# GESTATIONAL OBESITY/PREDIABETES AND FOLIC ACID SUPPLEMENTATION PROGRAM FETAL ONE-CARBON METABOLISM AND BETA CELL MASS

by

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## Abstract

**Background:** Folic acid supplementation is recommended for women of childbearing age to prevent birth defects. Women with pregestational obesity (BMI≥30kg/$m^2$) and/or diabetes are recommended to take up to 12x the recommended dose of folic acid. Concerns have been raised that elevated folic acid during pregnancy may have negative impacts on the cardiometabolic health of the mother and child. The objective of my thesis was to determine the effects of folic acid supplementation in gestational obesity/prediabetes on maternal and fetal health.

**Methods:** Female (C57BL/6J) mice were fed from weaning a control diet (10% kcal fat; control dams) or western diet (45% kcal fat; western dams; model of obesity/prediabetes). Diets contained supplemental (10mg/kg diet) or recommended level (2mg/kg diet) folic acid. Dams (n=11-14/diet) were fed for 13 weeks prior to breeding with control males. Tissue from dams and fetal offspring were collected at embryonic day (E)18.5.

**Results:** Prior to breeding, western dams had greater body weight and adiposity accompanied by glucose intolerance and impaired β cell function. Folic acid supplementation reduced insulin sensitivity in control dams and improved insulin sensitivity in western dams. At E18.5, western dams had larger livers and key methyl donor, *S*-adenosylmethionine (SAM), and it's demethylated product, *S*-adenosylhomocysteine (SAH), were lower in liver, compared to control dams. Male offspring from western dams had smaller livers and higher hepatic betaine and choline compared to offspring from control dams. Female offspring from western dams had smaller livers and higher hepatic betaine compared to those from control dams. Maternal folic acid supplementation increased hepatic SAM in female offspring, but not in male offspring.

Maternal diet did not affect fetal pancreas size, but male offspring from folic acid supplemented dams had greater β cell mass and β cell density compared to those from non-supplemented dams; no effect of maternal diet on β cell mass was observed in female offspring.

**Conclusion:** Folic acid supplementation does not exacerbate adiposity and glucose tolerance in dams with gestational obesity/prediabetes. However, maternal folic acid supplementation and gestational obesity/prediabetes has sex-specific effects on hepatic one-carbon metabolism and pancreatic β cell mass in fetal offspring.

## Lay Summary

Women of childbearing age are recommended to take a daily supplement containing 0.4mg of folic acid for the prevention of birth defects. There is concern that too much folic acid during pregnancy may be harmful for the health of the mother and offspring. My thesis research used a mouse model to investigate if folic acid supplementation during pregnancy influences changes in body fat, blood sugar regulation, and the amount of carbon-donating nutrients required for metabolic functions in the mother and fetus, particularly if the mother has obesity. I found that folic acid supplementation during pregnancy had little impact on the mother's health, but caused an increase in cells that control sugar levels in male fetuses and increased a key carbon-donating nutrient in female fetuses. These findings provide insight into how the mother's body responds to folic acid supplementation, and how it may affect the health of the fetal offspring.

## Preface

This thesis is submitted in partial fulfillment of the requirements for the Master of Science degree in the Reproductive and Developmental Sciences (RDS) program in the Department of Obstetrics and Gynaecology, University of British Columbia (UBC). Experimental procedures presented in this thesis were conducted in the laboratory of Dr. Angela Devlin at the BC Children's Hospital Research Institute (BCCHRI), except when stated otherwise. Animal work was conducted in the Animal Care Facility of BCCHRI and was approved by the UBC Animal Care Committee (certificate # A14-0246, A14-0030, and A18-0059) and the UBC Biosafety Committee (certificate # B18-0029).

Animal breeding, physiological assessments, and tissue collection of mice were performed by me. Genotyping for sex determination of fetal offspring was performed by me with the help of Rebecca Lim, Martina Stokes, and Danielle Cohen. Pancreas processing was completed by me, with some samples prepared by the Histology Core Lab at BCCHRI. Immunohistochemistry and imaging experiments were conducted by me; imaging was completed in the Imaging Suite at BCCHRI. Analysis of β cell mass was performed by me, and analysis of α cell mass was performed by me and Danielle Cohen. Quantification of liver triglyceride content was completed by me. Water soluble choline metabolites were quantified in the Analytics Core for Metabolomics and Nutrition at BCCHRI by Roger Dyer. Total *S*-adenosylmethionine and *S*-adenosylhomocysteine levels were quantified in the laboratory of Dr. Joshua Miller in the Department of Nutritional Sciences at Rutgers University, New Brunswick, NJ, USA.

Part of my initial research findings were presented as a virtual poster format at the Canadian Nutrition Society 2020 Annual Conference in May 2020; this meeting was hosted as a virtual

format due to the COVID-19 pandemic. My abstract from this meeting has been published: Mussai E, Boonpattrawong NP, Ramírez CY, Mehran AE, and Devlin AM. Maternal folic acid supplementation during pregnancy has sex-specific effects on pancreatic beta cell mass in fetal offspring. *Appl. Physiol. Nutr. Metab.* 2020;45:S36. Data from my thesis will be submitted for publication with the addition of RNA sequencing data from fetal liver and β cell mass analysis from dams; a manuscript is in preparation.

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| | |
|---|---|
| **10-formylTHF** | 10-formyltetrahydrofolate |
| **5-10-MTHF** | 5,10-methylenetetrahydrofolate |
| **5-MTHF** | 5-methyltetrahydrofolate |
| **ACC** | Acetyl-CoA carboxylase |
| **AIN** | American Institute of Nutrition |
| **AKT** | Protein kinase B |
| **ANOVA** | Analysis of variance |
| **AOC** | Area over the curve |
| **ATP** | Adenosine triphosphate |
| **AUC** | Area under the curve |
| **BHMT** | Betaine-homocysteine methyltransferase |
| **BMI** | Body mass index |
| **CBS** | Cystathionine-β-synthase |
| **CCT** | CTP:phosphocholine cytidylytransferase |
| **CD** | Control diet with adequate folic acid |
| **CDF** | Control diet with supplemental folic acid |
| **CDP** | Cytidine diphosphate |
| **ChREBP** | Carbohydrate-responsive element-binding protein |
| **CK** | Choline kinase |
| **CPT** | CDP-choline:1,2-diacylglycerol cholinephophotransferase |
| **CTH** | Cystathionine-γ-lyase |

| | |
|---|---|
| **CTP** | Choline-phosphate cytidylytransferase |
| **CVD** | Cardiovascular disease |
| **DHF** | Dihydrofolate |
| **DHFR** | Dihydrofolate reductase |
| **DIO/IGT** | Diet-induced obesity/impaired glucose tolerance |
| **DMG** | Dimethylglycine |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleoside triphosphate |
| **DOHaD** | Developmental Origins of Health and Disease |
| **E** | Embryonic day |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EtOH** | Ethanol |
| **FAS** | Fatty acid synthase |
| **FPGS** | Folylpolyglutamate synthetase |
| **GDM** | Gestational diabetes mellitus |
| **GLUT2** | Glucose transporter type 2 |
| **GLUT4** | Glucose transporter type 4 |
| **GPC** | Glycerophosphocholine |
| **GTT** | Glucose tolerance test |
| **GWG** | Gestational weight gain |
| **Hcy** | Homocysteine |
| **HOMA-IR** | Homeostatic model assessment of insulin resistance |

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|---|---|
| **HPLC** | High performance liquid chromatography |
| **IP** | Intraperitoneal |
| **IR** | Insulin receptor |
| **IRS-1** | Insulin receptor substrate-1 |
| **IST** | Insulin secretion test |
| **ITT** | Insulin tolerance test |
| **kcal** | Kilocalorie |
| **LC-MS/MS** | High performance liquid chromatography-tandem mass spectrometry |
| **LGA** | Large for gestational age |
| **MAT** | Methionine adenosyltransferase |
| **Met** | Methionine |
| **MRC** | Medical Research Council |
| **mRNA** | Messenger ribonucleic acid |
| **MTHFD1** | Methylenetetrahydrofolate dehydrogenase 1 |
| **MTHFR** | Methylenetetrahydrofolate reductase |
| **MTR** | Methionine synthase |
| **MTRR** | Methionine synthase reductase |
| **MTs** | Methyltransferases |
| **NAD** | Nicotinamide adenine dinucleotide |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **NAFLD** | Nonalcoholic fatty liver disease |
| **NTD** | Neural tube defects |

