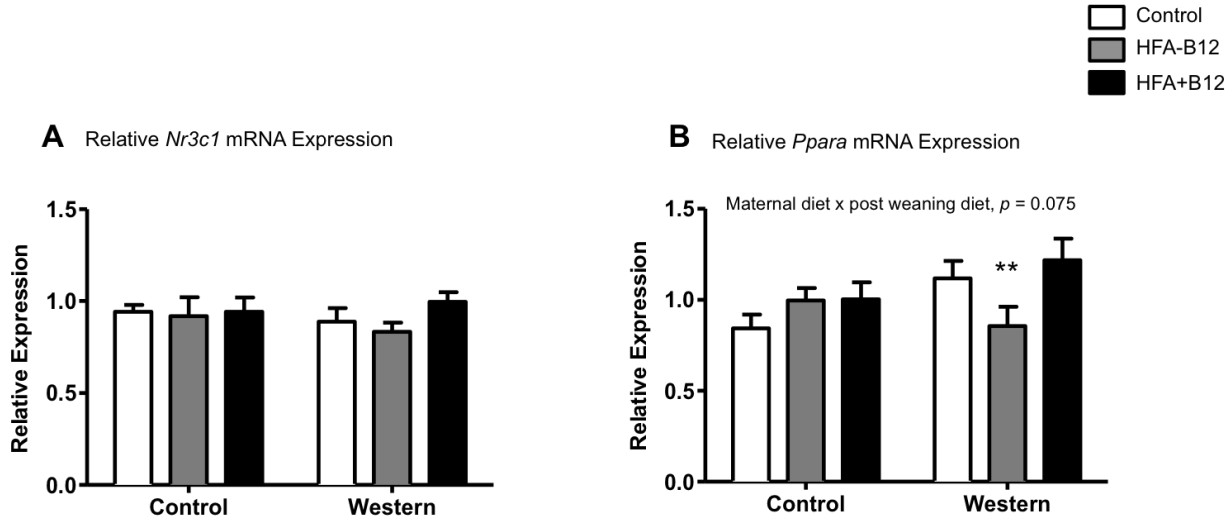

Figure 4.12: Hepatic *Nr3c1* (A) and *Ppara* (B) Expressions in Female Offspring
Data are presented as mean ± SEM (n=5-6/group). ANOVA was conducted to analyze differences between groups. ANOVA was conducted to analyze differences between groups. *$p < 0.05$, HFA-B12 vs. Control; **$p < 0.05$, HFA-B12 vs. HFA+B12.

4.7 Hepatic CBS Protein Expression in Male Offspring

Given the effect of maternal diet on hepatic *Cbs* mRNA expression in male offspring mice, I further explored whether similar effects of maternal diet are observed for Cbs protein expression. In contrast to what I found for hepatic *Cbs* mRNA expression, I found lower hepatic Cbs protein in male offspring fed the post weaning Western diet compared to male offspring fed the post weaning control diet (Figure 4.13). No effect of maternal diet was observed.

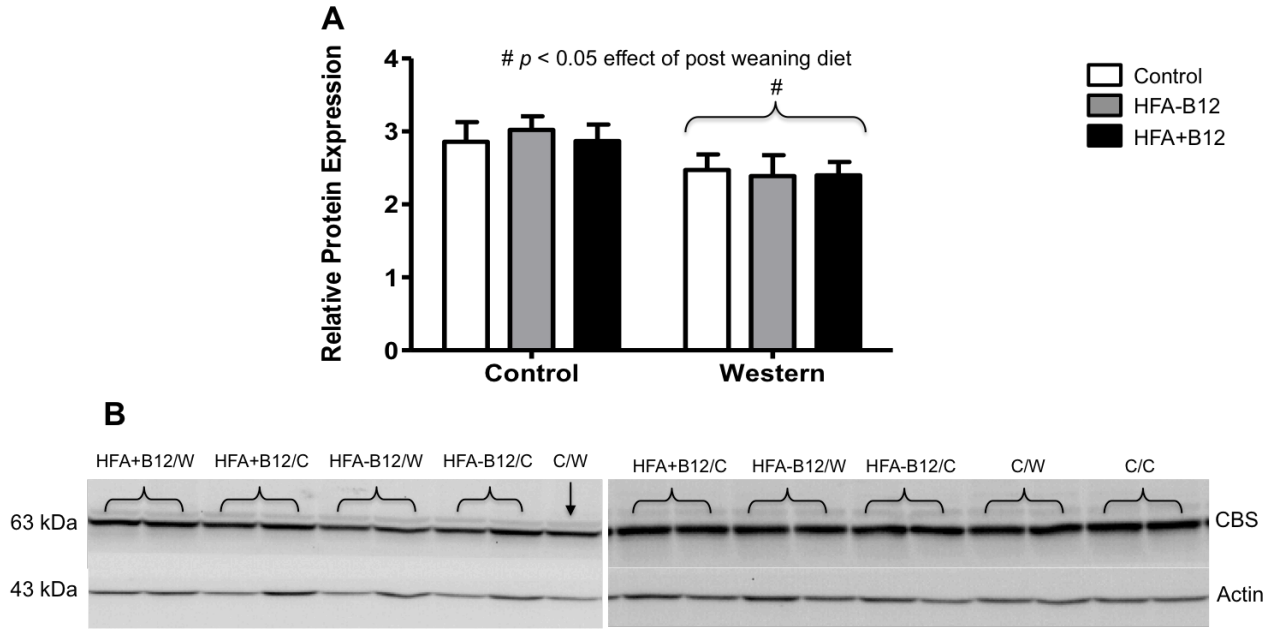

Figure 4.13: Hepatic CBS Protein Expression in Male Offspring
A. CBS protein expression as a ratio to beta actin levels. **B.** Representative blots illustrating protein bands with actin as a loading control; Offspring groups are presented as maternal diet/post weaning diet (C= Control diet, W= Western diet).
Data are presented as mean ± SEM (n=6/group). ANOVA was conducted to analyze differences between groups.

4.8 *Mthfr* DNA Methylation

Given that I observed a significant effect of maternal diet on *Mthfr* mRNA expression in both male and female offspring mice, the methylation status of this gene was quantified by bisulfite pyrosequencing[220] as described in Chapter 4. I assessed the methylation status of 9 CpGs sites in the *Mthfr* downstream promoter (Figure 4.2).

4.8.1 Male Offspring

Methylation at each of the 9 CpG sites were strongly correlated with each other, except for the methylation of CpG site 4 with site 7. I used results from all the 9 CpG sites to calculate the mean methylation status and found no effect of maternal diet or post weaning diet in male offspring (Figure 4.14). Lower methylation ($p = 0.015$) was observed at CpG site 4 in male offspring fed the post weaning Western diet compared to offspring fed the post weaning control diet regardless of maternal diet (Table 4.1). Male offspring from dams fed the control diet had lower methylation at CpG site 2 compared to those from dams fed the HFA+B12 diet ($p = 0.03$) or HFA-B12 diet ($p = 0.04$). No relationship between *Mthfr* mRNA expression and mean *Mthfr* downstream promoter methylation was observed (Figure 4.16A).

4.8.2 Female Offspring

In female offspring, methylation patterns at all 9 CpG sites were strongly correlated with each other. The mean methylation status of *Mthfr* downstream promoter was lower in offspring fed the Western diet compared to offspring fed the control diet (56.23 ± 0.78 vs 60.86 ± 0.80 %; $p < 0.001$) and was not affected by maternal diet (Figure 4.15). However, the methylation difference was less than 5%. When each CpG site was analyzed separately, all had lower methylation ($p < 0.01$) in female mice fed the post weaning Western diet compared to offspring fed the post weaning

control diet (Table 4.2). No relationship between hepatic *Mthfr* mRNA expression and *Mthfr* downstream promoter mean methylation was observed (Figure 4.16B).

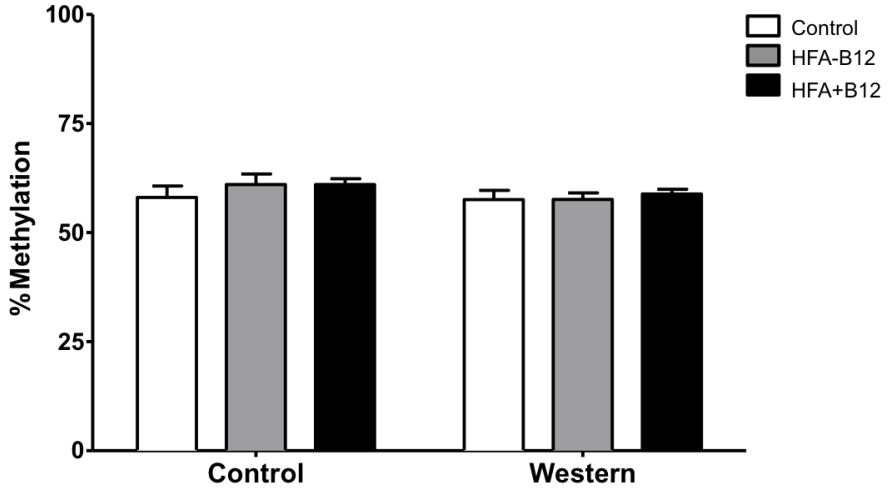

Figure 4.14: *Mthfr* DNA Methyaltion Status in Liver from Male Offspring
Data are presented as mean ± SEM (n = 6/group). ANOVA was conducted to analyze differences between groups.

Table 4.1: *Mthfr* DNA Methylation Status in Liver from Male Offspring

Maternal Diet	Post Weaning Diet	*Mthfr* Methylation Status (%methylation)								
		CpG 1	CpG 2	CpG 3	CpG 4*	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9
Control	Control	62.1± 1.78	46.53± 2.39	57.71± 4.16	72.47± 1.12	66.00± 2.05	49.73± 0.74	50.43± 4.00	49.20± 6.82	68.29± 0.83
HFA-B12		65.31± 2.23	52.90± 1.80	63.30± 2.04	74.60± 2.69	69.69± 2.82	52.10± 2.52	52.55± 3.67	48.16± 1.26	70.61± 2.56
HFA+B12		65.80± 1.59	52.14± 1.39	61.00± 1.51	75.24± 0.96	67.92± 1.23	49.77± 2.18	54.99± 0.86	48.50± 1.26	73.75± 1.12
Control	Western	61.88± 2.11	48.37± 2.69	56.73± 2.65	70.26± 1.19	65.88± 1.55	50.23± 1.72	49.90± 2.76	44.01± 2.17	70.91± 2.21
HFA-B12		61.75± 1.18	50.06± 1.62	57.10± 1.30	70.81± 1.87	65.77± 0.74	50.68± 1.04	50.17± 2.19	44.94± 1.87	67.22± 1.57
HFA+B12		63.64± 1.22	51.43± 0.92	59.45± 0.75	70.99± 1.22	65.43± 1.34	50.96± 0.89	52.57± 0.39	46.00± 0.86	69.33± 2.08

Data are presented as mean ± SEM. ANOVA was conducted to analyze differences between groups. *$p < 0.05$, effect of post weaning diet.

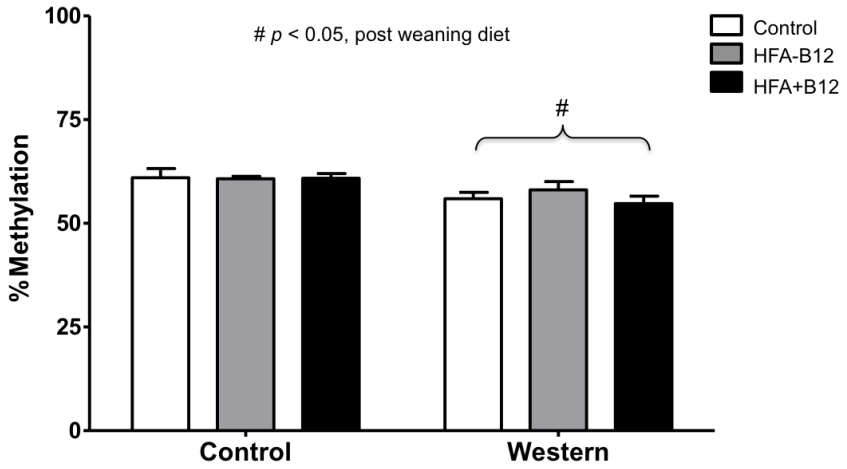

Figure 4.15: *Mthfr* DNA Methyaltion Status in Liver from Female Offspring
Data are presented as mean ± SEM (n = 6/group). ANOVA was conducted to analyze differences between groups.

