MATERNAL B-VITAMIN STATUS DURING DEVELOPMENT AND PROGRAMMING OF ADULT OFFSPRING ADIPOSITY

by

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Abstract

Background: Developmental programming suggests that perinatal environmental conditions can impact risk for chronic diseases. Population studies have reported greater insulin resistance and adiposity in offspring from mothers with adequate folate but low vitamin B12 (B12) status during pregnancy. Rodent studies have reported that these effects are sex-specific. Folate, a methyl nutrient, is metabolically linked to B12. Low B12 status, even when folate is adequate, can trap folate in a metabolically inactive form. Folate deficiency is rare in Canada due to folic acid fortification of grains, yet one in 20 Canadians are estimated to be B12-deficient. The objective of this thesis is to determine the mechanisms underlying the relationship between maternal B-vitamin status during pregnancy and offspring adiposity and glucose homeostasis.

Methodology/Results: *In vitro* experiments assessed direct effects of folic acid on adipocyte energy metabolism. In 3T3-L1 adipocytes, cells treated with 1.6μM folic acid had lower ($p \leq 0.05$) mitochondrial respiration rates than cells treated with 0.16μM folic acid. Adipocytes treated with 1.6μM 5-methyltetrahydrofolate (5-MTHF), the circulating form of folate, had higher ($p \leq 0.01$) mitochondrial respiration rates than cells treated with 0.16μM 5-MTHF.

Female mice (C57BL/6J) were fed one of three maternal diets six weeks prior to breeding and through pregnancy/lactation: control (M-CON), supplemental folic acid with adequate B12 (SFA+B12), or SFA without B12 (SFA-B12). One male and one female from each dam were weaned onto either a control or western diet (45% kcal fat). Sex-specific differences by maternal diet were observed in the offspring. Female control-fed SFA-B12 offspring had lower ($p \leq 0.05$) serum IGF-1 (insulin-like growth factor-1) concentrations than M-CON and SFA+B12 offspring. This was accompanied by higher ($p \leq 0.05$) hepatic *Cpt1a* mRNA in SFA-B12 and SFA+B12 offspring than M-CON offspring. Female western-fed SFA+B12 offspring had higher ($p \leq 0.05$)

hepatic FADS2 and ELOVL2 protein than M-CON offspring. Conversely, male control-fed SFA-B12 offspring had higher ($p \leq 0.05$) hepatic linoleic acid (C18:2*n*-6) and lower ($p \leq 0.05$) eicosapentaenoic acid (C20:5*n*-3) concentrations, and lower ($p = 0.08$) hepatic ELOVL2 protein than M-CON offspring.

Conclusion: These findings suggest programming of offspring adiposity and glucose homeostasis by maternal B-vitamin status occurs through sex-specific alterations in IGF-1, and adipose tissue and hepatic lipid metabolism.

Preface

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Pathology and Laboratory Medicine. All experiments pertaining to this thesis were performed under the guidance and supervision of Dr. Angela Devlin. This thesis was reviewed by Dr. Devlin, Dr. William Gibson, and Dr. Bruce Verchere.

The procedures and experiments presented in this thesis were conducted in Dr. Devlin's lab at the BC Children's Hospital Research Institute (BCCHR), at Dr. Yvonne Lamers' lab at the Department of Food, Nutrition and Health at the University of British Columbia (UBC), and at Dr. Sanjoy Ghosh's lab at the Department of Biology at UBC Okanagan. The animal portion of the project was part of a larger, previously established mouse cohort in the Devlin lab[1]. All animal work, including the planning and implementation of the maternal diet study, breeding for offspring, and mouse tissue harvest, was performed by Rika Aleliunas (M.Sc. 2013, UBC), with assistance from Abeer Aljaadi (M.Sc. 2014, UBC), and Melissa Glier (PhD 2016, UBC). The animal portion of the project was performed in the animal facility at BCCHR and was approved by the UBC Animal Care Committee (protocols: A09-0346 and A10-0179).

Offspring liver taurine concentrations and total folate concentrations in the cell culture media were quantified by Benny Chan in Dr. Lamers' lab. Liver fatty acids and protein (FADS2 and ELOVL2) were quantified in Dr. Ghosh's lab at with the assistance of Hoda Derakhshanian and Svetlana Simtchouk. All other molecular experiments, including quantifying serum IGF-1 concentrations, liver and adipose gene expression, and liver AKT protein were performed by me at BCCHR. I also planned and performed all *in vitro* experiments; for the Seahorse assays I received guidance from Dr. Michal Simon and Mitsuhiro Komba from Dr. Dan Luciani's lab (UBC Department of Surgery and BCCHR).

I presented the results of the serum IGF-1 concentrations, liver and adipose gene expression, and liver taurine concentrations in poster format at Experimental Biology in April 2016 in San Diego, CA. The abstract for this presentation was published in The FASEB Journal[2]. My findings were also presented orally at the FASEB Summer Research Conference: Folic Acid, Vitamin B12, and One-Carbon Metabolism conference in August 2016 in Steamboat Springs, CO. The cell culture experiments and results from the female offspring serum IGF-1 concentrations and adipose tissue gene expression are included in a manuscript that is currently in preparation[3].

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5-MTHF	5-methyltetrahydrofolate
ACC1	Acetyl-CoA carboxylase 1
AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosylmethionine
ANOVA	Analysis of variance
BHMT	Betaine-homocysteine S-methyltransferase
BMI	Body mass index
CDP-Choline	Cytidine diphosphate-choline
CpG	Cytosine-guanine dinucleotide
CPT1A	Carnitine palmitoyltransferase 1A
CVD	Cardiovascular disease
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DOHaD	Developmental Origins of Health and Disease
ECAR	Extracellular acidification rate
ELISA	Enzyme-linked immunosorbent assay
ELOVL2	Elongation of very long-chain fatty acids protein 2
FADS2	Fatty acid desaturase 2
FCCP	Carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrazone
GH	Growth hormone
GIF	Gastric intrinsic factor
GLUT4	Glucose transporter type 4
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor 1
IGFIR	Insulin-like growth factor 1 receptor
IGFBP2	Insulin-like growth factor binding protein 2
IR	Insulin receptor

LCPUFA	Long-chain polyunsaturated fatty acid
M-CON	Maternal control diet
MCM	Methylmalonyl-CoA mutase
MDI	Methylisobutylxanthine dexamethasone insulin
MS	Methionine synthase
NEFA	Non-esterified fatty acid
NTD	Neural tube defect
OCR	Oxygen consumption rate
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time polymerase chain reaction
SAT	Subcutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
SFA-B12	Supplemental folic acid with no vitamin B12 diet
SFA+B12	Supplemental folic acid with adequate vitamin B12 diet
T2D	Type 2 diabetes
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
TG	Triglyceride
THF	Tetrahydrofolate
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
WHO	World Health Organization
β-Me	Beta-mercaptoethanol

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The support of many individuals has been immeasurable during my time as a graduate student and I would like to take this opportunity to acknowledge them here. First and foremost, I would like to express my sincerest thanks and gratitude to my supervisor and mentor, Dr. Angela Devlin. Being a member of Dr. Devlin's lab has been an invaluable experience. Under the guidance of Dr. Devlin, I have had the opportunity to gain an understanding and appreciation for research, learn numerous laboratory techniques, and present at two international conferences. Furthermore, my time in the Devlin lab has allowed for significant personal growth, specifically in my confidence and presentation abilities, as a direct result of Dr. Devlin's encouragement.

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I would like to thank all previous and current members of the Devlin lab for their instruction, assistance, ideas, input, friendship, and moral support during this Master's degree. I would like to especially thank Rika Aleliunas for her work in establishing the mouse cohort, without which I would not have a project. I also thank Dr. Daven Tai, Dr. Melissa Glier, and Sarah Gerrard for teaching me laboratory techniques, and Nolan Chem for his help with gene expression analysis. I thank Nicha Boonpattrawong, Sarah Montgomery, Ei-Xia Mussai, Dr. Arya Mehran, and James Melanson for their support, friendship, insight, and helpful advice.

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CHAPTER 1: Introduction

1.1 Increasing Prevalence of Obesity

Obesity (in adults defined as body mass index ≥30.0kg/m^2) is characterized by abnormal or excessive fat accumulation and underlies numerous chronic health conditions. This disease, though traditionally thought to be an affliction of the western world, is rapidly becoming a global epidemic[4]. Over the last 30 years, economic and cultural shifts in the developing world have been accompanied by decreased physical activity and an increased intake of high-caloric food with poor nutritional value[5,6]. According to the World Health Organization (WHO), in 2008 more than half a billion adults were estimated to be obese worldwide[7]. The increasing prevalence of obesity is alarming because of its association with numerous comorbidities, including coronary heart disease, type 2 diabetes (T2D), hypertension, dyslipidemia, sleep apnea, stroke, respiratory problems, osteoarthritis, infertility, and various cancers[8]. Obesity is also a major risk factor for cardiovascular disease (CVD) and T2D[9].

Emerging evidence indicates that obesity is increasing at an alarming rate, not only in adults but in children and adolescents as well. This is occurring in both the developed and the developing world[4]. The rising rates in childhood obesity are paralleled by an emergence of T2D in children and adolescents. Once considered to be primarily a disease of adulthood, T2D was diagnosed in only 1-2% of diabetes cases in children two decades ago; however, today some countries have reported that up to 80% of all cases of diabetes in the pediatric population are of the type 2 nature[10,11].

Systematic reviews have indicated that childhood obesity increases the risk of obesity later in life and contributes to the early onset of comorbidities including T2D and CVD[12]. Based on the WHO age- and sex-specific cutoffs for children and adolescents 5 to 17 years of age, the

2009-2011 Canadian Health Measures Survey (CHMS) estimated that 31.5% of Canadian youth were classified as either overweight (19.8%) or obese (11.7%)[13]. These alarming statistics prompt the need for a better understanding of the pathophysiology behind the surge in obesity and T2D, particularly in younger populations, to help identify strategies for intervention and improvement.

1.2 Regional Fat Distribution

Body mass index (BMI) is used to define obesity. It is a simple weight-for-height calculation that classifies individuals as underweight, healthy weight, overweight, or obese[4]. It is calculated by weight (in kilograms) divided by the square of height (in metres). In adults, overweight is defined as having a BMI of 25.0-29.9kg/m^2 and obese is defined as having a BMI of ≥30.0kg/m^2. Though BMI is used to define obesity, and while population studies have reported its associated risk for T2D, CVD, and all-cause mortality[14,15], other indicators, such as waist circumference, are better predictors of T2D and CVD risk[16,17]. This is because BMI does not account for the wide variation in body composition due to factors such as age, sex, ethnicity, and lean mass. Among individuals, the amount and distribution of excess fat, and the associated health consequences can vary greatly[18,19]. Therefore, examining body fat distribution is more indicative of metabolic health than BMI or total body weight.

Individuals with excess fat in the intra-abdominal regions (visceral adipose tissue, VAT) are described as having an "android obesity" profile. These individuals are at particular risk for adverse health outcomes, such as CVD and insulin resistance[20,21]. In contrast, individuals with more evenly and peripherally distributed fat are described as having a "gynoid obesity" profile. In these individuals, fat accumulates in the subcutaneous compartment (subcutaneous adipose

tissue, SAT), often around the hips and thighs. Population studies have suggested that SAT is associated with a protective lipid and glucose profile, and lower CVD and T2D risk[18,22,23]. It has been reported that individuals with a gynoid obesity profile are more insulin-sensitive than individuals with an android obesity profile[24,25].

1.3 Visceral Adiposity and Cardiometabolic Risk

Although obesity, particularly in the visceral compartment, is a risk factor for insulin resistance and T2D, not every person with obesity develops insulin resistance to the same degree[19,26]. An important question to consider is whether excess visceral fat is causal of T2D and CVD or is simply a marker of a perturbed metabolic profile. Evidence suggests that visceral adiposity and the etiology of insulin resistance stem, in part, from dysfunctional adipocytes, the cells within adipose tissue that primarily function to store energy as fat. When adipocytes become dysfunctional and hypertrophied, they lose the ability to respond to insulin, decrease their glucose uptake, and enter a hyperlipolytic state[27,28]. This results in an increase of non-esterified fatty acid (NEFA) release from adipose tissue, a flux of NEFAs into the portal circulation, an accumulation of ectopic fat in other tissues (such as the liver), and altered glucose and lipid metabolism. All of these factors contribute to insulin resistance and other metabolic complications[27–30].

The flux of NEFAs has also been shown *in vitro* to contribute to the dysfunction of β-cells, the specialized cells within the pancreas that synthesize, store, and secrete insulin in response to blood glucose. It has been reported that NEFAs cause β-cell dysfunction by impairing glucose-stimulated insulin secretion, increasing expression of uncoupling protein 2 (a mitochondrial protein that regulates ATP production, UCP-2), and activating caspase-mediated

apoptotic pathways[31–33]. Similarly, chronic hyperglycemia impairs β-cell function by persistently stimulating the endoplasmic reticulum (ER) within the cells to produce insulin. This causes stress in the ER and disrupts the biosynthesis and folding of newly synthesized insulin. If ER stress is persistent, pro-apoptotic signals are generated[34]. Consequently, glucolipotoxicity derived from excess VAT can accelerate the progressive loss of β-cell mass and β-cell function, leading to insulin resistance and ultimately T2D.

In addition to impaired glucose and fatty acid metabolism, other factors have been implicated in the development of metabolic dysfunction stemming from excess VAT. Besides the role of adipose tissue as a depot for energy storage and lipid mobilization functions, it is a very important endocrine organ capable of releasing numerous cytokines (adipokines), including the proinflammatory molecules interleukin (IL)-6 and tumor-necrosis factor-α (TNF-α)[19]. It is well-known that obese adipose tissue undergoes macrophage infiltration, which may further contribute to the inflammatory profile observed in individuals with visceral adiposity[35]. Subjects with visceral adiposity also have elevated plasma levels of C-reactive protein (CRP), a proinflammatory marker that is predictive of risk for myocardial infarction[36]. Adipose tissue secretes adiponectin (an adipokine that increases insulin sensitivity), which circulates inversely proportional to adiposity[37]. In fact, adiponectin production by adipocytes has been shown to be inhibited by TNF-α *in vitro*[38]. Overall, factors including hyperglycemia, impaired adipose tissue and hepatic lipid metabolism, β-cell dysfunction, reduced circulating adiponectin concentrations, and elevated proinflammatory cytokines observed in subjects with visceral adiposity could collectively contribute to their metabolic risk factor profile.

1.4 Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) theory, spearheaded by Dr. David Barker, proposes that perinatal environmental conditions during development have the capacity to program chronic diseases later in life[39]. Both prenatal and early postnatal life fall in a critical period of developmental plasticity, wherein an organism is able to develop in various ways depending on the particular environment experienced. This phenomenon is thought to be advantageous from an evolutionary standpoint as it enables the developing organism to receive a "forecast" from the mother that predicts the environmental conditions the organism will ultimately inhabit[40]. For instance, if a developing offspring experiences undernourishment *in utero* due to poor maternal nourishment, the fetus may undergo adaptations, such as reduced body size and altered metabolism, to prioritize the development of essential tissues and prepare for a shortage of food after birth[41].

These growth and metabolic alterations, although advantageous for short-term survival, have the potential to lead to chronic diseases later in life[42,43]. This theory arose from epidemiological studies reporting that low birth weight is associated with poor infant growth and subsequently, elevated blood pressure, elevated plasma triglycerides (TGs), fasting hyperinsulinemia, and impaired glucose tolerance[44,45]. The Dutch Famine Birth Cohort, perhaps the most well-known of these studies, followed individuals who were conceived in the Netherlands between 1944 and 1945 and were born to women exposed during pregnancy to extreme stress and starvation during World War II. These individuals were reported to have reduced birth size and increased incidence of T2D and CVD in adulthood[46]. Moreover, studies of this cohort revealed differences in disease susceptibility depending on the stage of development in which famine was experienced. Exposure to famine during any stage of gestation was

associated with glucose intolerance, whereas exposure to famine specifically during early gestation was associated with obesity, coronary heart disease, and an atherogenic lipid profile[47–51]. A comparable study of the Chinese Famine from 1945 to 1964 observed that prenatal and early postnatal exposure to famine was associated with an increased risk of metabolic syndrome in adulthood, which was further exacerbated by a nutritionally-rich environment later in life[52].

It is evident that maternal nutrition during pregnancy and lactation plays an important role in later disease susceptibility in offspring. Much of the work in this field has focused on assessing the effects of maternal dietary energy intakes; however, less is known about how maternal nutritional imbalance of specific dietary factors during pregnancy, such as B-vitamins, could influence disease in offspring.

1.5 One-Carbon Metabolism

Folate and Vitamin B12

Folates (vitamin B9) comprise a family of chemically and structurally-related compounds that are involved in various metabolic functions in the body[53]. These nutrients are required for the transfer of one-carbon units (methyl groups) in numerous reactions, including synthesis of purines and pyrimidines and methylation of DNA, RNA, proteins,histones, phospholipids, and other molecules[53]. Mammals are incapable of synthesizing folates *de novo* and must therefore rely on dietary sources, such as dark green vegetables, certain fruits, legumes, and liver[54]. Naturally occurring folates typically exist in the reduced polyglutamyl form and must be cleaved by a brush border glutamylhydrolase to their monoglutamyl forms prior to absorption in the proximal small intestine[55]. Folic acid, a synthetic analogue found in vitamin supplements and fortified food products, is the most oxidized, stable and easily absorbed form of folate. Dietary

folates and folic acid are usually reduced and methylated to their active circulating form, 5-methyltetrahydrofolate (5-MTHF), in the intestinal mucosa following absorption. However, higher intakes of folic acid can be absorbed and secreted into the portal circulation as unmetabolized folic acid[53].

Vitamin B12 (B12) is a water-soluble vitamin that is required for proper red blood cell formation, neurological functions, and DNA synthesis. It acts as a cofactor for two known enzymes in the body: cytosolic methionine synthase (MS) and mitochondrial methylmalonyl-CoA mutase (MCM). Dietary sources of B12 include animal products (fish, meat, poultry, eggs, and milk products), of which B12 is bound to proteins in the food. Once consumed, B12 is released from the food proteins by pepsin and by the acidic environment of the stomach[53]. In the small intestine, free B12 binds to intrinsic factor (IF), a glycoprotein secreted by gastric parietal cells following a meal[53]. Absorption of B12 into enterocytes occurs at the distal ileum through receptor-mediated endocytic processes[53,56]. These receptors are only able to recognize and endocytose IF-B12 complexes; therefore, unbound B12 cannot be absorbed[56]. Intracellularly, endosomes containing the IF-B12 complexes fuse with lysosomes, degrade IF, and release vitamin B12 into the cytosol, allowing it to bind transcobalamin-II (TC-II) to permit entry into the portal circulation for delivery to various tissues[57].

Folate and Vitamin B12 in One-Carbon Metabolism

Methyl nutrients, such as folate and vitamin B12, are interconnected by three biochemical pathways, collectively termed one-carbon metabolism[58]. These pathways – the folate cycle, the methionine cycle, and the transsulfuration pathway – are summarized in Figure 1.

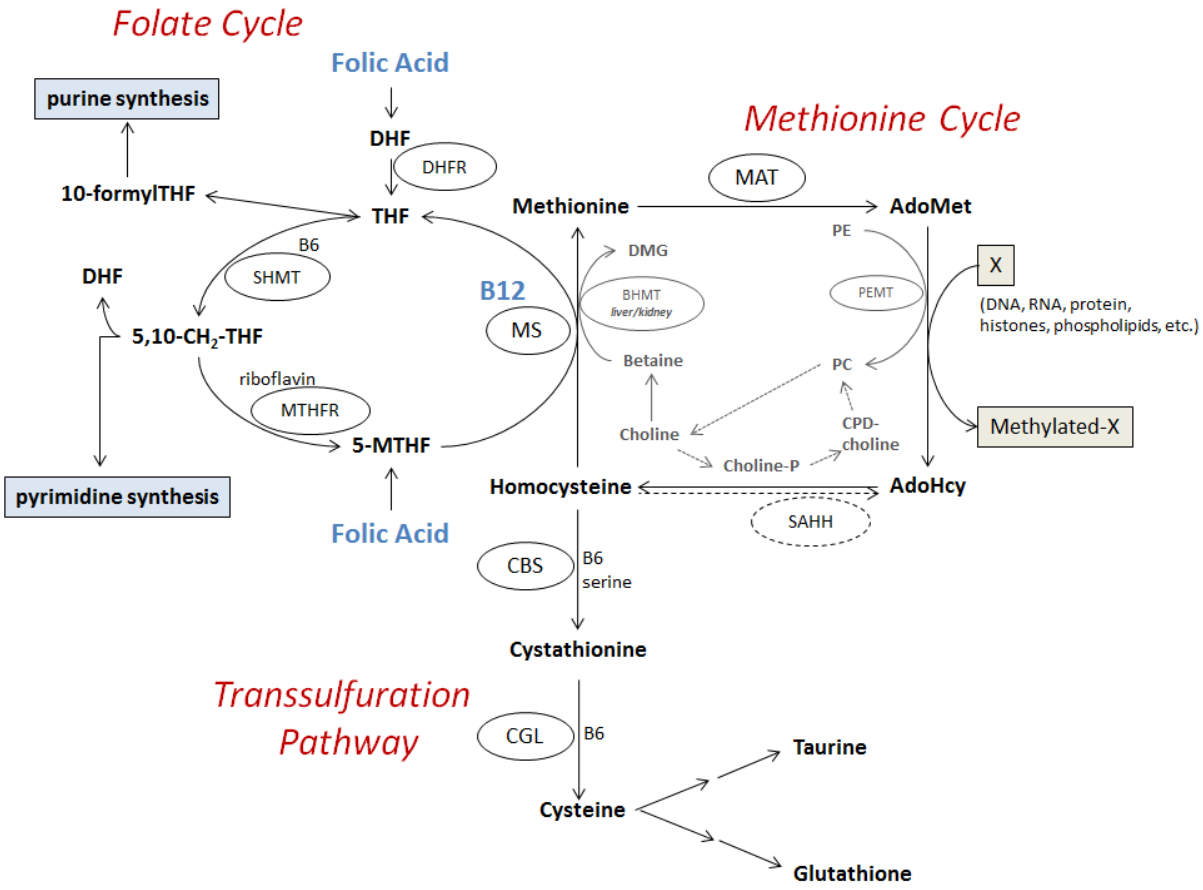

FIGURE 1: Methyl Nutrients and Metabolic Relationships

AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-beta-synthase; CGL, cystathionine-gamma-lyase; CDP-choline, cytidine diphosphate-choline; choline-P, phosphoryl-choline; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; 10-formylTHF, 10-formyltetrahydrofolate; MAT, methionine adenosyltransferase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; SAHH, *S*-adenosylhomocysteine hydrolase; THF, tetrahydrofolate. Adapted from: Glier *et al*. *Mol Nutr & Food Res* 58:172-182 (2014).

In the folate cycle, dietary folates and folic acid are normally metabolized (reduced and methylated) to 5-MTHF for entry into cells; 5-MTHF then serves as the methyl donor for the remethylation of homocysteine to methionine. This reaction is catalyzed by MS, a B12-dependent enzyme[53]. The donation of a methyl group to homocysteine results in the conversion of 5-MTHF to tetrahydrofolate (THF), which can subsequently be converted to 10-formyl-THF

or 5,10-methylene-THF for purine or pyrimidine synthesis, respectively[53]. In addition, 5,10-methylene-THF can be regenerated to 5-MTHF through the enzymatic activity of methylenetetrahydrofolate reductase (MTHFR) for re-entry into the methionine cycle.

The folate cycle intersects the methionine cycle at the remethylation of homocysteine to methionine by MS. Methionine adenosyltransferase (MAT) catalyzes the conversion of methionine to *S*-adenosylmethionine (AdoMet), a methyl donor in many cellular reactions involving DNA, RNA, histones, proteins, phospholipids, and other molecules. The product of methylation reactions, *S*-adenosylhomocysteine (AdoHcy), can be hydrolyzed back to homocysteine by AdoHcy hydrolase (SAHH). Homocysteine can re-enter the methionine cycle or enter the transsulfuration pathway where it is irreversibly degraded to cysteine. Cysteine is used for protein synthesis or for the generation of other metabolites, such as glutathione and taurine[59,60]. In certain tissues, such as the liver and kidney, homocysteine can be remethylated to methionine by betaine-homocysteine methyltransferase (BHMT) using betaine instead of 5-MTHF as the methyl donor[61]. Betaine can be obtained through diet or synthesized from choline, a dietary essential nutrient[62].

The Methyl Folate Trap

In vitamin B12 deficiency, folate accumulates and becomes "trapped" as 5-MTHF because MS is unable to function properly to transfer methyl groups from 5-MTHF for the remethylation of homocysteine to methionine. In turn, purine and pyrimidine synthesis become impaired[63,64]. However, when folic acid intake is high, it can serve as a substrate for dihydrofolate reductase (DHFR) for conversion to THF, still allowing participation in purine and pyrimidine synthesis. Consequently, despite sub-optimal B12 levels, high folic acid intakes can

mask the hematological signs of B12 deficiency, thus delaying the diagnosis of B12 deficiency and allowing the irreversible neurological implications of deficiency to progress[65].

1.6 Interactions between Methyl Nutrients and Lipid Metabolism

Lipid Metabolism

Lipids are a chemically diverse group of compounds that are involved in a wide array of biological functions, including maintenance of cellular structure, biological signaling, and energy storage[66]. The liver is a key metabolic organ, central to lipid metabolism, because it acts as a hub to various other metabolic tissues including adipose tissue and skeletal muscle. Thus, disordered lipid metabolism is a principal component of the etiology of obesity. Excess fat accumulation in the liver typically results from disturbed lipid metabolism pathways including increased hepatic synthesis, increased delivery of NEFAs to the liver (which get stored as TGs), impaired assembly and secretion of very low-density lipoproteins (VLDLs), and decreased β-oxidation of fatty acids[67].