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|---|---|
| **OGIS** | Oral glucose insulin sensitivity |
| **PBS** | Phosphate buffered saline |
| **PC** | Phosphatidylcholine |
| **PCFT** | Proton coupled folate transporter |
| **PCR** | Polymerase chain reaction |
| **PE** | Phosphatidylethanolamine |
| **PEMT** | Phosphatidylethanolamine *N*-methyltransferase |
| **PI3K** | Phosphoinositide 3-kinase |
| **RCF** | Reduced folate carrier |
| **RCT** | Randomized control trial |
| **RDA** | Recommended daily allowance |
| **rpm** | Revolutions per minute |
| **SAH** | *S*-adenosylhomocysteine |
| **SAHH** | *S*-adenosylhomocysteine hydrolase |
| **SAM** | *S*-adenosylmethionine |
| **SD** | Standard deviation |
| **SGA** | Small for gestational age |
| **SREBP-1c** | Sterol regulatory element-binding protein 1c |
| **T2D** | Type 2 diabetes |
| **THF** | Tetrahydrofolate |
| **UL** | Tolerable upper limit |
| **UV** | Ultraviolet |

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|---|---|
| **VLDL** | Very-low-density-lipoprotein |
| **WD** | Western diet with adequate folic acid |
| **WDF** | Western diet with supplemental folic acid |

## Acknowledgements

I would like to begin by thanking my supervisor, Dr. Angela Devlin, for her continuous guidance and support over the course of my graduate studies. I am grateful for the skills I have developed and the opportunities I have had as a member of her research team. I am especially grateful to have been introduced to the field of dietetics through Dr. Devlin's lab and for her encouragement and support as I begin to pursue my aspiration of becoming a registered dietitian. I would like to extend my appreciation to my supervisory committee, Dr. Alex Beristain, Dr. Julian Christians, Dr. Crystal Karakochuk, and Dr. Peter Leung, for their valuable insight and guidance throughout my program.

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Finally, I would like to thank all my friends and family, near and far, for their endless love and support throughout my graduate school journey. Whether it be through long hours of video or phone calling, surprise visits and treats, or a warm embrace, I am so grateful for each person who has encouraged me throughout this process. To my parents and sisters, thank you for believing in me and always holding me accountable for being the best that I can be.

# Chapter 1: Introduction

## 1.1 Developmental Origins of Health and Disease

The theory of developmental programing suggests that exposure to environmental factors during fetal and early postnatal development may contribute to the susceptibility of developing chronic disease later in life [1]. This theory, better known as the Developmental Origins of Health and Disease (DOHaD), was proposed by Dr. David Barker following epidemiological studies that revealed a high correlation between low birth weight and greater rates of mortality, as well as higher risk for coronary heart disease, type 2 diabetes, and hypertension [2–4]. The biological basis for this theory stems from the idea that organisms experience a plastic, adaptable period during their development *in utero* in which they are sensitive to environmental factors. This plastic state allows for short-term adaptations to its immediate environment with the goal of preparing for the long-term environment it anticipates living in [5]. Although this strategy may be advantageous in situations where the environment remains static, consequences arise when the postnatal environment changes, such as transitioning to an energy-rich setting following an initially energy-poor environment. A key illustration of the DOHaD theory is observed in the well-documented Dutch Famine Birth Cohort, a group of individuals conceived in the Netherlands between 1944 and 1945 during World War II when many experienced starvation and above-average stress. Studies from this cohort have shown that those who were exposed to famine and stress at any point during gestation were more likely to present with hypertension, glucose intolerance, and impaired insulin secretion during their middle-aged years [6–8]. The impairments in glucose homeostasis were further associated with famine if the exposure occurred during mid-to late gestation [7,8]. Furthermore, exposure during early gestation was associated with obesity, coronary heart disease, and an atherogenic lipid profile later in life [9–11]. It should be

noted that some studies from this cohort have reported no association with exposure to famine, including no association with offspring coronary heart disease or cognitive performance in middle-aged male and female offspring [12,13]. This suggests that the relationship between disease susceptibility and environmental exposures during gestation are not linear and may be influenced by other factors. Nevertheless, studies from this cohort provide evidence for the programming of offspring health by maternal health during gestation.

Studies of malnutrition have paved the way to understanding the DOHaD theory, however other aspects of maternal nutrition can affect the developmental progression of the offspring and cannot be excluded. Maternal obesity and folate status are two other nutritional environments that are of particular interest, especially in western countries where the rates of obesity [body mass index (BMI) ≥ 30 kg/m$^2$] are high [14] and folic acid consumption through supplement use, in addition to mandatory food fortification, are common.

## 1.2 One-Carbon Metabolism

### 1.2.1 Folate

Folates refer to a family of structurally and functionally similar compounds that are collectively known as vitamin B9 [15]. Folate is present in the food supply as naturally occurring folate or in fortified grain products as folic acid. Natural occurring folate is a reduced polyglutamylated form and sources of food with appreciable amounts include beef liver, leafy green vegetables, asparagus, and oranges [16]. Prior to intestinal absorption, the glutamate residues must be cleaved by the brush border membrane glutamylhydrolase, folate hydrolase (also known as glutamate carboxypeptidase 2), that is present on the proximal small intestine [17]. This monoglutamyl form can then be absorbed by the mucosal cells of the proximal small intestine via the transmembrane proton coupled folate transporter (PCFT) [15,17]. As it travels through the

mucosal cell, the monoglutamyl form is converted into 5-methyltetrahydrofolate (5-MTHF) before it is released into the portal vein, followed by entry into the peripheral circulation [15,17]. Once 5-MTHF is transported to peripheral tissues, it is taken up into the tissue via reduced folate carrier-1 (RCF), a transporter specific for reduced folates [17,18]. Some tissues, such as the liver, can additionally take up reduced and oxidized folate via PCFT [17,18]. Once in the cell, 5-MTHF can either be metabolized to tetrahydrofolate (THF) prior to polyglutamylation by folylpolyglutamate synthetase (FPGS) for tissue retention, or be directly used for one-carbon metabolism requirements [17].

### 1.2.2 Folic Acid

Folic acid is the synthetic, oxidized form of folate. It is more easily absorbed compared to naturally occurring folates because it is found in the monoglutamyl form and does not need to undergo glutamate hydrolysis [18]. Folic acid is the form that is added to fortified foods and supplements [15]. Similar to natural folates, folic acid is absorbed through the mucosal cells of the proximal small intestine. As it passes through the mucosal cell, folic acid is reduced to dihydrofolate (DHF) and then to THF by dihydrofolate reductase (DHFR), followed by further methylation to 5-MTHF before it is released into the portal vein and peripheral circulation (Figure 1.1) [18,19]. This sequential reduction is necessary for folic acid to become metabolically useful as oxidized folic acid lacks coenzyme activity [18,19].

### 1.2.3 Folates in One-Carbon Metabolism

One-carbon metabolism refers to a collective of interlinking metabolic pathways that generate methyl groups to be used for various cellular reactions including the synthesis of DNA, amino acids, and phospholipids [19]. One-carbon metabolism is active throughout the body, but a

major site is in the liver. Key pathways include the folate cycle, methionine cycle, and the transsulfuration pathway. These pathways are summarized in Figure 1.1.