Table 4.2: *Mthfr* DNA Methylation Status in Liver from Female Offspring

Maternal Diet	Post Weaning Diet	*Mthfr* Methylation Status (%methylation)								
		CpG 1*	CpG 2*	CpG 3*	CpG 4*	CpG 5*	CpG 6*	CpG 7*	CpG 8*	CpG 9*
Control	Control	63.95± 2.13	53.35± 3.06	61.53± 4.35	71.56± 2.91	68.69± 1.04	52.07± 0.76	55.81± 2.83	50.51± 2.23	71.34± 0.64
HFA-B12		64.19± 0.51	52.95± 0.67	61.77± 0.70	71.19± 0.31	68.10± 0.68	50.04± 0.43	56.84± 0.87	50.11± 0.62	71.42± 0.50
HFA+B12		64.69± 0.99	52.51± 1.50	62.77± 1.48	72.42± 1.07	68.79± 0.89	50.08± 1.01	56.22± 1.21	49.88± 0.96	70.32± 0.88
Control	Western	58.39± 1.07	48.93± 0.71	58.13± 1.24	67.18± 2.03	63.48± 1.51	45.89± 1.51	49.25± 3.21	46.06± 1.31	65.85± 1.15
HFA-B12		61.92± 2.28	47.76± 1.80	58.53± 1.51	69.21± 2.74	65.35± 1.52	48.22± 2.53	53.43± 1.44	48.81± 1.11	69.04± 3.04
HFA+B12		57.93± 1.81	47.24± 1.59	55.64± 1.38	65.20± 1.99	61.67± 1.73	45.29± 2.22	50.30± 1.85	46.77± 1.53	62.65± 2.04

Data are presented as mean ± SEM. ANOVA was conducted to analyze differences between groups. *$p < 0.01$, effect of post weaning diet

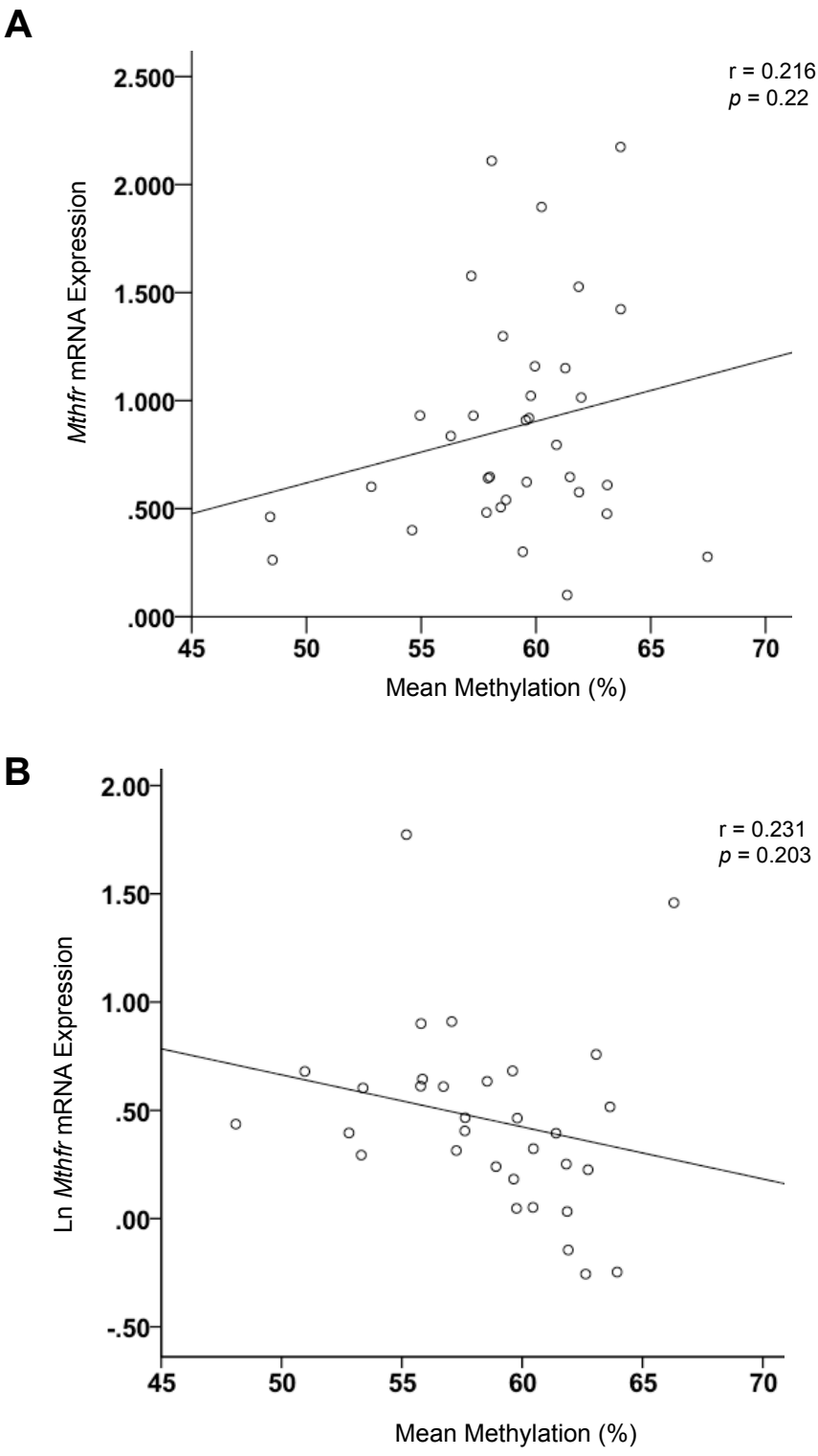

Figure 4.16: Relationship Between *Mthfr* mRNA Expression and Mean DNA Methylation
A. Male Offspring (n = 34). **B.** Female Offspring (n = 32).
Simple linear regression was used. Ln: natural log transformed.

Chapter 5: Discussion

An epidemiological study from South Asia has suggested a role for maternal high folate and low vitamin B12 status during pregnancy in programming of offspring adiposity and insulin resistance[12]. Two additional epidemiological studies in support of these findings have recently been published reporting independent roles for both maternal folate and vitamin B12 status during pregnancy on programming of adiposity and glucose homeostasis in children[13,14]. Given that most Canadian women have adequate to high folate status and that vitamin B12 deficiency is moderately prevalent in women of childbearing age [7,9,10], I sought to explore whether maternal high folic acid and low vitamin B12 intakes would program hepatic gene expression in the liver of adult offspring as a first step towards delineating the mechanisms accounting for the observations in the epidemiological studies[12-14]. I found three main findings in my thesis. First, maternal high folic acid without vitamin B12 altered hepatic gene expressions of enzymes in methyl metabolism in both male and female offspring. Second, the post weaning Western diet decreased hepatic AdoHcy concentrations and the developmental exposure to maternal high folic acid and no vitamin B12 decreased AdoMet /AdoHcy ratio in adult male offspring. Third, maternal folic acid and vitamin B12 imbalance during gestation and lactation did not affect offspring serum folate and vitamin B12 concentrations in adulthood. My findings are novel because they shed light on the interaction between *in utero* and early postnatal environment with post weaning diet as observed in hepatic gene expression and body weight.

Findings from the Dutch Famine Birth Cohort and other subsequent supporting studies demonstrated pivotal evidence for the impact of developmental exposure to environmental influences on future health[27-29,31-34,52-54]. The mechanistic bases of DOHaD are still poorly understood, but epigenetic mechanisms are potential candidates in mediating the effects of early life

cues on adult disease susceptibility. Factors, such as diet, have the ability to modify epigenetic mechanisms and alter gene expression[72,221-223]. Previous reports suggest a central role of the dietary methyl nutrients during pregnancy in the modulation of adult phenotype and epigenetic processes. Supplementation of the diet of female mice with methyl donors (folic acid, vitamin B12, choline, and betaine) prior to mating and throughout pregnancy and lactation altered coat colour in the offspring and *agouti* gene expression via changes in DNA methylation[110]. Sinclair et al. reported that periconceptional dietary restriction of folate, vitamin B12, and methionine in sheep was associated with alterations in plasma glucose and insulin concentrations and fat accumulation (determined by computed tomography), and DNA methylation in male offspring at 22 months of age[224]. Furthermore, the supplementation of diet with folic acid, vitamin B12, methionine, choline, and betaine in female rats during pregnancy and lactation was associated with lower plasma leptin in male and female offspring, and hypermethylation in the leptin gene promoter in male offspring at weaning[178]. Methyl donors are not limited to nutrients mentioned in these studies. Methyl nutrients can be vitamins, such as folate, vitamin B2, vitamin B6, vitamin B12 and choline, and amino acids, such as methionine, glycine, serine, and cysteine[222].

Folate is a key source of methyl groups for the synthesis of AdoMet and its metabolism is linked to vitamin B12. Thus, folate and vitamin B12 can play a role in the modulation of DNA methylation[110,111,178]. The current food fortification policies in North America and other countries mandate the addition of folic acid to grain and cereal products for the prevention of NTD, but there is not mandatory fortification for vitamin B12. Folic acid fortification resulted in high circulating concentrations of folate and unmetabolized folic acid post fortification[7,9], but the consequences are unknown. Although the majority of Canadians have adequate folate status and 40% have high RBC folate concentrations[7], approximately 5% of Canadian adults, including pregnant women, have

vitamin B12 deficiency[9,10]. Human studies have reported associations between folate and vitamin B12 status during pregnancy and children adiposity and insulin resistance[12-14]. Kumar et al. conducted a study on rats and examined the effects of maternal dietary adequate folate and restricted vitamin B12 on offspring adiposity and lipid metabolism, but offspring were weaned onto the same diet that they were exposed to *in utero* and lactation, which made it hard to rule out the effect of developmental diet from the weaning diet[225]. As such, I aimed to address this limitation in my thesis by investigating the effects of developmental exposure to folate and vitamin B12 imbalance using an animal model.

5.1 Offspring Body and Liver Weights

At weaning, body weight did not differ between groups in both male and female offspring. This is in contrast to two previous studies that reported that vitamin B12 deficiency during pregnancy and lactation to be associated with less body weight in the offspring at weaning[113,123]. Huang et al. did not find an effect of maternal folic acid intakes (2 mg, 5 mg, and 40 mg/ kg of diet) on offspring body weight at weaning[124].

There is an association between birth weight and rapid weight gain early in life and metabolic and cardiovascular risks[226,227]. Excess weight gain early in life (6-9 months) as a result of nutrient-dense formula feeding was reported to be associated with developing rapid weight gain and further adiposity and obesity risk at 6-8 years of age[228]. Leunissen et al. reported that rapid weight gain during the first 3 months of life was positively associated with central adiposity in early adulthood (18-24 years) [226]. Previous work in our lab showed similar growth trajectory in male offspring until 20 weeks on the post weaning diet when mice from dams fed the developmental HFA-B12 and HFA+B12 diets weighed less than mice from control-fed dams [229]. This was not

observed after 30 weeks on the post weaning diets[229]. I found a similar observation in my work, but this was only significant in female offspring who were fed the Western diet for 40 weeks post weaning. The finding that offspring weaned onto the Western diet gained more weight and had more liver weight than those weaned onto the control diet is expected and well established in C57BL/6J mice[179].

Although food intake and energy expenditure are important determinants of energy balance, I could not obtain these measurements because my research was part of a larger project that was not equipped to measure food intake or energy expenditure. Cannon et al. found that maternal high fat intake during pregnancy and lactation increased energy expenditure post weaning in male offspring [135]. Early postnatal nutrition may also have a role in determining physical activity and energy expenditure in adult female mice[230]. Dietary components in experimental diet can affect food palatability that can modify dietary intake. Mice on high fat diet (60% of energy) have been reported to have lower food intake compared to those fed the control diet[231]. Accordingly, the determination of caloric food intake and energy expenditure is informative in diet-related studies to ensure drawing a clearer picture in regard to differences between diet groups.