When nutrients are abundant, such as in the postprandial state, the liver uses glucose as fuel and converts excess energy into glycogen or into fatty acids, which are incorporated into TGs for storage. Nutrient-rich blood drains from the abdominal portion of the digestive tract through the hepatic portal vein and directly into the liver for processing. In the liver, fatty acids are esterified with glycerol-3-phosphate to form TGs or with cholesterol to form cholesterol esters[68]. Triglycerides and cholesterol esters may be stored in lipid droplets in hepatocytes or packed into VLDLs for mobilization and delivery to adipose tissue or other extrahepatic tissues. Additionally, NEFAs can be incorporated into phospholipids, which are an essential structural component of cell membranes and lipoproteins[66]. In the fasted state, hormone-sensitive lipase

(HSL) hydrolyzes TGs stored in adipose tissue, and lipoprotein lipase (LPL) hydrolyzes TGs stored in lipoproteins[69]; NEFAs are released into the circulation, and can then be oxidized in the mitochondria of hepatocytes for energy utilization[70].

Methyl Nutrients and Phosphatidylcholine Synthesis

Lipid metabolism is linked to one-carbon metabolism through the methionine cycle[58]. As illustrated in Figure 1, the methionine cycle is responsible for the synthesis of AdoMet, an important methyl donor for the methylation of many different macromolecules, and for the synthesis of hormones and other small molecules including carnitine and phosphatidylcholine (PC)[71]. There are two pathways in which PC can be synthesized in the liver: the PEMT pathway (synthesizes 30% of liver PC), and the CDP-choline pathway (synthesizes 70% of liver PC)[72]. Both pathways are linked to the methionine cycle. In the PEMT pathway, three AdoMet molecules are used for the methylation of phosphatidylethanolamine (PE) to form PC, a reaction catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT). The CDP-choline pathway involves the conversion of phosphocholine to an intermediate, cytidine diphosphate-choline (CDP-choline), by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15); CDP-choline is then converted to PC by cholinephosphotransferase (EC 2.7.8.2)[62]. The CDP-choline pathway requires pre-existing choline, derived from the PEMT pathway or obtained exogenously through the diet. Alternatively, choline can be oxidized to betaine, which can then serve as a methyl donor for the remethylation of homocysteine to methionine via BHMT[73].

Phosphatidylcholine is required for the synthesis and secretion of lipoproteins, and rodent studies have reported that alterations in PC synthesis affect hepatic lipid storage and secretion[74,75]. Lipoproteins are important for the transport of lipids to and from various tissues for

storage or energy utilization[66]. Thus, given that PC synthesized in the liver is required for the generation of lipoproteins, PC has an indirect role in the delivery of lipids, such as long-chain polyunsaturated fatty acids (LCPUFAs) to other tissues[58]. Further, PC derived from the PEMT pathway contains higher levels of LCPUFAs, such as arachidonic acid (AA, 20:4*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3). Alternatively, PC synthesized via the CDP-choline pathway contains more monounsaturated fatty acids, such as oleic acid (18:*1n*-9), and saturated fatty acids, such as stearic acid (18:0)[72].

Rodent Studies of Methyl Nutrients and Lipid Metabolism

Rodent studies have reported that mice with disturbances in one-carbon metabolism have alterations in hepatic lipid metabolism. Devlin *et al*. reported that mice with disordered methyl metabolism and hyperhomocysteinemia (HHcy), caused by a heterozygous targeted disruption of the gene encoding cystathionine-beta-synthase (*Cbs*$^{+/-}$ mice), have changes in methylation and expression of *Fads2* (encodes Δ-6 desaturase; also known as FADS2) in the liver. This is accompanied by lower PEMT activity, higher PE/PC ratios, alterations in the fatty acid composition of liver PE and PC, and impaired synthesis of LCPUFAs[73]. A further study by Ghosh *et al*. reported that *Cbs*$^{+/-}$ mice with diet-induced obesity have glucose intolerance, increased oxidative stress, higher cardiac TG concentrations, and markers of apoptosis in the heart compared to *Cbs*$^{+/+}$ mice[76]. Finally, Christensen *et al*. reported that disturbances in methyl nutrients, in this case folate deficiency, results in hepatic TG accumulation and hepatic steatosis in rodents[77,78]. Interestingly, this study reported that while male offspring were mild to moderately steatotic after exposure to folate-deficient diets, females were not steatotic,

suggesting sex-specific differences in hepatic lipid metabolism by modulations in one-carbon metabolism[78].

Methyl Nutrients and β-Oxidation of Fatty Acids

The liver is the major site for β-oxidation of fatty acids. Lipids are mobilized from stores during fasted states when energy production is required. Carnitine palmitoyltransferase 1A (CPT1A) is the rate-limiting enzyme for β-oxidation of fatty acids. It shuttles long-chain fatty acids (14 or more carbons) into the mitochondria to be oxidized[70]. During β-oxidation, fatty acids undergo successive removal of two-carbon units in the form of acetyl-CoA. The resulting acetyl-CoA molecules can then enter the citric acid cycle and ultimately lead to energy production. In β-oxidation of odd-chain fatty acids, the final cycle yields a three-carbon propionyl-CoA which must be converted to succinyl-CoA by an additional reaction prior to entry into the citric acid cycle[66]. Interestingly, this additional step in β-oxidation links back to methyl metabolism. As previously mentioned in Chapter 1.5, vitamin B12 is an important cofactor for methylmalonyl-CoA mutase (MCM), the enzyme that catalyzes the conversion of propionyl-CoA to succinyl-CoA. In vitamin B12 deficiency, MCM is unable to function properly and this results in an increase in methylmalonyl-CoA (intermediate between propionyl-CoA and succinyl-CoA), which can negatively feedback on CPT1A activity. Additionally, methylmalonyl-CoA can build up as methylmalonic acid (MMA) which can be damaging to tissues when accumulated.

1.7 Folic Acid Fortification

In 1976, Smithells *et al*. reported that lower levels of certain micronutrients, folate in particular, in the serum of women during the first trimester of pregnancy were associated with

neural tube defects (NTDs)[79]. This finding was the foundation for a series of small trials (n=200, n=454, n=198, n=226) in women who had previously been affected by an NTD pregnancy[80–83]. These small trials investigated periconceptional supplementation with folic acid and found that after administration of 400μg to 5.0mg folic acid per day, the recurrence risk of NTDs was significantly lower in women who had previously experienced an NTD-affected pregnancy. Subsequently, the Medical Research Council (MRC) Vitamin Study Research Group conducted a large, double-blind, placebo-controlled, randomized control trial in 1,817 women who had previously been affected by an NTD pregnancy. This study was able to demonstrate a 72% reduction in recurrent risk of NTDs after periconceptional supplementation with 4.0mg folic acid per day[84]. A similar large randomized control trial in Hungarian women reported a 93% reduction in the first occurrence of NTDs in women after daily supplementation with 800μg folic acid[85]. Furthermore, a public health campaign conducted in China from 1993 to 1995 demonstrated that periconceptional supplementation with 400μg folic acid per day reduced the risk of NTDs in regions with a high prevalence of these defects[86]. Growing evidence from these studies that sub-optimal folate status during pregnancy is associated with NTDs led to the recommendation that women of childbearing age consume 400μg folic acid per day to reduce their risk of a NTD-affected pregnancy[87]. Ensuring adequate folic acid intakes in women of childbearing age prior to conception is of utmost importance because the neural tube closes early during pregnancy (day 28 post-conception), and at this early stage a woman may be unaware of her pregnancy.

In 1998, Canada and the United States implemented mandatory folic acid fortification of cereal grain products for the prevention of NTDs [88,89]. While this policy has successfully reduced the incidence of NTDs by an estimated 46% in Canada, it has subsequently increased serum

folate concentrations in the general population by greater than twofold[90]. Currently, folate deficiency in Canada is estimated to be found in less than 1% of the general population; however, 40% of the population have erythrocyte folate concentrations (an indicator of long-term status) greater than 1,360nmol/L[91.] This is concerning because approximately one in 20 Canadians are estimated to be deficient in B12, and as previously mentioned, high folic acid intakes may mask the hematological signs of B12 deficiency[92,93]. Furthermore, when high doses of folic acid are consumed, the excess appears in the peripheral circulation in its unmetabolized form. The implications of circulating unmetabolized folic acid on health outcomes are not fully understood. Several groups have reported circulating unmetabolized folic acid in populations with mandatory folic acid fortification [94,95]. Circulating unmetabolized folic acid has also been reported in populations without mandatory fortification programs[96], and in serum and cord blood from pregnant women taking supplements[97].

1.8 One-Carbon Metabolism and Epigenetic Mechanisms

The molecular mechanisms underlying developmental programming are poorly understood, but may involve the interplay between perinatal environmental factors and epigenetic processes. Epigenetics can be defined as any modification of the genome or of gene expression that does not result from alteration in DNA nucleotide sequence. A number of epigenetic mechanisms have been identified; however, the three major processes of epigenetic modifications are direct DNA methylation, chromatin (histone) modifications, and non-coding RNAs (ncRNAs)[98]. These epigenetic processes have been targeted as potential mechanisms underlying developmental programming because patterns of DNA methylation and chromatin modifications can be inherited and they are particularly vulnerable to environmental stimuli

during development[99–102]. Environmental stimuli can include things like exposure to chemicals, pharmaceuticals or drugs, aging, diet, and other factors.

DNA methylation is quite stable[103], making it a feasible epigenetic marker to study. DNA methylation involves the covalent addition of a methyl group to the 5' position of cytosine in the context of a cytosine-guanine (CpG) dinucleotide. Most gene promoters have high CpG content and long stretches of CpGs, known as CpG islands[98]. In general, promoter methylation of CpG islands usually silences gene expression. A prime example of this is seen in the context of imprinted genes, where only one allele is expressed and the other is completely silenced by methylation depending on the parent from which allele originated[104].

DNA methylation undergoes a unique process of genome-wide demethylation and remethylation of the inherited parental genomes directly following fertilization and in the early stages of embryonic development[99]. In accordance with this phenomenon, the process of epigenetic reprogramming makes the developing embryo vulnerable to environmental factors that may modify the epigenetic patterns, such as maternal nutrition. Folate is often linked to epigenetic mechanisms because of its role in generating AdoMet (Figure 1), the primary methyl donor for many methyl acceptors including DNA. It has been reported that diet-induced changes in the ratio of AdoMet to AdoHcy are associated with changes in gene-specific DNA methylation patterns and gene expression in a tissue-specific manner[105]. Therefore, it is conceivable that periconceptional maternal folate levels may alter DNA methylation patterns established *in utero* that are vital for proper development. This could potentially impact later health outcomes in the offspring[53].

1.9 Epidemiological Studies of Maternal B-Vitamin Status and Offspring Health

Numerous human population-based studies have highlighted potential concerns about maternal B-vitamin status during pregnancy and the effects on offspring cardiometabolic health. Much of this work has centered in regions of the world, such as India and Nepal, where vitamin B12 deficiency is quite high, likely in part due to the prevalence of vegetarianism. It was reported that in some regions of India, 70% of adults have indicators of poor B12 status[106,107]. Moreover, it has been reported that there is an escalating epidemic of T2D and CVD in these regions[108]. Comparative studies have reported that adult Indians have a higher percentage of body fat for a given BMI, with more central adiposity and insulin resistance and a lower muscle mass, than Europeans[109]. There is little information on the origins of the adiposity and insulin resistant phenotype prevalent in these populations; however, it is quite possible that maternal factors, such as perinatal nutrition, are at play.

The Pune Maternal Nutrition Study in India reported that higher maternal erythrocyte folate concentrations (>1,144nmol/L) at 28 weeks of gestation were associated with greater adiposity in the children at age 6 years. Additionally, children that were born to women with adequate folate but poor serum B12 status (<150pmol/L) during pregnancy had greater insulin resistance (calculated using the homeostatic model assessment of insulin resistance [HOMA-IR]) at age 6. Children born to mothers who had a combination of high folate and low serum B12 concentrations (<114pmol/L) during pregnancy were the most insulin resistant[110]. Another study, the Parthenon Study in Mysore, India, aimed to replicate the findings from the Pune Maternal Nutrition Study by examining whether low maternal B12 and high plasma folate concentrations predicted higher insulin resistance and other cardiometabolic risk factors in children at 5, 9.5, and 13.5 years of age. They found that high maternal plasma folate concentrations were

associated with higher insulin resistance (HOMA-IR) in children at 9.5 and 13.5 years of age[111]. Finally, a study in Nepal reported that children whose mothers were deficient in vitamin B12 (serum B12 <148pmol/L) during early pregnancy had a 26.7% greater HOMA-IR at age 6-8 years; however, no association was found with maternal folate status in this cohort[112].

The effect of maternal B-vitamin status during pregnancy on offspring health has also been studied in other parts of the world. The Boston Birth Cohort (BBC) reported that low maternal plasma folate concentrations (<20.4nmol/L) during pregnancy were associated with an increased risk for overweight or obesity in children up 9 years of age[113]. Interestingly, this study also reported that the highest risk for overweight or obesity was found among children of obese mothers with folate concentrations in the lowest quartile (Q1, plasma folate ranging from 6.6-<20.4nmol/L) compared to children from obese mothers with folate concentrations in Q2-4 (plasma folate >20.5nmol/L)[113].

1.10 Rodent Studies of Maternal B-Vitamin Status and Offspring Health

The epidemiological evidence that maternal B-vitamin status affects offspring health has prompted further investigation in animal models. A study of maternal B-vitamin restriction in Wistar rats reported that male offspring from dams fed diets that were deficient in both folate and B12 during pregnancy have greater adiposity, higher plasma cholesterol and TGs, higher adipose tissue IL-6 and TNF-α, higher activity of hepatic lipogenic enzymes, and higher plasma cortisol levels at 12 months of age[114]. However, it should be noted that the offspring in this study were kept on the vitamin-deficient diets from weaning to 12 months; thus, it is difficult to tease out the effects of maternal B-vitamin status from the effects of the offspring diets.

Maternal B-vitamin status has been implicated in other aspects of offspring health outside the context of metabolism. For instance, Roman-Garcia *et al*. recently reported that maternal B12 deficiency results in impaired bone health in offspring[115]. In this study, the authors generated a mouse genetic model of B12 deficiency by knocking out the gene encoding gastric intrinsic factor (GIF), the glycoprotein required for absorption of B12 in the distal ileum[53]. Offspring from female *Gif*$^{-/-}$ mice (B12-deficient) were shown to have reduced bone mass, skeletal growth retardation, reduced serum osteocalcin, and increased bone fracture risk. It was determined that the exposure to maternal B12 deficiency during development resulted in this phenotype through disruptions in the offspring growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis.

Previous Findings in the Devlin Lab

The Devlin Lab previously established a mouse model to further understand the molecular and physiological effects of developmental exposure to maternal folate/vitamin B12 imbalance. Female mice were fed diets of supplemental folic acid, with or without B12, before breeding and through pregnancy and lactation, and programming of adiposity and glucose homeostasis was assessed in their offspring. As expected, dams fed the B12-deficient diet had lower serum B12 concentrations than dams fed diets containing B12. At the time of weaning of the offspring, no differences were observed in overall litter size or in the number of male and female pups per litter[1]. This is important to note because of known effects of variations in litter size on offspring growth and development in rodents[116]. Furthermore, no effect of maternal diet was observed on maternal body weight during breeding, pregnancy, or lactation[1].

The offspring were weaned onto diets containing identical amounts of folic acid and B12. No differences in serum total folate concentrations observed in the adult offspring mice[1,3]. Male

offspring fed the postweaning control diet from dams supplemented with folic acid had lower serum B12 concentrations, higher liver AdoMet concentrations, and no differences in liver AdoHcy concentrations. This was accompanied by differential expression of enzymes required for methyl metabolism in the liver in adult male offspring fed either postweaning diet[1]. Differential expression of methyl metabolism enzymes was also observed in the liver of adult female offspring; however, contrary to what was observed in males, no differences in serum B12, liver AdoMet, or liver AdoHcy concentrations were observed in female offspring[3].

Interestingly, the effects on adiposity and glucose homeostasis observed in offspring were sex-specific. Folic acid supplementation during pregnancy resulted in larger visceral (gonadal and retroperitoneal) and subcutaneous (inguinal) adipose tissue depots, fasting hyperglycemia, glucose intolerance, and lower β-cell mass in adult female offspring[3]. In contrast, adult male offspring had smaller visceral and subcutaneous adipose tissue depots, no changes in glucose homeostasis, and lower expression of NADPH oxidase 2 (NOX2) in aorta (responsible for generation of reactive oxygen species)[1]. Together, these findings suggest that developmental exposure to maternal supplemental folic acid adversely programs adiposity and glucose homeostasis in female offspring, whereas it promotes a lean phenotype and reduced oxidative stress in male offspring.

CHAPTER 2: Rationale and Thesis Objective

Epidemiological and rodent studies have demonstrated that maternal B-vitamin status programs sex-specific differences in adiposity and glucose homeostasis in offspring, although the underlying mechanisms are not understood. Disturbances in methyl metabolism alter hepatic lipid metabolism in mice[73,76,78]. However, it is not known whether this occurs in the context of developmental programming; that is, if alterations in hepatic lipid metabolism occur in offspring if the mother has disturbed methyl metabolism. Further, maternal B12 deficiency disrupts the GH/IGF-1 axis in offspring[115]. Finally, we do not fully understand the consequences of circulating unmetabolized folic acid on health outcomes.

Taking this information into consideration, the overall **objective** of my thesis is to determine how maternal B-vitamin status during pregnancy programs offspring adiposity and glucose homeostasis. To accomplish this objective, I addressed the following specific aims:

AIM 1: To determine the direct effects of folic acid supplementation on adipocyte energy metabolism.

AIM 2: To determine if maternal B-vitamin status during pregnancy programs offspring adiposity through disturbances in the GH/IGF-1 axis.

AIM 3: To determine if maternal B-vitamin status during pregnancy programs offspring adiposity through altered adipose tissue and hepatic lipid metabolism.

CHAPTER 3: Effects of Folic Acid Supplementation on Adipocyte Energy Metabolism

AIM 1: To determine the direct effects of folic acid supplementation on adipocyte energy metabolism. This aim will be addressed in two parts:

PART A: To determine if folic acid supplementation directly affects adipose tissue distribution in mice.

PART B: To determine if folic acid supplementation directly alters energy metabolism of 3T3-L1 adipocytes *in vitro*.

PART A: Body Fat Distribution in Female Mice Supplemented with Folic Acid

3.1 Rationale

As discussed in the literature review, it is recommended that women of childbearing age consume 400μg folic acid per day to reduce their risk of a NTD-affected pregnancy[87]. In 1998, Canada and the United States mandated that grain products be fortified with folic acid to aid in the prevention of NTDs [88,89]. Consequently, folate deficiency is rare in Canada (found in less than 1% of the general population); however, 40% of the population have erythrocyte folate concentrations greater than 1,360nmol/L[91]. In the 2007-2009 CHMS it was reported that 25% of women of childbearing age used dietary folic acid supplements[117]. Furthermore, data from the 2003-2006 National Health and Nutrition Examination Survey (NHANES) reported that more than one third of children 1 to 13 years of age in the United States used dietary folic acid supplements, and that the majority of these children exceeded the upper limit (UL) of intake of folic acid (set at 1,000μg/day) by greater than 50%[118]. Similarly, data from the 2003-2006

NHANES reported that 5% of adults >50 years of age also exceeded the UL of intake of folic acid[119].

When higher intakes of folic acid are consumed, some appears in peripheral circulation unmetabolized[95–97]. Circulating unmetabolized folic acid has been reported in populations with mandatory folic acid fortification [94,95], in populations without mandatory fortification programs[96], and in serum and cord blood from pregnant women taking supplements[97]. Little is known regarding consequences of circulating unmetabolized folic acid on specific tissue functions and on overall health outcomes. However, Kelly *et al*. recently reported that rats fed diets of excess folic acid had increased weight gain, larger adipose tissue depots, and markers of adipose tissue inflammation[120]. Further studies are warranted to elucidate the consequences of higher intakes of folic acid and unmetabolized folic acid in circulation. In Aim 1 Part A, I sought to investigate the direct effects of folic acid supplementation on adipose tissue distribution in mice.

3.2 Methods

Mouse Cohort

The animal study design, animal care and breeding, and tissue collection for this project were conducted by Rika Aleliunas[1]. Female and male C57BL/6J mice were purchased from the UBC Centre for Disease Modeling and Charles River, respectively. The mice were housed in the Animal Facility at the BC Children's Hospital Research Institute and fed standard laboratory chow (5058-PicoLab Mouse Diet 20, Lab Diet®, PMI Nutrition International). All mice had *ad libitum* access to food and water and were housed under a standard 12-hour light-dark cycle. The mice were acclimated for one week prior to breeding and virgin dams were then bred with age-matched males at 6 weeks of age.

After weaning of the first litter, the same dams were then assigned to one of the following diets: maternal control (M-CON; adequate folic acid/adequate vitamin B12), supplemental folic acid/no vitamin B12 (SFA-B12), or supplemental folic acid/adequate vitamin B12 (SFA+B12). The maternal diets were identical in composition except for folic acid and vitamin B12 content. The M-CON diet contained 2mg of folic acid per kg of diet, as recommended for the AIN-93G diet[121]. The SFA-B12 and SFA+B12 diets contained 10mg of folic acid per kg of diet, which was 5 times more than the M-CON diet. The M-CON and SFA+B12 diets contained 50μg of vitamin B12 per kg of diet, which is 2 times the amount recommended for the AIN-93G diet[121]. The SFA-B12 diet contained no vitamin B12. All animal diets met requirements set by the American Institute of Nutrition and the National Research Council[121,122].

The dams were fed the maternal diets six weeks before breeding of their second litter and throughout mating, pregnancy, and lactation. One week after weaning of the offspring, the dams were anesthetized with isoflurane, sacrificed by cervical dislocation, and tissue was harvested, flash frozen in liquid nitrogen, and stored at -80°C. The animal breeding scheme and maternal diet compositions are depicted in Figure 2 and Table 1, respectively. Details regarding the offspring postweaning diets are described in Chapter 4.2.

Statistical Analyses

Statistical analyses of results were tested by a one-way analysis of variance (ANOVA) with maternal diet as the independent variable. The least significant difference test for multiple comparisons was used to compare findings between groups (M-CON, SFA-B12, and SFA+B12). Data were normally distributed and statistical analyses were performed using SPSS Statistics

software (version 12) with $p \leq 0.05$ considered significant. Experimental results were graphed on GraphPad Prism 5 software and are presented as mean ± standard deviation (SD).

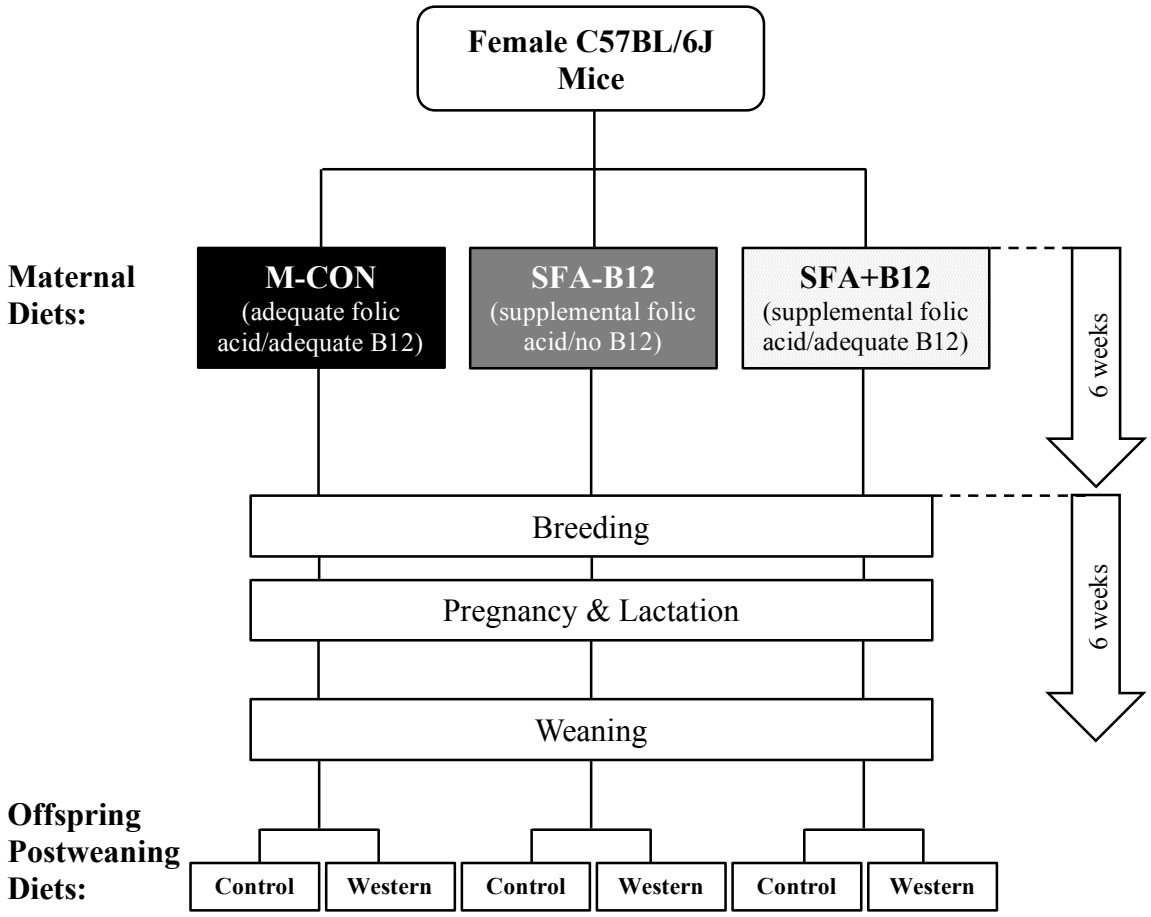

FIGURE 2: Animal Diets and Breeding Scheme
Female C57BL/6J mice were fed one of three diets, 6 weeks prior to breeding and throughout mating, pregnancy, and lactation. The maternal diets were as follows: maternal control (M-CON; adequate folic acid/adequate vitamin B12), supplemental folic acid/no vitamin B12 (SFA-B12), or supplemental folic acid/adequate vitamin B12 (SFA+B12).

TABLE 1: Maternal Diet Compositions

	Maternal Diets		
Nutrient	M-CON	SFA-B12	SFA+B12
Folic Acid (mg/kg diet)	2.0	10.0	10.0
Vitamin B12 (µg/kg diet)	50.0	0.0	50.0
Carbohydrate (% energy)	64%	64%	64%
Protein (% energy)	20%	20%	20%
Fat (% energy)	16%	16%	16%

All diets contained 3g/kg cysteine and 50g/kg pectin. All other dietary components were consistent between groups. Source of protein is vitamin-free casein; source of fat is soybean oil.