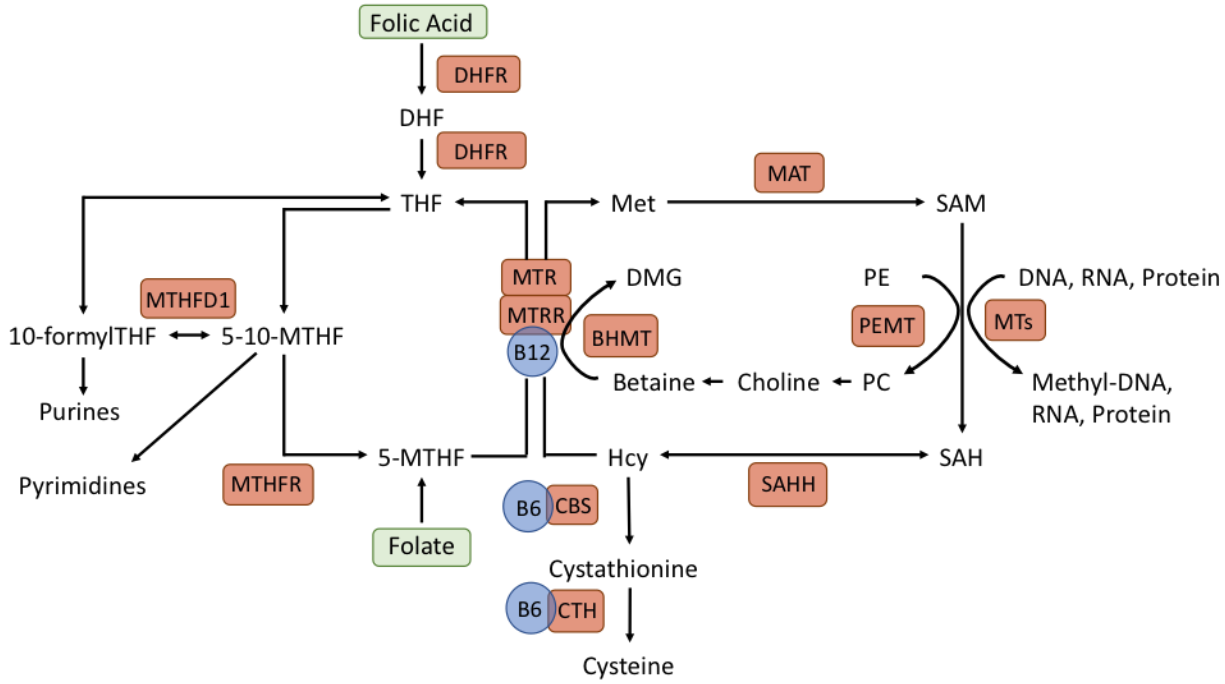


**Figure 1.1. Schematic Representation of One-Carbon Metabolism.** One-carbon metabolism is primarily formed by the folate cycle, methionine cycle, and the transsulfuration pathway. DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; 5-10-MTHF, 5,10-methylenetetrahydrofolate; 10-formylTHF, 10-formyltetrahydrofolate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; 5-MTHF, 5-methyltetrahydrofolate; MTR, methionine synthase; MTRR, methionine synthase reductase; Hcy, homocysteine; Met, methionine; MAT, methionine adenosyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SAHH, *S*-adenosylhomocysteine hydrolase; MTs, methyltransferases; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase; BHMT, betaine-homocysteine methyltransferase; DMG, dimethylglycine; CBS, cystathionine-β-synthase; CTH, cystathionine-γ-lyase. Some enzymatic activities and pathways are tissue specific: DHFR reduction of folic acid occurs predominantly in the small intestine and to a lesser amount in the liver [19]. BHMT is limited to the liver and kidneys [19] while PEMT is specific to the liver only [20]. CBS and the transsulfuration pathway occur in the liver, brain, and pancreas [19].

Folate is one of many methyl nutrients that feed into one-carbon metabolism. Folate and folic acid are metabolized to 5-MTHF before entering the folate cycle where it will serve as the

methyl donor for the remethylation of homocysteine to methionine, a reaction catalyzed by methionine synthase (MTR) and cofactors methionine synthase reductase (MTRR) and vitamin B12 [19]. Following methyl donation to homocysteine, 5-MTHF produces THF which can subsequently be converted to 10-formylTHF or 5,10-methyleneTHF for the synthesis of purines or pyrimidines, respectively [19]. In addition, 5-MTHF is regenerated from 5,10-methyleneTHF through methylene-tetrahydrofolate reductase (MTHFR) to continue the folate cycle [19]. Folate metabolism occurs in several compartments of the cell including the cytosol and mitochondria. In order to conserve cytosolic NADPH and uncouple one-carbon metabolism from glycolysis as both pathways require electron acceptors ($NAD^+$/$NADP^+$), folate metabolism is compartmentalized in the cytosol and the mitochondria [19]. To enable mitochondrial one-carbon metabolism reactions to ensue, a mitochondrial folate transporter (SLC25A32) facilitates translocation of THF from the cytosol to the mitochondria [19].

The generation of methionine through 5-MTHF is the connection between the folate and methionine cycles. Methionine is converted into *S*-adenosylmethionine (SAM) through the enzymatic activity of methionine adenosyltransferase (MAT) [21]. *S*-adenosylmethionine subsequently serves as the primary methyl donor in the body for several cellular reactions [20]. Following its methyl donation, *S*-adenosylhomocysteine (SAH) is produced and can be hydrolyzed back to homocysteine via SAH hydrolase (SAHH) to continue the methionine cycle, or it can enter the transsulfuration pathway where it will be transsulfurated to cystathionine followed by cysteine through the catalytic activities of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CTH), respectively [15,20].

A folate-independent, alternative source of methyl groups for the remethylation of homocysteine to methionine is betaine. This methylation reaction is catalyzed by betaine-

homocysteine *S*-methyltransferase (BHMT) and predominantly occurs in the liver and kidneys [20]. Betaine is an intermediate of choline oxidation [20]. Choline is an essential nutrient predominantly obtained through the diet in which it is found as both water-soluble forms (free choline, phosphocholine, and glycerophosphocholine) and lipid-soluble forms (phosphatidylcholine and sphingomyelin) [22]. Choline may also be synthesized in the liver as phosphatidylcholine (PC) through the methylation of phosphatidylethanolamine (PE) via phosphatidylethanolamine *N*-methyltransferase (PEMT) or through the cytidine diphosphate (CDP)-choline pathway [23]. This pathway utilizes pre-existing dietary choline to produce PC through a three step process [24]. First, choline is phosphorylated by choline kinase (CK) into phosphocholine. This is followed by CTP:phosphocholine cytidylytransferase (CCT) converting phosphocholine into the high-energy donor, CDP-choline; this is a rate limiting reaction. In the final step, CDP-choline is converted to PC by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT).

### 1.3 Folic Acid Supplementation

Folate is essential for proper fetal growth and development. To support this healthy development, folic acid is recommended to women prior to, and during pregnancy due to its role in preventing birth defects such as neural tube defects (NTDs) [25]. During pregnancy, there is a greater need for folate due to many physiological changes that are occurring in the mother's body, particularly rapid cell division for the growth of the fetus and uteroplacental organs [26]. Other factors may contribute to the greater need for folate to a lesser extent, these include increased dilution of folate concentration due to blood volume expansion, increased urinary folate excretion, and increased folate catabolism [26].

### 1.3.1 Neural Tube Defects

Birth defects affect between 1-3% of births around the world and are considered one of the major causes of infant mortality [27]. Neural tube defects are defined as a defect in the brain, spine, or spinal cord, and occur due to failed closure of the neural tube during embryogenesis. This closure occurs within the first 28 days of gestation, and initiates the development of the central and peripheral nervous systems [27,28]. The most common type of NTD is spina bifida. Spina bifida refers to an incomplete closure of the spinal column, resulting in a partial exposure of the spinal cord through a hernia [27]. Neural tube defects may lead to death of the offspring, however there is a significantly higher chance of survival in those with spina bifida (7%) as compared to other types of NTDs [27].

### 1.3.2 Prevention of Neural Tube Defects

In 1976, Smithells and colleagues reported that NTDs were associated with lower serum levels of several micronutrients, including folate, in women during pregnancy [29]. This lead to a series of small trials of periconceptional supplementation during pregnancy and a foundation for investigation into the role of folate in the prevention of NTDs [28,30,31]. The British Medical Research Council (MRC) and Hungarian randomized control trials (RCTs) were launched in 1983 and 1984, respectively. The MRC study (n=1817 women) sought to study the prevention of NTD recurrence in women with a previous NTD-affected pregnancy by using a 4.0 mg supplement of folic acid [25]. A 72% reduction in the risk of a recurring NTD was observed, suggesting that if a second NTD-affected pregnancy could be prevented using folic acid, supplementation must likely prevent a first occurrence as well. In the Hungarian RCT (n=4704 women), prevention of a first NTD occurrence was assessed using a vitamin mix that included 0.8 mg of folic acid [32]. This study found that 93% of NTDs were prevented in first time

pregnancies. In both studies, women took supplements during the periconceptional period and continued throughout pregnancy. Another similar study was conducted in China between 1993 and 1995 in which they supplemented women (n=247,283) with 0.4 mg of folic acid in two regions of China; the northern region known to have high rates of NTDs, and the southern region, an area with lower rates [33]. Findings from this study were consistent with the RCTs such that supplementation with folic acid resulted in a reduction in the risk of NTD-affected pregnancies, particularly in the northern region of China. Taken together, these studies highlighted the importance of folic acid in the prevention of NTDs for all women of child-bearing age and recommendations were made for women to take 0.4 mg/day of folic acid.