5.2 Offspring Serum Folate and Vitamin B12

I quantified serum folate and vitamin B12 concentrations in the offspring to observe the long-term effect of developmental exposure to different intakes of folic acid and vitamin B12. Offspring serum folate concentrations were not affected by the exposure to different maternal folic acid and vitamin B12 intakes nor by the post weaning diet. Offspring received the same amount of folic acid and vitamin B12 in their diets post weaning until adulthood, so this could have compensated for any differences in folate and vitamin B12 concentrations at birth or at weaning

due to exposure to maternal high folic acid and low vitamin B12 intake. Female mice fed a diet deficient in vitamin B12 from mid gestation and during lactation had offspring with lower serum vitamin B12 concentration at weaning compared to offspring from control-fed dams (219 vs 508 pmol/L)[123]. This suggests that maternal vitamin B12 intake during pregnancy and lactation can influence offspring serum vitamin B12 at weaning. Serum folate concentrations in this study are in accordance with previously published work, which indicates elevated folate concentrations in rodent sera could be 10 fold higher than levels found in human sera[232-234].

Further, serum vitamin B12 concentrations in male and female offspring were not affected by maternal or post weaning diet. However, I observed a trend toward higher serum vitamin B12 concentrations in Western-fed offspring, particularly in male offspring in which the effect of post weaning diet approached statistical significance ($p = 0.063$). A repeat of the study using a larger sample size will increase the statistical power, which may then reveal important effects of post weaning diet on serum vitamin B12 concentrations. To my knowledge this is the first report of an effect of a Western diet on serum vitamin B12 concentrations. How a diet high in fat and sugar may affect circulating vitamin B12 concentrations remains to be determined.

A possible explanation for the slight increase in serum vitamin B12 concentrations in offspring mice fed the Western diet is the bioavailability of vitamin B12 in a high fat diet. Given that vitamin B12 is mainly obtained from animal sources that usually contain considerable fat content, I speculate that vitamin B12 might be more bioavailable in a high fat diet, which was used in the Western-fed group. According to the National Nutrient Database from the US Department of Agriculture, butter contains 0.17 μg of vitamin B12 per 100 g of butter while soybean oil, which was the only source of fat in the control-fed group, contains no vitamin B12. This suggests the slight increase in serum vitamin B12 concentrations in the offspring fed the post weaning Western

diet may be due to the presence of vitamin B12 in fat sources, such as butter, adding more amounts of vitamin B12 in the Western diet than the control diet. One study conducted on male C57BL/6 mice reported higher vitamin B12 intake in the group fed high fat diet compared to the control[231], although the group fed the high fat diet had lower food intake compared to the control. Overall, the vitamin B12 concentrations I observed in this cohort were consistent with previous reports in rodents[206,207,235].

The higher serum vitamin B12 concentrations in Western-fed offspring mice is in contrast to what has been found in humans where an inverse association between BMI and serum B12 concentrations has been reported[9,236,237]. In a Canadian population fewer obese individuals had adequate vitamin B12 status (<0.220 pmol/L) as compared to healthy and overweight individuals[9]. Multiple linear regression analysis of data from NHANES (2003–2006) also revealed lower serum vitamin B12 concentrations with increased BMI in the US population after adjusting for sociodemographic factors[236]. The metabolism of water-soluble vitamins in overweight and obese individuals warrants further investigation. Lower serum folate, but higher RBC folate, was found in obese individuals, suggesting that excess adiposity may affect the tissue distribution of folate in the body [236,238]. Accordingly, quantifying hepatic vitamin B12 concentrations in my cohort would be useful.

The presence of measurable quantities of vitamin B12 in dams lacking vitamin B12 in their diet can be attributed to the production of vitamin B12 by gut bacteria, especially because antibiotics were not used and mice were housed in groups[239,240].

5.3 Programming of Offspring Methyl Metabolism Gene Expression in the Liver

Methyl metabolism is essential for cell growth and development and plays a predominant role in the generation of the methyl-donor AdoMet. It is estimated that ~85% of all methylation reactions occurs in the liver[180,181]. Disturbances in hepatic methyl metabolism have been implicated in hyperhomocysteinemia, CVD, oxidative stress, certain types of cancer, and insulin resistance[94,104,212,213]. Here I report expression of four genes encoding main enzymes in methyl metabolism: *Mtr*, *Cbs*, *Mthfr*, and *Mat1a*.

Expression of Mtr mRNA

Mtr encodes one of the only two vitamin B12-dependent enzymes, MTR. This enzyme remethylates homocysteine and regenerates methionine. Methionine is an essential amino acid in mammals and plays a role in protein synthesis as well as in AdoMet synthesis. I found higher expression of *Mtr* mRNA in male mice fed the Western diet. Data on the effect of the high fat diet or a Western diet on *Mtr* gene expression are limited. One study reported that male C57BL/6 mice fed a high fat diet (60% of energy from fat) for 12 weeks did not change hepatic *Mtr* expression compared to the control-fed group[231]. Similarly, Yun et al. did not find significant changes in hepatic MTR activity and protein expression following high fat feeding (40% of energy) in male C57BL/6 mice[241]. These studies, however, fed the diets for 12 weeks compared to my thesis: 20 weeks of feeding. Accordingly, the 12 weeks feeding period may not be long enough to induce changes in *Mtr* mRNA expression. One study on a human colorectal adenocarcinoma cell line (Caco-2) found increased *Mtr* mRNA levels upon treatment of these cells with homocysteine, suggesting a role of homocysteine in regulating *Mtr* mRNA expression in these cell lines[242].

Another study on human liver cirrhosis and hepatocellular carcinoma found lower *MTR* mRNA expression in these conditions, compared to liver from healthy individuals[243].

Studies on MTR have mainly focused on genetic variants and association with metabolic and disease risks[244]. An *MTR* variant (-186 T>G) was associated with increased CHD risk in 3 case-control studies (2,340 patients and 2,270 matched controls) conducted on a Chinese population[245]. Further analysis from the same group revealed that *MTR* mRNA expression was reduced by almost half in cardiovascular tissue samples (n = 28, collected from patients who had heart catheterisation or a cardiac operation) carrying -186GG variant compared to samples carrying the major allele TT[245]. Heterozygous deficiency of *Mtr* in mice ($Mtr^{+/-}$) resulted in endothelial dysfunction and oxidative stress in cerebral arterioles[246]. I observed lower *Mtr* mRNA expression in female offspring who were developmentally exposed to HFA-B12. Furthermore, oxidizing conditions reduces MTR enzymatic activity in porcine liver[247]. Altogether, these studies suggest adverse effects of reduced MTR gene expression, activity, or deficiency. However, it is crucial to note that MTR expression can be regulated post-translationally. Additional experiments are essential to add more information regarding its protein expression and enzymatic activity in the liver.

Expression of Cbs mRNA

The vitamin-B6 dependent enzyme, CBS, is a key enzyme in the homocysteine catabolism, expressed in the liver, kidney, and brain, but CBS expression during embryogenesis has also been reported[248,249]. Analysis of hepatic *Cbs* mRNA showed lower relative expression in male offspring from dams fed HFA-B12. However, in the present study, changes at the transcript level did not correspond to changes at the protein level; there was no effect of maternal diet on CBS expression in liver from male offspring although the Western-fed offspring had decreased CBS protein

expression. The observation that the Western diet caused decreased CBS expression in male offspring is in agreement with other studies. One study revealed feeding mice high fat diet (60% of energy) for 12 weeks was associated with a significant reduction in hepatic CBS protein expression, although the reduction in mRNA levels did not reach statistical significance[231]. Also, feeding C57BL/6J mice high fat diet (45% of energy) for 12 weeks decreased *Cbs* mRNA expression in the liver[250]. Fonseca et al. fed female rats high fat (39.5% of energy) and high sucrose diet (39.5% of energy) and observed a significant reduction in CBS activity at 6-month and 2-years of age and these rats were hyperinsulinemic[251]. An association has been previously reported between *Cbs* and CBS expressions and diabetes. Streptozotocin-induced diabetic rats have increased hepatic CBS protein expression and enzymatic activity[252,253]. Further work on the same animal model showed high hepatic *Cbs* mRNA expression in diabetic rats that was reduced by insulin administration[254]. Non-obese diabetic (Goto-Kakizaki) rats were also reported to have lower CBS activity compared to non-diabetic rats with no change in CBS protein expression[255]. Interestingly, previous work in our lab revealed increased fasting plasma insulin in male offspring from dams fed HFA-B12; however, this was only observed in post weaning control-fed offspring, not Western-fed[229].

By catalyzing the first and rate-limiting step in homocysteine degradation, CBS contributes to the formation of cysteine. Cysteine is a precursor for glutathione synthesis, which is an antioxidant that plays a major role in oxidative stress. Almost 50% of hepatic glutathione is supplied by this transsulfuration pathway[256]. It has been reported that diabetic rats have a 3-fold increase in hepatic cysteine, compared to non-diabetic[257]. Moreover, decreased *Cbs* mRNA has been found in human cirrhotic liver, compared to normal liver[243]. Mice heterozygous for disruption of CBS ($Cbs^{+/-}$) were reported to have lower hepatic CBS activity and 2 fold increase in plasma homocysteine compared to $Cbs^{+/+}$ [258]. Ghosh et al. reported similar weight gain between $Cbs^{+/-}$ and

Cbs$^{+/+}$ that were fed high fat diet (60% of energy), but *Cbs*$^{+/-}$ mice were more glucose intolerant and more susceptible to cardiac lipotoxicity[259]. This illustrates the importance of optimal CBS status to maintain normal metabolic processes, but also indicates that the exact mechanisms are not well established yet. Overall, CBS has a complex structure that adds complexity to its regulation. It can be regulated transcriptionally as well as post-translationally. AdoMet, for example, is an allosteric activator of CBS and increases its activity ~3 fold[260].

As illustrated in Figure 4.4B, male offspring from dams fed the high folic acid diet, independent of vitamin B12 content, had lower *Cbs* mRNA expression compared to offspring from control-fed dams. Similar findings have been reported in Wistar rats whose moms were supplemented with 5 mg/kg folic acid, compared to offspring from dams supplemented with only 2 mg/kg folic acid[261]. In female offspring, I did not find any significant difference in *Cbs* mRNA expression between groups.

Expression of Mthfr mRNA

I was also interested in whether *Mthfr* mRNA expression could be developmentally programmed. The expression of *Mthfr* mRNA in mice is ubiquitous, but there are tissue-specific differences in adult mice[262]. The highest expression of *Mthfr* is in adult testis while brain and kidney have intermediate expression levels[262]. The activity of MTHFR enzyme in the liver is lower than what observed in testis, brain, and kidney[262]. I found lower expression of hepatic *Mthfr* mRNA in male offspring exposed developmentally to high folic acid and low vitamin B12 intakes. Given that MTHFR catalyzes the formation of 5-methylTHF, which serves as a methyl donor for homocysteine remethylation into methionine, I speculate that decreased *Mthfr* mRNA expression may have adverse effects. A common MTHFR variant in humans, 677C>T, is associated with

decreased MTHFR activity and increased risk for vascular diseases[244,263,264]. Lower MTHFR activity results in elevated homocysteine[265]. Hyperhomocysteinemia by itself is a risk factor for atherosclerosis and CHD[266,267]. Further, *Mthfr* $^{+/-}$ mice on a control diet exhibited lower AdoMet:AdoHcy ratio compared to *Mthfr*$^{+/+}$ mice, but low folate diet resulted in similar reductions in AdoMet:AdoHcy ratios among both *Mthfr* $^{+/-}$ and *Mthfr* $^{+/+}$ [268].

I observed an interaction between maternal diet and post weaning diet on the expression of *Mthfr* mRNA in liver from female offspring. This indicates that the response to maternal diet can be modified by the exposure to different post weaning diets. Up-regulation of *Mthfr* mRNA expression as well as protein levels in neuroblastoma cells has been reported as a result of endoplasmic reticulum (ER) stress[269]. Abnormal metabolic conditions, such as diabetes and inflammation, can impair folding and transport of proteins in the ER, causing ER stress as a cellular defense mechanism[270]. Female rats fed a high fat (39.5%) and high sucrose diet (39.5%) had increased hepatic MTHFR activity at 6 months and 2 years of age[251]. I expect both down-regulation (as observed in male offspring) and up-regulation (as observed in female offspring) can be detrimental depending on what caused these alterations. After all, further work is warranted to examine MTHFR activity and protein expression in offspring from dams fed the HFA-B12 diet. At the protein level, mammalian MTHFR activity can be regulated by AdoMet and AdoHcy[212,263]. In humans, AdoMet/AdoHcy ratio has been found to regulate MTHFR post-translationally by phosphorylation[208]. Tissue *Mthfr* mRNA levels, including liver tissue, were positively correlated with tissue homocysteine and AdoHcy, while MTHFR enzyme was negatively correlated with AdoHcy [271] There is limited research on the effect of high fat/high sugar diet on *Mthfr* mRNA expression, but current data suggest no significant effect of high fat diet on *Mthfr* gene expression[231,250] and MTHFR protein expression[241].