3.3 Results

Body Fat Distribution in Female Mice Supplemented with Folic Acid

Dams that were fed the SFA-B12 diet had greater ($p \leq 0.05$) total body weight than those fed the M-CON diet (Figure 3A). Given that body fat distribution rather than total body weight is more indicative of metabolic health, individual adipose tissue depots were harvested and weighed. Dams fed the SFA-B12 and SFA+B12 diets had larger visceral (gonadal and retroperitoneal) fat depots (Figures 3B & C); although, these differences did not reach statistical significance. Additionally, dams that were fed the SFA-B12 diet had larger ($p \leq 0.05$) subcutaneous (inguinal) fat depots than dams fed the M-CON diet (Figure 3D).

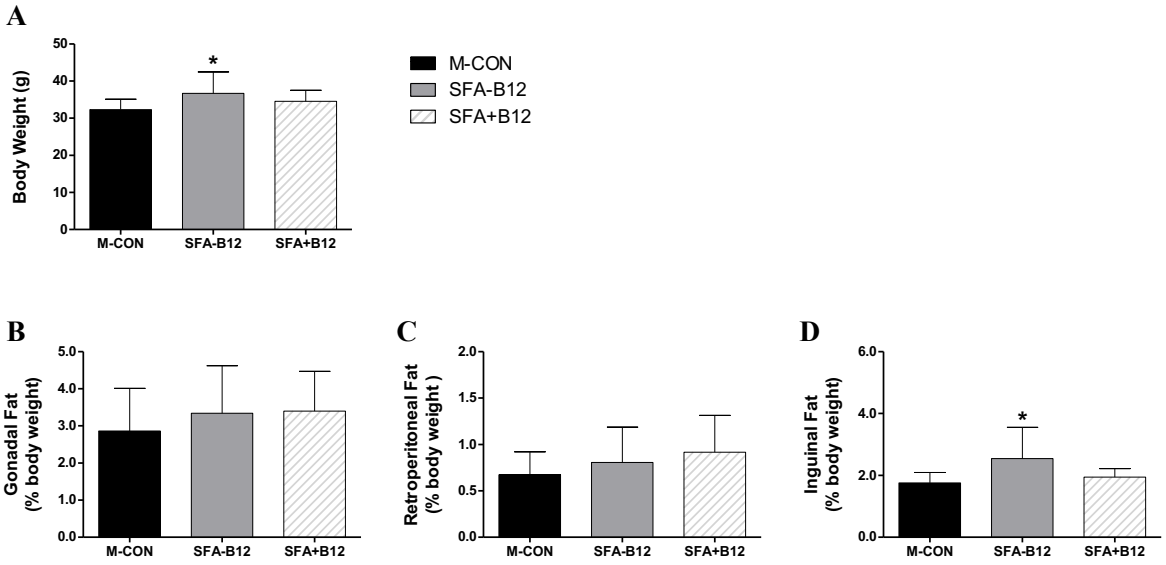

FIGURE 3: Body Weight and Adiposity in Female Mice Supplemented with Folic Acid
A. Total body weight in female mice at 20 weeks postweaning. **B.** Gonadal fat depot. **C.** Retroperitoneal fat depot. **D.** Inguinal (subcutaneous) fat depot. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=7-8; *$p \leq 0.05$ vs. M-CON.

3.4 Summary of Findings

My observations in Aim 1A indicate that folic acid supplementation results in alterations in adipose tissue distribution and adiposity in female mice. This raises the possibility that higher intakes of folic acid could lead to greater adipose tissue deposition. *In vitro* studies have reported more TG accumulation in 3T3-L1 adipocytes treated with excess folic acid concentrations[120]. It is possible that supplemental folic acid may disturb energy metabolism in adipocytes, promoting more storage of nutrients, and ultimately leading to larger adipose tissue depots. In Aim 1B, I sought to investigate how exposure to different concentrations of folic acid may alter energy metabolism in 3T3-L1 adipocytes. Furthermore, I investigated whether treating adipocytes with different forms of folate would elicit different effects on energy metabolism.

PART B: Energy Metabolism in 3T3-L1 Adipocytes Supplemented with Folic Acid

3.5 Rationale

In Aim 1 Part A, I determined that female mice fed diets supplemented with folic acid for 13 weeks had greater total body weight and larger adipose tissue depots than mice fed the control diet (Figure 3). Building from these findings, I sought to investigate whether folic acid supplementation alters adipocyte energy metabolism, possibly contributing to the larger adipose tissue depots I observed in the mice. I conducted *in vitro* experiments to assess how exposure to two forms of folate (folic acid and 5-MTHF) affects energy metabolism in 3T3-L1 adipocytes. Folic acid in fortified foods and supplements is normally metabolized to 5-MTHF in the small intestine; however, when high doses of folic acid are consumed, some appears in peripheral circulation unmetabolized[95–97]. I chose to treat the 3T3-L1 adipocytes with the following concentrations of either folic acid or 5-MTHF: 0.005 (trace concentration in 10% FBS), 0.08, 0.16, 0.8, or 1.6μM. These concentrations were selected because serum total folate concentrations in control mice are approximately 0.16μM[1]. Accordingly, treatments were selected to be the trace concentration (0.005 μM) and half (0.08μM) the normal circulating concentration to assess how lower folate concentrations may affect energy metabolism; or five times (0.8μM) and ten times (1.6μM) the normal circulating concentration to assess how higher folate concentrations may affect energy metabolism in adipocytes. In comparison to humans, women at risk for an NTD-affected pregnancy may be recommended to consume up to 10 times (4-5mg/day) the recommended 400μg/day supplement for a healthy pregnancy[54,1]

The predominant physiological function of mitochondria is the generation of energy (in the form of ATP) from nutrients[123]. Given mitochondria are major producers of energy in the cell, impaired mitochondrial respiration leading to deficits in energy production readily impact

other cellular processes. It is evident that dysfunctional mitochondria are implicated in many chronic pathologies, including CVD, T2D, neurodegeneration, and cancer[124]. Extracellular flux methods have recently been established to monitor changes in oxygen concentration and pH in cultures of live adherent cells in multiple sample wells simultaneously. This technique provides information on bioenergetic functions of intact cells maintained in a physiologically relevant context. Using the Seahorse XFe96 Extracellular Flux Analyzer and the Seahorse XF Mito Stress Test, I assessed mitochondrial respiration and glycolysis in live 3T3-L1 adipocytes in real-time under different conditions of folic acid or 5-MTHF to determine if energy metabolism is affected by these two forms of folate.

3.6 Methods

The mouse embryonic 3T3-L1 cell line has a fibroblast-like morphology, but under appropriate conditions, can undergo a pre-adipose to adipose-like conversion by rapidly accumulating lipids[125,126]. This cell line is commonly used for studies on adipose tissue physiology. For this thesis, differentiated 3T3-L1 adipocytes were cultured in various concentrations of folic acid or 5-MTHF and analyzed for alterations in energy metabolism by assessing mitochondrial respiration and glycolytic rates using the Seahorse XF Mito Stress Test (Agilent Technologies). The complete 3T3-L1 adipocyte cell culture experimental design is summarized in Figure 4.

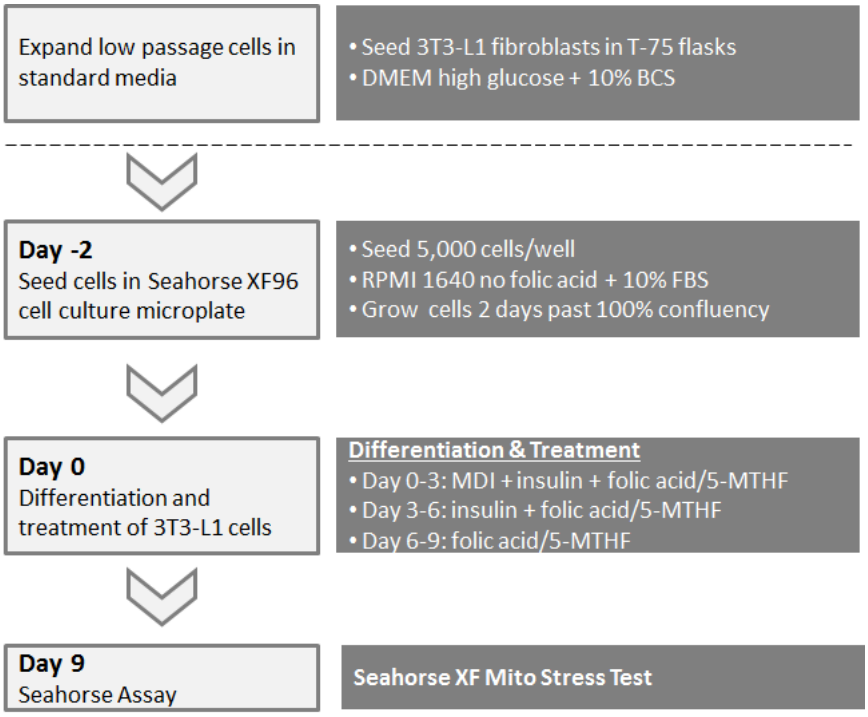

FIGURE 4: 3T3-L1 Adipocyte Cell Culture Experimental Design
The 3T3-L1 mouse embryonic fibroblast cell line was expanded in complete DMEM high glucose. The 3T3-L1 cells were switched to folic acid-free RPMI 1640 media; differentiation and folic acid/5-MTHF treatment of the 3T3-L1 cells was initiated 2 days after 100% confluency. After the 9-day differentiation and treatment regiment, energy metabolism was assessed in the 3T3-L1 adipocytes using the Seahorse XFe96 Extracellular Flux Analyzer and the Seahorse XF Mito Stress Test.

Expansion of 3T3-L1 Fibroblast Population

Cryopreserved 3T3-L1 murine embryonic fibroblasts (CL-173™, American Type Culture Collection®) were purchased at passage 2. Cells were immediately seeded in a T-25 flask with vent cap (Corning®) and maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5g/mL) (Gibco®), supplemented with heat-inactivated 10% bovine calf serum (Gibco®) and 1% penicillin/streptomycin (Sigma-Aldrich®). During this time, the cells were fed with fresh complete DMEM high glucose medium every two days. Cells reached 70-80% confluency in 2-3 days and were split into one T-75 flask with vent cap and three cryovials with

Recovery™ Cell Culture Freezing Medium (Gibco®) for storage in liquid nitrogen. Expansion of the 3T3-L1 fibroblast population continued until passage 12, allowing for storage of 26 cryovials of cells at different passage numbers archived in liquid nitrogen.

Cell cultures intended for Oil Red O staining were seeded in 12-well tissue culture plates (Falcon™) at a density of $8x10^4$ cells/well. Cell cultures intended for the Seahorse XF Mito Stress Test were seeded in Seahorse XF96 Cell Culture Microplates (Agilent Technologies) at a density of $5x10^3$ cells/well. In preparation for 3T3-L1 fibroblast differentiation into mature adipocytes, all tissue cultureware (12-well plates and Seahorse XF96 Cell Culture Microplates) were coated with poly-D-lysine (Sigma-Aldrich®) to optimize and maintain adherence of differentiating 3T3-L1 cells. Cultureware surfaces were incubated for 5 minutes with 0.5-1.0mL of poly-D-lysine solution per $25cm^2$. The poly-D-lysine was then aspirated off and the cultureware surfaces were rinsed with sterile tissue culture-grade dH_2O. The cultureware was left to dry for 2 hours before seeding cells in RPMI 1640-no folic acid (Gibco®), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown two days past 100% confluency before differentiation into 3T3-L1 adipocytes was induced.

The recommended growth medium for 3T3-L1 cells most often used in the literature is DMEM[127]. However, because the objective of these experiments was to determine the effects of folic acid/5-MTHF supplementation on adipocyte energy metabolism, DMEM was substituted instead with commercially-available folic acid-free medium (RPMI 1640-no folic acid, Gibco®). The composition of RPMI 1640-no folic acid is similar to that of DMEM, and both types of media support growth of a wide array of cell types[128]. The advantage of using RPMI 1640-no folic acid in lieu of DMEM is that it permitted re-supplementation of the media with folic acid or 5-MTHF in controlled and specific concentrations. In addition, it allowed for some cultures to be

grown in trace levels of folic acid, sourced only from 10% FBS supplementation of the complete growth medium. Total folate concentration in FBS was quantified by Benny Chan in Dr. Yvonne Lamers' lab at the University of British Columbia, Vancouver Campus. The complete RPMI 1640-no folic acid medium supplemented with 10% FBS and 1% penicillin/streptomycin contained basal levels of folic acid, at an approximate concentration of 0.005μM.

Differentiation and Treatment of 3T3-L1 Adipocytes

Two days after the 3T3-L1 fibroblasts were 100% confluent, differentiation into mature adipocytes was induced (Day 0) by supplementing the complete RPMI 1640-no folic acid medium with a differentiation cocktail (MDI), consisting of 1μM dexamethasone, 500μM 3-isobutyl-1-methylxanthine (IBMX), 1μM rosiglitazone, and 1.5μg/mL insulin (3T3-L1 Differentiation Kit-DIF001, Sigma-Aldrich®). The cells were cultured with MDI for 3 days (Day 0-3) and then switched to complete RPMI 1640-no folic acid medium with just 1μL/mL insulin for 3 days (Day 3-6). After differentiation, the adipocytes were maintained in complete RPMI 1640-no folic acid medium for an additional 3 days (Day 6-9). During the course of differentiation (Day 0-9), the adipocytes were treated alongside the MDI or insulin with either folic acid or 5-MTHF (Sigma-Aldrich®) at the concentrations previously described in Chapter 3.5.

Confirmation of Differentiation in 3T3-L1 Adipocytes

Differentiation of 3T3-L1 fibroblasts into adipocytes was confirmed by Oil Red O staining to quantify lipid accumulation in the cells[129]. A stock solution of Oil Red O was prepared by dissolving 60mg of Oil Red O (Sigma-Aldrich®) in 20mL of 100% isopropanol. The

stock solution was incubated at room temperature for 20 minutes. To make a working Oil Red O solution, 3 parts of the stock solution was added to 2 parts dH_2O and was left to sit for 10 minutes at room temperature. The working solution was filtered through a 0.2μm syringe filter. Differentiated 3T3-L1 adipocytes were washed twice with 1x phosphate-buffered saline (PBS) and then fixed with 10% formalin for 30 minutes. After cell fixation, the cells were washed twice with dH_2O and incubated for 10 minutes with 60% isopropanol. Upon removal of the isopropanol, Oil Red O working solution was applied to the cells for 10 minutes. The Oil Red O solution was then removed and the cells were washed 5 times with dH_2O to remove excess stain. To elute Oil Red O bound to lipids in the cells, 100% isopropanol was applied to the cells, the eluted stain was collected, and the absorbance was read at 492nm. Confirmation of differentiation by Oil Red O staining of 3T3-L1 adipocytes is shown in Figure 6.

Seahorse XF Mito Stress Test in 3T3-L1 Adipocytes

The Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies) uses a piston to temporarily enclose a small fixed volume of media (forming a transient micro-chamber) above the cells in a 96-well plate. The piston measures fluctuations in extracellular oxygen concentrations (oxygen consumption rate, OCR) and extracellular pH (extracellular acidification rate, ECAR) of live cells in real-time[124]. These OCR and ECAR measurements are indicators of mitochondrial respiration and glycolytic capacity, respectively[130]. Using the Seahorse XFe96 Analyzer, differentiated 3T3-L1 adipocytes were assessed for alterations in energy metabolism in response to various folic acid and 5-MTHF concentrations.

Cells were seeded in a Seahorse XF96 Cell Culture Microplate coated with poly-D-lysine as described above. Differentiation of the 3T3-L1 cells was induced alongside the folic acid/5-

MTHF treatments. One day prior to each Seahorse experiment, a Seahorse XFe96 FluxPak (Agilent Technologies) containing a sensor cartridge and utility plate was prepared. The sensor cartridge was hydrated by filling each well of the 96-well utility plate with 200μL of Seahorse XF Calibrant (Agilent Technologies) and lowering the sensor cartridge onto the utility plate, ensuring full submersion of each sensor probe of the cartridge into the calibrant. The utility plate/cartridge was placed in a 37°C non-CO_2 incubator overnight.

On the day of the Seahorse experiment, assay medium was prepared by supplementation of Seahorse XF Base Medium with 1mM of sodium pyruvate and 10mM of glucose. The pH of the assay medium was adjusted to 7.4 and the medium was warmed to 37°C until use. The Seahorse Mito Stress Test Kit (Agilent Technologies), containing stock oligomycin, carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrazone (FCCP), and rotenone/antimycin A, was removed from storage at -20°C and the compounds were reconstituted with the prepared Seahorse assay medium to obtain the following final concentrations: 100μM oligomycin, 100μM FCCP, and 50μM rotenone/antimycin A. The effects of each drug on the electron transport chain (ETC) and mitochondrial respiration are summarized in Figure 5 and Table 2.

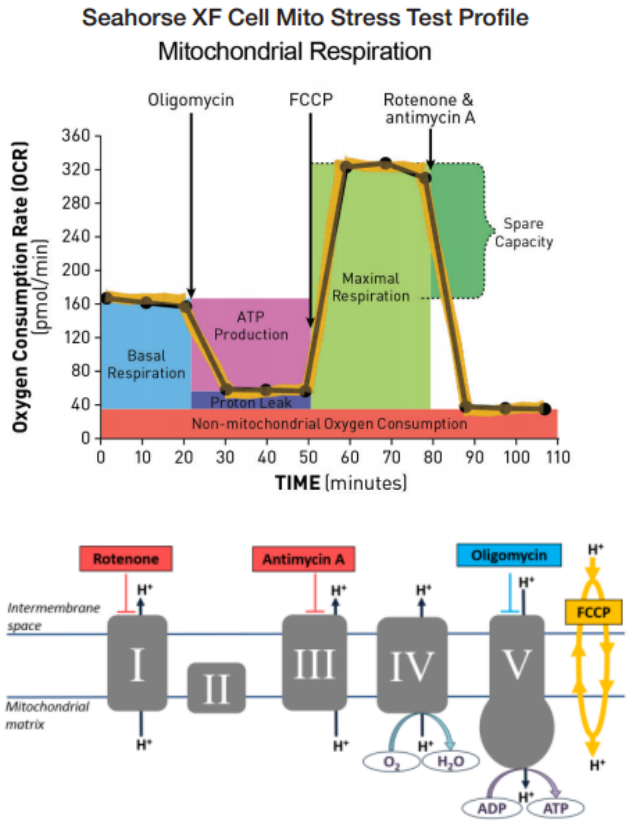

FIGURE 5: Seahorse XF Cell Mito Stress Test
Sequential injections of the Seahorse Mito Stress Test Kit modulators (oligomycin, FCCP, and rotenone/antimycin A) allow for measurement of basal mitochondrial respiration, ATP production, proton leak, maximal respiration, spare respiration capacity, and non-mitochondrial oxygen consumption.
Image obtained from: Seahorse XF Cell Mito Stress Test Kit User Guide.

TABLE 2: Seahorse XF Cell Mito Stress Test Modulators of the ETC

Mitochondrial Respiration Modulator	Electron Transport Chain Target	Effect on Mitochondrial Respiration	Final Concentration (per well)
Oligomycin	ATP Synthase (Complex V)	Decrease	1μM
FCCP	Inner Mitochondrial Membrane	Increase	1μM
Rotenone/Antimycin A	Complexes I and III	Decrease	0.5μM

One hour prior to the Seahorse experiment, the Seahorse XF96 Cell Culture Microplate containing the differentiated 3T3-L1 adipocytes was removed from the incubator and the cell culture growth medium was exchanged with warmed Seahorse assay medium. The plate was placed in the non-CO_2 incubator for 1 hour. During the incubation time, the utility plate/sensor cartridge from the previous day was removed from the non-CO_2 incubator and the utility plate was discarded. Each well of the sensor cartridge was loaded with the three Seahorse Mito Stress Test compounds; port A of the cartridge was loaded with 25μL of oligomycin for a final concentration of 1μM per well, port B was loaded with 25μL of FCCP for a final concentration of 1μM per well, port C was loaded with 25μL of rotenone/antimycin A for a final concentration of 0.5μM per well, and port D was left empty. The cartridge was calibrated in the Seahorse XFe96 Analyzer for 20 minutes. Following calibration, the Seahorse XF96 Cell Culture Microplate containing the 3T3-L1 adipocytes was removed from the non-CO_2 incubator and loaded into the Seahorse XFe96 Analyzer. Each Seahorse experiment was designed on Wave 2.3.0 software. Data was normalized to protein concentration per well, determined by the Bradford Protein Assay using Quick Start™ Bradford 1x Dye Reagent (Bio-Rad)[131].

Sex Determination of 3T3-L1 Adipocytes

The origin of the 3T3-L1 murine embryonic fibroblast cell line (CL-173™, American Type Culture Collection®) is unknown. Because sex-specific differences in programming of offspring adiposity and glucose homeostasis by maternal folate/vitamin B12 imbalance have been observed in mice[1,3], it was important to determine the sex of the 3T3-L1 murine embryonic fibroblast cell line. DNA was extracted from the 3T3-L1 fibroblasts using the DNeasy Blood & Tissue Kit (Qiagen©). Multiplex polymerase chain reaction (PCR) was used to amplify a

sequence of the male-specific *Sry* gene, located on the Y chromosome, and a sequence of the *Actb* gene (encodes β-actin), used as an internal control. The *Sry* sequence was amplified with a forward primer, 5'-TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA-3', and a reverse primer, 5'-CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA-3' (Integrated DNA Technologies®), to yield a 273-base pair product. The *Actb* sequence was amplified with a forward primer, 5'-AGC TCA GTA ACA GTC CGC CTA-3', and a reverse primer, 5'-CAG AGA GCT CAC CAT TCA CCA T-3' (Integrated DNA Technologies®), to yield a 203-base pair product. The primers were designed using Primer-BLAST software (NCBI). Extracted DNA samples from male and female C57BL/6J mouse livers were used as positive and negative controls, respectively. The PCR reactions were performed in tubes containing 2μL of the extracted genomic DNA and 23μL of a master mixture [10x PCR buffer (Qiagen®), *Taq* DNA polymerase (Invitrogen™), 10mM dNTPs, 10mM *Sry*-forward primers, 10mM *Sry*-reverse primers, 10mM *Actb*-forward primers, 10mM *Actb*-reverse primers, dH_2O]. The PCR cycling conditions set on the thermal cycler were as follows: denaturation at 95°C for 2 minutes; amplification (35 cycles) at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; extension at 72°C for 4 minutes; and hold at 4°C. Figure 7 shows the PCR products observed on a 2.5% agarose gel, run at 100V for 40 minutes.

Statistical Analyses

Repeated measures ANOVA was used to analyze OCR and ECAR in 3T3-L1 adipocytes across time points, with time as the within-subjects (dependent) variable and folic acid/5-MTHF treatment as the between-subjects (independent) variable. Statistical analyses of the OCR parameters were tested by one-way ANOVA with folic acid/5-MTHF treatment as the

independent variable. The least significant difference test for multiple comparisons was used to compare findings between groups. Statistical analyses were performed using SPSS Statistics software (version 12) with $p \leq 0.05$ considered significant. Data were normally distributed and analyzed separately in cells treated with folic acid and 5-MTHF. Experimental results were graphed on GraphPad Prism 5 software and are presented as mean ± standard deviation (SD).

3.7 Results

Differentiation of 3T3-L1 Adipocytes

Differentiation of 3T3-L1 mouse embryonic fibroblasts into mature adipocytes was assessed by visualization of intracellular lipid droplets (Figure 6A) and by Oil Red O incorporation into the cells. Oil Red O is a fat-soluble diazol dye that stains neutral lipids and cholesteryl esters, but not biological membranes[132]. This allows for easy estimation of lipid accumulation in cells and in this case, permitted confirmation of differentiation of the 3T3-L1 fibroblasts into adipocytes. Incorporation of Oil Red O was visualized (Figure 6B) and quantified by reading the absorbance at 492nm (Figure 6C).

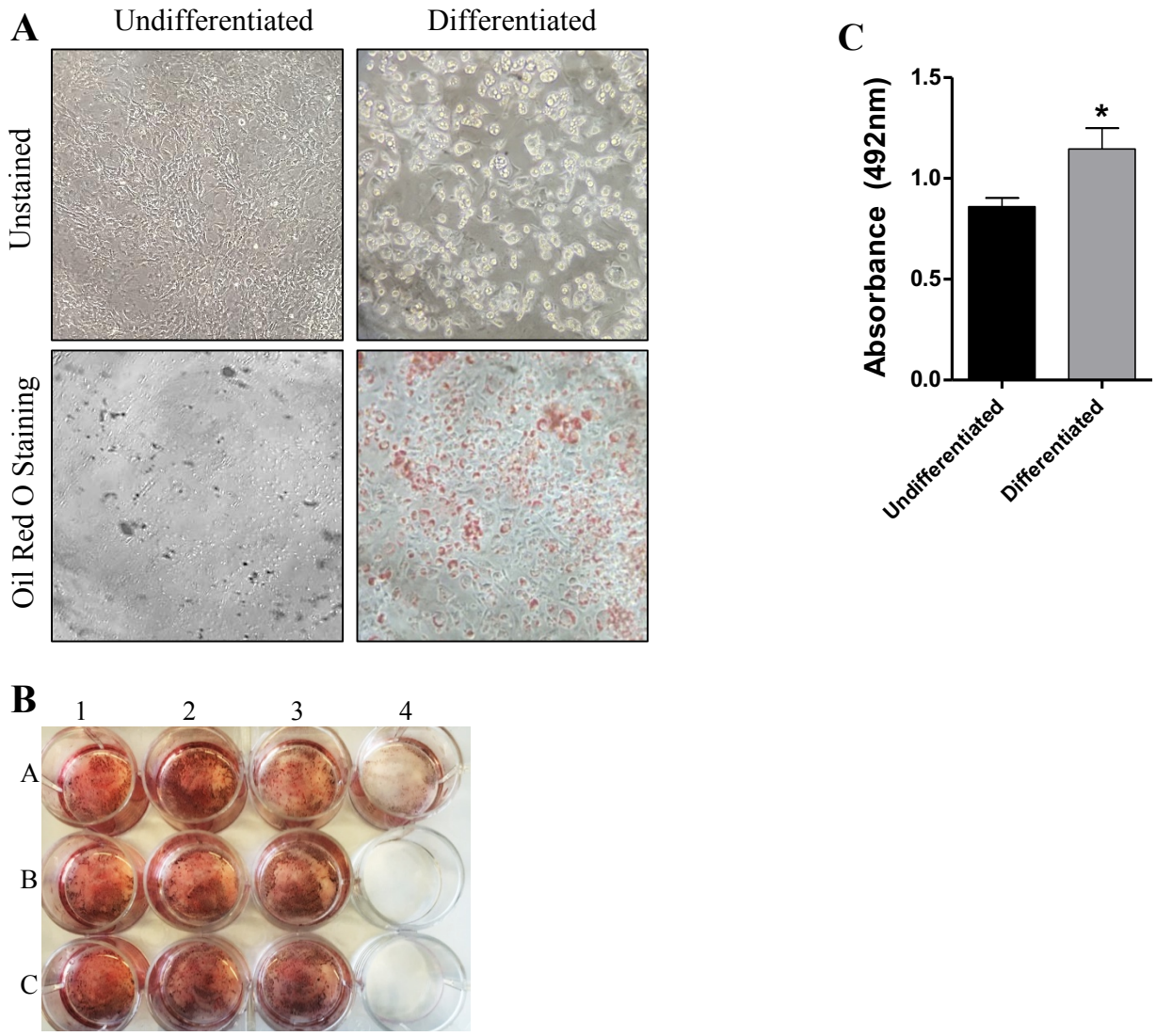

FIGURE 6: Confirmation of Differentiation of 3T3-L1 Cells into Adipocytes

A. Visualization of lipid droplet formation (top) and lipid staining with Oil Red O (bottom) in differentiated 3T3-L1 adipocytes (right) compared to undifferentiated 3T3-L1 cells (left). **B**. Oil Red O staining in a 12-well plate; well A4 contains undifferentiated 3T3-L1 cells; wells A1-C3 contain differentiated 3T3-L1 cells treated with the different folic acid or 5-MTHF concentrations. **C.** Absorbance reading at 492nm of eluted Oil Red O stain from undifferentiated and differentiated 3T3-L1 cells. Data presented as mean±SD; n=3-8; *$p \leq 0.05$ vs. undifferentiated.