### 1.3.3 Folic Acid Fortification and Recommendations

Ensuring adequate folic acid intake prior to conception is critical as the neural tube closes very early in the first trimester; a period in which a women may be unaware that she is pregnant. Compliance towards folic acid supplementation was difficult to achieve in women of child-bearing age despite public campaigns. This therefore prompted the proposal for fortification of folic acid in staple foods in many countries [25,32]. Fortification of grain products became mandated in Canada on November 11, 1998 [34]. This included the fortification of all types of white flour, enriched pasta, and cornmeal. Another form of obtaining adequate folic acid intake is through oral supplement capsules. Currently, the recommended dietary allowance (RDA) for folic acid, determined by the National Academy of Medicine (formerly the Institute of Medicine), is 0.4 mg/ day for all women of child-bearing age to be consumed before and during pregnancy and lactation; this amount is also recommended by the Society of Obstetricians and Gynaecologists of Canada (SOGC) Clinical Practice Guidelines [35]. Daily prenatal supplements in Canada however, typically contain between 0.6-1.0 mg of folic acid, in which 1.0 mg is the

tolerable upper limit (UL) [36]. Furthermore, the SOGC Clinical Practice Guidelines recommend that women who are at a higher risk of having a NTD-affected pregnancy, such as those with pregestational obesity or diabetes, take up to 5.0 mg of folic acid per day; this is 12.5X the recommended RDA, and 5X the UL [35]. To meet the RDA and SOGC Clinical Practice Guidelines, folates should be obtained from fortified foods and supplements, in addition to folate obtained from natural dietary sources [37,38].

### 1.3.4 Folate Status of Canadians

Between food fortification and supplement use, women are consuming higher-than-recommended doses of folic acid. Fortification alone in the general Canadian population has resulted in an almost complete lack of folate deficiency (red blood cell folate < 305 nmol/L) [39]. A study from Alberta (n= 599 women) reported that over 40% of pregnant women are considered to have high red blood cell folate concentrations (> 1360 nmol/L) [40]. Folic acid must be reduced by DHFR to DHF, followed by THF before being sequentially methylated into 5-MTHF in order to become metabolically useful [19]. The activity of DHFR in the intestinal mucosal cells however, has limited ability to reduce folic acid, therefore resulting in circulating unmetabolized folic acid following folic acid intakes > 0.2 mg [41,42]. Unmetabolized folic acid has been detected in populations with [43,44] and without fortification [45], as well as in the serum and cord blood, and breast milk of women taking supplements during pregnancy and lactation [46,47]. The potential adverse effects of unmetabolized folic acid are not understood.

## 1.4 Epidemiological Studies of Maternal Folate Status and Offspring Health

The successful implementation of folic acid supplementation in Canada and several other countries has resulted in elevated folate status, particularly during pregnancy [48]. There is epidemiological evidence to suggest that high maternal folate status can lead to adverse

metabolic outcomes for offspring. The Pune Maternal Nutrition Study in India is a longitudinal prospective cohort study that was established in 1994 to assess maternal dietary intakes and micronutrient status during pregnancy and continuously assess body composition and cardiometabolic risk factors in offspring every 6 months. In a study from this cohort (n=653 mother-child dyads) investigating maternal folate status and offspring adiposity and insulin resistance at 6 years of age, authors reported that high maternal erythrocyte folate (> 1144 nmol/L) at 28 weeks of pregnancy was associated with greater adiposity and indicators of insulin resistance (calculated by homeostatic model assessment of insulin resistance, HOMA-IR), in children at age 6 years [49]. Children had the highest HOMA-IR when their mothers had high erythrocyte folate but low plasma vitamin B12 (<114 pmol/L) at 18 weeks of pregnancy [49]. Another study conducted in India, the Parthenon Study (n=533-539 mother-child dyads), aimed to replicate findings of the Pune study. This study reported that maternal folate concentrations around 30 weeks of pregnancy were positively associated with insulin resistance (calculated by HOMA-IR) in the children at ages 9.5 and 13.5 years [50]. In contrast to the Pune study, there was no interaction between folate and vitamin B12 in relation to childhood insulin resistance as both vitamin B12 deficient mothers, with or without high folate concentrations, had children that presented with insulin resistance [50].

The majority of pregnant and non-pregnant women of reproductive age residing in countries with mandatory folic acid fortification have improved folate status and a significant decline in the prevalence of folate deficiency (erythrocyte folate <305 nmol/L) [40,51–53]. However, there remain some women who are considered to have low maternal folate status in which their offspring can display negative metabolic outcomes [54]. The Boston Birth Cohort study (n=1517 mother-child dyads) reported an L-shaped relationship between maternal plasma folate at 48-72

hours post-delivery and child BMI z-scores at age 9 years [54]. Children from mothers in the lowest quartile of plasma folate (6.6- <20.4 nmol/L) had the highest BMI z-scores and greatest incidence of overweight or obesity (BMI ≥ 85$^{th}$ percentile for sex and age) compared to children from women with plasma folate in the 2$^{nd}$- 4$^{th}$ quartiles [54]. These studies investigating maternal folate status provide evidence of a role for folate in the development of metabolic risk in offspring.

## 1.5 Rodent Studies of Maternal Folic Acid Supplementation and Offspring Health

### 1.5.1 Effects on Adult Offspring Adiposity and Glucose Homeostasis

Rodent models of maternal folic acid supplementation provide further insight into the mechanisms underlying the effects of maternal folate status on offspring metabolism. Rodent models are advantageous due to their short gestational period and life span, as well as the ability to control various aspects of the pre- and postnatal environment, including diet. Several studies have sought to confirm and further understand the impact of maternal high folic acid supplementation on offspring adiposity and glucose homeostasis [55–60]. The Devlin lab previously established a mouse model of maternal folate and vitamin B12 imbalance to further understand the molecular and physiological effects on adult offspring [55,56]. Female C57BL/6J mice were fed a folic acid supplemented diet [10 mg/kg diet; 5X American Institute of Nutrition (AIN) recommendations for mice] with, or without vitamin B12 before and during pregnancy and lactation. Sex-specific differences in adiposity and glucose homeostasis were observed. Male offspring from folic acid supplemented dams gained less weight, had smaller retroperitoneal and subcutaneous fat pads, and had no changes in glucose tolerance (as assessed by an intraperitoneal glucose tolerance test, IPGTT) [55]. In contrast, female offspring from folic acid supplemented

dams had greater fat mass, larger gonadal fat pads, fasting hyperglycemia, and increased glucose intolerance [increased IPGTT area under the curve (AUC)] [56].

Similar to observations made in the Devlin Lab, others have investigated differences in offspring adiposity and glucose homeostasis from mothers fed a diet supplemented with folic acid. A study of Sprague-Dawley rat offspring from dams supplemented with folic acid (5 mg/kg; 2.5X AIN recommendations) reported that male offspring gained more body weight and female offspring gained less body weight compared to male and female offspring from un-supplemented dams, respectively [57]. Similar to reports from the Devlin Lab, Hoile *et al.* reported sex-specific differences in fasting plasma glucose in offspring from Wistar rat dams supplemented with folic acid (5 mg/kg diet; 2.5X AIN recommendations) in which female, but not male offspring, displayed elevated fasting blood glucose [58]. In another study, investigators fed female Sprague-Dawley rats a diet supplemented with folic acid (40 mg/kg diet; 20X AIN recommendations) and reported reduced glucose tolerance (as assessed by oral GTT) in both male and female adult offspring [59]. Greater glucose intolerance was also reported in high fat diet-fed male C57BL/6 mouse offspring from folic acid supplemented dams (20 mg/kg diet; 20X AIN recommendations); females were not assessed in this study [60]. Despite the variable findings observed in rodent models, it is clear that the key findings suggest a role for maternal folate status on the development of adiposity and impaired glucose homeostasis in adult offspring.

### 1.5.2 Effects on Offspring One-Carbon Metabolism

Maternal folic acid supplementation during pregnancy may alter one-carbon metabolism in the offspring. Few studies have investigated changes in methyl metabolism in offspring from dams supplemented with folic acid. The Devlin Lab previously reported differential mRNA expression of enzymes involved in methyl nutrient metabolism in the liver of adult male and

female offspring from folic acid supplemented dams (10 mg/kg diet; 5X AIN recommendations) [55,56]. Male offspring expressed higher *Mthfr* mRNA [55] whereas female offspring expressed lower *Mtr* mRNA and had increased liver SAH [56]. In another study, Bahous *et al.* quantified several hepatic one carbon enzymes and choline metabolites in fetal (embryonic day 17) and 3 week old weanling male C57BL/6 mouse offspring from dams fed a diet supplemented with folic acid (20 mg/kg diet; 10X AIN recommendations). Fetal offspring liver had reduced MTHFR protein and mRNA, decreased *Pemt* and *Mat1a* mRNA, a trend for reduced *Bhmt* mRNA, and decreased betaine, choline, and phosphocholine [61]. At 3 weeks of age, male offspring continued to have reduced MTHFR protein and phosphocholine in the liver [61]. Although observations vary between studies, these findings suggest that maternal folic acid supplementation disturbs offspring one-carbon metabolism, beginning during embryonic development and continuing into adulthood.