According to VISTA, a group of tools and databases for comparative genomics, Pickell et al. found both *Mthfr* promoters have binding sites for NF-κB family, a group of transcription factors, which regulate expression of genes implicated in inflammation, immune regulation, apoptosis, and liver abnormalities[272-274]. However, they also found that only the downstream promoter activity, not the upstream one, was enhanced following NF- κB co-transfection into neuroblastoma cell lines and that NF-κB activates *Mthfr* expression through the *Mthfr* downstream promoter[202]. In the liver, it remains unclear which promoter is more active and whether NF-κB plays a regulatory role. Hepatic *Mthfr* mRNA expression observed in my study showed no relationship to *Mthfr* methylation in the downstream promoter. The primers I used measure the two major transcripts of *Mthfr*, therefore the assay could not distinguish between them. It is possible that the upstream promoter is more involved in hepatic *Mthfr* transcriptional regulation. It is also possible that the regulation of *Mthfr* mRNA expression can be through other layers of epigenetic regulation, such as histone modifications and RNA-based mechanisms.

Expression of Mat1a mRNA

Lastly, the expression of *Mat1a* mRNA was not affected by maternal and post weaning diet in both male and female offspring. One study reported an increase of *Mat1a* mRNA expression after feeding mice a high fat diet[250], but two other studies reported no significant changes[231,241]. I propose that mice in my study had normal liver based on the unchanged *Mat1a* gene expression, which is mostly down-regulated in hepatocellular carcinoma, liver cirrhosis and inflammation[104]. However, the assumptions that offspring in my thesis have healthy liver needs to be confirmed histologically. Jung et al. reported reduced levels of hepatic MAT activity in Goto-Kakizaki rats, a non-obese diabetic rat model, but there were no changes in the gene or protein expression[275].

Regulation of MAT is not clearly understood, but current research suggests it is regulated at different levels: transcription, post-transcription, and translation [as reviewed in104].

5.4 Methylation Status of *Mthfr*

Given the differences I observed in *Mthfr* mRNA expression in both sexes, I investigated the methylation status of this gene. DNA methylation is one of the epigenetic processes that can regulate gene expression. The main proposed mechanisms regarding the role of DNA methylation in gene repression include interference with nuclear factors binding to regulatory elements that are required for transcriptional activation, recruitment of methyl-binding proteins that can impede transcription, and modification of chromatin structure that limit DNA accessibility[72,276]. Studies of murine *Mthfr* gene revealed the presence of two major promoters[202,205], multiple transcription start sites, and alternative splicing patterns, which result in the formation of two isoforms of MTHFR enzyme, 70kDa and 77kDa[201,202,205,262]. I chose to quantify the methylation status in the downstream promoter because *Mthfr* expression was enhanced by nuclear factor-κB (NF-κB) via its binding to the downstream promoter. Due to lack of publications on *Mthfr* methylation and the difficulty of designing high quality primers to cover the binding site of NF-κB, I sequenced a region between NF-κB binding site and transcription start site within the downstream promoter (Figure 3.2). This region has been reported by Pickell et al. to be necessary for minimal promoter activity[202].

The significant difference found in *Mthfr* methylation at 2 CpG sites in male offspring apppears too small to be biologically relevant and requires further validation. In female offspring, the mean methylation of all CpG sites was significantly higher among post weaning control-fed offspring compared to Western-fed, 60.86% and 56.23%, respectively. All individually analyzed CpG sites were significantly more methylated in control-fed offspring. The difference in

methylation percentage ranged from 4.26% to 5.3%, except for CpG site 8 (2.96%). Although DNA methylation of all 9 CpG sites in female offspring was statistically significant, this difference may not great enough to be biologically relevant and is considered a small difference in methylation studies using the bisulfie pyrosequencing method similar to what I used[277]. Given that the percent methylation in the selected region was high overall (~45-70%), I expected a higher methylation difference to be of greater biological relevance.

The relationship between consumption of high fat or high sugar diet on *Mthfr* DNA methylation has not been previously reported. *Mthfr* methylation has been mainly studied in cancer and male infertility. For example, high *MTHFR* promoter methylation (up to 60% methylation) was seen in human cervical samples with high-grade intraepithelial lesions and tumors[278]. Two studies reported an association between *MTHFR* promoter hypermethylation in sperm and infertility of unknown cause and sperm morphology in males[279,280].

5.5 Hepatic Methylation Metabolites

The status of methionine cycle and methylation can be assessed by measuring their metabolites and cofactors. This includes folate, vitamin B12, vitamin B6, methionine, homocysteine, AdoMet, and AdoHcy. AdoMet acts as an allosteric activator for CBS and inhibitor of MTHFR[104]. AdoHcy does not affect MTHFR directly, but it blocks the effect of AdoMet on MTHFR[208]. AdoHcy is also an inhibitor of methylation reactions[210,211]. For my study, I quantified concentrations of both AdoMet and AdoHcy in the liver of adult offspring. The Western diet led to lower hepatic AdoHcy concentrations in male offspring, but did not affect AdoMet concentrations. The evidence is inconclusive on whether high fat diet influences AdoMet and AdoHcy concentrations. For example, Yun et al. reported an increase in hepatic AdoHcy and a decrease in

hepatic AdoMet concentrations after 12 weeks of feeding mice a high fat diet[241]. However, Dahlhoff et al. used higher amounts of fat in the diet, but found no changes in hepatic AdoMet and AdoHcy contents following feeding the mice a high fat diet for 12 weeks[231]. Both studies were conducted on male C57BL/6 mice, but Yun et al. used 40% of energy from fat while Dahlhoff et al. used 60%.

Given that AdoMet in the liver activates CBS and inhibits MTHFR, it is suggested that homocysteine is channeled toward the remethylation pathway in case of hepatic AdoMet depletion and channeled toward the transsfulfuration pathway when hepatic AdoMet is high[105]. Hepatic AdoMet concentrations were not changed in male offspring, but AdoMet:AdoHcy ratios were lower in the group exposed to the maternal HFA-B12 diet. I believe the reduction in AdoMet;AdoHcy ratio in this group is due to high AdoHcy concentrations, especially in the post weaning Western-fed offspring. Although AdoMet:AdoHcy ratio is not a good marker for methylation capacity, disturbances in this ratio can indicate possible changes at the cellular level.

I speculate the small reduction in hepatic AdoMet concentrations in female offspring exposed developmentally to HFA-B12 ($p = 0.068$) partially explains the increase in *Mthfr* mRNA expression observed in female offspring from dams fed the HFA-B12 and weaned onto the Western diet. This is because depleted AdoMet in the liver is suggested to increase homocysteine remethylation to regenerate AdoMet[105], so higher levels of MTHFR are needed for the production of 5-methyl THF for homocysteine remethylation. However, this speculation warrants investigation given that I did not find any relationship between AdoMet and AdoHcy concentrations and gene expression. Quantification of other metabolites in the liver, such as folate, vitamin B12, methionine, cysteine and homocysteine, will allow a more detailed interpretation of the results.

5.6 Expression of *Nr3c1* and *Ppara* in the Liver

Nr3c1 and *Ppara* are good targets for studying developmental programming as their expressions are affected by maternal influences[19,215,216]. Glucocorticoids are steroid hormones that when bind to GR, encoded by *Nr3c1*, regulate several cardiometabolic and immunological functions as well as mediate stress responses[214]. Disruption of liver GR improved hepatic steatosis in mice[281]. In addition, PPARα is involved in fatty acid synthesis and oxidation, and gluconeogenesis. PPARa-null mice had more fat accumulation in the liver when fed a high fat diet (39%) compared to control-fed mice, and had disturbed glucose and fat metabolism when fasted[219].

In my study I found that *Nr3c1* mRNA did not change as a result of maternal or post weaning diet in liver from both male and female offspring. The primers used were designed to amplify a region of mRNA corresponding to exon 2 in the *Nr3c1* gene that is ubiquitously expressed[282]. There could be an isoform-specific effect that was not detected in my work. Similar findings were seen in the hepatic *Ppara* mRNA expression of male and female offspring. However, maternal diet interacts with post weaning diet in female offspring. The developmental exposure to HFA-B12 diet decreased the expression of *Ppara* only in the post weaning Western-fed group. Decreased *Ppara* mRNA has been reported previously in animals exposed *in utero* to maternal obesity[216,283].

5.7 Strengths and Limitations

I believe there are several strengths in this work that are supported with a good research design. Having an animal model allowed me to control the amount of nutrients given, including folic acid and vitamin B12. All diets used in this experiment were made in the lab based on the recommendations of NRC of Canada. I had the opportunity to explore the effect of maternal high

folic acid with and without adequate vitamin B12 in both sexes, but at different ages. To my knowledge, my work is one of the first reports exploring the consequences maternal folic acid and vitamin B12 imbalance on hepatic methyl metabolism in adult offspring.

Nevertheless, my study has some limitations. Despite the similarities between human and mice as mammals, there are still some metabolic differences that make it hard to extrapolate results to humans. For example, DHFR, which metabolizes folic acid into THF, is more active in rodents than in humans, suggesting that humans are less efficient at handling high amounts of folic acid compared to rodents[284]. My thesis mainly addressed changes at the transcription level, which hampers drawing a complete picture. Examining more post-transcriptional and translational mechanisms will facilitate more accurate interpretations and understanding of the results. I focused more on the vitamin B12-dependant pathway of homocysteine remethylation, so I did not examine the expression of *Bhmt*, which is equally important in the liver and contributes to almost half of homocysteine remethylation[99].

Chapter 6: Conclusion and Future Directions

I have demonstrated that exposure to maternal high folic acid and low vitamin B12 intakes *in utero* and early in life can program expression of genes in the liver from adult offspring. These changes are not related to differences in serum folate and vitamin B12 concentrations of the offspring. Alterations in hepatic gene expression of male offspring can be partially attributed to changes in hepatic AdoHcy and AdoMet:AdoHcy ratio, but these findings require further confirmation by examining changes in enzyme activity and other methyl metabolites. I further report that adult diet can modify the response to maternal folic acid and vitamin B12 imbalance. The metabolic consequences of these alterations require further investigations. In general, my work adds to the present knowledge and helps in clarifying important aspects. Future work supporting the present findings will add to the current human evidence, and by doing so can inform the debate about fortifying food with vitamin B12 and assist making recommendations for vitamin B12 screening before/during pregnancy.

Future work can focus on epidemiological studies as well as on mechanistic trials. Current epidemiological data on high folate and low vitamin B12 status during pregnancy are primarily available from South Asian women. Further work in non-South Asian populations will inform as to whether the previously reported outcomes are ethnic-specific. Additionally, human studies can consider the consequences of folic acid and vitamin B12 imbalance on pregnant women and their health, and whether there are critical periods in pregnancy when the mother or fetus are most susceptible to adverse outcomes. Thorough investigations on other metabolic parameters, such as lipid profile, organ functions, and hormones, will provide better understanding of possible mechanisms driving the reported responses. Animal models can provide valuable mechanistic explanations with fewer confounders. Possible areas to explore include quantification of other

nutrients involved in methyl metabolism, such as vitamin B6, choline, and betaine, monitoring of dietary intake and physical activity level, measurement of tissue lipid contents, and examination of more genes implicated in cardiometabolic dysfunction.

References

1. Aljaadi A, Aleliunas RE, Olson JD, Sulistyoningrum DC, Green TJ, Devlin AM. Programming of liver gene expression by gestational exposure to folic acid/vitamin B12 imbalance, Abstracts of the 20th International Congress of Nutrition. Granada, Spain. September 15-20, 2013. *Ann Nutr Metab*. 2013;63 Suppl 1:394.

2. World Health Organization. Obesity and overweight Fact sheet N°311. http://www.who.int/mediacentre/factsheets/fs311/en/. Updated 2013. Accessed 5/2, 2014.

3. Ng M, Fleming T, Robinson M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: A systematic analysis for the global burden of disease study 2013. *Lancet*. 2014.

4. World Health Organization. WHO maps noncommunicable disease trends in all countries. http://www.who.int/mediacentre/news/releases/2011/NCDs_profiles_20110914/en/. Updated 2011. Accessed 5/19, 2014.

5. Canadian Food and Drugs Act. Regulations amending the food and drug regulations (1066). *Canada Gazette*. 1998;24:98-550.

6. US Food and Drug Administration. Food standards: Amendment of standards of identity for enriched grain products to require addition of folic acid: Final rule. *Fed Reg*. 1996;61:8781-8797.