Sex Determination of 3T3-L1 Cells

To corroborate the finding that female mice fed supplemental folic acid diets have greater adiposity (Figure 3), it was important to identify the sex of the 3T3-L1 cells to draw comparisons between the mouse cohort and the *in vitro* experiments. The sex of the 3T3-L1 cells was determined by detecting the male-specific *Sry* gene by multiplex PCR of genomic DNA with *Actb* as an internal control. Liver DNA samples from female and male mice were used as negative (Figure 7, Lanes 4 & 5) and positive (Figure 7, Lanes 6 & 7) controls, respectively. It was determined that the 3T3-L1 mouse embryonic cell line did not contain the *Sry* gene (Figure 7, Lanes 8 & 9), suggesting female origin. However, it is important to consider the possibility of chromosomal rearrangements resulting in loss of the Y chromosome by the cells, as this has been reported on occasion to occur in known male-derived cell lines[133].

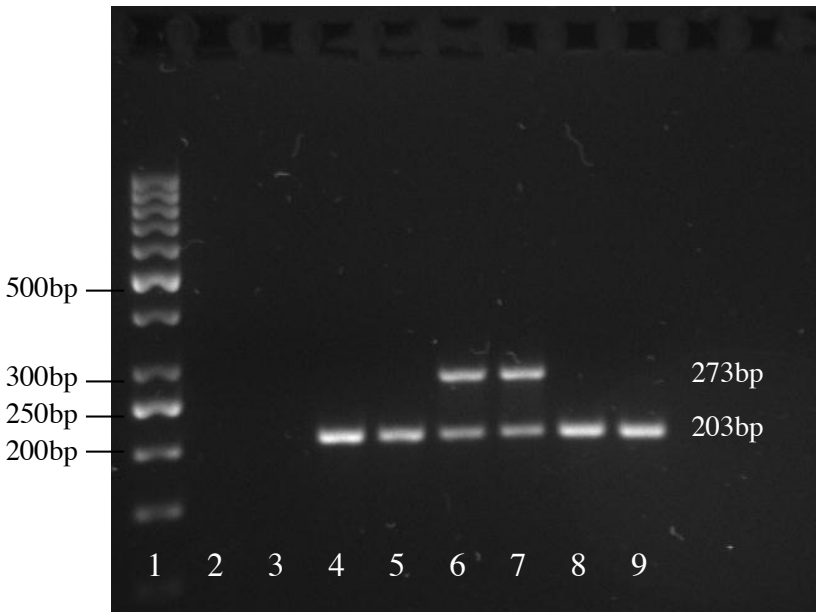

FIGURE 7: Sex Determination of 3T3-L1 Cells

Multiplex PCR of *Sry* (273bp) and *Actb* (203bp). Lane 1: ladder (GeneRuler™ 50bp DNA ladder, ThermoFisher Scientific); Lanes 2 & 3: non-template H_2O control; Lanes 4 & 5: liver genomic DNA from female mice; Lanes 6 & 7: liver genomic DNA from male mice; Lanes 8 & 9: genomic DNA from 3T3-L1 cells.

Oxygen Consumption Rates of 3T3-L1 Adipocytes

I observed that 3T3-L1 adipocytes treated with 1.6μM folic acid had lower ($p \leq 0.05$) mitochondrial respiration rates across the time points of the Seahorse XF Mito Stress Test than cells treated with the control folic acid concentration of 0.16μM (Figure 8A). Conversely, 3T3-L1 adipocytes treated with 1.6 μM 5-MTHF had higher ($p \leq 0.01$) mitochondrial respiration rates than cells treated with 0.16 μM 5-MTHF (Figure 8B). These analyses were performed using repeated measures ANOVA. My findings demonstrate differential effects of exposure to folic acid and 5-MTHF treatment on energy metabolism in adipocytes. High folic acid concentrations lower, whereas high 5-MTHF concentrations raise, mitochondrial respiration rates in adipocytes.

The OCR curves (Figure 8) can be used to calculate different OCR parameters, such as basal respiration, maximal respiration, and spare respiratory capacity, as depicted in Figure 5. No effect of folic acid or 5-MTHF treatment was observed on basal respiration rates of the 3T3-L1 adipocytes, indicating that the treatments did not alter the energy metabolism of adipocytes at baseline conditions (Figure 9A). However, I did observe that the different forms of folate elicit differential effects on maximal respiration rates of the adipocytes upon addition of the proton gradient uncoupler FCCP to the cells during the assay. As illustrated in Figure 5 and Table 2, addition of FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, representing the maximal rate of mitochondrial respiration that the cells can achieve. I observed that 3T3-L1 adipocytes treated with 1.6μM 5-MTHF had higher ($p \leq 0.05$) maximal respiration rates than cells treated with 0.16μM 5-MTHF (Figure 9B). This effect was not observed in cells treated with folic acid. Taking together the basal and maximal respiration rates, the spare respiratory capacity can be calculated as an indicator of how closely the cells are respiring to their theoretic maximum (Figure 5). The 3T3-L1 adipocytes treated with

1.6μM 5-MTHF had a higher ($p \leq 0.05$) spare respiratory capacity than cells treated with 0.16μM 5-MTHF (Figure 9C); again, this effect was not observed in cells treated with folic acid. Despite the alterations in mitochondrial respiration rates observed in 3T3-L1 adipocytes treated with 1.6μM 5-MTHF, no differences were seen in ATP production or non-mitochondrial respiration rates in these cells (Figures 9D & E).

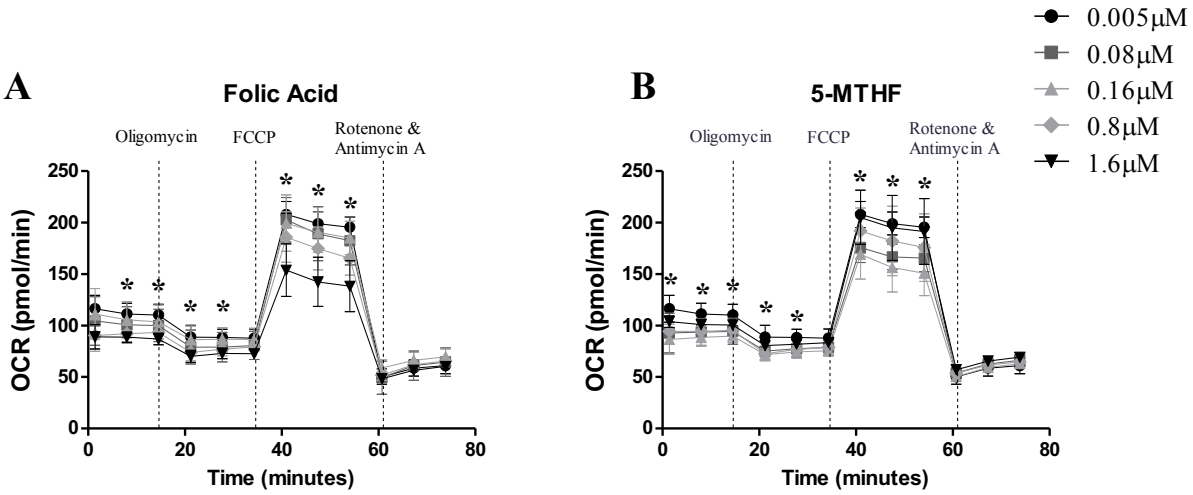

FIGURE 8: Oxygen Consumption Rates of 3T3-L1 Adipocytes
A. OCR of 3T3-L1 adipocytes treated with folic acid or **B.** 5-MTHF. Data were analyzed by repeated measures ANOVA and presented as mean ± SD; n=4; *$p \leq 0.05$ 0.16μM vs. 1.6μM-treated cells.

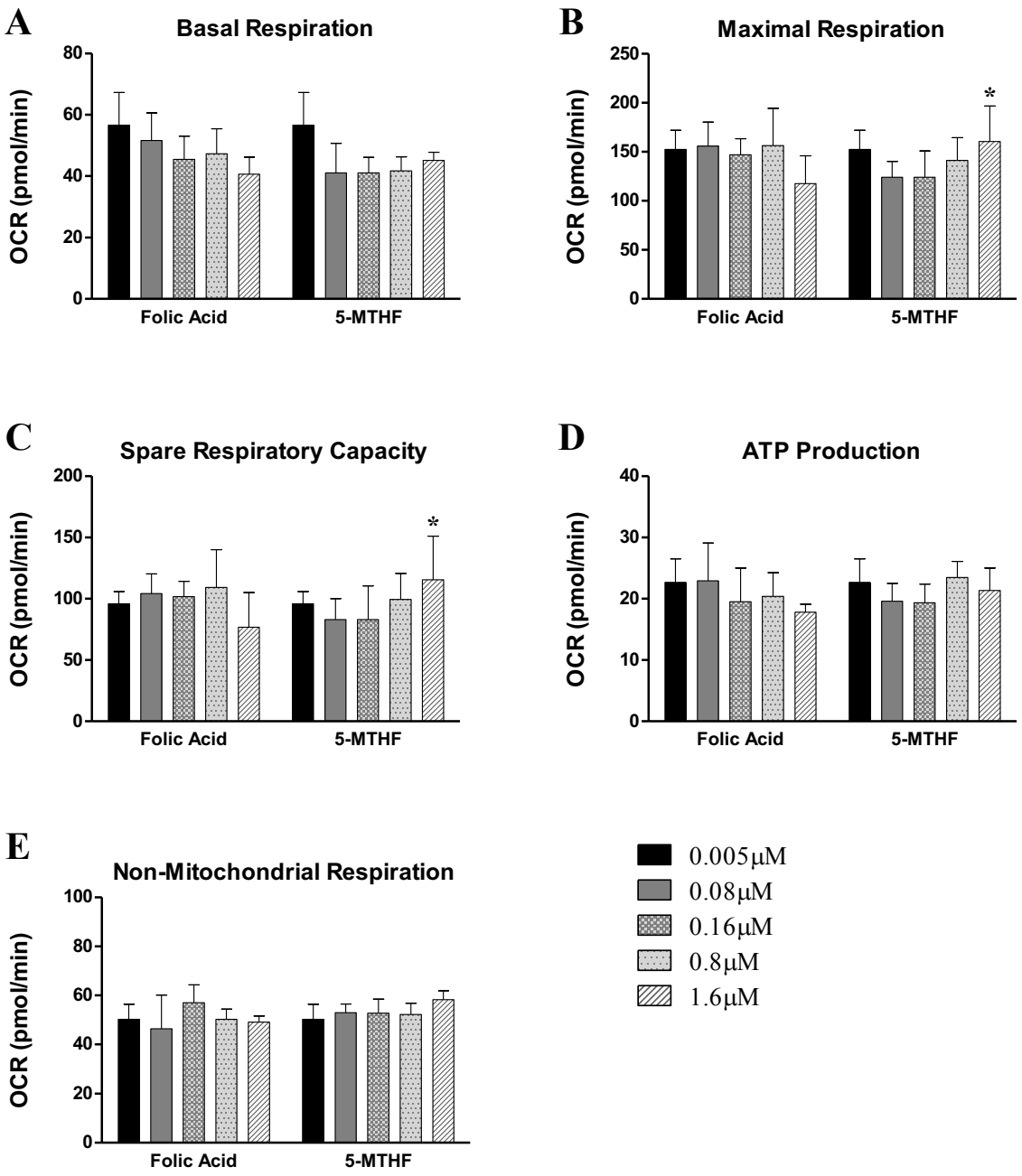

FIGURE 9: Parameters of Mitochondrial Respiration in 3T3-L1 Adipocytes

A. Basal respiration rates, **B.** Maximal respiration rates, **C.** Spare respiratory capacity, **D.** ATP production, and **E.** Non-mitochondrial respiration rates in 3T3-L1 adipocytes treated with folic acid or 5-MTHF. Data were analyzed by one-way ANOVA and presented as mean ± SD; n=4; *$p \leq 0.05$ vs. 0.16μM-treated cells.

Extracellular Acidification Rates of 3T3-L1 Adipocytes

In addition to evaluating how folic acid or 5-MTHF treatment alters mitochondrial respiration rates in adipocytes, I also assessed how these treatments affect glycolysis by measuring pH changes in the media within the transient micro-chambers. As glycolysis progresses, the piston can measure the ECAR that results from lactate excretion from the cells. I observed that the adipocytes treated with 0.16μM folic acid had lower ($p \leq 0.05$) glycolytic rates than cells treated with the 0.16μM folic acid (Figure 10A). I did not observe an effect of 5-MTHF treatment on glycolysis (Figure 10B) in the 3T3-L1 adipocytes. These findings suggest that high folic acid concentrations may disturb glycolysis in the adipocytes and alter their ability to utilize glucose for energy production.

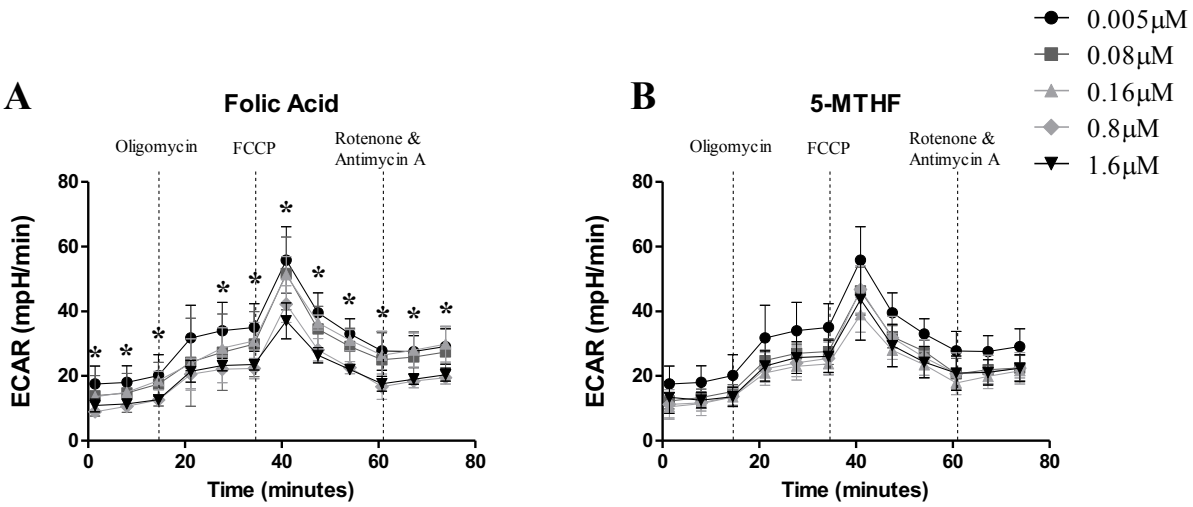

FIGURE 10: Extracellular Acidification Rates of 3T3-L1 Adipocytes
A. ECAR of 3T3-L1 adipocytes treated with folic acid or **B.** 5-MTHF. Data were analyzed by repeated measures ANOVA and presented as mean ± SD; n=4; *$p \leq 0.05$ 0.16μM vs. 1.6μM-treated cells.

3.8 Summary of Findings

My findings from Aim 1 indicate that folic acid supplementation directly affects adipose tissue distribution and adipocyte energy metabolism. In Aim 1A, I observed that female mice supplemented with folic acid for 13 weeks had greater total body weight and larger adipose tissue depots. Similarly, folic acid supplementation of 3T3-L1adipocytes *in vitro* resulted in alteration in energy metabolism; high folic acid concentrations lowered mitochondrial respiration and glycolytic rates, whereas high 5-MTHF raised mitochondrial respiration rates. The differential effects of folic acid and 5-MTHF observed on adipocyte energy metabolism suggest that excess intakes of folic acid, perhaps leading to unmetabolized folic acid in circulation, could contribute to greater adiposity. Taken together, my observations that folic acid supplementation alters adipose tissue distribution and adipocyte energy metabolism may indicate that supplemental folic acid diminishes the adipocytes' ability to utilize nutrient sources (glucose and fatty acids) for energy metabolism, instead promoting storage of nutrients as lipids. This corresponds to findings by Kelly *et al*. that reported greater TG accumulation in 3T3-L1 adipocytes treated with excess folic acid[120].

The overall objective of my thesis is to determine how maternal B-vitamin status during pregnancy programs offspring adiposity. My findings from Aim 1 provide evidence that programming of offspring adiposity by maternal B-vitamin status may involve changes in adipose tissue and glucose metabolism in the mother, subsequently contributing to metabolic alterations of the developing offspring. Human and rodent studies have reported greater adiposity in offspring from mothers with pre-gestational obesity and/or greater than recommended weight gain during pregnancy[134–136].

CHAPTER 4: Effects of Maternal B-Vitamin Status on the Offspring GH/IGF-1 Axis

AIM 2: To determine if maternal B-vitamin status during pregnancy programs offspring adiposity through disturbances in the GH/IGF-1 axis.

4.1 Rationale

As illustrated in Chapter 3, folic acid directly affects adipose tissue metabolism. Female mice had greater total body weight and larger fat pads after fed diets supplemented with folic acid for 13 weeks. Correspondingly, *in vitro* experiments showed that 3T3-L1 adipocytes were less energetically active, with lower mitochondrial respiration rates, after exposure to higher concentrations of folic acid but not 5-MTHF.

To bring these findings back to the context of developmental programming, I sought to investigate potential mechanisms involved in programming of offspring adiposity by maternal B-vitamin status during pregnancy. I wanted to determine if maternal B-vitamin status during pregnancy programs offspring adiposity through disturbances in the GH/IGF-1 axis because this has previously been demonstrated in mice using a genetic model of maternal B12 deficiency[115]. Growth hormone (a well-known regulator of somatic growth, development, and lipid metabolism) stimulates IGF-1 production in the liver, and together GH and IGF-1 regulate adipogenesis and adipose tissue metabolism[137,138]. To determine if maternal B-vitamin status during development affects the GH/IGF-1 axis in offspring, I quantified hepatic *Igf1* mRNA and serum IGF-1 concentrations.

In circulation, IGFs are normally bound to IGF binding proteins (IGFBPs) that act as carriers to IGFs, stabilizing them and prolonging their half-life[139]. Although IGFBP-2 is the second-most abundant IGF binding protein, its physiological role remains poorly understood. It

has been shown that genetic overexpression of *Igfbp2* in mice results in increased insulin sensitivity, though the underlying mechanisms are not understood[140, 141]. Studies have also indicated a role for IGFBP2 in the developmental programming of metabolic disorders. In Wistar rats, alterations in *Igfbp2* in offspring liver have been observed from developmental exposure to maternal undernutrition[142, 143]. Based on these reported findings, I quantified hepatic *Igfbp2* mRNA in the offspring.

Insulin and IGF-1 are closely related hormones that are involved in both growth and metabolism. They act on their own specific tyrosine kinase receptors, the insulin receptor (IR) and the IGF-1 receptor (IGF1R); however, they also have the capacity to bind and activate each other's receptors[144]. I sought to investigate IR/IGFR1 signaling pathways in visceral adipose tissue based on the phenotypic observations that maternal folic acid supplementation during pregnancy results in larger adipose tissue depots, glucose intolerance, fasting hyperglycemia, and lower serum IGF-1 in female but not male offspring[3, 1]. Following ligand binding, IR/IGF1R elicits the activation of a cascade of downstream intracellular proteins including phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT), ultimately leading to various cellular responses including lipogenesis[145]. Activation of PI3K by IR/IGF1R stimulates the translocation of GLUT4 transporters from intracellular vesicles towards the plasma membrane[146]. Translocated GLUT4 has a central role in whole-body glucose metabolism, as it is responsible for the transport of glucose out of peripheral circulation and into tissues such as skeletal muscle and adipose tissue. Consistent with its major role in glucose disposal, mice with whole-body heterozygous knockout of GLUT4 display decreased GLUT4 protein expression in skeletal muscle and adipose tissue, and subsequently develop insulin resistance and propensity towards diabetes[147,148]. Moreover, it has been reported that mice with adipose tissue-specific depletion of

GLUT4 exhibit muscle and liver insulin resistance[149]. In addition to GLUT4 translocation, activation of PI3K by IR/IGF1R results in phosphorylation of AKT at serine 473. Western blot analysis of AKT and phosphorylated AKT (pAKT) is a well-established method for determining insulin signaling in tissues[150]. To determine if maternal B-vitamin status during development affects offspring adipose tissue insulin signaling, I quantified *Slc2a4* (encodes GLUT4) mRNA and AKT/pAKT protein.

4.2 Methods

Mouse Cohort

The tissue used for analysis in this chapter was harvested from offspring mice from dams described in Chapter 3.2 (Figure 2 and Table 1) fed the maternal diets (M-CON, SFA-B12, SFA+B12). At weaning, one male and one female pup from each dam were randomly assigned to either a postweaning control diet (16% energy from fat, 64% energy from carbohydrate) or postweaning western diet (45% energy from fat, 35% energy from carbohydrate). The offspring postweaning diet compositions are depicted in Table 3. The western (obesogenic) diet was selected to induce excess adiposity in the mice and parallels the fat content of the diet commonly observed in westernized human populations. The C57BL/6J mouse strain is a particularly good model mimicking human metabolic derangements that are observed in obesity because when fed a control diet these mice remain lean and metabolically healthy, but when fed a western diet they develop excess adiposity, hyperglycemia, and hyperinsulinemia that closely resembles that of human obesity progression[151,152]. It is important to note that the westernized diet observed in humans typically consists of a higher carbohydrate content than that of the rodent western diet. In humans, higher intakes of fat and carbohydrate both contribute to obesity progression[153].

However, in rodents excess adiposity is induced by a higher fat content in the diet and therefore, in order to raise the fat content in the rodent western diet the carbohydrate content must be reduced.

Offspring mice were housed in cages of 3-5 animals per cage. Male offspring were fed for 20 weeks postweaning and female offspring were fed for 30 weeks postweaning. The female offspring were fed the postweaning diets longer than the males because it has been previously shown that female rodents do not develop glucose intolerance and fasting hyperglycemia as quickly as male rodents on obesogenic diets[154]. Following the end of the offspring feeding periods, the mice were anesthetized with isoflurane and blood was collected via cardiac puncture. The blood was allowed to clot at room temperature for 15 minutes, centrifuged at 12,000 rpm at 4°C for 15 minutes, and serum was collected and stored at -80°C until further analysis. After cervical dislocation, tissue was harvested, flash frozen in liquid nitrogen, and stored at -80°C. The offspring tissue and serum samples used for analysis in this chapter were selected from the same mice that had been used for analysis in previous experiments when possible[1,3]. This was to ensure that the molecular effects observed in the offspring would be matched to the same offspring in which the phenotypes and previous results were observed.

TABLE 3: Offspring Postweaning Diet Compositions

	Postweaning Diets	
Nutrient	Control	Western
Folic Acid (mg/kg diet)	2.0	2.0
Vitamin B12 (µg/kg diet)	50.0	50.0
Carbohydrate (% energy)	64%	35%
Protein (% energy)	20%	20%
Fat (% energy)	16%	45%

Source of protein is vitamin-free casein; source of fat in control diet is soybean oil; source of fat in the western diet is 11.2% soybean oil, 29.6% butter, 29.6% lard, 29.6% vegetable shortening.

Quantification of Serum IGF-1 Concentrations

Serum samples from male and female offspring were diluted in dH_2O by a factor of 1:100, which I determined to be the optimal dilution factor for quantification of IGF-1. Fasting serum IGF-1 concentrations were quantified by the Mouse/Rat IGF-1 ELISA Kit (ALPCO Diagnostics®).

Isolation of Mature Adipocytes from Gonadal Fat Pads

Mature adipocytes were isolated from gonadal fat pads using the method modified from Rodbell[155]. The frozen gonadal fat pads were removed from -80°C storage and placed immediately in falcon tubes containing 2mL of Krebs-Ringer buffer with 2% bovine serum albumin (BSA) and 0.25% type I collagenase. The fat pads were briefly minced with scissors and then incubated at 37°C with shaking for 45 minutes. The collagenase-digested samples were then

filtered through 250μm nylon mesh cell strainers and the filtrates were centrifuged at 1,000 rpm at 4°C for 10 minutes to obtain floating primary mature adipocytes. The mature adipocytes were transferred to 2mL Eppendorf tubes containing 5 times the volume of RNAprotect Cell Reagent (Qiagen©) for every 1 volume of sample. The RNAprotect Cell Reagent stabilized RNA in the samples and allowed for long-term storage at -80°C. The samples were archived at -80°C for later RNA extraction.