### 1.6 Maternal Obesity and Obstetrical and Neonatal Outcomes

Obesity is a human condition defined by a BMI of ≥30 kg/m$^2$ and is characterized as an accumulation of excess body fat [62]. This condition is associated with an increased risk of several adverse health conditions including cardiovascular disease (CVD) and type 2 diabetes (T2D) [62]. Obesity is becoming increasingly prevalent around the world [62]. This is particularly of concern for women of childbearing age, due to the effects on pregnancy and offspring health. In 2016, the World Health Organization reported that 40% of adult women (aged 18 years and older) had overweight (BMI 25-29.9 kg/m$^2$) and 15% had obesity [62]. Women with overweight or obesity before pregnancy are at an increased risk of having an obstetrical complication such as gestational hypertension, preeclampsia, or gestational diabetes mellitus (GDM) [63]. A secondary analysis of a prospective study conducted in Canada (n=1996 singleton pregnancies) reported that women with increasing prepregnancy BMI had increasing risk of developing GDM [64].

Furthermore, pregnancy is accompanied by gestational weight gain (GWG) which is necessary to ensure proper fetal growth. It encompasses multiple characteristics, including maternal fat accumulation, fluid expansion, and the growth of the fetus, placenta, and uterus [65,66]. Guidelines from The US National Academy of Medicine (formerly the Institute of Medicine) are in place for GWG and take into consideration maternal prepregnancy BMI; women who weigh less prior to pregnancy should gain more weight during gestation compared to those who have prepregnancy overweight or obesity [65]. Similar to maternal prepregnancy BMI, excessive GWG has also been associated with adverse outcomes for both the mother and offspring [63,67]. The risk for adverse obstetrical complications increases with increasing prepregnancy BMI and GWG across the full range of BMI and weight gain, with the highest risk for women with obesity who experience excessive GWG [63,67].

Maternal prepregnancy BMI and GWG contribute to adverse neonatal outcomes including preterm birth (<32 weeks of gestation), stillbirth, or large for gestational age (LGA) at birth [63,68]. A cross-sectional study from the Canadian Maternity Experience Survey (n=71,200 women) investigated the risk of prepregnancy BMI and GWG on preterm birth and LGA from Canadian pregnancies and reported that maternal weight, and GWG in particular, significantly increased the risk of these adverse neonatal outcomes [68]. Similarly in the United States of America (USA), a retrospective study of pregnant women (n=112,309 deliveries) reported an increase in preterm birth, LGA, and intensive care unit admission as maternal BMI increased [69]. An individual participant data meta-analysis of European, North American, and Australian cohorts investigated the impact of maternal prepregnancy BMI and GWG on obstetrical and neonatal outcomes and reported that both maternal parameters were associated with increased risk of preterm birth and LGA [63]. Findings from these reports investigating obstetrical and

neonatal outcomes identify the importance of promoting and maintaining a healthy weight prior to, and during pregnancy, in order to have a successful pregnancy and birth for both mother and child.

## 1.7 Maternal Obesity and Offspring Cardiometabolic Health

Cardiometabolic disease is a cluster of metabolic derangements such as obesity, insulin resistance, impaired glucose tolerance, dyslipidemia, and hypertension that promote the development of CVD and/or T2D [70]. Aside from lifestyle choices and genetic predisposition, research has provided ample evidence to support the theory of developmental programming as a factor driving the development of cardiometabolic disease. This evidence will be discussed in the following sub-sections.

### 1.7.1 Maternal Obesity and Offspring Adiposity

Maternal obesity increases the likelihood that the offspring will also present with obesity beginning in childhood and often carrying into adulthood. A prospective, longitudinal study by Fraser *et al.* (n=5154 mother-child dyads) sought to investigate the association between maternal prepregnancy BMI and GWG, and offspring health in 9-year-old children [71]. They reported that children whose mothers gained more than the recommended amount of weight during pregnancy had greater BMI, waist circumference, and fat mass. Additionally, GWG before 14 weeks was positively associated with offspring adiposity. Many studies have observed the association between maternal adiposity and offspring health not only in childhood, but in adulthood as well, indicating that exposure *in utero* can persist much later into life. A prospective study (n=1400 adults) investigated adiposity and cardiometabolic outcomes in 32-year-old offspring and correlated them to maternal prepregnancy BMI and GWG [72]. Maternal prepregnancy BMI and GWG were both independently positively associated with offspring BMI, waist circumference,

and several other cardiometabolic health risks. These studies and others [73,74] indicate that maternal overweight and obesity are influential factors in the progression of cardiometabolic disease. Furthermore, disease progression is associated with greater all-cause premature mortality and cardiovascular events [75], adding to the evidence that maternal BMI status can influence offspring health during development and have lasting consequences in adulthood.

Several rodent studies have replicated similar findings of offspring adiposity from mothers with diet-induced obesity. Samuelsson *et al.* fed female C57BL/6J mice either a standard chow or an obesogenic diet [20% kcal fat as lard with *ad libitum* access to sweetened condensed milk (8% kcal fat)] for 6 weeks before breeding and assessing male and female offspring [76]. At 3 and 6 months of age, offspring from dams fed the obesogenic diet displayed increased body weight, larger fat pads, and had elevated fasting plasma triglycerides. Another study fed female C57BL/6J mice either a control or high fat diet (62.2% kcal fat as soybean oil + lard) during gestation and male and female offspring were assessed [77]. Body weight in both male and female offspring from dams fed the high fat diet were greater at birth and continued from weaning until 6 weeks of age. Male offspring additionally had increased liver triglyceride concentrations. Other studies in C57BL/6J male mouse offspring from dams fed obesogenic diets have similarly reported increased body weight at 3 weeks and at 3 months of age [78,79].

### 1.7.2 Maternal Obesity and Offspring Glucose Homeostasis

#### 1.7.2.1 Insulin Signalling and Glucose Uptake

Other cardiometabolic factors leading to CVD and/or T2D are related to derangements in glucose homeostasis. Glucose homeostasis refers to the tight regulation of blood glucose levels by the pancreatic hormones insulin and glucagon. Insulin is secreted by β cells, and glucagon is secreted by α cells. These two cells, along with other cell types make up a cluster of cells known

as an islet of Langerhans [80]. The β cell is responsible for producing, storing, and releasing insulin to maintain glucose homeostasis. When plasma glucose levels rise, glucose enters the β cells through insulin-independent glucose transporter type 2 (GLUT2) where it will be metabolized, resulting in the generation of ATP [81]. This will lead to the closure of ATP-dependent potassium channels, depolarization, and an influx of calcium into the β cells [81]. Calcium will subsequently initiate the exocytosis of insulin-secreting vesicles which will release insulin into circulation, proportional to the concentration of blood glucose [81]. Insulin promotes the uptake of glucose in peripheral tissues including skeletal muscle and adipose tissue. When insulin binds to insulin receptor (IR) on the surface of these tissues, a signaling cascade occurs to phosphorylate insulin receptor substrate-1 (IRS-1) and subsequently activate phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) [82]. Activation of these kinases initiates the translocation of glucose transporter 4 (GLUT4) to the plasma membrane for glucose uptake [83]. When insulin signalling is impaired and cells no longer adequately respond to insulin, this is referred to as insulin resistance; a condition that is associated with obesity and the development of T2D [84]. Insulin resistance can cause hyperglycemia, which promotes increased demand on pancreatic β cells to produce and secrete more insulin. In the presence of obesity where β cells are chronically exposed to excess nutrients including glucose and free fatty acids, insulin release is augmented [81]. This hyperinsulinemia can lead to β cell compensation, followed by peripheral insulin resistance, or vice versa [80]. β cell compensation includes further secretion of insulin and increased β cell mass. Ultimately, these events will result in β cell dysfunction if hyperinsulinemia is prolonged and β cell compensation is unable to be sustained [80].