7. Colapinto CK, O'Connor DL, Tremblay MS. Folate status of the population in the Canadian Health Measures Survey. *CMAJ*. 2011;183(2):E100-6.

8. Rolfes SR, Pinna K, Whitney EN. *Understanding normal and clinical nutrition.* 7th ed. Belmont, CA: Thomson/Wadsworth; 2006.

9. MacFarlane AJ, Greene-Finestone LS, Shi Y. Vitamin B-12 and homocysteine status in a folate-replete population: Results from the Canadian health measures survey. *Am J Clin Nutr*. 2011;94(4):1079-1087.

10. Ray JG, Goodman J, O'Mahoney PR, Mamdani MM, Jiang D. High rate of maternal vitamin B12 deficiency nearly a decade after Canadian folic acid flour fortification. *QJM*. 2008;101(6):475-477.

11. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am J Clin Nutr*. 2007;85(1):193-200.

12. Yajnik CS, Deshpande SS, Jackson AA, et al. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: The Pune Maternal Nutrition Study. *Diabetologia*. 2008;51(1):29-38.

13. Stewart CP, Christian P, Schulze KJ, et al. Low maternal vitamin B-12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural nepal. *J Nutr*. 2011;141(10):1912-1917.

14. Krishnaveni GV, Veena SR, Karat SC, Yajnik CS, Fall CH. Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia*. 2014;57(1):110-121.

15. Roseboom TJ, Painter RC, van Abeelen AF, Veenendaal MV, de Rooij SR. Hungry in the womb: What are the consequences? Lessons from the Dutch famine. *Maturitas*. 2011;70(2):141-145.

16. Alfaradhi MZ, Ozanne SE. Developmental programming in response to maternal overnutrition. *Front Genet*. 2011;2:27.

17. de Boo HA, Harding JE. The developmental origins of adult disease (Barker) hypothesis. *Aust N Z J Obstet Gynaecol*. 2006;46(1):4-14.

18. Yehuda R, Engel SM, Brand SR, Seckl J, Marcus SM, Berkowitz GS. Transgenerational effects of posttraumatic stress disorder in babies of mothers exposed to the world trade center attacks during pregnancy. *J Clin Endocrinol Metab*. 2005;90(7):4115-4118.

19. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics*. 2008;3(2):97-106.

20. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS. Exposure to bisphenol A advances puberty. *Nature*. 1999;401(6755):763-764.

21. Samuelsson AM, Matthews PA, Argenton M, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: A novel murine model of developmental programming. *Hypertension*. 2008;51(2):383-392.

22. *Malnutrition and starvation in Western Netherlands; September 1944 - July 1945.* Vol 2. General State Printing Office: The Hague; 1948.

23. Stein Z, Susser M, Saenger G, Marolla F. Nutrition and mental performance. *Science*. 1972;178(4062):708-713.

24. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure *in utero* and early infancy. *N Engl J Med*. 1976;295(7):349-353.

25. Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet*. 1989;2(8663):577-580.

26. de Rooij SR, Painter RC, Phillips DI, et al. Hypothalamic-pituitary-adrenal axis activity in adults who were prenatally exposed to the Dutch famine. *Eur J Endocrinol*. 2006;155(1):153-160.

27. de Rooij SR, Painter RC, Phillips DI, et al. Impaired insulin secretion after prenatal exposure to the Dutch famine. *Diabetes Care*. 2006;29(8):1897-1901.

28. Stein AD, Kahn HS, Rundle A, Zybert PA, van der Pal-de Bruin K, Lumey LH. Anthropometric measures in middle age after exposure to famine during gestation: Evidence from the Dutch famine. *Am J Clin Nutr*. 2007;85(3):869-876.

29. de Rooij SR, Painter RC, Roseboom TJ, et al. Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. *Diabetologia*. 2006;49(4):637-643.

30. Lumey LH, Stein AD, Kahn HS, Romijn JA. Lipid profiles in middle-aged men and women after famine exposure during gestation: The Dutch Hunger Winter families study. *Am J Clin Nutr*. 2009;89(6):1737-1743.

31. Ravelli AC, van der Meulen JH, Michels RP, et al. Glucose tolerance in adults after prenatal exposure to famine. *Lancet*. 1998;351(9097):173-177.

32. Lorenzo C, Wagenknecht LE, Rewers MJ, et al. Disposition index, glucose effectiveness, and conversion to type 2 diabetes: The Insulin Resistance Atherosclerosis Study (IRAS). *Diabetes Care*. 2010;33(9):2098-2103.

33. Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr*. 1999;70(5):811-816.

34. Painter RC, de Rooij SR, Bossuyt PM, et al. Early onset of coronary artery disease after prenatal exposure to the Dutch famine. *Am J Clin Nutr*. 2006;84(2):322-7; quiz 466-7.

35. Whincup PH, Kaye SJ, Owen CG, et al. Birth weight and risk of type 2 diabetes: A systematic review. *JAMA*. 2008;300(24):2886-2897.

36. Bryce J, Coitinho D, Darnton-Hill I, Pelletier D, Pinstrup-Andersen P, Maternal and Child Undernutrition Study Group. Maternal and child undernutrition: Effective action at national level. *Lancet*. 2008;371(9611):510-526.

37. Ong KK, Emmett PM, Noble S, Ness A, Dunger DB, ALSPAC Study Team. Dietary energy intake at the age of 4 months predicts postnatal weight gain and childhood body mass index. *Pediatrics*. 2006;117(3):e503-8.

38. Singhal A, Cole TJ, Fewtrell M, Lucas A. Breastmilk feeding and lipoprotein profile in adolescents born preterm: Follow-up of a prospective randomised study. *Lancet*. 2004;363(9421):1571-1578.

39. Shields M, Tjepkema M. Trends in adult obesity. *Health Rep*. 2006;17(3):53-59.

40. Lawlor DA, Fraser A, Lindsay RS, et al. Association of existing diabetes, gestational diabetes and glycosuria in pregnancy with macrosomia and offspring body mass index, waist and fat mass in later childhood: Findings from a prospective pregnancy cohort. *Diabetologia*. 2010;53(1):89-97.

41. Dabelea D, Hanson RL, Lindsay RS, et al. Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: A study of discordant sibships. *Diabetes*. 2000;49(12):2208-2211.

42. Canadian Diabetes Association Clinical Practice Guidelines Expert Committee. 2008 clinical practice guidelines for the prevention and management of diabetes in Canada. *Can J Diabetes*. 2008;32.

43. Li CC, Young PE, Maloney CA, et al. Maternal obesity and diabetes induces latent metabolic defects and widespread epigenetic changes in isogenic mice. *Epigenetics*. 2013;8(6):602-611.

44. Oken E, Levitan EB, Gillman MW. Maternal smoking during pregnancy and child overweight: Systematic review and meta-analysis. *Int J Obes (Lond)*. 2008;32(2):201-210.

45. Toschke AM, Montgomery SM, Pfeiffer U, von Kries R. Early intrauterine exposure to tobacco-inhaled products and obesity. *Am J Epidemiol*. 2003;158(11):1068-1074.

46. Leary SD, Smith GD, Rogers IS, Reilly JJ, Wells JC, Ness AR. Smoking during pregnancy and offspring fat and lean mass in childhood. *Obesity (Silver Spring)*. 2006;14(12):2284-2293.

47. Chen A, Pennell ML, Klebanoff MA, Rogan WJ, Longnecker MP. Maternal smoking during pregnancy in relation to child overweight: Follow-up to age 8 years. *Int J Epidemiol*. 2006;35(1):121-130.

48. Power C, Jefferis BJ. Fetal environment and subsequent obesity: A study of maternal smoking. *Int J Epidemiol*. 2002;31(2):413-419.

49. Oken E, Taveras EM, Kleinman KP, Rich-Edwards JW, Gillman MW. Gestational weight gain and child adiposity at age 3 years. *Am J Obstet Gynecol*. 2007;196(4):322.e1-322.e8.

50. Schack-Nielsen L, Michaelsen KF, Gamborg M, Mortensen EL, Sorensen TI. Gestational weight gain in relation to offspring body mass index and obesity from infancy through adulthood. *Int J Obes (Lond)*. 2010;34(1):67-74.

51. Remacle C, Dumortier O, Bol V, et al. Intrauterine programming of the endocrine pancreas. *Diabetes Obes Metab*. 2007;9 Suppl 2:196-209.

52. Ozanne SE, Hales CN. The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. *Proc Nutr Soc*. 1999;58(3):615-619.

53. Petry CJ, Dorling MW, Pawlak DB, Ozanne SE, Hales CN. Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int J Exp Diabetes Res*. 2001;2(2):139-143.

54. Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, Ozanne SE. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *Am J Physiol Regul Integr Comp Physiol*. 2005;288(2):R368-73.

55. van Straten EM, Bloks VW, Huijkman NC, et al. The liver X-receptor gene promoter is hypermethylated in a mouse model of prenatal protein restriction. *Am J Physiol Regul Integr Comp Physiol*. 2010;298(2):R275-82.

56. Petrik J, Reusens B, Arany E, et al. A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology*. 1999;140(10):4861-4873.

57. Nivoit P, Morens C, Van Assche FA, et al. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia*. 2009;52(6):1133-1142.

58. Zhu MJ, Du M, Nathanielsz PW, Ford SP. Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. *Placenta*. 2010;31(5):387-391.

59. Zhu MJ, Ma Y, Long NM, Du M, Ford SP. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(5):R1224-31.

60. Muhlhausler BS, Duffield JA, McMillen IC. Increased maternal nutrition stimulates peroxisome proliferator activated receptor-gamma, adiponectin, and leptin messenger ribonucleic acid expression in adipose tissue before birth. *Endocrinology*. 2007;148(2):878-885.

61. Power C, Li L, Manor O, Davey Smith G. Combination of low birth weight and high adult body mass index: At what age is it established and what are its determinants? *J Epidemiol Community Health*. 2003;57(12):969-973.

62. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature*. 2010;467(7318):963-966.

63. Mahan LK, Escott-Stump S. *Krause's food & nutrition therapy.* 12th ed. St. Louis, Mo.: Saunders/Elsevier; 2008:1352.

64. Muoio DM, Newgard CB. Obesity-related derangements in metabolic regulation. *Annu Rev Biochem*. 2006;75:367-401.

65. McCurdy CE, Bishop JM, Williams SM, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest*. 2009;119(2):323-335.

66. Pruis MG, Lendvai A, Bloks VW, et al. Maternal western diet primes non-alcoholic fatty liver disease in adult mouse offspring. *Acta Physiol (Oxf)*. 2014;210(1):215-227.

67. Bruce KD, Cagampang FR, Argenton M, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology*. 2009;50(6):1796-1808.

68. Bouchard L, Thibault S, Guay SP, et al. Leptin gene epigenetic adaptation to impaired glucose metabolism during pregnancy. *Diabetes Care*. 2010;33(11):2436-2441.

69. Brons C, Jacobsen S, Nilsson E, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. *J Clin Endocrinol Metab*. 2010;95(6):3048-3056.

70. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004;429(6990):457-463.

71. Rodenhiser D, Mann M. Epigenetics and human disease: Translating basic biology into clinical applications. *CMAJ*. 2006;174(3):341-348.

72. Yan MS, Matouk CC, Marsden PA. Epigenetics of the vascular endothelium. *J Appl Physiol*. 2010;109(3):916-926.

73. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 2009;10(2):126-139.

74. Ehrlich M, Wang RY. 5-methylcytosine in eukaryotic DNA. *Science*. 1981;212(4501):1350-1357.

75. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462(7271):315-322.

76. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet*. 2000;9(16):2395-2402.

77. Turner BM. Cellular memory and the histone code. *Cell*. 2002;111(3):285-291.

78. Margueron R, Trojer P, Reinberg D. The key to development: Interpreting the histone code? *Curr Opin Genet Dev*. 2005;15(2):163-176.

79. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell*. 2007;128(4):669-681.

80. Rothbart SB, Strahl BD. Interpreting the language of histone and DNA modifications. *Biochim Biophys Acta*. 2014.

81. Phillips T. Small non-coding RNA and gene expression. *Nature Educ*. 2008;1(1):115.

82. Greene ND, Stanier P, Copp AJ. Genetics of human neural tube defects. *Hum Mol Genet*. 2009;18(R2):R113-29.

83. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet*. 1991;338(8760):131-137.

84. Berry RJ, Li Z, Erickson JD, et al. Prevention of neural-tube defects with folic acid in China. China-U.S. collaborative project for neural tube defect prevention. *N Engl J Med*. 1999;341(20):1485-1490.

85. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med*. 1992;327(26):1832-1835.

86. Centers for Disease Control and Prevention (CDC). CDC grand rounds: Additional opportunities to prevent neural tube defects with folic acid fortification. *MMWR Morb Mortal Wkly Rep*. 2010;59(31):980-984.

87. Food Fortification Initiative. Global progress: Mandatory cereal grain legislation - May 2014. http://www.ffinetwork.org/global_progress/index.php. Updated 2014. Accessed 5/15, 2014.

88. Health Canada. Prenatal nutrition guidelines for health professionals: Folate contributes to a healthy pregnancy. . 2009;978-1-100-12208-3.

89. De Wals P, Tairou F, Van Allen MI, et al. Spina bifida before and after folic acid fortification in Canada. *Birth Defects Res A Clin Mol Teratol*. 2008;82(9):622-626.

90. Bell KN, Oakley GP,Jr. Update on prevention of folic acid-preventable spina bifida and anencephaly. *Birth Defects Res A Clin Mol Teratol*. 2009;85(1):102-107.

91. Pfeiffer CM, Johnson CL, Jain RB, et al. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988 2004. *Am J Clin Nutr*. 2007;86(3):718-727.

92. Ganji V, Kafai MR. Trends in serum folate, RBC folate, and circulating total homocysteine concentrations in the United States: Analysis of data from national health and nutrition examination surveys, 1988-1994, 1999-2000, and 2001-2002. *J Nutr*. 2006;136(1):153-158.

93. Kalmbach RD, Choumenkovitch SF, Troen AM, D'Agostino R, Jacques PF, Selhub J. Circulating folic acid in plasma: Relation to folic acid fortification. *Am J Clin Nutr*. 2008;88(3):763-768.

94. Shane B. Folate and vitamin B12 metabolism: Overview and interaction with riboflavin, vitamin B6, and polymorphisms. *Food Nutr Bull*. 2008;29(2 Suppl):S5-16; discussion S17-9.

95. McNulty H, Scott JM. Intake and status of folate and related B-vitamins: Considerations and challenges in achieving optimal status. *Br J Nutr*. 2008;99 Suppl 3:S48-54.

96. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: Acute studies in subjects consuming fortified food and supplements. *Am J Clin Nutr*. 1997;65(6):1790-1795.

97. Miller JW, Garrod MG, Allen LH, Haan MN, Green R. Metabolic evidence of vitamin B-12 deficiency, including high homocysteine and methylmalonic acid and low holotranscobalamin, is more pronounced in older adults with elevated plasma folate. *Am J Clin Nutr*. 2009;90(6):1586-1592.

98. Quay T. *Assessment of the rate and determinants of vitamin B12 deficiency in South Asian and European women of childbearing age in Metro Vancouver.* [Master of Science]. University of British Columbia; 2014.

99. Blom HJ. Folic acid, methylation and neural tube closure in humans. *Birth Defects Res A Clin Mol Teratol*. 2009;85(4):295-302.

100. Food and Agriculture Organization of the United Nations, World Health Organization. Folate and folic acid. In: *Vitamin and mineral requirements in human nutrition: [Report of a joint FAOWHO expert consultation, Bangkok, Thailand, 21-30 September 1998]*. 2nd ed. Geneva: World Health Organization; 2004:341.

101. Webster-Gandy J, Madden A, Holdsworth M. *Oxford handbook of nutrition and dietetics.* Oxford ; Toronto: Oxford University Press; 2006:730. http://www.loc.gov/catdir/toc/ecip0615/2006019103.html.

102. Kozyraki R, Cases O. Vitamin B12 absorption: Mammalian physiology and acquired and inherited disorders. *Biochimie*. 2013;95(5):1002-1007.

103. Nielsen MJ, Rasmussen MR, Andersen CB, Nexo E, Moestrup SK. Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol*. 2012;9(6):345-354.

104. Lu SC, Mato JM. S-adenosylmethionine in liver health, injury, and cancer. *Physiol Rev*. 2012;92(4):1515-1542.

105. Lu SC, Mato JM. S-adenosylmethionine in cell growth, apoptosis and liver cancer. *J Gastroenterol Hepatol*. 2008;23(Suppl 1):S73-7.

106. Selhub J. Homocysteine metabolism. *Annu Rev Nutr*. 1999;19:217-246.

107. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. *JAMA*. 2002;288(16):2015-2022.

108. Herrmann M, Peter Schmidt J, Umanskaya N, et al. The role of hyperhomocysteinemia as well as folate, vitamin B(6) and B(12) deficiencies in osteoporosis: A systematic review. *Clin Chem Lab Med*. 2007;45(12):1621-1632.

109. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med*. 2002;346(7):476-483.

110. Waterland RA, Jirtle RL. Transposable elements: Targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol*. 2003;23(15):5293-5300.

111. Wolff GL, Kodell RL, Moore SR, Cooney CA. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *FASEB J*. 1998;12(11):949-957.

112. McKay JA, Wong YK, Relton CL, Ford D, Mathers JC. Maternal folate supply and sex influence gene-specific DNA methylation in the fetal gut. *Mol Nutr Food Res*. 2011;55(11):1717-1723.

113. Meher A, Joshi A, Joshi S. Differential regulation of hepatic transcription factors in the Wistar rat offspring born to dams fed folic acid, vitamin B12 deficient diets and supplemented with omega-3 fatty acids. *PLoS One*. 2014;9(2):e90209.

114. Weaver BP, Zhang Y, Hiscox S, et al. Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration. *PLoS One*. 2010;5(10):10.1371/journal.pone.0013158.

115. Zhang Y, Bharadwaj U, Logsdon CD, Chen C, Yao Q, Li M. ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB. *Clin Cancer Res*. 2010;16(5):1423-1430.

116. Li M, Zhang Y, Liu Z, et al. Aberrant expression of zinc transporter ZIP4 (SLC39A4) significantly contributes to human pancreatic cancer pathogenesis and progression. *Proc Natl Acad Sci U S A*. 2007;104(47):18636-18641.

117. Michalik L, Auwerx J, Berger JP, et al. International union of pharmacology. LXI. peroxisome proliferator-activated receptors. *Pharmacol Rev*. 2006;58(4):726-741.

118. Knauf C, Rieusset J, Foretz M, et al. Peroxisome proliferator-activated receptor-alpha-null mice have increased white adipose tissue glucose utilization, GLUT4, and fat mass: Role in liver and brain. *Endocrinology*. 2006;147(9):4067-4078.

119. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr*. 2005;135(6):1382-1386.

120. Langie SA, Achterfeldt S, Gorniak JP, et al. Maternal folate depletion and high-fat feeding from weaning affects DNA methylation and DNA repair in brain of adult offspring. *FASEB J*. 2013;27(8):3323-3334.

121. McKay JA, Groom A, Potter C, et al. Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: Role for folate gene variants and vitamin B12. *PLoS One*. 2012;7(3):e33290.

122. Muthayya S, Kurpad AV, Duggan CP, et al. Low maternal vitamin B12 status is associated with intrauterine growth retardation in urban South Indians. *Eur J Clin Nutr*. 2006;60(6):791-801.

123. Molina V, Medici M, Taranto MP, Font de Valdez G. Effects of maternal vitamin B12 deficiency from end of gestation to weaning on the growth and haematological and immunological parameters in mouse dams and offspring. *Arch Anim Nutr*. 2008;62(2):162-168.

124. Huang Y, He Y, Sun X, He Y, Li Y, Sun C. Maternal high folic acid supplement promotes glucose intolerance and insulin resistance in male mouse offspring fed a high-fat diet. *Int J Mol Sci*. 2014;15(4):6298-6313.

125. Leal Vde O, Mafra D. Adipokines in obesity. *Clin Chim Acta*. 2013;419:87-94.

126. Nawrocki AR, Rajala MW, Tomas E, et al. Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem*. 2006;281(5):2654-2660.

127. Luo ZC, Nuyt AM, Delvin E, et al. Maternal and fetal leptin, adiponectin levels and associations with fetal insulin sensitivity. *Obesity (Silver Spring)*. 2013;21(1):210-216.

128. da Silva VC, Fernandes L, Haseyama EJ, et al. Effect of vitamin B deprivation during pregnancy and lactation on homocysteine metabolism and related metabolites in brain and plasma of mice offspring. *PLoS One*. 2014;9(4):e92683.

129. Dwarkanath P, Barzilay JR, Thomas T, Thomas A, Bhat S, Kurpad AV. High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: A prospective observational cohort study. *Am J Clin Nutr*. 2013;98(6):1450-1458.

130. Krishnaveni GV, Hill JC, Veena SR, et al. Low plasma vitamin B12 in pregnancy is associated with gestational 'diabesity' and later diabetes. *Diabetologia*. 2009;52(11):2350-2358.

131. Krishnaveni GV, Veena SR, Hill JC, Kehoe S, Karat SC, Fall CH. Intrauterine exposure to maternal diabetes is associated with higher adiposity and insulin resistance and clustering of cardiovascular risk markers in Indian children. *Diabetes Care*. 2010;33(2):402-404.

132. Duque-Guimaraes DE, Ozanne SE. Nutritional programming of insulin resistance: Causes and consequences. *Trends Endocrinol Metab*. 2013;24(10):525-535.

133. Gluckman PD, Hanson MA, Pinal C. The developmental origins of adult disease. *Matern Child Nutr*. 2005;1(3):130-141.

134. Li CC, Young PE, Maloney CA, et al. Maternal obesity and diabetes induces latent metabolic defects and widespread epigenetic changes in isogenic mice. *Epigenetics*. 2013;8(6):602-611.

135. Cannon MV, Buchner DA, Hester J, et al. Maternal nutrition induces pervasive gene expression changes but no detectable DNA methylation differences in the liver of adult offspring. *PLoS One*. 2014;9(3):e90335.

136. Canadian Institute for Health Information, Public Health Agency of Canada. *Obesity in Canada: A joint report from the Public Health Agency of Canada and the Canadian Institute for Health Information.* Ottawa: Public Health Agency of Canada; 2011:54.

137. Centres for Disease Control and Prevention. Defining Overweight and Obesity. http://www.cdc.gov/obesity/adult/defining.html. Updated 2012. Accessed 5/3, 2014.

138. Yusuf S, Hawken S, Ounpuu S, et al. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: A case-control study. *Lancet*. 2005;366(9497):1640-1649.

139. Hamdy O, Porramatikul S, Al-Ozairi E. Metabolic obesity: The paradox between visceral and subcutaneous fat. *Curr Diabetes Rev*. 2006;2(4):367-373.

140. McCarty MF. A paradox resolved: The postprandial model of insulin resistance explains why gynoid adiposity appears to be protective. *Med Hypotheses*. 2003;61(2):173-176.

141. Gallagher D, Visser M, Sepulveda D, Pierson RN, Harris T, Heymsfield SB. How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups? *Am J Epidemiol*. 1996;143(3):228-239.

142. Prado CM, Siervo M, Mire E, et al. A population-based approach to define body-composition phenotypes. *Am J Clin Nutr*. 2014.

143. Tillin T, Hughes AD, Mayet J, et al. The relationship between metabolic risk factors and incident cardiovascular disease in Europeans, South Asians, and African Caribbeans: SABRE (Southall and Brent revisited) -- a prospective population-based study. *J Am Coll Cardiol*. 2013;61(17):1777-1786.

144. Wang D, Li Y, Lee SG, et al. Ethnic differences in body composition and obesity related risk factors: Study in Chinese and White males living in China. *PLoS One*. 2011;6(5):e19835.

145. Shuster A, Patlas M, Pinthus JH, Mourtzakis M. The clinical importance of visceral adiposity: A critical review of methods for visceral adipose tissue analysis. *Br J Radiol*. 2012;85(1009):1-10.

146. Wang Y, Rimm EB, Stampfer MJ, Willett WC, Hu FB. Comparison of abdominal adiposity and overall obesity in predicting risk of type 2 diabetes among men. *Am J Clin Nutr*. 2005;81(3):555-563.

147. Janssen I, Katzmarzyk PT, Ross R. Body mass index, waist circumference, and health risk: Evidence in support of current national institutes of health guidelines. *Arch Intern Med*. 2002;162(18):2074-2079.

148. National Heart, Lung, and Blood Institute. Guidelines on overweight and obesity: Electronic textbook. http://www.nhlbi.nih.gov/guidelines/obesity/e_txtbk/txgd/4142.htm. Accessed 5/7, 2014.