Quantification of Liver and Adipocyte mRNA

Total RNA was extracted from liver and isolated mature adipocytes with the RNeasy Mini Kit (Qiagen©). The isolated mature adipocytes were previously stabilized in RNAprotect Cell Reagent; therefore, to remove the RNAprotect reagent the samples were centrifuged at 8,000 rpm for 5 minutes and the supernatant was decanted. The pellet was re-dissolved in buffer RLT containing β-mercaptoethanol (β–Me) and the adipocytes were lysed using the QiaShredder spin columns (Qiagen©). The RNeasy Mini Kit was then followed as per standard protocol with the addition of on column-DNase digestion for the removal of DNA contamination (RNase-free DNase Set, Qiagen©). Extracted RNA was assessed for purity and concentration using a nanodrop spectrophotometer. Purity was determined by a 260/280 measurement of approximately 1.8. Integrity of the RNA was determined by observation of 18s and 28s ribosomal RNA (rRNA) bands on a 1.5% agarose gel. Conversion of total RNA to 500ng of cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Liver *Igf1* and *Igfbp2*, and adipocyte *Slc2a4* mRNA were quantified by TaqMan® real-time PCR (RT-PCR) using the $^{\Delta\Delta}$Ct method of relative quantification[156]. The TaqMan® primer/probes were specific for murine *Igf1* (Mm00439560_m1), *Igfbp2* (Mm00492632_m1),

and *Slc2a4* (Mm00436615_m1). The endogenous control used for all experiments was 18s rRNA (VIC®/MGB probe, Applied Biosystems®). Each sample was run in duplicate to determine intra-assay variability, and each experiment was repeated.

Quantification of Liver Taurine Concentrations

To quantify liver taurine concentrations, approximately 50mg of each frozen liver sample was weighed and quickly transferred to 600μL of cold 1x PBS. The liver samples were sonicated on ice with three pulses (15 seconds/pulse) and centrifuged at 8,000 rpm at 4°C for 10 minutes. The supernatant was aliquoted, diluted in dH_2O by a factor of 1:20, and placed on dry ice. Taurine concentrations were quantified by HPLC-MS/MS using an Agilent 1260 HPLC system and AB Sciex API 4000 mass spectrometer. This analysis was performed by Benny Chan in Dr. Yvonne Lamers' lab. Taurine concentrations were normalized to protein concentration as determined by the Bradford Protein Assay using the Quick Start™ Bradford 1x Dye Reagent (Bio-Rad)[131].

Western Blotting

Protein was extracted from adult offspring gonadal fat pads, and AKT and phosphorylated AKT were quantified. Approximately 50mg of each sample was weighed and placed immediately in 500μL of cold 1x PBS. The samples were centrifuged at 8,000 rpm for 30 seconds, the supernatant was discarded, and the tissue was resuspended in 500μL of 1x RIPA buffer (Cell Signaling Technology®: 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/mL leupeptin) containing an EDTA-free protease inhibitor

tablet (Roche Diagnostics). The samples were sonicated on ice with three pulses (15 seconds/pulse) and then centrifuged at 9,000 rpm at 4°C for 4 minutes. The supernatants were aliquoted and archived in -80°C for later analyses. Protein concentrations were quantified in each sample by the Bradford Protein Assay using the Quick Start™ Bradford 1x Dye Reagent (Bio-Rad)[131].

For western blotting, the protein samples were resolved on hand-casted 10% resolving and 4% stacking gels [dH_2O, 1.5M Tris-HCl (pH 8.8), 30% Acrylamide/Bis Solution (Bio-Rad), 10% SDS (Gibco), 10% APS (Sigma-Aldrich®), and TEMED (Bio-Rad)]. The resolving gels were poured into vertical 18-well cassettes (Bio-Rad), covered with a thin layer of isopropanol to remove bubbles, and allowed to polymerize for 1 hour. The isopropanol was then decanted off and the stacking gels were allowed to polymerize for 30-45 minutes with the 18-well comb.

The protein samples were prepared in 1.5mL safe-lock Eppendorf tubes. Each sample contained 30μg of protein, 5μL of working Laemmli buffer (from a stock solution of 950μL Laemmli Sample Buffer (Bio-Rad) + 50μL β-Me), and a variable volume of dH_2O to bring the total volume to 30μL. The safe-lock lids were secured with parafilm and snap caps, and the samples were boiled in a water bath for 20 minutes. The protein samples, 10μL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Bio-Rad), and 10μL of SDS-PAGE Molecular Weight Standards, Low Range (Bio-Rad) were loaded on the gels and run in 1x Tris/Glycine/SDS Buffer (Bio-Rad). The gels were run at 110V for 120 minutes.

The resolved proteins were transferred in ice-cold 1x transfer buffer (25mM Tris base, 190mM glycine) from the gels onto polyvinylidine difluoride (PVDF) membranes (Bio-Rad) at 110V overnight. The transfer apparatus was kept in a bucket of ice and a magnetic spin bar was used to facilitate movement of the transfer buffer in the apparatus. Transfer of protein to the

PVDF membranes was confirmed with Ponceau S Solution (Sigma-Aldrich®) and the membranes were then washed 3 times with dH_2O to remove the Ponceau S stain. The membranes were blocked in 5% milk powder or 5% BSA (if probing for phosphorylated proteins) dissolved in Tris-buffered saline (20mM Tris base, 250mM NaCl) with 0.05% Tween-20 (Fisher BioReagents™) (TBST) for 2 hours at 4°C with rocking. After blocking, the membranes were rinsed three times (10 minutes/rinse) in TBST and cut for separate incubation in TBST with 5% BSA and the primary antibodies: AKT (C-20) goat polyclonal, 1:500 (sc-1618); pAKT1/2/3 (Ser 473)-R rabbit polyclonal, 1:500 (sc-7985-R); Actin (I-19) rabbit polyclonal, 1:1000 (sc-1616-R) (Santa Cruz Biotechnology Inc.). Actin expression was used as an internal control. After overnight incubation, the primary antibodies were decanted, the membranes were rinsed three times (10 minutes/rinse) in TBST, and incubated for 2.5 hours in TBST with 5% BSA and the secondary antibodies: goat anti-rabbit IgG-AP, 1:2000 (sc-2007); donkey anti-goat IgG-AP, 1:2000 (sc-2022) (Santa Cruz Biotechnology Inc.). The membranes were rinsed three times (10 minutes/rinse) in TBST, incubated for 2-3 minutes in CDP-Star® Chemiluminescent Substrate (Sigma-Aldrich®), and exposed in a ChemiGenius2 gel doc for 10 minutes under "no light", "no filter" and "high resolution" settings.

Statistical Analyses

Statistical analyses of results were tested by a one-way ANOVA with maternal diet as the independent variable. The least significant difference test for multiple comparisons was used to compare findings between groups (M-CON offspring, SFA-B12 offspring, and SFA+B12 offspring). Data was normally distributed and no outliers were removed from the data sets. Statistical analyses were performed using SPSS Statistics software (version 12) with $p \leq 0.05$

considered significant. Data were analyzed separately in male and female offspring, and separately in offspring fed the postweaning control diet and postweaning western diet. Gene expression results were normalized to findings in the M-CON offspring fed the postweaning control diet and in the M-CON offspring fed the postweaning western diet, respectively. Experimental results were graphed on GraphPad Prism 5 software and are presented as mean ± SD.

4.3 Results

Quantification of Serum IGF-1 Concentrations

Circulating IGF-1 concentrations were quantified in serum from adult female and male offspring by ELISA. In postweaning control-fed females, offspring from SFA-B12 dams had lower ($p \leq 0.05$) serum IGF-1 concentrations than offspring from M-CON and SFA+B12 (Figure 11A). Similarly, postweaning western-fed females from SFA-B12 dams tended to have lower ($p=0.07$) serum IGF-1 concentrations than offspring from SFA+B12 (Figure 11A). Interestingly, the differences in offspring serum IGF-1 concentrations resulting from maternal diet were sex-specific; no effect of maternal diet on serum IGF-1 concentrations in adult male offspring was observed (Figure 11B).

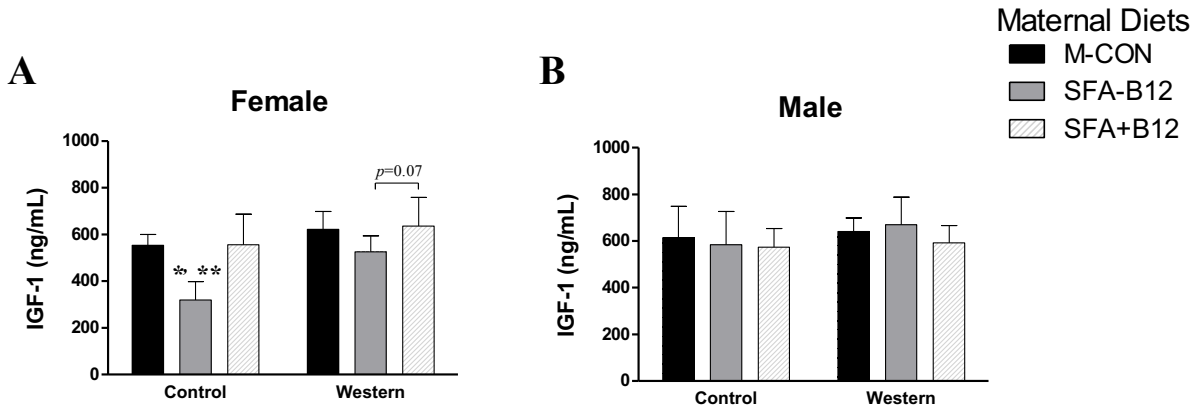

FIGURE 11: Serum IGF-1 Concentrations in Offspring
A. Serum IGF-1 concentrations in adult female and **B.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-7; *$p \leq 0.05$ vs. M-CON, **$p \leq 0.05$ vs. SFA+B12.

Quantification of Liver *Igf1* and *Igfbp2* mRNA

To determine whether the effect of maternal diet on serum IGF-1 concentrations in female offspring occurs at the level of gene expression, hepatic *Igf1* mRNA was quantified. Additionally, I chose to quantify hepatic *Igfbp2* mRNA because of its potential role in glucose metabolism and its implications in developmental programming[140–142]. Interestingly, the sex-specific effects of maternal diet observed in circulating IGF-1 concentrations did not match the expression of *Igf1* in the liver; I did not observe an effect of maternal diet in postweaning control-fed female offspring (Figure 12A). However, I did observe that in postweaning western-fed females, offspring from SFA+B12 dams had higher ($p \leq 0.05$) *Igf1* mRNA than offspring from M-CON dams (Figure 12A). No effect of maternal diet on liver *Igf1* mRNA was observed in male offspring fed either the postweaning control or western diet (Figure 12B). In postweaning control-fed males, those from SFA-B12 dams had higher ($p \leq 0.05$) *Igfbp2* mRNA than offspring

from M-CON dams (Figure 12D). No differences in hepatic *Igfbp2* mRNA were observed in postweaning western-fed male offspring or in female offspring fed either the postweaning control or western diet (Figures 12C & D).

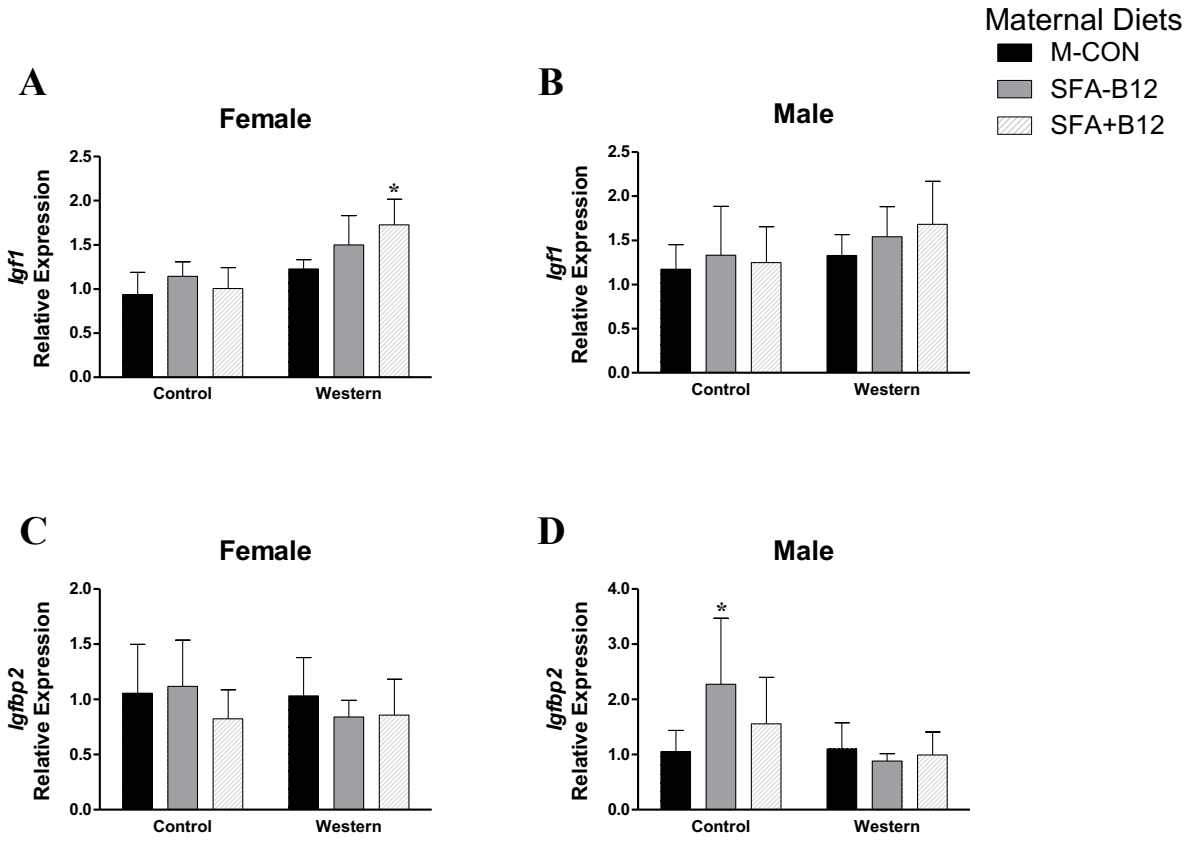

FIGURE 12: Hepatic *Igf1* and *Igfbp2* mRNA in Offspring
A. Liver *Igf1* mRNA in adult female and **B.** male offspring. **C.** Liver *Igfbp2* mRNA in adult female and **D.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-7; *$p \leq 0.05$ vs. M-CON.

Quantification of Liver Taurine Concentrations

The effects of maternal B12 deficiency on the offspring GH/IGF-1 axis reported that the reduced serum IGF-1 concentrations in offspring from B12-deficient dams was dependent on decreased production of taurine in the liver[115]. Taurine is a sulfur-containing semi-essential amino acid that is synthesized primarily in the liver, accumulates in various tissues, and plays a role in growth and metabolism[157]. Taurine is linked to one-carbon metabolism because it is a downstream product of the transsulfuration pathway[59,60]. Taurine deficiency is associated with both pre- and post-natal growth retardation[158]. Contrary to what Roman-Garcia *et al*. observed, quantification of taurine in our mouse cohort revealed no effect of maternal diet on hepatic taurine concentrations in female or male offspring fed either the postweaning control or western diet (Figures 13A & B). These observations indicate that programming of offspring adiposity by maternal B-vitamin status does not involve alterations in offspring taurine concentrations.

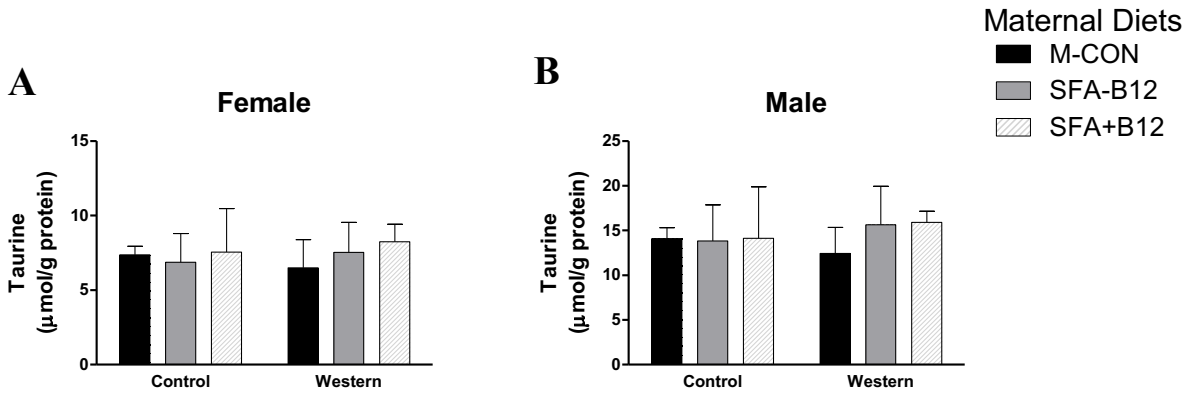

FIGURE 13: Liver Taurine Concentrations in Offspring
A. Liver taurine concentrations in adult female and **B.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-6.

Quantification of Adipose *Slc2a4* mRNA and AKT/pAKT Protein

To investigate IR/IGF1R signaling in offspring adipose tissue, relative *Slc2a4* mRNA expression was quantified in mature adipocytes isolated from gonadal fat depots. Moreover, western blot analysis was undertaken to quantify AKT and pAKT protein levels. No effect of maternal diet was observed in *Slc2a4* mRNA, nor in AKT and pAKT protein, in female or male offspring fed either the postweaning control or western diet (Figures 14A & B, Figures 15 A-D).

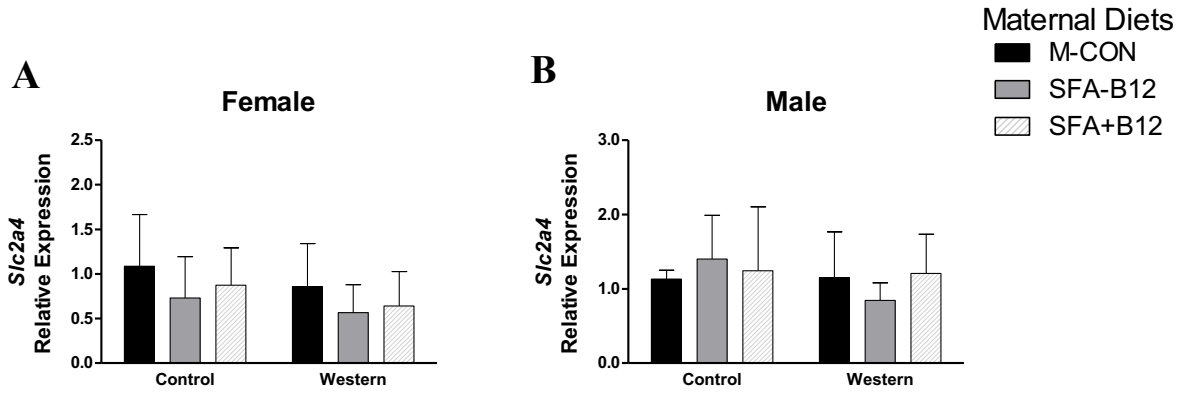

FIGURE 14: Adipocyte *Slc2a4* mRNA in Offspring
A. Gonadal adipocyte *Slc2a4* mRNA in adult female and **B.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-6.

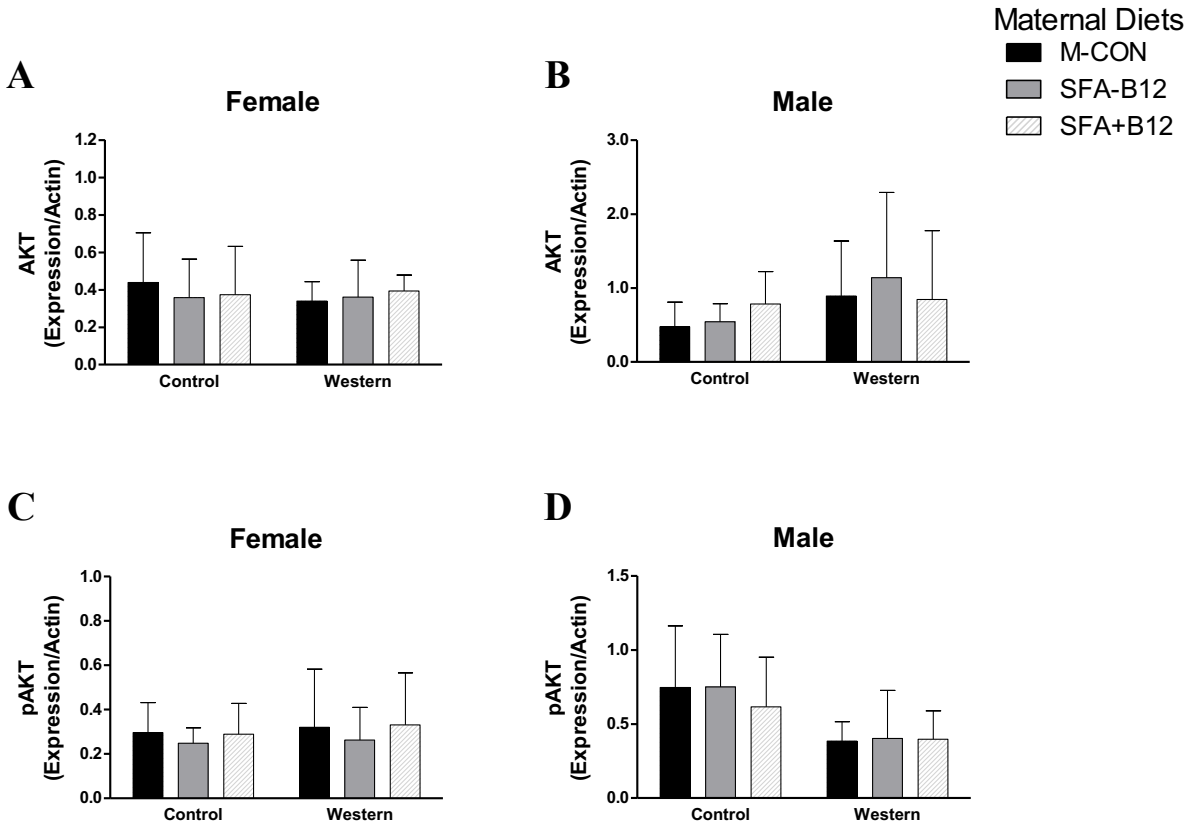

FIGURE 15: Adipose Tissue AKT Protein Expression in Offspring

A. Gonadal adipose tissue AKT protein in adult female and **B.** male offspring. **C.** Phosphorylated (p)AKT protein in adult female and **D.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=4-6.

4.4 Summary of Findings

My findings from Aim 2 indicate that maternal B-vitamin status results in some disturbances in IGF-1, but these effects were predominantly seen in adult female offspring only. I observed that maternal folate/B12 imbalance during pregnancy resulted in lower serum IGF-1 concentrations in female offspring. Interestingly, these effects did not correspond with expression of *Igf1* mRNA in the liver, the site of IGF-1 synthesis. Therefore, it is possible that maternal B-vitamin status may not play a role in altering IGF-1 synthesis, but may perhaps modulate its clearance from circulation by peripheral tissues. I observed no differences in IR/IGF1R signaling in offspring adipose tissue, suggesting that other tissues, such as skeletal muscle, may be more important for IGF-1 clearance from the circulation. Therefore, further investigation is warranted to determine how maternal B-vitamin status may affect IR/IGF1R signaling in skeletal muscle.

CHAPTER 5: Effects of Maternal B-Vitamin Status on Offspring Lipid Metabolism

AIM 3: To determine if maternal B-vitamin status during pregnancy programs offspring adiposity through altered adipose tissue and hepatic lipid metabolism.

5.1 Rationale

Maternal folic acid supplementation during pregnancy resulted in sex-specific differences in adiposity and glucose homeostasis. Whereas adult female offspring had greater adiposity and glucose intolerance, male offspring observed a leaner phenotype and had no changes in glucose homeostasis[1,3]. Further exploration into potential metabolic alterations was undertaken by quantifying relative expression of key lipogenic genes in offspring adipocytes isolated from gonadal fat pads. Acetyl-CoA carboxylase 1 (ACC1), the rate-limiting enzyme for *de novo* fatty acid biosynthesis, carboxylates acetyl-CoA to form malonyl-CoA[70]. While ACC1 is expressed in all tissues, expression is highest in lipogenic tissues such as adipose tissue and liver[70]. Previous studies have reported that adipose tissue-specific ACC1 knockout mice have reduced lipid accumulation in white adipose tissue depots[159]. Stearoyl-CoA desaturase 1 (SCD1), the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids (mainly oleic acid from desaturation of stearic acid), has also been implicated in adiposity. Mice with targeted disruption of SCD1 have reduced body adiposity, increased insulin sensitivity, and are resistant to diet-induced weight gain[160].

The inter-relationship between methyl metabolism and lipid metabolism is well-understood. Numerous studies have published evidence linking disruptions in methyl metabolism to alterations in hepatic lipid metabolism in mice[58,73,76,78,]. As described in Chapter 1.6, methyl metabolism is linked to lipid metabolism through the synthesis of PC and LCPUFAs. Evidence

from various human and mouse studies have demonstrated the benefits of *n*-3 LCPUFAs, namely eicosapentaenoic acid (EPA, C20:5*n*-3) and docosahexaenoic acid (DHA, C22:6*n*-3), in improving inflammation, insulin resistance, reducing plasma TGs, and reducing the incidence of CVD[161–164]. For these reasons, I investigated LCPUFA concentrations in the offspring mice to determine if developmental exposure to maternal B-vitamin status results in differences in fatty acid metabolism in the offspring, altering the concentrations of *n*-6 and *n*-3 fatty acids and possibly contributing to the observed adiposity and changes in glucose homeostasis.

Figure 16 summarizes the metabolism of the dietary *n*-6 and *n*-3 essential fatty acids into various LCPUFAs in the liver. Linoleic acid (LA, C18:2*n*-6) is desaturated to γ-linolenic acid (GLA, C18:3*n*-6) through the enzymatic activity of Δ-6 desaturase (FADS2). Further desaturation and elongation to arachidonic acid (AA, C20:4*n*-6) occurs via elongase (ELOVL2) and Δ-5 desaturase (FADS1). Similarly, alpha-linolenic acid (ALA, C18:3*n*-3) is desaturated and elongated to EPA (C20:5*n*-3) via FADS2, ELOVL2, and FADS1. Further elongation to docosapentaenoic acid (DPA, C22:5*n*-3) and desaturation to DHA (C22:6*n*-3) occurs through ELOVL2 and Δ-4 desaturase, respectively. In our animal study, the amounts of the dietary essential fatty acids (LA and ALA) were consistent across all maternal diets, and the source of fat in these diets was soybean oil (Table 1).