#### 1.7.2.2 Obesity and Diabetes During Pregnancy and Offspring Glucose Homeostasis

Impairments in offspring glucose homeostasis can stem from maternal obesity and/or GDM. Gestational diabetes mellitus refers to the development of any degree of hyperglycemia recognized for the first time during pregnancy [85]. There is controversy over the definition of GDM based on diagnostic criteria and screening method [85]. Two types of screening methods exist, universal and selective screening [86]. Selective screening is centered on the presence of specific risk factors based on ethnicity [87], whereas universal screening, the approach used in Canada, screens all pregnant women for GDM between 24-28 weeks of gestation [86]. There are several risk factors that can lead to the development of GDM, including the presence of pregestational overweight or obesity [63,67,88]. A healthy pregnancy involves a change in insulin sensitivity, usually resulting in a mild state of insulin resistance that is reversed following birth [89]. This change in insulin sensitivity is due to fetal growth, GWG, greater food intake, and placental hormone production [88]. Maternal pancreatic islets respond to this demand by enhancing insulin secretion by β cells, augmenting β cell proliferation, and β cell mass expansion [90]. In the case of GDM, impaired β cell function is a result of inadequate secretion of, and responsiveness to insulin, therefore leading to insulin resistance and glucose intolerance [88].

Exposure to elevated glucose concentrations during pregnancy can program the offspring to have adverse health risks such as obesity, impaired glucose tolerance, and T2DM in childhood and adulthood [91–94]. For example, a prospective study investigated adiposity and insulin resistance in offspring of mothers who had GDM (n=232 mother-child dyads) and reported that at 11 years of age, children exposed to hyperglycemia during gestation were overweight and had insulin resistance (calculated by HOMA-IR) [94]. This insulin resistance was further associated with the child's BMI. Studies have similarly investigated changes in offspring glucose

homeostasis in relation to maternal obesity. A family-based study of adult offspring from mothers with obesity during pregnancy (n=67 individuals) assessed insulin sensitivity and secretion from 1-3 siblings of each family [95]. All offspring from mothers with obesity had a greater BMI, waist circumference, and fat mass in adulthood. Following an oral GTT, offspring were glucose intolerant and insulin resistant [calculated using an oral glucose insulin sensitivity (OGIS) index [96]]. Interestingly, only male offspring exposed to maternal obesity were able to compensate for the insulin resistance by increasing insulin secretion (calculated using a method developed by Mari *et al.* [97]), whereas females were not able to do the same.

It is clear that maternal obesity and hyperglycemia are capable of impairing offspring glucose homeostasis. Rodent studies have further identified the sex-specific differences in offspring glucose homeostasis. Reports from these studies have been variable largely due to differences in fat source and fat content in the obesogenic diets employed in each study. This is in addition to species and study design differences. Nevertheless, information from these studies continue to provide understanding into the programming of offspring metabolic health following maternal obesity. A study fed female C57BL/6J mice either a control or high fat diet (62.2% kcal fat as soybean oil + lard) during gestation and male and female offspring were assessed [77]. Glucose tolerance was assessed by IPGTT in which male offspring were glucose intolerant at 6, 14, and 20 weeks of age whereas female offspring were glucose intolerant at 6 weeks only. Furthermore, insulin tolerance tests conducted at 20 weeks revealed that both males and females were unable to clear insulin after 60 minutes following an insulin bolus. Another study of adult female Sprague-Dawley rat offspring from dams fed a fat-rich diet (20% kcal fat as lard) reported that female offspring were more insulin resistant as assessed by a euglycemic-hyperinsulinemic clamp [98]. Moreover, *ex vivo* glucose-stimulated insulin secretion from isolated

islets from this same study was reduced in offspring from dams fed the fat-rich diet compared to offspring from chow-fed dams. In a study of male C57BL/6 mouse offspring from dams fed a high fat diet (49% kcal fat as soybean oil + lard), male offspring were glucose intolerant (as assessed by oral GTT AUC), had elevated fasting plasma insulin concentrations, and had increased β and α cell mass [79]. Other studies in male rodent offspring from high fat diet-fed dams have reported similar findings in glucose intolerance following an IPGTT [99,100].

### 1.8 Thesis Hypotheses and Specific Aims

Folic acid is required for proper development and NTD prevention. However, little is known about the metabolic effects of high folic acid intakes, particularly in women with gestational obesity and/or prediabetes, on the consequences for the developing fetus. Individually, maternal gestational obesity and prediabetes have several negative consequences for offspring health [71,76,79]. Many studies have examined the individual effects of maternal gestational obesity/prediabetes or folic acid supplementation in adult offspring [55,56,77], however what remains unclear is the timing at which the adverse effects manifest and how both maternal environments work together to influence offspring health. The **objective** of my MSc research was therefore to use a mouse model to determine the effect of maternal folic acid supplementation in a healthy pregnancy and a pregnancy complicated by obesity and glucose intolerance on fetal offspring liver and pancreas, and on the metabolic health of the mother.

I **hypothesize** that supplemental folic acid during pregnancy will exacerbate i) the adverse metabolic health outcomes associated with gestational obesity and prediabetes in the mother; and ii) the adverse effects of maternal gestational obesity and prediabetes on fetal offspring liver and pancreas. These hypotheses were addressed by the following three specific aims:

AIM 1: To determine if maternal folic acid supplementation before and during pregnancy affect maternal adiposity, glucose and insulin tolerance, and β cell function in dams with or without diet-induced obesity (Chapter 3).

AIM 2: To determine if maternal diet-induced obesity/glucose intolerance and folic acid supplementation affect liver one-carbon metabolism in fetal offspring (Chapter 4).

AIM 3: To determine if maternal diet-induced obesity/glucose intolerance and folic acid supplementation affect β and α cell mass in fetal offspring (Chapter 5).

# Chapter 2: Materials and Methods

## 2.1 Mice and Study Design

Female and male C57BL/6J mice were gifted by Dr. Francis Lynn (Associate Professor, Department of Surgery, UBC). All mice were housed in cages of 2-4 animals per cage in the Animal Care Facility at the BC Children's Hospital Research Institute (BCCHRI) under a standard 12-hour light-dark cycle with *ad libitum* access to food and water. Female mice were fed from weaning (3 weeks of age) one of four diets formulated by Research Diets Inc. (New Brunswick, NJ, USA); details on the composition of the diets are provided in Table 2.1. The four diets were as follows: control diet with recommended level folic acid (CD), control diet with supplemental folic acid (CDF), western diet with recommended level folic acid (WD), and western diet with supplemental folic acid (WDF). The control diets (CD, CDF; control dams) contained 10% energy from fat, and the western diets (WD, WDF; western dams) contained 45% energy from fat to induce excess adiposity and glucose intolerance in the mice as a model of gestational obesity and prediabetes. The western diet was chosen to mimic the fat content commonly observed in westernized human populations. The control and western diets with recommended folic acid (CD, WD) contained approximately 2 mg/kg diet of folic acid, as recommended for rodents by the AIN-93G diet [101,102]. Diets with supplemental folic acid (CDF, WDF) contained approximately 10 mg/kg diet of folic acid which is 5X the AIN-93G recommendations. All diets met the nutrition requirements for rodents set by the AIN and the National Research Council [101–103].

**Table 2.1. Diet Composition**

| Ingredients (g/kg diet) | Control Diet (CD) D12450K | Western Diet (WD) D12451 | Control Diet + Folic Acid (CDF) D17032103 | Western Diet + Folic Acid (WDF) D17032104 |
|---|---|---|---|---|
| **Fat (% kcal)** | 10 | 45 | 10 | 45 |
| **Lard** | 20 | 177.5 | 20 | 177.5 |
| **Soybean Oil** | 25 | 25 | 25 | 25 |
| **Protein (% kcal)** | 20 | 20 | 20 | 20 |
| **Casein, 30 Mesh** | 200 | 200 | 200 | 200 |
| **L-Cystine** | 3 | 3 | 3 | 3 |
| **Carbohydrate (% kcal)** | 70 | 35 | 70 | 35 |
| **Cornstarch** | 550 | 72.8 | 550 | 72.8 |
| **Sucrose** | 0 | 172.8 | 0 | 172.8 |
| **Maltodextrin 10** | 150 | 100 | 150 | 100 |
| **Cellulose, BW200** | 50 | 50 | 50 | 50 |
| **Vitamin Mix V10001*** | 10 | 10 | 10 | 10 |
| **Choline Bitartrate** | 2 | 2 | 2 | 2 |
| **Folic Acid** | 0 | 0 | 0.008 | 0.008 |
| **Folic Acid (mg/kg diet)** | 1.97 | 2.42 | 9.55 | 11.75 |
| **Total Energy (kcal/kg diet)** | 3800 | 4700 | 3800 | 4700 |

* Contains 2 mg folic acid per 10 g V10001.