149. Neeland IJ, Turer AT, Ayers CR, et al. Dysfunctional adiposity and the risk of prediabetes and type 2 diabetes in obese adults. *JAMA*. 2012;308(11):1150-1159.

150. Wajchenberg BL, Giannella-Neto D, da Silva ME, Santos RF. Depot-specific hormonal characteristics of subcutaneous and visceral adipose tissue and their relation to the metabolic syndrome. *Horm Metab Res*. 2002;34(11-12):616-621.

151. Chiba Y, Saitoh S, Takagi S, et al. Relationship between visceral fat and cardiovascular disease risk factors: The Tanno and Sobetsu Study. *Hypertens Res*. 2007;30(3):229-236.

152. Harvard School of Public Health. Obesity prevention source. http://www.hsph.harvard.edu/obesity-prevention-source/. Accessed 5/6, 2014.

153. Karelis AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET. Metabolic and body composition factors in subgroups of obesity: What do we know? *J Clin Endocrinol Metab*. 2004;89(6):2569-2575.

154. Hill JO, Wyatt HR. The myth of healthy obesity. *Ann Intern Med*. 2013;159(11):789-790.

155. Kramer CK, Zinman B, Retnakaran R. Are metabolically healthy overweight and obesity benign conditions?: A systematic review and meta-analysis. *Ann Intern Med*. 2013;159(11):758-769.

156. Centres for Disease Control and Prevention. Genomics and heallth. http://www.cdc.gov/genomics/resources/diseases/obesity/index.htm. Updated 2013. Accessed 5/14, 2014.

157. Hu FB. Genetic predictors of obesity. In: *Obesity epidemiology*. Oxford ; New York: Oxford University Press; 2008:437-460.

158. Guo DF, Rahmouni K. Molecular basis of the obesity associated with Bardet-Biedl syndrome. *Trends Endocrinol Metab*. 2011;22(7):286-293.

159. Rocha CF, Paiva CL. Prader-Willi-like phenotypes: A systematic review of their chromosomal abnormalities. *Genet Mol Res*. 2014;13(1):2290-2298.

160. Tounian P. Programming towards childhood obesity. *Ann Nutr Metab*. 2011;58 Suppl 2:30-41.

161. Cordain L, Eaton SB, Sebastian A, et al. Origins and evolution of the western diet: Health implications for the 21st century. *Am J Clin Nutr*. 2005;81(2):341-354.

162. World Health Organization. New physical activity guidance can help reduce risk of breast, colon cancers. http://www.who.int/mediacentre/news/notes/2011/world_cancer_day_20110204/en/. Updated 2011. Accessed 5/7, 2014.

163. Public Health Agency of Canada, Canadian Institute for Health Information, Canadian Health Research Collection, Canadian Public Policy Collection. Obesity in Canada. . 2011.

164. Allison DB, Fontaine KR, Manson JE, Stevens J, VanItallie TB. Annual deaths attributable to obesity in the United States. *JAMA*. 1999;282(16):1530-1538.

165. Bogers RP, Bemelmans WJ, Hoogenveen RT, et al. Association of overweight with increased risk of coronary heart disease partly independent of blood pressure and cholesterol levels: A meta-analysis of 21 cohort studies including more than 300 000 persons. *Arch Intern Med*. 2007;167(16):1720-1728.

166. Moller DE, Kaufman KD. Metabolic syndrome: A clinical and molecular perspective. *Annu Rev Med*. 2005;56:45-62.

167. Colditz GA, Willett WC, Rotnitzky A, Manson JE. Weight gain as a risk factor for clinical diabetes mellitus in women. *Ann Intern Med*. 1995;122(7):481-486.

168. Koh-Banerjee P, Wang Y, Hu FB, Spiegelman D, Willett WC, Rimm EB. Changes in body weight and body fat distribution as risk factors for clinical diabetes in US men. *Am J Epidemiol*. 2004;159(12):1150-1159.

169. Cardiometabolic Risk Working Group: Executive Committee, Leiter LA, Fitchett DH, et al. Cardiometabolic risk in Canada: A detailed analysis and position paper by the Cardiometabolic Risk Working Group. *Can J Cardiol*. 2011;27(2):e1-e33.

170. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: Old questions and new insights. *Science*. 2011;332(6037):1519-1523.

171. Adams LA, Angulo P. Recent concepts in non-alcoholic fatty liver disease. *Diabet Med*. 2005;22(9):1129-1133.

172. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med*. 2002;346(16):1221-1231.

173. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity. *Hepatology*. 2004;40(6):1387-1395.

174. Bellentani S, Saccoccio G, Masutti F, et al. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med*. 2000;132(2):112-117.

175. Franzese A, Vajro P, Argenziano A, et al. Liver involvement in obese children. Ultrasonography and liver enzyme levels at diagnosis and during follow-up in an Italian population. *Dig Dis Sci*. 1997;42(7):1428-1432.

176. Tominaga K, Kurata JH, Chen YK, et al. Prevalence of fatty liver in Japanese children and relationship to obesity. An epidemiological ultrasonographic survey. *Dig Dis Sci*. 1995;40(9):2002-2009.

177. Sinclair KD, Allegrucci C, Singh R, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A*. 2007;104(49):19351-19356.

178. Giudicelli F, Brabant AL, Grit I, Parnet P, Amarger V. Excess of methyl donor in the perinatal period reduces postnatal leptin secretion in rat and interacts with the effect of protein content in diet. *PLoS One*. 2013;8(7):e68268.

179. Gallou-Kabani C, Vige A, Gross MS, et al. C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity (Silver Spring)*. 2007;15(8):1996-2005.

180. Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. *Metabolism*. 1975;24(6):721-735.

181. Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology*. 2007;45(5):1306-1312.

182. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004;114(2):147-152.

183. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: Lessons from genetically engineered mice. *J Clin Invest*. 2008;118(3):829-838.

184. Targher G. Non-alcoholic fatty liver disease, the metabolic syndrome and the risk of cardiovascular disease: The plot thickens. *Diabet Med*. 2007;24(1):1-6.

185. National Research Council. Subcommittee on Laboratory Animal Nutrition. *Nutrient requirements of laboratory animals*. 4th rev ed. Washington, D.C.: National Academy of Sciences; 1995:173.

186. Liu Z, Choi SW, Crott JW, et al. Mild depletion of dietary folate combined with other B vitamins alters multiple components of the Wnt pathway in mouse colon. *J Nutr*. 2007;137(12):2701-2708.

187. Intapad S, Tull FL, Brown AD, et al. Renal denervation abolishes the age-dependent increase in blood pressure in female intrauterine growth-restricted rats at 12 months of age. *Hypertension*. 2013;61(4):828-834.

188. Tao H, Rui C, Zheng J, et al. Angiotensin II-mediated vascular changes in aged offspring rats exposed to perinatal nicotine. *Peptides*. 2013;44:111-119.

189. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. *J Clin Pathol*. 1992;45(4):344-347.

190. Grossowicz N, Waxman S, Schreiber C. Cryoprotected Lactobacillus casei: An approach to standardization of microbiological assay of folic acid in serum. *Clin Chem*. 1981;27(5):745-747.

191. Wilson SD, Horne DW. Use of glycerol-cryoprotected Lactobacillus casei for microbiological assay of folic acid. *Clin Chem*. 1982;28(5):1198-1200.

192. Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin Chem*. 1988;34(11):2357-2359.

193. Kelleher BP, Broin SD. Microbiological assay for vitamin B12 performed in 96-well microtitre plates. *J Clin Pathol*. 1991;44(7):592-595.

194. Fell D, Benjamin LE, Steele RD. Determination of adenosine and S-adenosyl derivatives of sulfur amino acids in rat liver by high-performance liquid chromatography. *J Chromatogr*. 1985;345(1):150-156.

195. Miller JW, Nadeau MR, Smith J, Smith D, Selhub J. Folate-deficiency-induced homocysteinaemia in rats: Disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. *Biochem J*. 1994;298 (Pt 2)(Pt 2):415-419.

196. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001;25(4):402-408.

197. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3(6):1101-1108.

198. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.

199. Dupont JM, Tost J, Jammes H, Gut IG. De novo quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem*. 2004;333(1):119-127.

200. Frosst P, Zhang Z, Pai A, Rozen R. The methylenetetrahydrofolate reductase (Mthfr) gene maps to distal mouse chromosome 4. *Mamm Genome*. 1996;7(11):864-865.

201. Gaughan DJ, Barbaux S, Kluijtmans LA, Whitehead AS. The human and mouse methylenetetrahydrofolate reductase (MTHFR) genes: Genomic organization, mRNA structure and linkage to the CLCN6 gene. *Gene*. 2000;257(2):279-289.

202. Pickell L, Tran P, Leclerc D, Hiscott J, Rozen R. Regulatory studies of murine methylenetetrahydrofolate reductase reveal two major promoters and NF-kappaB sensitivity. *Biochim Biophys Acta*. 2005;1731(2):104-114.

203. Li LC, Dahiya R. MethPrimer: Designing primers for methylation PCRs. *Bioinformatics*. 2002;18(11):1427-1431.

204. UCSC Genome Bioinformatics. Mouse genome browser gateway. http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=315692569&clade=mammal&org=Mouse&db=mm10. Updated 2011. Accessed 12/3, 2012.

205. Pickell L, Wu Q, Wang XL, et al. Targeted insertion of two Mthfr promoters in mice reveals temporal- and tissue-specific regulation. *Mamm Genome*. 2011;22(11-12):635-647.

206. Troen AM, Shea-Budgell M, Shukitt-Hale B, Smith DE, Selhub J, Rosenberg IH. B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice. *Proc Natl Acad Sci U S A*. 2008;105(34):12474-12479.

207. McNeil CJ, Beattie JH, Gordon MJ, Pirie LP, Duthie SJ. Nutritional B vitamin deficiency disrupts lipid metabolism causing accumulation of proatherogenic lipoproteins in the aorta adventitia of ApoE null mice. *Mol Nutr Food Res*. 2012;56(7):1122-1130.

208. Yamada K, Strahler JR, Andrews PC, Matthews RG. Regulation of human methylenetetrahydrofolate reductase by phosphorylation. *Proc Natl Acad Sci U S A*. 2005;102(30):10454-10459.

209. Miles EW, Kraus JP. Cystathionine beta-synthase: Structure, function, regulation, and location of homocystinuria-causing mutations. *J Biol Chem*. 2004;279(29):29871-29874.

210. Chiang PK, Gordon RK, Tal J, et al. S-adenosylmethionine and methylation. *FASEB J*. 1996;10(4):471-480.

211. Chiang PK. Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. *Pharmacol Ther*. 1998;77(2):115-134.

212. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. *Vitam Horm*. 2008;79:1-44.

213. Liu AY, Scherer D, Poole E, et al. Gene-diet-interactions in folate-mediated one-carbon metabolism modify colon cancer risk. *Mol Nutr Food Res*. 2013;57(4):721-734.

214. Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease. *Front Behav Neurosci*. 2009;3:19.

215. Slater-Jefferies J, Lillycrop K, Townsend P, et al. Feeding a protein-restricted diet during pregnancy induces altered epigenetic regulation of peroxisomal proliferator-activated receptor-α in the heart of the offspring. *J Dev Orig Health Dis*. 2011;2(4):250-255.

216. Shankar K, Kang P, Harrell A, et al. Maternal overweight programs insulin and adiponectin signaling in the offspring. *Endocrinology*. 2010;151(6):2577-2589.

217. Weaver IC, Cervoni N, Champagne FA, et al. Epigenetic programming by maternal behavior. *Nat Neurosci*. 2004;7(8):847-854.

218. Abdelmegeed MA, Yoo SH, Henderson LE, Gonzalez FJ, Woodcroft KJ, Song BJ. PPARalpha expression protects male mice from high fat-induced nonalcoholic fatty liver. *J Nutr*. 2011;141(4):603-610.

219. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest*. 1999;103(11):1489-1498.

220. Dupont JM, Tost J, Jammes H, Gut IG. De novo quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem*. 2004;333(1):119-127.

221. Devlin AM, Singh R, Wade RE, Innis SM, Bottiglieri T, Lentz SR. Hypermethylation of Fads2 and altered hepatic fatty acid and phospholipid metabolism in mice with hyperhomocysteinemia. *J Biol Chem*. 2007;282(51):37082-37090.

222. Glier MB, Green TJ, Devlin AM. Methyl nutrients, DNA methylation, and cardiovascular disease. *Mol Nutr Food Res*. 2014;58(1):172-182.