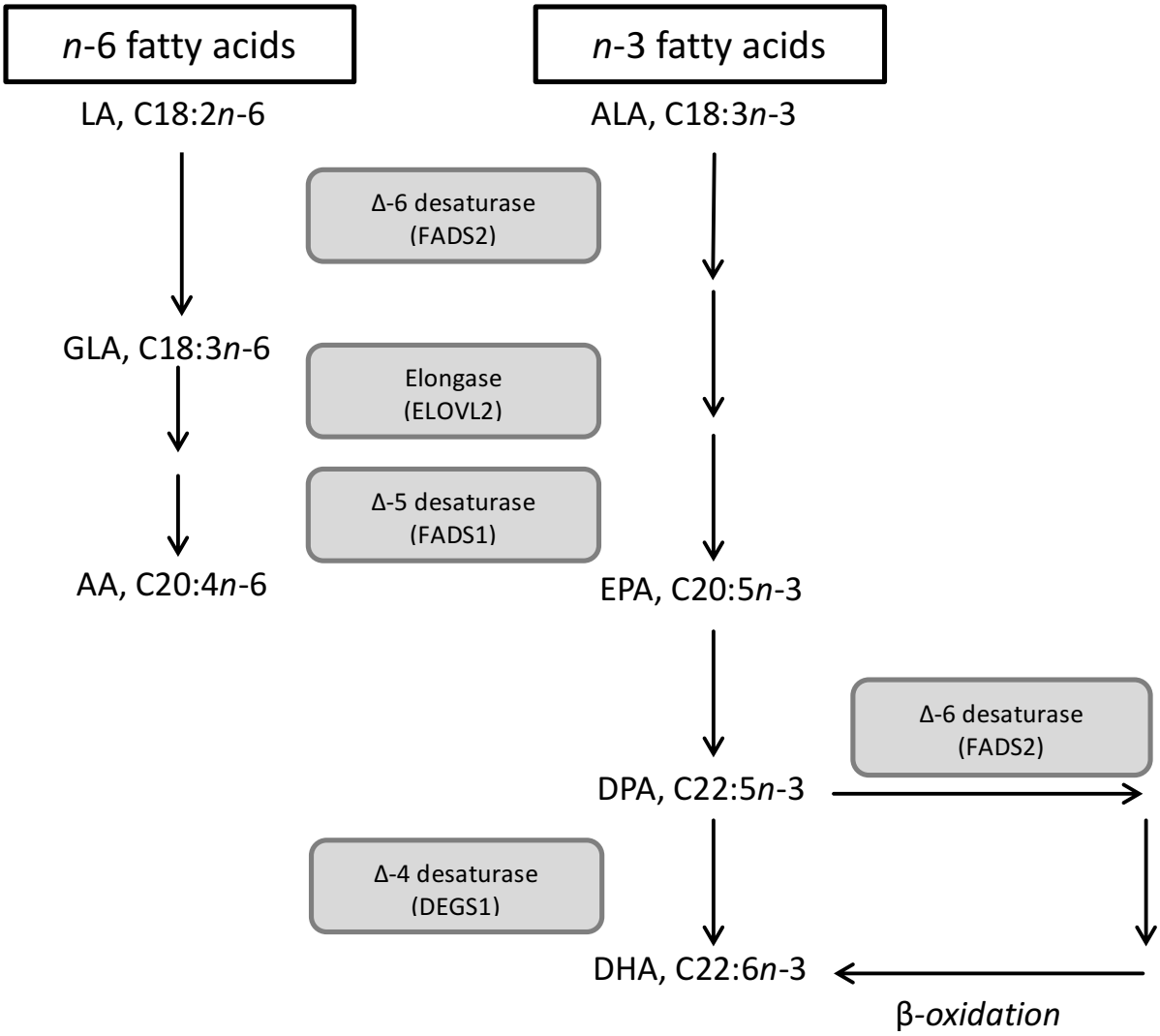

FIGURE 16: Desaturation and Elongation of the *n*-6 and *n*-3 Fatty Acid Series
The dietary *n*-6 and *n*-3 essential fatty acids can be desaturated and elongated to various long-chain polyunsaturated fatty acids (LCPUFAs).

5.2 Methods

The tissues used for analysis in this chapter were harvested from offspring from the mouse cohort previously described in Chapter 3.2 (maternal diets and breeding scheme) and Chapter 4.2 (offspring postweaning diets). Statistical analyses of results were tested by a one-way ANOVA and presented as described in Chapter 4.2.

Liver and adipocyte mRNA were quantified by RT-PCR and liver proteins were quantified by western blotting, as described in Chapter 4.2. For mRNA quantification, liver *Cpt1a,* and adipocyte *Acaca* and *Scd1* mRNA were quantified with the TaqMan® primer/probes specific for murine *Cpt1a* (Mm01231183_m1), *Acaca (*Mm01304283_g1), and *Scd1* (Mm00772290_m1). For western blotting protein, the following primary antibodies were used: FADS2 (N-12) goat polyclonal, 1:200 (sc-109272); ELOVL2 (S-16) goat polyclonal, 1:200 (sc-54974); GAPDH (V-18) goat polyclonal, 1:1000 (sc-20357) (Santa Cruz Biotechnology Inc.). GAPDH expression was used as an internal control. The following secondary antibodies were used: donkey anti-goat IgG-AP, 1:2000 (sc-2022) (Santa Cruz Biotechnology Inc.).

Quantification of Liver Fatty Acids

Liver fatty acids were quantified in offspring liver samples. Whole frozen liver samples were packaged on dry ice and sent to Dr. Sanjoy Ghosh's lab at The University of British Columbia, Okanagan Campus. Total lipids were extracted from the tissue by the method of Folch *et al*.[165]. The organic phase was evaporated under nitrogen and the lipids were solubilized by chloroform/methanol/acetone/hexane (2.0:3.0:0.5:0.5, v/v). Individual lipids were separated with a 2690 Alliance HPLC and the separated fatty acids were quantified by evaporative light scattering detection (model 2000, Alltech, Mandel Scientific, Guelph, ON)[76].

5.3 Results

Quantification of Adipocyte *Acaca* and *Scd1* mRNA

Western diet-fed female offspring from SFA-B12 and SFA+B12 dams had lower ($p \leq 0.05$) adipocyte *Acaca* (encodes ACC1) mRNA than offspring from M-CON dams (Figure

17A), indicating that maternal supplemental folic acid results in less endogenous fatty acid synthesis in female offspring. This effect of maternal diet was not observed in postweaning control diet-fed female offspring, nor was it observed in male offspring, regardless of postweaning diet (Figures 17A &B). Quantification of adipocyte *Scd1* (encodes SCD1) mRNA showed no effect of maternal diet on gene expression in female or male offspring fed either the postweaning control or western diet (Figures 17C &D). Though SCD1 has previously been implicated in adiposity in rodents[160], my observations suggest that it is likely not involved in programming of offspring adiposity by maternal B-vitamin status.

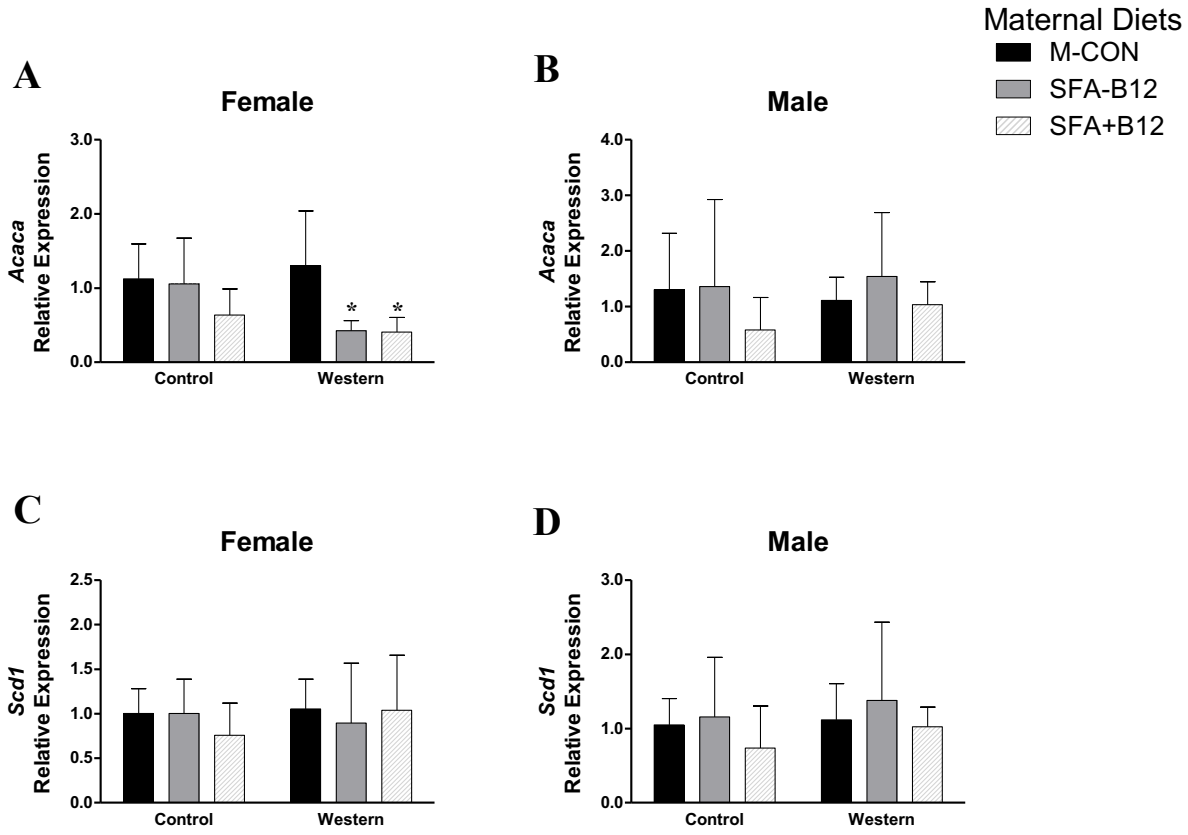

FIGURE 17: Adipocyte *Acaca* and *Scd1* mRNA in Offspring
A. Gonadal adipocyte *Acaca* mRNA in adult female and **B.** male offspring. **C.** Gonadal adipocyte *Scd1* mRNA in adult female and **D.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-6; *$p \leq 0.05$ vs. M-CON.

Quantification of Liver Fatty Acid Concentrations

I observed sex-specific effects of maternal diet on offspring hepatic lipid metabolism. Whereas male offspring were affected by maternal B-vitamin status, females had no alterations in hepatic fatty acid concentrations (Table 4). Male offspring from SFA-B12 dams fed the postweaning control diet had higher ($p \leq 0.05$) LA (C18:2*n*-6) and lower ($p \leq 0.05$) EPA (C20:5*n*-3) concentrations than M-CON offspring and SFA+B12 offspring, respectively (Table 5). Also, male offspring from SFA+B12 dams fed the postweaning western diet had lower ($p \leq 0.05$) concentrations of the saturated fatty acid stearic acid (C18:0) than M-CON offspring (Table 5).

TABLE 4: Hepatic Fatty Acid Concentrations in Female Offspring

	Female Control			**Female Western**		
Fatty Acid (% of total fatty acids)	M-CON	SFA-B12	SFA+B12	M-CON	SFA-B12	SFA+B12
Stearic Acid (C18:0)	12.5±2.55	13.0±2.66	12.3±1.60	11.4±2.57	11.9±1.81	11.9±1.67
Oleic Acid (C18:1*n*-9)	23.9±3.84	23.1±3.03	25.0±2.96	27.9±4.86	27.3±2.75	27.8±3.25
Linoleic Acid (LA, C18:2*n*-6)	17.6±2.75	18.9±1.75	17.6±2.05	14.1±2.61	14.4±1.51	13.9±1.74
Arachidonic Acid (AA, C20:4*n*-6)	8.5±1.33	8.44±1.35	7.9±1.02	8.31±1.60	8.00±1.16	8.30±1.04
Alpha-Linolenic Acid (ALA, C18:3*n*-3)	0.76±0.24	0.84±0.23	0.79±0.19	0.49±0.15	0.48±0.12	0.42±0.08
Eicosapentaenoic Acid (EPA, C20:5*n*-3)	0.26±0.05	0.30±0.04	0.27±0.04	0.19±0.05	0.18±0.06	0.17±0.05
Docosahexaenoic Acid (DHA, C22:6*n*-3)	2.07±0.44	2.16±0.34	2.08±0.37	1.91±0.44	1.95±0.46	1.94±0.34

Data presented as mean ± SD; n=6.

TABLE 5: Hepatic Fatty Acid Concentrations in Male Offspring

	Male Control			Male Western		
Fatty Acid (% of total fatty acids)	M-CON	SFA-B12	SFA+B12	M-CON	SFA-B12	SFA+B12
Stearic Acid (C18:0)	12.3±2.47	11.8±2.36	12.7±2.13	13.8±1.86	12.7±1.99	11.3±1.82*
Oleic Acid (C18:1*n*-9)	18.9±3.29	17.1±4.73	16.1±2.71	21.2±3.73	21.0±2.25	23.6±4.02
Linoleic Acid (LA, C18:2*n*-6)	18.9±3.95	24.1±3.42*	21.9±1.88	15.3±1.40	16.3±0.87	16.3±1.88
Arachidonic Acid (AA, C20:4*n*-6)	7.56±1.71	8.12±1.12	8.22±0.22	8.67±1.45	8.25±1.31	7.02±1.44
Alpha-Linolenic Acid (ALA, C18:3*n*-3)	0.85±0.27	0.95±0.22	0.99±0.10	0.53±0.11	0.57±0.04	0.64±0.15
Eicosapentaenoic Acid (EPA, C20:5*n*-3)	0.25±0.07	0.19±0.05**	0.27±0.05	0.22±0.06	0.24±0.06	0.21±0.04
Docosahexaenoic Acid (DHA, C22:6*n*-3)	1.81±0.51	1.81±0.19	2.08±0.26	1.80±0.32	1.61±0.38	1.50±0.27

Data presented as mean ± SD; n=6; *$p \leq 0.05$ vs. M-CON, **$p \leq 0.05$ vs. SFA-B12.

Given an effect of maternal diet was observed in hepatic *n*-6 and *n*-3 fatty acid concentrations in male offspring, I assessed two key proteins in this pathway: FADS2 (Δ-6 desaturase, 52kDA) and ELOVL2 (35kDa). As mentioned in Chapter 1.6, it has been reported that mice with disturbances in methyl metabolism have altered expression and activity of FADS2 in the liver[73]. Thus, I wanted to assess whether developmental exposure to maternal B-vitamin status affects hepatic lipid metabolism in offspring through altered FADS2 and ELOVL2 expression.

Consistent with the observed differences in hepatic fatty acid concentrations, postweaning control-fed male offspring from SFA-B12 dams tended to have lower (p=0.08) hepatic ELOVL2 protein in the than M-CON offspring (Figure 18B), suggesting they have disturbed fatty acid elongation. Postweaning control-fed male offspring from SFA+B12 dams had lower ($p \leq 0.05$) hepatic FADS2 protein than M-CON offspring (Figure 18D), suggesting they

have disturbed fatty acid desaturation. No effect of maternal diet was observed in hepatic FADS2 and ELOVL2 protein in male offspring fed the postweaning western diet (Figures 18B & D). In contrast, maternal B-vitamin status has diverse effects on hepatic FADS2 and ELOVL2 protein levels in adult female offspring. Postweaning western-fed female offspring from SFA+B12 dams had higher ($p \leq 0.05$) hepatic FADS2 and ELOVL2 protein than M-CON offspring (Figures 18A & C). This indicates that maternal supplemental folic acid augments fatty acid desaturation and elongation in female offspring.

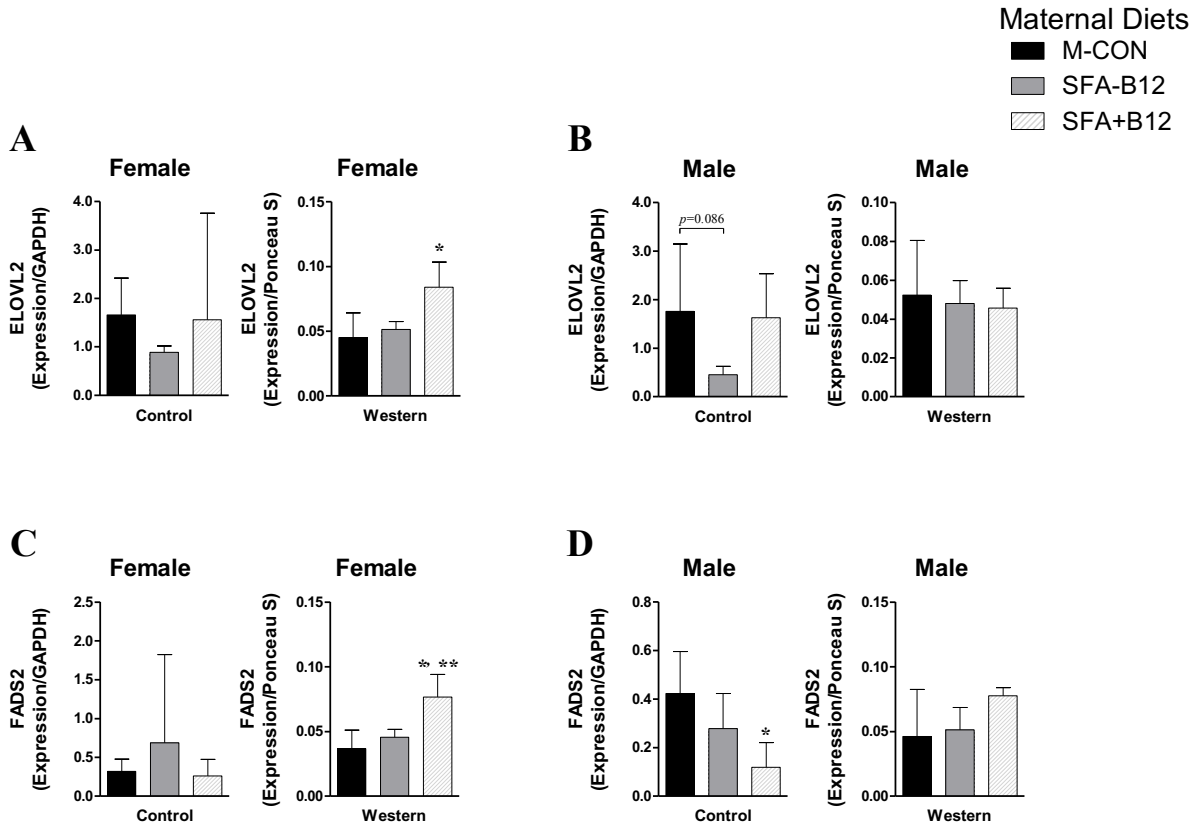

FIGURE 18: Hepatic ELOVL2 and FADS2 Protein Expression in Offspring
A. Liver ELOVL2 protein in adult female and **B.** male offspring. **C.** Liver FADS2 (Δ-6 desaturase) protein in adult female and **D.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-6; *$p \leq 0.05$ vs. M-CON, **$p \leq 0.05$ vs. SFA-B12.

Quantification of Liver *Cpt1a* mRNA

Fatty acids are β-oxidized, predominantly in the liver, to release energy from storage when required. As discussed in Chapter 1.6, CPT1A is the rate-limiting enzyme for β-oxidation of fatty acids and is linked to methyl nutrients through the enzymatic activity of MCM. I quantified gene expression of *Cpt1a* and observed that postweaning control-fed female offspring from SFA-B12 and SFA+B12 dams had higher ($p \leq 0.05$) hepatic *Cpt1a* mRNA than offspring from M-CON dams (Figure 19A). This effect of maternal diet was not observed in postweaning western-fed female offspring (Figure 19A). Conversely, postweaning western-fed male offspring from SFA-B12 dams had lower ($p \leq 0.05$) hepatic *Cpt1a* mRNA than both M-CON and SFA+B12 offspring (Figure 19B). No effect of maternal diet was observed in postweaning control-fed male offspring (Figure 19B). These findings show that maternal folic acid supplementation promotes more β-oxidation of fatty acids in female offspring, whereas maternal folate/B12 imbalance lowers β-oxidation of fatty acids in male offspring.

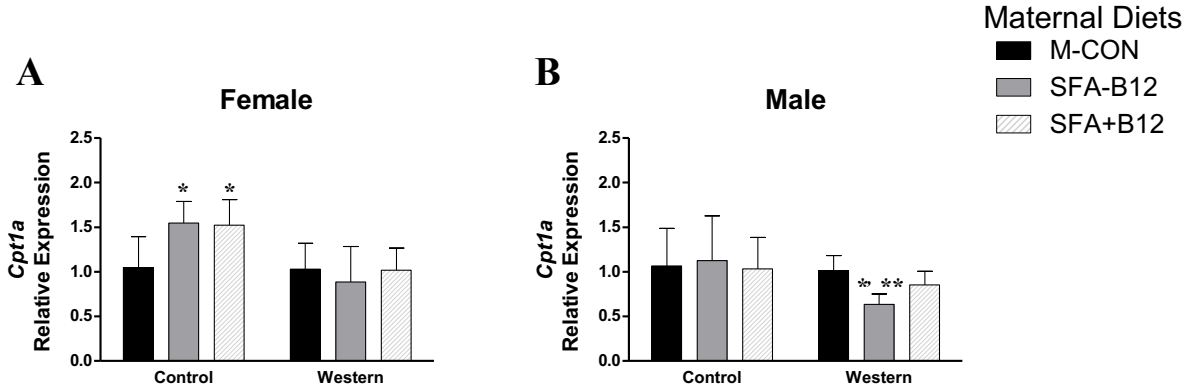

FIGURE 19: Hepatic *Cpt1a* mRNA in Offspring
A. Liver *Cpt1a* mRNA in adult female and **B.** male offspring from maternal diet-fed dams. Black bars, control mice; grey bars, supplemental folic acid/no B12 mice; white bars, supplemental folic acid/adequate B12 mice. Data presented as mean ± SD; n=5-6; *$p \leq 0.05$ vs. M-CON, **$p \leq 0.05$ vs. SFA+B12.

5.4 Summary of Findings

My findings from Aim 3 indicate that maternal B-vitamin status during pregnancy results in sex-specific differences in adipose tissue and hepatic lipid metabolism. Overall, adult female offspring from folic acid supplemented dams had augmented hepatic fatty acid desaturation and elongation, as well as alterations in expression of key hepatic and adipocyte lipid metabolism genes. Conversely, adult male offspring had disturbed hepatic fatty acid desaturation and elongation, and no changes in expression of lipid metabolism genes.

Taken together, these findings indicate that perhaps greater hepatic LCPUFA synthesis in female offspring results in more delivery of these lipids to adipose tissue depots for storage. Subsequently, this could contribute to larger adipose tissue depots and could also lead to reduced endogenous fatty acid synthesis in adipocytes, as observed by lower *Acaca* mRNA levels. On the other hand, it is possible that the disturbed hepatic LCPUFA synthesis observed in male offspring results in reduced delivery of lipids to the adipose tissue for storage, overall contributing to smaller adipose tissue depots observed in these mice. These sex-specific alterations in LCPUFA synthesis prompt the need for further investigation of how maternal B-vitamin status may play a role in lipid metabolism and adipose tissue distribution in offspring.

CHAPTER 6: General Discussion

6.1 Overall Summary

The developmental origins of health and disease theory proposes that prenatal and early postnatal environment plays an important role in later disease susceptibility in offspring[39]. Both prenatal and early postnatal life fall within a critical period of developmental plasticity, wherein the *in utero* and early-life environmental conditions can influence the trajectory of an organism's development and health. Hence, disturbances in these environments could significantly impact long-term offspring health. Maternal nutrient status is one example of such a disturbance. Epidemiological evidence suggests that alterations of specific dietary factors, such as folate and vitamin B12, during pregnancy impact cardiometabolic health of children[110–113]. The Pune Maternal Nutrition Study in India demonstrated that higher maternal erythrocyte folate concentrations paired with sub-optimal B12 status during pregnancy was associated with greater adiposity and insulin resistance in children at age 6[110]. Similarly, rodent studies have shown that maternal B-vitamin status during pregnancy programs offspring adiposity and glucose homeostasis in a sex-specific context[1,3]. Given the evidence that adiposity and glucose homeostasis are programmed through exposure to maternal B-vitamin status during development, the objective of my thesis was to determine the underlying mechanisms involved in programming of these offspring phenotypes.

As a first step to understanding how maternal B-vitamin status programs offspring adiposity, I sought to investigate whether folic acid directly affects adipocyte energy metabolism. Given my observations that female mice fed diets supplemented with folic acid for 13 weeks had greater total body weight and larger adipose tissue depots than mice fed the control diet, I conducted experiments *in vitro* to assess whether folic acid supplementation alters energy

metabolism in adipocytes. I chose to investigate the effects of two different forms of folate (folic acid and 5-MTHF) on adipocyte energy metabolism because folic acid is normally reduced and methylated to 5-MTHF (the circulating form of folate) in the small intestine; however, when folic acid intakes are high, some of it appears in peripheral circulation unmetabolized[95–97]. Little is known about the consequences of circulating unmetabolized folic acid on tissues.

My *in vitro* experiments demonstrate that the different forms of folate (folic acid and 5-MTHF) elicit effects on mitochondrial respiration in 3T3-L1 adipocytes. Higher concentrations of folic acid appear to disturb mitochondrial respiration, whereas higher concentrations of 5-MTHF augment mitochondrial respiration in 3T3-L1 adipocytes. These findings are novel; there are no published studies demonstrating differential effects of folic acid and MTHF on adipocyte energy metabolism. Taking these data alongside the larger adipose tissue depots observed in female mice supplemented with folic acid, it is possible that unmetabolized folic acid in circulation results in mitochondrial dysfunction in adipose tissue and contributes to metabolic complications, such as adiposity and glucose intolerance. Mitochondrial dysfunction is associated with adipose tissue dysfunction observed in obesity[166]. In fact, previous studies in humans have reported that markers of mitochondrial biogenesis and mitochondrial respiration are lower in overweight and obese insulin-resistant subjects[167–169].