Female mice were assigned to a diet group at weaning and fed for 13 weeks with body weight measured weekly. At 13 weeks of feeding, female mice were singly-housed and bred with age-matched male mice fed the control diet. A male was placed into each female cage in the evening and separated the following morning; this was repeated until pregnancy was confirmed by the presence of a vaginal plug, indicating embryonic day (E) 0.5. On E18.5 of pregnancy, dams were anesthetized with isoflurane for blood collection by cardiac puncture, followed by immediate sacrifice by cervical dislocation and collection of maternal and fetal tissue. To collect fetal tissues, the uterine horn was dissected and placed in ice cold 1X phosphate buffered saline (PBS) before individually dissecting each fetus on a petri dish filled with ice. The total number of viable and non-viable (fetal resorption) pups were counted to calculate embryonic loss as the

percent of resorptions per total litter size. All maternal and fetal tissues were either flash frozen in liquid nitrogen to be stored in -80°C, or fixed in 4% paraformaldehyde overnight. Blood was allowed to clot on ice for 30 mins before centrifugation at 8000 rpm for 10 mins at 4°C. Serum was separated into 100 μL aliquots and stored at -80°C. The experimental design is illustrated in Figure 2.1.

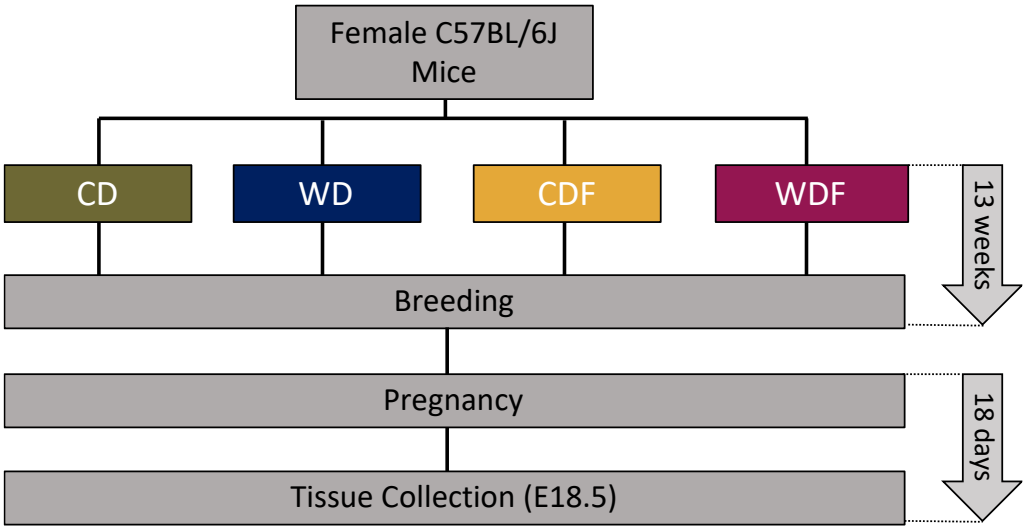


**Figure 2.1. Experimental Design.** Female C57BL/6J mice (dams) were fed either a control or western diet (CD, WD), with or without supplemental folic acid (CDF, WDF) from weaning (3 weeks of age). At 13 weeks of feeding, dams were bred and pregnancy was confirmed by presence of a vaginal plug to mark embryonic day (E) 0.5. Pregnancy was carried out for 18 days before collecting maternal and fetal tissue.

## 2.2 Body Composition

Body composition was quantified in conscious female mice by quantitative magnetic resonance (EchoMRI-100; Echo Medical Systems, Houston, TX, USA) after 10 weeks on diet; 2-3 days before undergoing physiological assessments of glucose homeostasis. For each mouse, 2-3 scans were taken and averaged to calculate the percentage of fat and lean mass.

## 2.3 Physiological Assessments of Glucose Homeostasis

Glucose homeostasis was assessed in females prior to breeding. A glucose stimulated insulin secretion test (IST) was conducted as a physiological indicator of β cell function at 11 weeks on diet, an intraperitoneal insulin tolerance test (IPITT) was conducted as a physiological indicator of insulin sensitivity at 12 weeks on diet, and an IPGTT was carried out to assess glucose excursion at 13 weeks on diet. Assessments were conducted with one week between tests to provide sufficient recovery time.

For ISTs, D-dextrose (Sigma-Aldrich) was dissolved in 0.9% NaCl and filtered through a 0.2 μm syringe filter to a final concentration of 0.15 g/mL. Animals were fasted for 5 hrs prior to injection, fasting blood glucose was measured from a tail prick with a glucometer (OneTouch Verio Meter; LifeScan, Malvern, PA, USA), and 70 μL of blood from the tail vein was collected. Mice were subsequently injected with 0.75 g D-dextrose/kg lean mass and 35 μL of blood from the saphenous vein was collected at 2, 15, and 30 mins post-injection; blood glucose concentrations were also measured at 15 and 30 mins by glucometer. Blood samples were allowed to clot on ice for 30 mins, centrifuged at 8000 rpm at 4°C for 10 mins, and serum was collected and stored at -80°C. Fasting serum insulin concentrations were quantified by the Mouse Ultrasensitive Insulin ELISA kit (ALPCO) as per manufacturer's instructions.

For IPITTs, insulin (Novolin® ge Toronto; Novo Nordisk Canada Inc., Mississauga, ON, CAN) was diluted in filtered 1X PBS to 0.1 U/mL. Mice were fasted for 5 hrs and fasting blood glucose was measured by glucometer. Mice were then injected with 0.75 U insulin/kg lean mass and blood glucose concentrations were measured by tail prick at 15, 30, 60, 90, and 120 mins post-injection. Similarly, for IPGTTs, animals were fasted for 5 hrs and fasting blood glucose

measured. Mice were injected with 0.75 g D-dextrose/kg lean mass and blood glucose concentrations were measured by tail prick at 15, 30, 60, 90, and 120 mins post-injection.

### 2.4 Genotyping of the *Sry* Gene for Fetal Sex Determination

Genomic DNA was extracted from fetal tail snips using the Gentra Puregene Tissue Kit (Qiagen) following the manufacturer's protocol. The purity and concentration of extracted DNA was assessed with a NanoDrop™ spectrophotometer (NanoDrop 2000c; NanoDrop Technologies, Wilmington, DE, USA) in which a 260/280 nm absorbance ratio of approximately 2.0 was considered pure DNA. Multiplex polymerase chain reaction (PCR) was used to simultaneously amplify a 273 base pair sequence of the *Sry* gene (encoding sex-determining region of the Y chromosome) and a 203 base pair sequence of the *Actb* gene (encoding β-Actin), which was used as an internal control. The forward and reverse primers for the *Sry* and *Actb* sequences were as follows: *Sry* forward, 5'-TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA-3'; *Sry* reverse, 5'-CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA-3'; *Actb* forward, 5'-AGC TCA GTA ACA GTC CGC CTA-3'; and *Actb* reverse, 5'-CAG AGA GCT CAC CAT TCA CCA T-3' (Integrated DNA Technologies). Extracted liver DNA from a known male C57BL/6N mouse was used as a positive control. Preparation of the PCR reaction comprised of 2 μL of extracted DNA and 23 μL of a master mix [10X PCR buffer (Qiagen), *Taq* DNA polymerase (Invitrogen), 10 mM dNTPs, 10 mM each of forward and reverse *Sry* and *Actb* primers, $dH_2O$]. Thermal cycler conditions for the PCR were as follows: denaturation at 95°C for 2 mins; amplification (35 cycles) at 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; extension at 72°C for 4 mins; and hold at 4°C. Reactions were subsequently run on a 2.5%

agarose gel to visualize bands. The presence of both *Sry* and *Actb* bands indicated a male offspring, whereas the presence of only the *Actb* band indicated a female offspring.

### 2.5 Pancreas Processing

Dissected pancreata were immediately fixed in 4% paraformaldehyde at 4°C overnight, washed with 1X PBS, and stored in 70% EtOH at 4°C until embedding in paraffin. Embedding consisted of dehydrating the tissue at room temperature through a series of graded ethanol baths (2x 95% EtOH, 3x 100% EtOH; 30 mins each) to displace water, clearing the ethanol with xylene (2x 30 mins each), and infiltrating with paraffin wax (2x 1 hr each) before embedding the tissue in wax blocks for long term storage.

Sections of pancreas were cut from paraffin blocks using a microtome to prepare 5 μm thick slices. Tissue sections were floated in a 40°C distilled water bath, mounted on glass microscope slides (VWR), air-dried overnight, and stored at room temperature.