223. Lim U, Song MA. Dietary and lifestyle factors of DNA methylation. *Methods Mol Biol*. 2012;863:359-376.

224. Sinclair KD, Allegrucci C, Singh R, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A*. 2007;104(49):19351-19356.

225. Kumar KA, Lalitha A, Pavithra D, et al. Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. *J Nutr Biochem*. 2013;24(1):25-31.

226. Leunissen RW, Kerkhof GF, Stijnen T, Hokken-Koelega A. Timing and tempo of first-year rapid growth in relation to cardiovascular and metabolic risk profile in early adulthood. *JAMA*. 2009;301(21):2234-2242.

227. Eriksson JG, Forsen T, Tuomilehto J, Osmond C, Barker DJ. Early growth and coronary heart disease in later life: Longitudinal study. *BMJ*. 2001;322(7292):949-953.

228. Singhal A, Kennedy K, Lanigan J, et al. Nutrition in infancy and long-term risk of obesity: Evidence from 2 randomized controlled trials. *Am J Clin Nutr*. 2010;92(5):1133-1144.

229. Aleliunas R. *Programming of adult metabolic phenotype by maternal dietary folic acid and vitamin B12 imbalance in mice.* [Master of Science]. University of British Columbia; 2013.

230. Li G, Kohorst JJ, Zhang W, et al. Early postnatal nutrition determines adult physical activity and energy expenditure in female mice. *Diabetes*. 2013;62(8):2773-2783.

231. Dahlhoff C, Desmarchelier C, Sailer M, et al. Hepatic methionine homeostasis is conserved in C57BL/6N mice on high-fat diet despite major changes in hepatic one-carbon metabolism. *PLoS One*. 2013;8(3):e57387.

232. Leamon CP, Reddy JA, Dorton R, et al. Impact of high and low folate diets on tissue folate receptor levels and antitumor responses toward folate-drug conjugates. *J Pharmacol Exp Ther*. 2008;327(3):918-925.

233. Sontag JM, Nunbhakdi-Craig V, Montgomery L, Arning E, Bottiglieri T, Sontag E. Folate deficiency induces in vitro and mouse brain region-specific downregulation of leucine carboxyl

methyltransferase-1 and protein phosphatase 2A B(alpha) subunit expression that correlate with enhanced tau phosphorylation. *J Neurosci*. 2008;28(45):11477-11487.

234. Venn BJ, Mann JI, Williams SM, et al. Assessment of three levels of folic acid on serum folate and plasma homocysteine: A randomised placebo-controlled double-blind dietary intervention trial. *Eur J Clin Nutr*. 2002;56(8):748-754.

235. Troen AM, Lutgens E, Smith DE, Rosenberg IH, Selhub J. The atherogenic effect of excess methionine intake. *Proc Natl Acad Sci U S A*. 2003;100(25):15089-15094.

236. Pfeiffer CM, Sternberg MR, Schleicher RL, Rybak ME. Dietary supplement use and smoking are important correlates of biomarkers of water-soluble vitamin status after adjusting for sociodemographic and lifestyle variables in a representative sample of U.S. adults. *J Nutr*. 2013;143(6):957S-65S.

237. Kimmons JE, Blanck HM, Tohill BC, Zhang J, Khan LK. Associations between body mass index and the prevalence of low micronutrient levels among US adults. *MedGenMed*. 2006;8(4):59.

238. Tinker SC, Hamner HC, Berry RJ, Bailey LB, Pfeiffer CM. Does obesity modify the association of supplemental folic acid with folate status among nonpregnant women of childbearing age in the United States? *Birth Defects Res A Clin Mol Teratol*. 2012;94(10):749-755.

239. Albert MJ, Mathan VI, Baker SJ. Vitamin B12 synthesis by human small intestinal bacteria. *Nature*. 1980;283(5749):781-782.

240. Capozzi V, Russo P, Duenas MT, Lopez P, Spano G. Lactic acid bacteria producing B-group vitamins: A great potential for functional cereals products. *Appl Microbiol Biotechnol*. 2012;96(6):1383-1394.

241. Yun KU, Ryu CS, Oh JM, et al. Plasma homocysteine level and hepatic sulfur amino acid metabolism in mice fed a high-fat diet. *Eur J Nutr*. 2013;52(1):127-134.

242. Ortiou S, Alberto JM, Gueant JL, Merten M. Homocysteine increases methionine synthase mRNA level in caco-2 cells. *Cell Physiol Biochem*. 2004;14(4-6):407-414.

243. Avila MA, Berasain C, Torres L, et al. Reduced mRNA abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma. *J Hepatol*. 2000;33(6):907-914.

244. Molloy AM. Genetic aspects of folate metabolism. *Subcell Biochem*. 2012;56:105-130.

245. Zhao JY, Qiao B, Duan WY, et al. Genetic variants reducing MTR gene expression increase the risk of congenital heart disease in Han Chinese populations. *Eur Heart J*. 2014;35(11):733-742.

246. Dayal S, Devlin AM, McCaw RB, et al. Cerebral vascular dysfunction in methionine synthase-deficient mice. *Circulation*. 2005;112(5):737-744.

247. Chen Z, Chakraborty S, Banerjee R. Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded. *J Biol Chem*. 1995;270(33):19246-19249.

248. Quere I, Paul V, Rouillac C, et al. Spatial and temporal expression of the cystathionine beta-synthase gene during early human development. *Biochem Biophys Res Commun*. 1999;254(1):127-137.

249. Robert K, Vialard F, Thiery E, et al. Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. *J Histochem Cytochem*. 2003;51(3):363-371.

250. Rubio-Aliaga I, Roos B, Sailer M, et al. Alterations in hepatic one-carbon metabolism and related pathways following a high-fat dietary intervention. *Physiol Genomics*. 2011;43(8):408-416.

251. Fonseca V, Dicker-Brown A, Ranganathan S, et al. Effects of a high-fat—sucrose diet on enzymes in homocysteine metabolism in the rat. *Metab Clin Exp*. 2000;49(6):736-741.

252. Jacobs RL, House JD, Brosnan ME, Brosnan JT. Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. *Diabetes*. 1998;47(12):1967-1970.

253. Li S, Arning E, Liu C, et al. Regulation of homocysteine homeostasis through the transcriptional coactivator PGC-1alpha. *Am J Physiol Endocrinol Metab*. 2009;296(3):E543-8.

254. Ratnam S, Maclean KN, Jacobs RL, Brosnan ME, Kraus JP, Brosnan JT. Hormonal regulation of cystathionine beta-synthase expression in liver. *J Biol Chem*. 2002;277(45):42912-42918.

255. Jung YS, Yun KU, Ryu CS, et al. Alterations in hepatic metabolism of sulfur amino acids in non-obese type-2 diabetic Goto-Kakizaki rats. *Chem Biol Interact*. 2013;204(2):80-87.

256. Mosharov E, Cranford MR, Banerjee R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. *Biochemistry*. 2000;39(42):13005-13011.

257. Brosnan JT, Man KC, Hall DE, Colbourne SA, Brosnan ME. Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidotic rat. *Am J Physiol*. 1983;244(2):E151-8.

258. Vitvitsky V, Dayal S, Stabler S, et al. Perturbations in homocysteine-linked redox homeostasis in a murine model for hyperhomocysteinemia. *Am J Physiol Regul Integr Comp Physiol*. 2004;287(1):R39-46.

259. Ghosh S, Sulistyoningrum DC, Glier MB, Verchere CB, Devlin AM. Altered glutathione homeostasis in heart augments cardiac lipotoxicity associated with diet-induced obesity in mice. *J Biol Chem*. 2011;286(49):42483-42493.

260. Finkelstein JD, Kyle WE, Martin JL, Pick AM. Activation of cystathionine synthase by adenosylmethionine and adenosylethionine. *Biochem Biophys Res Commun*. 1975;66(1):81-87.

261. Chmurzynska A, Malinowska AM. Homocysteine homeostasis in the rat is maintained by compensatory changes in cystathionine beta-synthase, betaine-homocysteine methyltransferase, and phosphatidylethanolamine N-methyltransferase gene transcription occurring in response to maternal protein and folic acid intake during pregnancy and fat intake after weaning. *Nutr Res*. 2011;31(7):572-578.

262. Tran P, Leclerc D, Chan M, et al. Multiple transcription start sites and alternative splicing in the methylenetetrahydrofolate reductase gene result in two enzyme isoforms. *Mamm Genome*. 2002;13(9):483-492.

263. Thomas P, Fenech M. Methylenetetrahydrofolate reductase, common polymorphisms, and relation to disease. *Vitam Horm*. 2008;79:375-392.

264. Klerk M, Verhoef P, Clarke R, et al. MTHFR 677C-->T polymorphism and risk of coronary heart disease: A meta-analysis. *JAMA*. 2002;288(16):2023-2031.

265. Rosenblatt DS. Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver CR, Beaudet AL, Sly S, Valle D, eds. *Metabolic and molecular bases of inherited diseases.* New York: McGraw-Hill; 1995:3111-3128.

266. Clarke R, Daly L, Robinson K, et al. Hyperhomocysteinemia: An independent risk factor for vascular disease. *N Engl J Med*. 1991;324(17):1149-1155.

267. Danesh J, Lewington S. Plasma homocysteine and coronary heart disease: Systematic review of published epidemiological studies. *J Cardiovasc Risk*. 1998;5(4):229-232.

268. Devlin AM, Arning E, Bottiglieri T, Faraci FM, Rozen R, Lentz SR. Effect of Mthfr genotype on diet-induced hyperhomocysteinemia and vascular function in mice. *Blood*. 2004;103(7):2624-2629.

269. Leclerc D, Rozen R. Endoplasmic reticulum stress increases the expression of methylenetetrahydrofolate reductase through the IRE1 transducer. *J Biol Chem*. 2008;283(6):3151-3160.

270. Yoshida H. ER stress and diseases. *FEBS J*. 2007;274(3):630-658.

271. Chen NC, Yang F, Capecci LM, et al. Regulation of homocysteine metabolism and methylation in human and mouse tissues. *FASEB J*. 2010;24(8):2804-2817.

272. Muriel P. NF-kappaB in liver diseases: A target for drug therapy. *J Appl Toxicol*. 2009;29(2):91-100.

273. Luedde T, Schwabe RF. NF-kappaB in the liver--linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol*. 2011;8(2):108-118.

274. Hoffmann A, Leung TH, Baltimore D. Genetic analysis of NF-kappaB/rel transcription factors defines functional specificities. *EMBO J*. 2003;22(20):5530-5539.

275. Jung YS, Yun KU, Ryu CS, et al. Alterations in hepatic metabolism of sulfur amino acids in non-obese type-2 diabetic Goto-Kakizaki rats. *Chem Biol Interact*. 2013;204(2):80-87.

276. Glier MB, Green TJ, Devlin AM. Methyl nutrients, DNA methylation, and cardiovascular disease. *Mol Nutr Food Res*. 2014;58(1):172-182.

277. Hogg K, Price EM, Robinson WP. Improved reporting of DNA methylation data derived from studies of the human placenta. *Epigenetics*. 2014;9(3):333-337.

278. Botezatu A, Socolov D, Iancu IV, et al. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and promoter methylation in cervical oncogenic lesions and cancer. *J Cell Mol Med*. 2013;17(4):543-549.

279. Botezatu A, Socolov R, Socolov D, Iancu IV, Anton G. Methylation pattern of methylene tetrahydrofolate reductase and small nuclear ribonucleoprotein polypeptide N promoters in oligoasthenospermia: A case-control study. *Reprod Biomed Online*. 2014;28(2):225-231.

280. Wu W, Shen O, Qin Y, et al. Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR). *PLoS One*. 2010;5(11):e13884.

281. Lemke U, Krones-Herzig A, Berriel Diaz M, et al. The glucocorticoid receptor controls hepatic dyslipidemia through Hes1. *Cell Metab*. 2008;8(3):212-223.

282. Freeman AI, Munn HL, Lyons V, Dammermann A, Seckl JR, Chapman KE. Glucocorticoid down-regulation of rat glucocorticoid receptor does not involve differential promoter regulation. *J Endocrinol*. 2004;183(2):365-374.

283. Borengasser SJ, Kang P, Faske J, et al. High fat diet and in utero exposure to maternal obesity disrupts circadian rhythm and leads to metabolic programming of liver in rat offspring. *PLoS One*. 2014;9(1):e84209.

284. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. *Proc Natl Acad Sci U S A*. 2009;106(36):15424-15429.