In this thesis, I chose to treat the 3T3-L1 cells with different folic acid or 5-MTHF concentrations while simultaneously inducing their differentiation into adipocytes. I chose this experimental design with the goal of mimicking how developing offspring adipose tissue would be exposed to maternal B-vitamin status *in utero*. Adipogenesis is a multistep process controlled by various transcription factors. Many pro-adipogenic factors function by activating PPARγ (peroxisome proliferator-activated receptor gamma), a master regulator of adipogenesis that is

sensitive to the nutrient composition of the diet, including folic acid[170]. The capacity of folic acid to modulate PPARγ expression in adipose tissue has previously been established[171]. In fact, it has been reported that maternal folic acid supplementation during pregnancy alters epigenetic processes in offspring rat liver at weaning through effects on PPARγ promoter methylation, leading to higher PPARγ gene expression [172]. Recently, Kelly *et al*. reported that 3T3-L1 adipocytes treated with excess folic acid have higher *Pparg* mRNA (encodes PPARγ) and higher TG accumulation[120]. Moreover, a previous study in 3T3-L1 adipocytes reported that inhibiting mitochondrial respiration with antimycin A induces greater accumulation of TGs in the cells[173]. Taken together, the results from these studies further validate my findings that folic acid supplementation alters adipocyte energy metabolism through disruptions in mitochondrial respiration, and it is possible that this may lead to greater lipid accumulation in the adipocytes.

Future work assessing TG accumulation in 3T3-L1 adipocytes treated with folic acid or 5-MTHF would be an interesting avenue to explore. Additionally, though investigating epigenetic processes is outside the scope of my thesis, it would be beneficial to explore whether folic acid supplementation in 3T3-L1 adipocytes induces changes in methylation status of regulatory regions of the *Pparg* gene and other pro-adipogenic genes. This may help to further characterize the link between PPARγ, TG accumulation, and altered mitochondrial respiration in 3T3-L1 adipocytes supplemented with folic acid.

My novel findings in Aim 1 demonstrate that supplementing adipocytes with folic acid *in vitro* alters their energy metabolism. Building from these findings, I sought to investigate potential mechanisms involved in programming of offspring adiposity by maternal B-vitamin status. I chose to explore the GH/IGF-1 axis as a potential mechanism because it was reported that developmental exposure to maternal B12 deficiency disrupts the offspring GH/IGF-1 axis[115].

It is well-known that GH stimulates IGF-1 secretion from the liver and together, GH and IGF-1 elicit effects on adipose tissue metabolism[138]. To my knowledge, I am the first to report that maternal B-vitamin status during pregnancy is associated with sex-specific disturbances in IGF-1 in offspring. I observed that adult female offspring from SFA-B12 dams had lower serum IGF-1 concentrations than offspring from either M-CON or SFA+B12 dams. Conversely, no effect of maternal diet on serum IGF-1 concentrations was observed in adult male offspring. These observations parallel the phenotypes observed in the offspring from dams supplemented with folic acid; females had greater adiposity, glucose intolerance, and fasting hyperglycemia, whereas males were leaner and had no changes in glucose homeostasis[1,3]. Together, these findings support the possibility that low IGF-1 concentrations perturb glucose metabolism and contribute to greater adiposity.

My findings are supported by other rodent studies of low circulating IGF-1 concentrations and glucose homeostasis. Yakar *et al*. reported that mice with low circulating levels of IGF-1 due to knockout of the *Igf1* gene in liver exhibited hyperinsulinemia and insulin resistance, suggesting a key role of IGF-1 in glucose metabolism[174]. The authors of this study further extended their findings by overexpressing a dominant negative form of IGF1R in skeletal muscle to attenuate its function. They reported that these mice had a marked reduction in IGF-1-mediated glucose uptake into skeletal muscle and subsequently developed severe insulin resistance and overt diabetes[175]. In my thesis, I explored IR/IGF-1 signaling pathways in adipose tissue to determine if the adiposity, glucose intolerance, and fasting hyperglycemia observed in adult female offspring could, in part, be explained by lower circulating IGF-1 concentrations. Surprisingly, I did not see an effect of maternal B-vitamin status on AKT/pAKT protein levels or in *Slc2a4* mRNA in offspring adipose tissue. Future studies are warranted to further examine

pathophysiology of the glucose intolerance and fasting hyperglycemia in the female offspring. It would be beneficial to investigate skeletal muscle IR/IGF-1 signaling due to the predominant role of IGF-1 in skeletal muscle insulin-stimulated glucose uptake[176].

Low circulating IGF-1 concentrations could indicate disturbances in GH signaling in the liver. Roman-Garcia *et al*. reported that a genetic mouse model of maternal B12 deficiency (*Gif*$^{-/-}$) resulted in lower levels of circulating IGF-1 concentrations in offspring[115]. Interestingly, this was accompanied by higher circulating GH concentrations, suggesting that offspring from B12-deficient dams exhibit GH resistance in the liver. In my study, assessing GH concentrations could have been useful in determining if offspring are GH resistant; however, this assessment is rather challenging due to GH's known diurnal rhythms of secretion from the anterior pituitary. Humans exhibit multiple GH peaks per day depending on sleep state, stress, sex, nutritional status, body composition and age[177, 178]; rodents also exhibit diurnal rhythms of secretion[179]. As such, I did not quantify serum GH concentrations in the offspring.

Results from additional studies by Roman-Garcia *et al*. elucidated a mechanism whereby maternal B12 deficiency attenuated offspring growth hormone-induced signaling of signal transducer and activator of transcription 5 (STAT5) and diminished hepatic production of taurine, which in turn lowered the hepatic synthesis of IGF-1[115]. In accordance with these observations, the authors found that after administering exogenous taurine, the F2 generation from the *Gif*$^{-/-}$ dams had normal levels of circulating GH and IGF-1, and were indistinguishable from their wild-type littermates fed a vehicle control[115]. Despite these reported findings, I found no effect of maternal diet on liver taurine concentrations in female or male offspring. This suggests that the programming of IGF-1 that I observed in female offspring from maternal folate/B12 imbalance occurred through taurine-independent pathways. These observations

prompt the need for future work to further illuminate the mechanisms underlying alterations in IGF-1 in female offspring from exposure to maternal folate/B12 imbalance during development.

My objective for Aim 3 was to explore lipid metabolism as another potential mechanism involved in programming of offspring adiposity by maternal B-vitamin status during pregnancy. Methyl metabolism is linked to lipid metabolism through the synthesis of PC in the liver[58]. It has previously been shown that disturbances in methyl metabolism result in altered synthesis of PC and LCPUFAs, and expression of FADS2 in the liver of mice[73]. Considering this, I chose to investigate whether alterations in hepatic lipid metabolism from disturbances in methyl metabolism occur in the context of developmental programming. Accordingly, my findings are the first to demonstrate that maternal B-vitamin status during pregnancy is associated with sex-specific alterations in hepatic lipid metabolism in offspring.

Liver PC and fatty acid metabolism are central to governing the levels and composition of circulating lipoproteins and the subsequent delivery of lipids to other tissues[73]. Synthesis of PC occurs in the liver by two pathways (the PEMT pathway and the CDP-choline pathway), both of which are linked to methyl metabolism[72]. Given that the PEMT pathway is directly linked to methyl metabolism through the utilization of AdoMet, I sought to explore whether altered methyl metabolism in dams during pregnancy results in differences in LCPUFA metabolism in offspring. Male offspring fed the postweaning control diet from SFA-B12 dams had higher LA (C18:2*n*-6) and lower EPA (C20:5*n*-3) concentrations. This suggests that male offspring have disturbances in fatty acid elongation and desaturation. This was accompanied by lower levels of hepatic ELOVL2, an important enzyme involved in elongation of the dietary essential fatty acids LA (C18:2*n*-6) and ALA (C18:3*n*-3) to the LCPUFAs AA (20:4*n*-6) and EPA (C20:5*n*-3), respectively. Together, these findings suggest that lower ELOVL2 activity results in higher

concentrations of hepatic LA (C18:2*n*-6) due to disturbed *n*-3 fatty acid metabolism, and lower concentrations of EPA (C20:5*n*-3) due to disturbed *n*-6 fatty acid metabolism.

I observed differential effects of maternal B-vitamin status on offspring hepatic lipid metabolism in female offspring. Contrary to what I observed in male offspring, female offspring had no differences in hepatic fatty acid concentrations. Nevertheless, female offspring fed the postweaning western diet from SFA+B12 dams had higher hepatic FADS2 and ELOVL2 protein levels. This suggests the possibility that maternal folic acid supplementation promotes greater desaturation and elongation of hepatic fatty acids to LCPUFAs in female offspring.

An important caveat to highlight in my study is that when investigating lipid metabolism, I was only able to quantify offspring hepatic fatty acid concentrations. Due to the feasibility of the analysis, I was unable to quantify other lipid classes, such as phospholipids, TGs, and cholesteryl esters. Given that LCPUFAs are incorporated into phospholipids such as PC, and because PC plays a major role in the synthesis of lipoproteins for delivery of lipids to other tissues, assessing phospholipids would be essential for gaining insight into the bigger picture of how maternal B-vitamin status affects lipid metabolism in offspring. The PEMT pathway, which uses three AdoMet molecules to methylate PE for PC synthesis, is thought to preferentially utilize PE that is acetylated with LCPUFAs, such as AA (20:4*n*-6) and DHA (22:6*n*-3)[72]. Conversely, PC synthesized from the CDP-choline pathway contains higher monounsaturated and saturated fatty acids in the *sn*-1 position[72]. Therefore, assessing fatty acid compositions of liver PC could assist in delineating functional relevance of the alterations in LCPUFA metabolism I observed in the offspring. For instance, if liver PC in offspring contained lower levels of AA (20:4*n*-6) and DHA (22:6*n*-3), this could reflect that altered methyl metabolism in the dams disturbs synthesis of liver PC through the PEMT pathway in offspring. Furthermore, it

would be interesting to quantify lipids classes in extrahepatic tissues, such as skeletal muscle and adipose tissue, to determine if maternal B-vitamin status results in greater ectopic lipid accumulation or alterations in fatty acid concentrations in these tissues. Evidence from various human and mouse studies have demonstrated the benefits of *n*-3 LCPUFAs, namely EPA (C20:5*n*-3) and DHA (C22:6*n*-3), in improving inflammation, insulin resistance, reducing plasma TGs, and reducing the incidence of CVD[161–164].

In addition to measuring hepatic fatty acid concentrations in the offspring, I also quantified relative expression of some key genes involved in lipid metabolism; I quantified *Acaca* (encodes ACC1) mRNA in adipocytes and *Cpt1a* (encodes CPT1A) mRNA in liver. Acetyl-CoA carboxylase is the rate-limiting enzyme for *de novo* fatty acid biosynthesis and CPT1A is the rate-limiting enzyme for β-oxidation of fatty acids. Once again, I found sex-specific differences in the relative expression of these genes based on maternal B-vitamin status. In female offspring from dams supplemented with folic acid, I observed lower *Acaca* mRNA in adipocytes and higher *Cpt1a* mRNA in liver, suggesting a preference towards catabolism of fatty acids in females. This is surprising given that the female offspring have larger adipose tissue depots. My observation of lower *Acaca* mRNA contradicts previous studies that have reported reduced lipid accumulation in adipose tissue in mice with adipose tissue-specific knockout of ACC1 (FACC1KO mice)[159]. Strikingly, however, this study also reported that FACC1KO mice have a 30% reduction in serum IGF-1 concentrations, an interesting finding that parallels my results in Aim 2. Thus, it is possible that disturbances in ACC1 activity are linked to reductions in circulating IGF-1 concentrations.

Contrary to what I observed in the female offspring, male offspring from dams supplemented with folic acid had no differences in adipocyte *Acaca* mRNA, but lower *Cpt1a*

mRNA in liver. It has been reported that inhibition of CPT1A in mice with diet-induced obesity results in improved insulin sensitivity, greater insulin signaling in skeletal muscle, and increased glucose metabolism[180]. The authors suggest that though inhibiting mitochondrial fatty acid uptake with a CPT1A inhibitor decreases fatty acid oxidation, these mice have enhanced skeletal muscle glucose metabolism, a phenomenon known as the Randle Cycle[181]. As previously mentioned in my discussion of Aim 2, investigating skeletal muscle IR/IGF1R signaling in the offspring would be beneficial to further elucidate why female offspring from dams supplemented with folic acid are glucose intolerant, but males have no changes in glucose homeostasis.

6.2 Conclusions and Limitations

The overarching summary of my findings is depicted in Figure 20. My work demonstrates three novel findings: 1) folic acid supplementation directly alters adipocyte energy metabolism; 2) maternal B-vitamin status during development programs sex-specific differences in GH/IGF-1 in offspring; and 3) maternal B-vitamin status during development programs sex-specific differences in hepatic lipid metabolism in offspring.

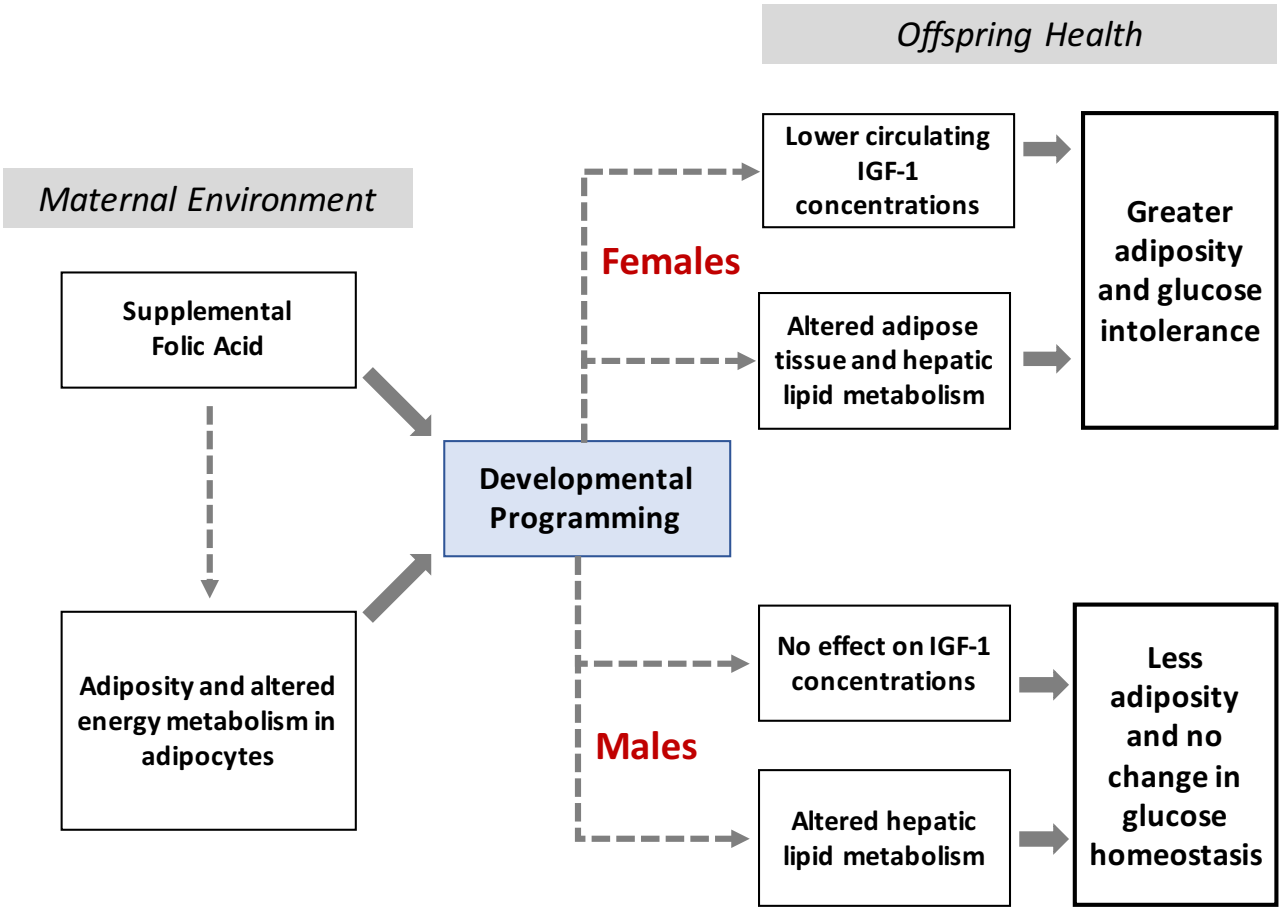

FIGURE 20: Overarching Summary of Thesis
Maternal B-vitamin status during development and programming of adult offspring adiposity.

The main findings from this project suggest potential mechanisms by which maternal B-vitamin status during pregnancy programs offspring adiposity and glucose homeostasis. I observed that female mice supplemented with folic acid for 13 weeks (throughout pregnancy and lactation) had greater total body weight and adiposity. Additionally, *in vtiro* experiments revealed that 3T3-L1 adipocytes had altered energy metabolism following exposure to supplemental folic acid. In the context of developmental programming, adult female offspring from dams supplemented with folic acid during pregnancy had greater adiposity, glucose intolerance, and fasting hyperglycemia. I postulate that this occurs through mechanisms

involving lower circulating IGF-1 concentrations and altered hepatic and adipose tissue lipid metabolism. Conversely, male offspring from dams supplemented with folic acid had less adiposity and no changes in glucose homeostasis. I postulate that this occurs through mechanisms involving altered hepatic lipid metabolism.

As previously discussed, programming of offspring adiposity and glucose homeostasis by maternal B-vitamin status has been reported in both human and rodent studies[1,110–114]. Though my findings suggest potential mechanisms underlying these offspring phenotypes, it is important to take into consideration the differences in folic acid metabolism in mice verses humans when drawing comparisons. It is known that rodents are capable of handling larger intakes of folic acid than humans due to higher activity of DHFR in the liver[182,183]. Therefore, the supplemental folic acid diets (5-fold higher folic acid) used in my study may not be considered a "high" intake for a mouse. Nevertheless, I observed physiological and molecular effects of the maternal diets in the offspring. These findings suggest the possibility that excess maternal folic acid intakes in humans may have even more profound effects on offspring health.

6.3 Future Directions

Health Canada recommends that women of childbearing age and pregnant women consume 400μg folic acid per day for the prevention of NTDs[54,84–86]. The risk for having an NTD-affected pregnancy is higher in obese women. Subsequently, women with pre-gestational obesity (with a BMI of >35kg/m^2) have been recommended to consume as much as 5mg folic acid per day[184]. Beneficial effects on vascular function and reductions in oxidative stress have been reported in obese women with metabolic syndrome following supplementation with 5mg folic acid per day[185]. Therefore, it is reasonable to consider that perhaps folic acid

supplementation mitigates the adverse effects of obesity on cardiometabolic health; however, this is not known. It is unclear whether obese women metabolize folate differently, but a recent population study reported that obese women have higher erythrocyte folate concentrations, regardless of supplement use, than women of a healthy BMI[186]. Conceivably, obese women may distribute folate among body compartments differently than women of a healthy BMI. My findings from Aim 1 hint towards the possibility that high intakes of folic acid could actually be detrimental to health. This raises the question that perhaps circulating unmetabolized folic acid accumulates in adipose tissue and disturbs metabolism, an effect which is possibly exacerbated by obesity. These findings warrant the need for future studies focusing on folic acid supplementation in the context of obesity, looking both at the direct effects on obese individuals, and in offspring exposed to these environmental conditions during development.

Epigenetic processes of gene regulation, such as DNA methylation, would be a relevant area of investigation due to the link between folate metabolism and methylation reactions[82]. A rodent study reported that disturbances in folate metabolism from a mutation in the methionine synthase reductase (*Mtrr*) enzyme in the maternal grandparent generation results in intrauterine growth restriction, developmental delay, and congenital malformations in the F2 generation[187]. In humans, a large epigenome-wide meta-analysis of newborns reported associations between maternal plasma folate and DNA methylation in cord blood at 443 CpGs[188]. Uncovering epigenetic patterns in specific tissues of offspring exposed to altered maternal B-vitamin status during development may provide more insight into the mechanisms involved in programming of offspring adiposity.

Finally, recent reports have demonstrated that in addition to maternal perinatal environmental conditions, paternal stimuli may also be involved in developmental programming

of offspring metabolic health. For instance, paternal obesity has been associated with adverse effects on childhood adiposity in humans, and in glucose homeostasis and β-cell function in mice[189–191]. Very little is known about paternal B-vitamin status and offspring health; however, one study did report that low paternal dietary folate alters epigenetic patterns in mouse sperm resulting in negative pregnancy outcomes[192]. Much work remains to be done in this field to uncover the long-term impact on health outcomes of the offspring resulting from paternal B-vitamin status.

In conclusion, the maternal environmental conditions to which one is exposed during prenatal and early postnatal life influence the trajectory of offspring development and long-term health. It is evident that maternal B-vitamin status programs adiposity and glucose homeostasis in offspring. The benefits of folic acid supplementation of grains for human consumption in preventing NTDs are undeniable. Nevertheless, it is essential that we gain a better understanding of the health implications of developmental exposure to maternal B-vitamin status to improve the health of future generations.

References

1. Aleliunas, R. E. *et al*. Folic acid supplementation of female mice, with or without vitamin B12, before and during pregnancy and lactation programs adiposity and vascular health in adult male offspring. *Am. J. Clin. Nutr.* **146,** 688–696 (2016).
2. Henderson, A. *et al*. The role of insulin-like growth factor-1 in programming of offspring adiposity by maternal folate/ vitamin B12 imbalance. *FASEB J.* **30,** (2016).
3. Henderson, A. & Tai, D. In preparation. (2017).
4. World Health Organization. Obesity: preventing and managing the global epidemic. *WHO Tech. Rep. Ser.* **894,** (2000).
5. Bhurosy, T. & Jeewon, R. Overweight and obesity epidemic in developing countries: a problem with diet, physical activity, or socioeconomic status. *Sci. World J.* **2014,** 964236 (2014).
6. Yach, D., Stuckler, D. & Brownell, K. D. Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nat. Med.* **12,** 62–66 (2006).
7. World Health Organization. Obesity and overweight: Geneva, Switzerland. *WHO Media Cent*. (2013).
8. Centers for Disease Control. Overweight and obesity. http://www.cdc.gov/obesity/adult/causes/ index.htm (2013).
9. Poirier, P. *et al*. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. *Circulation* **113,** 898–918 (2006).
10. American Diabetes Association. Type 2 diabetes in children and adolescents. *Pediatrics* **105,** 671–680 (2000).
11. Yoon, K. H. *et al*. Epidemic obesity and type 2 diabetes in Asia. *Lancet* **368,** 1681–1688

(2006).

12. Alberti, G. *et al*. Type 2 diabetes in the young: the evolving epidemic. *Diabetes Care* **27,** 1798–1811 (2004).

13. Roberts, K., Shields, M., DeGroh, M., Aziz, A. & Gilbert, J. Overweight and obesity in children and adolescents: results from the 2009 to 2011 Canadian Health Measures Survey. *Heal. Reports* **9,** 37–41 (2012).

14. Alberti, G., Zimmet, P. & Shaw, J. Metabolic syndrome: a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet. Med.* **23,** 469–80 (2006).

15. Lee, I., Manson, J. E., Hennekens, C. H. & Paffenbarger, R. S. Body weight and mortality. *JAMA* **270,** 2823–2828 (1993).

16. Ohlson, L. *et al*. The infuence of body fat distribution on the indicence of diabetes mellitus: 13.5 years of follow-up of the participants in the study of men born in 1913. *Diabetes* **34,** 1055–1058 (1985).

17. Rexrode, K. M. *et al*. Abdominal adiposity and coronary heart disease in women. *JAMA* **280,** 1843–1848 (1998).

18. Després, J. P. Body fat distribution and risk of cardiovascular disease: An update. *Circulation* **126,** 1301–1313 (2012).

19. Despres, J. & Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **444,** 881–887 (2006).

20. Zimmet, P., Magliano, D., Matsuzawa, Y., Alberti, G. & Shaw, J. The metabolic syndrome: a global public health problem and a new definition. *J. Atheroscler. Thromb.* **12,** 295–300 (2005).

21. Yusuf, S. *et al*. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. *Lancet* **366,** 1640–1649 (2005).

22. Seidell, J. C., Pérusse, L., Després, J. P. & Bouchard, C. Waist and hip circumferences have independent and opposite effects on cardiovascular disease risk factors: the Quebec Family Study. *Am. J. Clin. Nutr.* **74,** 315–321 (2001).

23. Terry, R. B., Stefanick, M. L., Haskell, W. L. & Wood, P. D. Contributions of regional adipose tissue depots to plasma lipoprotein concentrations in overweight men and women: Possible protective effects of thigh fat. *Metabolism* **40,** 733–740 (1991).

24. Misra, A. *et al*. Relationship of anterior and posterior subcutaneous abdominal fat to insulin sensitivity in nondiabetic men. *Obes. Res.* **5,** 93–99 (1997).

25. Snijder, M. B. *et al*. Association of hip and thigh circumferences independent of waist circumference with the incidence of type 2 diabetes: The Horn Study. *Am. J. Clin. Nutr.* **77,** 1192–1197 (2003).

26. Abbasi, F., Brown, B. W., Lamendola, C., McLaughlin, T. & Reaven, G. M. Relationship between obesity, insulin resistance, and coronary heart disease risk. *J. Am. Coll. Cardiol.* **40,** 937–943 (2002).

27. Mauriege, P. *et al*. Regional variation in adipose tissue metabolism of severely obese premenopausal women. *J. Lipid Res.* **36,** 684 (1995).

28. Bergman, R. N. *et al*. Why visceral fat is bad: mechanisms of the metabolic syndrome. *Obes. (Silver Spring)* **14 Suppl 1,** 16S–19S (2006).

29. Wu, H. & Ballantyne, C. Skeletal muscle inflammation and insulin resistance in obesity. *J. Clin. Invest.* **4,** 22–25 (2017).

30. Mittelman, S. D., Citters, G. W. Van, Kirkman, E. L. & Bergman, R. N. Extreme insulin

resistance of the central adipose depot in vivo. *Diabetes* **51,** 755–761 (2002).

31. Gravena, C., Mathias, P. C. & Ashcroft, S. J. H. Acute effects of fatty acids on insulin secretion from rat and human islets of Langerhans. *J. Endocrinol.* **173,** 73–80 (2002).

32. Joseph, J. W. *et al.* Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression. *J. Biol. Chem.* **279,** 51049–51056 (2004).

33. Lupi, R. *et al.* Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets. *Diabetes* **51,** 1437–1442 (2002).

34. Back, S. & Kaufman, R. Endoplasmic reticulum stress and type 2 diabetes. *Annu. Rev. Biochem.* **81,** 767–793 (2012).

35. Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112,** 1796–1808 (2003).

36. Yudkin, J., Stehouwer, C., Emeis, J. & Coppack, S. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction. *Arterioscler. Thromb. Vasc. Biol.* **19,** 972–978 (1999).

37. Côté, M. *et al.* Adiponectinemia in visceral obesity: impact on glucose tolerance and plasma lipoprotein and lipid levels in men. *J. Clin. Endocrinol. Metab.* **90,** 1434–1439 (2005).

38. Wang, B., Jenkins, J. R. & Trayhurn, P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. *Am. J. Physiol. Endocrinol. Metab.* **288,** E731–E740 (2005).

39. Barker, D. J. P. The developmental origins of adult disease. *J. Am. Coll. Nutr.* **23,** 588S–595S (2004).

40. Bateson, P. Fetal experience and good adult design. *Int. J. Epidemiol.* **30,** 928–934 (2001).

41. Hales, C. N. & Barker, D. J. P. The thrifty phenotype hypothesis. *Br. Med. Bull.* **60,** 5–20 (2001).

42. Gluckman, P. D. *et al.* Towards a new developmental synthesis: adaptive developmental plasticity and human disease. *Lancet* **373,** 1654–1657 (2009).

43. Vaag, A. A., Grunnet, L. G., Arora, G. P. & Brøns, C. The thrifty phenotype hypothesis revisited. *Diabetologia* **55,** 2085–2088 (2012).

44. Reinehr, T., Kleber, M. & Toschke, A. M. Small for gestational age status is associated with metabolic syndrome in overweight children. *Eur. J. Endocrinol.* **160,** 579–584 (2009).

45. Chen, W. *et al.* Low birth weight is associated with higher blood pressure variability from childhood to young adulthood: the Bogalusa Heart Study. *Am. J. Epidemiol.* **176,** 99–105 (2012).

46. Roseboom, T., Rooij, S. De & Painter, R. The Dutch famine and its long-term consequences for adult health. *Early Hum. Dev.* **82,** 485–491 (2006).

47. Ravelli, A. C. J. *et al.* Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351,** 173–177 (1998).

48. de Rooij, S. *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* **49,** 637–643 (2006).

49. Roseboom, T. J. *et al.* Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. *Am. J. Clin. Nutr.* **72,** 1101–6 (2000).

50. Roseboom, T. J. *et al.* Coronary heart disease after prenatal exposure to the Dutch famine, 1944 – 1945. *Heart* **84,** 595–598 (2000).

51. Painter, R. C. *et al*. Early onset of coronary artery disease after prenatal exposure to the Dutch famine. *Am. J. Clin. Nutr.* **84,** 322–327 (2006).

52. Li, Y. *et al*. Exposure to the Chinese famine in early life and the risk of metabolic syndrome in adulthood. *Diabetes Care* **34,** 1014–1018 (2011).

53. Shane, B. Folate and vitamin B12 metabolism: overview and interaction with riboflavin, vitamin B6, and polymorphisms. *Food Nutr. Bull.* **29,** S5–S16 (2008).

54. Guidelines Expert Advisory Group on National Nutrition in Pregnancy. Prenatal nutrition guidelines for health professionals: folate contributes to a healthy pregnancy. *Heal. Canada, Gov. Canada* (2009).

55. Devlin, A. M. *et al*. Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. *Hum. Mol. Genet.* **9,** 2837–2844 (2000).

56. Fedosov, S. N., Fedosova, N. U., Berglund, L., Moestrup, S. K. & Nexø, E. Composite organization of the cobalamin binding and cubilin recognition sites of intrinsic factor. *Biochemistry* **44,** 3604–3614 (2005).

57. Seetharam, B. Receptor-mediated endocytosis of cobalamin. *Annu. Rev. Nutr.* **19,** 173–95 (1999).

58. Glier, M. B., Green, T. J. & Devlin, A. M. Methyl nutrients, DNA methylation, and cardiovascular disease. *Molecular Nutrition and Food Research* **58,** 172–182 (2014).

59. Selhub, J. Homocysteine metabolism. *Annu. Rev. Nutr.* **19,** 217–246 (1999).

60. Brosnan, J. T. & Brosnan, M. E. The sulfur-containing amino acids: an overview. *J. Nutr.* **136,** 1636–1640 (2006).

61. Mato, M., Mart, M. L. & Lu, S. C. Methionine metabolism and liver disease. *Annu. Rev.*

Nutr. **28,** 273–93 (2008).

62. Vance, J. E. & Vance, D. E. Phospholipid biosynthesis in mammalian cells. *Biochem. Cell Biol*. **128,** 113–128 (2004).

63. Scott, J. M. & Weir, D. G. The methyl folate trap. *Lancet* **2,** 337–40 (1981).

64. Scott, J. M. Folate and vitamin B12. *Proc. Nutr. Soc*. **58,** 441–448 (1999).

65. Reynolds, E. & Road, C. Vitamin B12, folic acid, and the nervous system. *Lancet Neurol*. **5,** 949–60 (2006).

66. Nelson, D. & Cox, M. *Lehninger Principles of Biochemistry, 6th Edition*. (2013).

67. Dowman, J. K., Tomlinson, J. W. & Newsome, P. N. Pathogenesis of non-alcoholic fatty liver disease. *QJM* **103,** 71–83 (2010).

68. Rui, L. Energy metabolism in the liver. *Compr. Physiol*. **4,** 177–197 (2014).

69. Reynisdottir, S. *et al*. Adipose tissue lipoprotein lipase and hormone-sensitive lipase. *Arterioscler. Thromb. Vasc. Biol*. **17,** 2287–2292 (1997).

70. Wakil, S. J. & Abu-Elheiga, L. A. Fatty acid metabolism: target for metabolic syndrome. *J. Lipid Res*. **50,** S138–S143 (2008).

71. da Silva, R. P., Kelly, K. B., Al Rajabi, A. & Jacobs, R. L. Novel insights on interactions between folate and lipid metabolism. *Biofactors* **40,** 277–283 (2014).

72. Delong, C. J., Shen, Y., Thomas, M. J. & Cui, Z. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J. Biol. Chem*. **274,** 29683–29688 (1999).

73. Devlin, A. M. *et al*. Hypermethylation of Fads2 and altered hepatic fatty acid and phospholipid metabolism in mice with hyperhomocysteinemia. *J. Biol. Chem*. **282,** 37082–37090 (2007).

74. Jacobs, R. L., Lingrell, S., Zhao, Y., Francis, G. A. & Vance, D. E. Hepatic CTP:phosphocholine cytidylyltransferase-alpha is a critical predictor of plasma HDL and VLDL. *J. Biol. Chem.* **283,** 2147–2155 (2008).

75. Vance, D. E. & Li, Z. Hepatic phosphatidylethanolamine N-methyltransferase, unexpected roles in animal biochemistry and physiology. *J. Biol. Chem.* **282,** 33237–33241 (2007).

76. Ghosh, S., Sulistyoningrum, D. C., Glier, M. B., Verchere, C. B. & Devlin, A. M. Altered glutathione homeostasis in heart augments cardiac lipotoxicity associated with diet-induced obesity in mice. *J. Biol. Chem.* **286,** 42483–42493 (2011).

77. Akesson, B., Fehling, C., Jagerstad, M. & Stenram, U. Effect of experimental folate deficiency on lipid metabolism in liver and brain. *Br. J. Nutr.* **47,** 505 (1982).

78. Christensen, K. E. *et al*. Steatosis in mice is associated with gender, folate intake, and expression of genes of one-carbon metabolism. *J. Nutr.* **140,** 1736–41 (2010).

79. Smithells, R. W., Sheppard, S. & Schorah, C. J. Vitamin deficiencies and neural tube defects. *Arch. Dis. Child.* **51,** 944–951 (1976).

80. Smithells, R. et al. Possible prevention of neural tube defects by periconceptional vitamin supplementation. *Lancet* **1,** 339–340 (1980).

81. Smithells, R. W. *et al*. Further experience of vitamin supplementation for prevention of neural tube defect recurrences. *Lancet* **321,** 1027–1031 (1983).

82. Blom, H. J. Folic acid, methylation, and neural tube closure in humans. *Birth Defects Res. (Part A) Clin. Mol. Teratol.* **85,** 295–302 (2009).

83. Seller, M. & Nevin, N. Periconceptional vitamin supplementation and the prevention of neural tube defects in south-east England and Northern Ireland. *J. Med. Genet.* **12,** 325–330 (1984).

84. MRC Vitamin Study Research. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. *Lancet* **338,** 131–37 (1991).

85. Czeizel, A. & Dudas, I. Prevention of the first occurence of neural tube defects by periconceptional vitamin supplementation. *N. Engl. J. Med.* **327,** 1832–5 (1992).

86. Berry, R., Li, Z. & Erickson, J. Prevention of neural tube defects with folic acid in China. *N. Engl. J. Med.* **341,** 1485–1490 (1999).

87. Centers for Disease Control. Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Recomm. Rep.* **41,** (1992).

88. Health Canada. Food and Drug Regulations, Ammendment Schedule No. 1066. **No. 1066,** (1997).

89. Food and Drug Administration USA. vol 61 No. 44. **61,** 8781 (1996).

90. De Wals, P., Tairou, F., Van Allen, M. & UH, S. Reduction in neural tube defects after folic acid fortification in Canada. *N. Engl. J. Med.* **357,** 135–142 (2007).

91. Colapinto, C. K., Connor, D. L. O. & Tremblay, M. S. Folate status of the population in the Canadian Health Measures Survey. *Can. Med. Assoc. J.* **183,** E100–E106 (2011).

92. Ray, J. G., Goodman, J., Mahoney, P. R. A. O., Mamdani, M. M. & Jiang, D. High rate of maternal vitamin B12 deficiency nearly a decade after Canadian folic acid flour fortification. *QJM* **101,** 475–477 (2008).

93. MacFarlane, A. J., Greene-Finestone, L. S. & Shi, Y. Vitamin B-12 and homocysteine status in a folate-replete population: results from the Canadian Health Measures Survey. *Am. J. Clin. Nutr.* **94,** 1079–1087 (2011).

94. Bailey, R. L. *et al.* Unmetabolized serum folic acid and its relation to folic acid intake

from diet and supplements in a nationally representative sample of adults aged >60 years in the United States. *Am. J. Clin. Nutr.* **92,** 383–9 (2010).

95. Morris, M. S., Jacques, P. F., Rosenberg, I. H. & Selhub, J. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. *Am. J. Clin. Nutr.* **91,** 1733–44 (2010).

96. Boilson, A. *et al*. Unmetabolized folic acid prevalence is widespread in the older Irish population despite the lack of a mandatory fortification program. *Am. J. Clin. Nutr.* **96,** 613–21 (2012).

97. Obeid, R., Kasoha, M., Kirsch, S. H., Munz, W. & Herrmann, W. Concentrations of unmetabolized folic acid and primary folate forms in pregnant women at delivery and in umbilical cord blood. *Am. J. Clin. Nutr.* **92,** 1416–1422 (2010).

98. Hagood, J. Beyond the genome: epigenetic mechanisms in lung remodeling. *Physiology* **29,** 177–85 (2014).

99. Reik, W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* **447,** 425–432 (2007).

100. Probst, A. V. & Almouzni, G. Heterochromatin establishment in the context of genome-wide epigenetic reprogramming. *Trends Genet.* **27,** 177–185 (2011).

101. Waterland, R. A. & Jirtle, R. L. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol. Cell. Biol.* **23,** 5293–300 (2003).

102. Weaver, I. C. G. *et al*. Epigenetic programming by maternal behavior. *Nat. Neurosci.* **7,** 847–54 (2004).

103. Bird, A. DNA methylation patterns and epigenetic memory DNA methylation patterns and epigenetic memory. *Genes Dev.* **16,** 6–21 (2002).

104. Li, E., Beard, C. & Jaenisch, R. Role for DNA methylation in genomic imprinting. *Nature* **366,** 362–5 (1993).

105. Glier, M. B. *et al*. Tissue-specific relationship of S-adenosylhomocysteine with allele-specific H19/Igf2 methylation and imprinting in mice with hyperhomocysteinemia. *Epigenetics* **8,** 44–53 (2013).

106. Refsum, H. *et al*. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. *Am. J. Clin. Nutr.* **74,** 233–241 (2001).

107. Allen, L. H. How common is vitamin B12 deficiency? *Am. J. Clin. Nutr.* **89,** 693S (2009).

108. Ramachandran, A. *et al*. High prevalence of diabetes and impaired glucose tolerance in India: National Urban Diabetes Survey. *Diabetologia* **44,** 1094–1101 (2001).

109. Chowdhury, B., Lantz, H. & Sjöström, L. Computed tomography-determined body composition in relation to cardiovascular risk factors in Indian and matched Swedish males. *Metabolism*. **45,** 634–644 (1996).

110. Yajnik, C. S., Deshpande, S. S. & Jackson, A. A. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. *Diabetologia* **51,** 29–38 (2008).

111. Krishnaveni, G. V., Veena, S. R., Karat, S. C., Yajnik, C. S. & Fall, C. H. D. Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia* **57,** 110–121 (2014).

112. Stewart, C. P. *et al*. Low maternal vitamin B12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural Nepal. *J. Nutr.* **141,** 1912–1917 (2011).

113. Wang, G. *et al*. Association between maternal pre-pregnancy body mass index and plasma folate concentrations with child metabolic health. *JAMA Pediatr*. **21205,** e160845 (2016).

114. Kumar, K. A. *et al*. Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. *J. Nutr. Biochem*. **24,** 25–31 (2013).

115. Roman-Garcia, P. *et al*. Vitamin B12-dependent taurine synthesis regulates growth and bone mass. *J. Clin. Invest*. **124,** 2988–3002 (2014).

116. Mendl, M. The effects of litter size variation on mother-offspring relationships and behavioral and physical development in several mammalian species (principally rodents). *J. Zool*. **215,** 15–34 (1988).

117. Colapinto, C. K., O'Connor, D. L., Dubois, L. & Tremblay, M. S. Folic acid supplement use is the most significant predictor of folate concentrations in Canadian women of childbearing age. *Appl. Physiol. Nutr. Metab*. **37,** 284–92 (2012).

118. Bailey, R. L. *et al*. Total folate and folic acid intakes from foods and dietary supplements of US children aged 1-13 y. *Am. J. Clin. Nutr*. **92,** 353–358 (2010).

119. Bailey, R. L. *et al*. Total folate and folic acid intake from foods and dietary supplements in the United States: 2003-2006. *Am. J. Clin. Nutr*. **91,** 231–237 (2010).

120. Kelly, K. B. *et al*. Excess folic acid increases lipid storage, weight gain, and adipose tissue inflammation in high fat diet-fed rats. *Nutrients* **8,** (2016).

121. Reeves, P., Nielsen, F. & Fahey, G. Committee Report AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. *J. Nutr*. **123,** 1939–1951 (1993).

122. Subcommittee on Laboratory Animal Nutrition. Committee on Animal Nutrition. Board on Agriculture. Nutrient requirements of the mouse. Nutrient requirements of laboratory animals. *Natl. Acad. Press* 80–102 (1995).

123. O'Rourke, B. Metabolism: beyond the power of mitochondria. *Nat. Rev. Cardiol.* **13,** 386–388 (2016).

124. Dranka, B. P. *et al.* Assessing bioenergetic function in response to oxidative stress by metabolic profiling. *Free Radic. Biol. Med.* **51,** 1621–1635 (2011).

125. Green, H. & Meuth, M. An established pre-adipose cell line and its differentiation in culture. *Cell* **3,** 127–133 (1974).

126. Green, H. & Kehinde, O. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1,** 113–116 (1974).

127. Zebisch, K., Voigt, V., Wabitsch, M. & Brandsch, M. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Anal. Biochem.* **425,** 88–90 (2012).

128. Arora, M. Cell culture media: a review. *Mater. Methods* **3,** 175 (2013).

129. Ramirez-Zacarias, J., Castro-Munozledo, F. & Kuri-Harcuch, W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil Red O. *Histochemistry* **97,** 493–497 (1992).

130. Brand, M. D. & Nicholls, D. G. Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435,** 297–312 (2011).

131. Bradford, M. M. A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254 (1976).

132. Mehlem, A., Hagberg, C. E., Muhl, L., Eriksson, U. & Falkevall, A. Imaging of neutral

lipids by Oil Red O for analyzing the metabolic status in health and disease. *Nat. Protoc.* **8,** 1149–1154 (2013).

133. Shah, K., McCormack, C. E. & Bradbury, N. A. Do you know the sex of your cells? *Am. J. Physiol. Cell Physiol.* **306,** C3–C18 (2014).

134. Fraser, A. *et al.* Association of maternal weight gain in pregnancy with offspring obesity and metabolic and vascular traits in childhood. *Circulation* **121,** 2557–2564 (2010).

135. Reynolds, R. M., Osmond, C., Phillips, D. I. W. & Godfrey, K. M. Maternal BMI, parity, and pregnancy weight gain: Influences on offspring adiposity in young adulthood. *J. Clin. Endocrinol. Metab.* **95,** 5365–5369 (2010).

136. Samuelsson, A. 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* **51,** 383–392 (2008).

137. Reynolds, C. M., Li, M., Gray, C. & Vickers, M. H. Preweaning growth hormone treatment ameliorates adipose tissue insulin resistance and inflammation in adult male offspring following maternal undernutrition. *Endocrinology* **154,** 2676–2686 (2013).

138. Berryman, D. E., Glad, C. a M., List, E. O. & Johannsson, G. The GH/IGF-1 axis in obesity: pathophysiology and therapeutic considerations. *Nat. Rev. Endocrinol.* **9,** 346–56 (2013).

139. Hwa, V., Oh, Y. & Rosenfeld, R. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr. Rev.* **20,** 761–787 (1999).

140. Hedbacker, K. *et al.* Antidiabetic effects of IGFBP2, a leptin-regulated gene. *Cell Metab.* **11,** 11–22 (2010).

141. Neumann, U. H. *et al.* IGFBP2 is neither sufficient nor necessary for the physiological

actions of leptin on glucose homeostasis in male ob/ob mice. *Endocrinology* **155,** 716–725 (2014).

142. Smith, T., Sloboda, D. M., Saffery, R., Joo, E. & Vickers, M. H. Maternal nutritional history modulates the hepatic IGF-IGFBP axis in adult male rat offspring. *Endocrine* **46,** 70–82 (2014).

143. Li, M., Reynolds, C. M., Gray, C. & Vickers, M. H. Preweaning GH treatment normalizes body growth trajectory and reverses metabolic dysregulation in adult offspring after maternal undernutrition. *Endocrinology* **156,** 3228–3238 (2015).

144. Boucher, J., Tseng, Y. & Kahn, C. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J. Biol. Chem.* **285,** 17235–17245 (2010).

145. Badalà, F., Nouri-Mahdavi, K. & Raoof, D. A. Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia* **56,** 949–964 (2013).

146. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. & Ui, M. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J. Biol. Chem.* **269,** 3568–3573 (1994).

147. Stenbit, A. *et al*. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. *Nat. Med.* **3,** 1096–1101 (1997).

148. Li, J., Houseknecht, K., Stenbit, A., Katz, E. & Charron, M. Reduced glucose uptake precedes insulin signaling defects in adipocytes from heterozygous GLUT4 knockout mice. *FASEB J.* **14,** 1117–25 (2000).

149. Abel, E. *et al*. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* **409,** 729–733 (2001).

150. Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R. & Kahn, B. B. Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J. Clin. Invest.* **104,** 733–741 (1999).

151. Wang, C. & Liao, J. K. A mouse model of diet-induced obesity and insulin resistance. *Methods Mol. Biol.* **821,** 1–11 (2012).

152. Collins, S., Martin, T. L., Surwit, R. S. & Robidoux, J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol. Behav.* **81,** 243–248 (2004).

153. Cordain, L. *et al*. Origins and evolution of the Western diet: health implications for the 21st century. *Am. J. Clin. Nutr.* **81,** 341–354 (2005).

154. Pettersson, U. S., Waldén, T. B., Carlsson, P.-O., Jansson, L. & Phillipson, M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS One* **7,** e46057 (2012).

155. Rodbell, M. Metabolism of isolated fat cells: effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239,** 375–380 (1961).

156. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta delta CT method. *Methods* **25,** 402–408 (2001).

157. Sturman, J. A. Taurine in development. *Physiol. Rev.* **73,** 119–147 (1993).

158. Verner, A., Craig, S. & McGuire, W. Effect of taurine supplementation on growth and development in preterm or low birth weight infants. *Cochrane Database Syst. Rev.* **4,** CD006072 (2007).

159. Mao, J. *et al*. aP2-Cre-mediated inactivation of acetyl-CoA carboxylase-1 causes growth

retardation and reduced lipid accumulation in adipose tissues. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 17576–81 (2009).

160. Ntambi, J. M. *et al.* Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11482–6 (2002).

161. Kris-Etherton, P. M., Harris, W. S. & Appel, L. J. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* **106,** 2747–2757 (2002).

162. Chapkin, R. S., Kim, W., Lupton, J. R. & McMurray, D. N. Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. *Prostaglandins Leukot. Essent. Fat. Acids* **81,** 187–191 (2009).

163. Talukdar, S., Oh, S., E, B. & Imamura, T. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin sensitizing effects. *Cell* **142,** 687–698 (2011).

164. Flachs, P., Rossmeisl, M., Bryhn, M. & Kopecky, J. Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin. Sci.* **116,** 1–16 (2009).

165. Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226,** 497–509 (1957).

166. Vernochet, C. *et al.* Adipose tissue mitochondrial dysfunction triggers a lipodystrophic syndrome with insulin resistance, hepatosteatosis, and cardiovascular complications. *FASEB J.* **28,** 4408–4419 (2014).

167. Heilbronn, L. K., Seng, K. G., Turner, N., Campbell, L. V. & Chisholm, D. J. Markers of mitochondrial biogenesis and metabolism are lower in overweight and obese insulin-resistant subjects. *J. Clin. Endocrinol. Metab.* **92,** 1467–1473 (2007).

168. Kaaman, M. *et al*. Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. *Diabetologia* **50,** 2526–2533 (2007).

169. De Pauw, A., Tejerina, S., Raes, M., Keijer, J. & Arnould, T. Mitochondrial (dys)function in adipocyte (de)differentiation and systemic metabolic alterations. *Am. J. Pathol.* **175,** 927–39 (2009).

170. Symonds, M. *Adipose Tissue Biology*. (2012).

171. Burdge, G. C. *et al*. Folic acid supplementation during the juvenile-pubertal period in rats modifies the phenotype and epigenotype induced by prenatal nutrition. *J. Nutr.* **139,** 1054–60 (2009).

172. Sie, K. K. Y. *et al*. Effect of maternal and postweaning folic acid supplementation on global and gene-specific DNA methylation in the liver of the rat offspring. *Mol. Nutr. Food Res.* **57,** 677–685 (2013).

173. Vankoningsloo, S. *et al*. Mitochondrial dysfunction induces triglyceride accumulation in 3T3-L1 cells: role of fatty acid beta-oxidation and glucose. *J. Lipid Res.* **46,** 1133–1149 (2005).

174. Yakar, S. *et al*. Liver-specific Igf1 gene deletion leads to muscle insulin insensitivity. *Diabetes* **50,** 1110–1118 (2001).

175. Fernández, A. M. *et al*. Functional inactivation of the IGF-I and insulin receptors in skeletal muscle causes type 2 diabetes. *Genes Dev.* **15,** 1926–1934 (2001).

176. Leney, S. E. & Tavare, J. M. The molecular basis of insulin-stimulated glucose uptake: signalling, trafficking and potential drug targets. *J. Endocrinol.* **203,** 1–18 (2009).

177. Sinah, Y. N., Salocks, C. B. & Vanderlaan, W. P. Prolactin and growth hormone levels in different inbred strains of mice: patterns in association with estrous cycle, time of day, and

perphenazine stimulation. *Endocrinology* **97,** 112–22 (1975).

178. Klerman, K. B. & et al. A statistical model of diurnal variation in human growth hormone. *Am. J. Physiol. Endocrinol. Metab.* **285,** E1118-1126 (2003).

179. Sinha, Y., Salocks, C., Wickes, M. & Vanderlaan, W. Serum and pituitary concentrations of prolactin and growth hormone in mice during a twenty-four hour period. *Endocrinology* **100,** 786–791 (1977).

180. Keung, W. *et al*. Inhibition of carnitine palmitoyltransferase-1 activity alleviates insulin resistance in diet-induced obese mice. *Diabetes* **62,** 711–720 (2013).

181. Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E. A. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **281,** 785–789 (1963).

182. Bailey, S. W. & Ayling, J. E. 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.* **106,** 15424–9 (2009).

183. Whitehead, V., Kamen, B. & Beaulieu, D. Levels of dihydrofolate reductase in livers of birds, animals, primates, and man. *Cancer Drug Deliv.* **4,** 185–189 (1987).

184. Kennedy, D. & Koren, G. Identifying women who might benefit from higher doses of folic acid in pregnancy. *Can. Fam. Physician* **58,** 394–397 (2012).

185. Schneider, M. P. *et al*. Folic acid treatment normalizes NOS-dependence of vascular tone in the metabolic syndrome. *Obesity (Silver Spring).* **19,** 960–967 (2011).

186. Tinker, S. C., Hamner, H. C., Berry, R. J., Bailey, L. B. & Pfeiffer, C. M. 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. (Part A) Clin. Mol.*

Teratol. **94,** 749–755 (2012).

187. Padmanabhan, N. *et al*. Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. *Cell* **155,** 81–93 (2013).

188. Joubert, B. R. *et al*. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nat. Commun.* **7,** 10577 (2016).

189. Soubry, A. *et al*. Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC Med* **11,** 29 (2013).

190. Fleten, C. *et al*. Parent-offspring body mass index associations in the Norwegian Mother and Child Cohort Study: a family-based approach to studying the role of the intrauterine environment in childhood adiposity. *Am. J. Epidemiol.* **176,** 83–92 (2012).

191. Ng, S.-F. *et al*. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* **467,** 963–966 (2010).

192. Lambrot, R. *et al*. Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nat. Commun.* **4,** 2889 (2013).