### 2.6 Immunohistochemical Analysis of Insulin and Glucagon

For each fetal pup, 3 pancreatic sections separated by 30 μm each were stained for insulin and glucagon. Slides containing pancreatic sections were deparaffinized in xylene (3x 10 mins each) and rehydrated in a series of graded ethanol baths and water (2x 100% EtOH, 1x 95% EtOH, 1x 70% EtOH; 10 mins each, 1x 50% EtOH, 1x 30% EtOH; 2 mins each, 1x distilled water; 1 min, 1x 1X PBS; 10 mins). Slides were then submerged in 500 mL of antigen retrieval buffer (10 mM sodium citrate dihydrate, 0.05% Tween-20, pH 6.0) and heated to 95-100°C in the microwave for 6 mins at high power followed by 20 mins on a timed defrost setting to expose antigenic sites for antibody binding. Once the buffer containing the slides was cooled to room temperature, sections were briefly dried off and placed in a humid chamber. An outline was

traced around the perimeter of each section using a hydrophobic pen (Diagnostic BioSystems) followed by the addition of blocking solution (Dako) for 1 hr at room temperature to block non-specific binding. Blocking solution was removed and a primary antibody cocktail of insulin (1:100, rabbit anti-mouse; Cell Signaling) and glucagon (1:500, mouse monoclonal anti-mouse; Sigma-Aldrich) in antibody diluent (Dako) was added for an overnight incubation at 4°C. Slides were subsequently washed 3x in 1X PBS with gentle shaking at room temperature. Sections were briefly dried off, and a secondary antibody cocktail (4 μg/mL, Alexa Fluor™ 594 goat anti-rabbit IgG and Alexa Fluor™ 488 goat anti-mouse IgG; Invitrogen) in antibody diluent was added to incubate for 1 hr at room temperature in a dark humid chamber. Slides were then washed 3x in 1X PBS with gentle shaking and briefly dried off. Mounting medium with DAPI (Vectashield) was added to each section, followed by the addition of a coverslip and a clear nail polish seal. As secondary antibodies are light-sensitive, all steps following overnight incubation with primary antibodies were carried out in a dark room. Images of whole pancreas and insulin and glucagon-positive cells were visualized and tiled with a BX61 fluorescence microscope (Olympus; Tokyo, Japan) and quantified by Fiji ImageJ software [104]. β cell mass and α cell mass were calculated as the insulin- or glucagon-positive area expressed as a percentage of the whole pancreas area, respectively. β cell density was calculated as the number of insulin-positive particles per 100 $mm^2$ of whole pancreas area.

### 2.7 Quantification of Fetal Liver Triglyceride Concentrations

Whole frozen fetal livers (40-70 mg) were homogenized in 100 μL of $dH_2O$ by sonication followed by centrifugation at 8000 rpm for 10 mins at 4°C. The supernatant was diluted 1:100 in $ddH_2O$ and protein concentration was immediately determined by the Bradford

Protein Assay using the Quick Start™ Bradford 1X Dye Reagent (Bio-Rad) [105]. Total lipids were extracted from homogenized liver using a modified Folch *et al*. method [106]. A solvent proportion of 2:1:0.75 v/v/v chloroform/methanol/homogenate was thoroughly vortexed followed by centrifugation at 2500 rpm for 5 mins at room temperature. The lower organic layer was then transferred to a new microcentrifuge tube to dry the lipids using a nitrogen evaporator with additional heat for 15 mins. Dried lipids were subsequently resuspended in 75 μL 1:1 v/v Triton™ X-100 (Sigma-Aldrich)/methanol, vortexed thoroughly, and sonicated for 15 mins at 40°C. Lipid extracts were stored at -80°C.

Triglycerides were quantified using a colorimetric kit (Triglyceride Reagent Set; Pointe Scientific, Canton, MI, USA) and a triglyceride standard (Pointe Scientific) was diluted to create a standard curve (200, 160, 100, 50, and 25 mg/dL). In a 96-well plate, 10 μL of sample was mixed with 180 μL of triglyceride reagent prewarmed to 37°C. Samples were incubated for 5 mins with constant shaking at 700 rpm before reading the absorbance at 500 nm. Triglyceride concentrations were normalized to protein concentration.

### 2.8 Quantification of Fetal Liver Water Soluble Choline Metabolite Concentrations

Hepatic water soluble choline metabolites (free choline, phosphocholine, glycerophosphocholine, and betaine) were quantified by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using stable isotope-labeled internal standards. These analyses were performed by Roger Dyer, Senior Laboratory Technician at the Analytical Core for Metabolomics and Nutrition at BCCHRI.

## 2.9 Quantification of Liver SAM and SAH Concentrations

Maternal and fetal livers were dissected and weighed. To prevent enzymatic conversion of SAM and SAH during the procedure [107], stabilize SAM from degradation using an acidic pH [108], and deproteinize the tissue, 0.4 M perchloric acid (50 μL per 0.01 g tissue) was immediately added to excised tissue and homogenized in a Bullet Blender® Tissue Homogenizer (Next Advance Inc., Troy, NY, USA). Homogenates were centrifuged at 13,000 rpm for 10 mins at 4°C and the supernatant was flash frozen in liquid nitrogen followed by storage at -80°C.

Tissue extracts were packaged on dry ice and sent to Dr. Joshua Miller's laboratory at Rutgers University, New Brunswick, NJ, USA for the quantification of SAM and SAH by high-performance liquid chromatography (HPLC) with UV detection using the methods of Fell *et al.* [109] with modifications by Miller *et al.* [110].

## 2.10 Statistical Analyses

Two-way analysis of variance (ANOVA) was used to determine the effect of maternal diet and folic acid supplementation. If a significant ($p<0.05$) interaction between maternal diet and folic acid supplementation was observed, a subsequent t-test was performed to determine the effect of folic acid supplementation separately in dams fed the control diet (CD vs CDF) and dams fed the western diet (WD vs WDF). Data from one male and one female offspring per litter were used for analyses and were conducted separately. Analyses were performed using SPSS Statistics Software (version 27, IBM) with $p<0.05$ considered statistically significant. Individual data points were graphed using GraphPad Prism 5 software and bar graphs represent mean ± standard deviation (SD).

# Chapter 3: Effects of Folic Acid Supplementation on Maternal Adiposity and Glucose Homeostasis

This chapter presents the findings for Aim 1: To determine if maternal folic acid supplementation before and during pregnancy affect maternal adiposity, glucose and insulin tolerance, and β cell function in dams with or without diet-induced obesity.

## 3.1 Rationale

Folic acid has been identified to reduce the risk of having a NTD-affected pregnancy. As such, women of childbearing age are recommended to consume 0.4 mg of folic acid per day to reduce this risk [35]. Furthermore, those with pregestational obesity or diabetes are at a higher risk of having a NTD-affected pregnancy and are recommended to take up to 5.0 mg of folic acid per day which is 12.5X the recommended dose for women at low risk [35]. There is evidence in both population-based and rodent studies to suggest that consuming high levels of folic acid can have negative consequences for the offspring [49,50,55,56]. Moreover, there may be additional risks for the mother herself. Epidemiological studies have outlined contrasting associations between folic acid supplementation during pregnancy and risk for GDM [111,112]. In a prospective cohort study of Chinese women (n=4353 pregnancies), high doses of folic acid supplements (≥ 0.8 mg/day) during pregnancy were positively associated with increased risk for GDM [112]. In contrast, a prospective cohort study from the Nurses' Health Study II (n=20,199 pregnancies) reported that increasing habitual intakes of supplemental folic acid (up to ≥ 0.6 mg/day) before pregnancy were associated with lower risk for GDM [111]. Another adverse consequence for the mother is increased adiposity. In female Sprague-Dawley rats supplemented with folic acid during mating, pregnancy, and lactation (40 mg/kg diet, 20X AIN recommendations), dams gained more weight

after weaning of their offspring but had smaller gonadal fat pads compared to control dams [59]. Persistent exposure to glucose intolerance either as GDM or milder forms of glucose intolerance, as well as increased adiposity can lead to the development of T2D later in life as well as other cardiometabolic diseases [113–115]. Women with obesity and/or diabetes are being prescribed very high doses of folic acid prior to pregnancy despite the lack of large-scale epidemiological evidence to suggest that this is necessary. Investigation into the effects on maternal health has been minimal. In particular, the effect of folic acid supplementation in combination with obesity on maternal adiposity and glucose homeostasis has not been studied.

## 3.2 Results

### 3.2.1 Western diet-fed dams had increased body weight prior to pregnancy

Female mice were weaned on to the experimental diets at age 3 weeks and weighed weekly throughout the study until 13 weeks on diet. From weeks 1-13 on diet, western dams had significantly greater body weight ($p<0.05$) compared to control dams (Figure 3.1). There was no effect of folic acid supplementation on body weight.

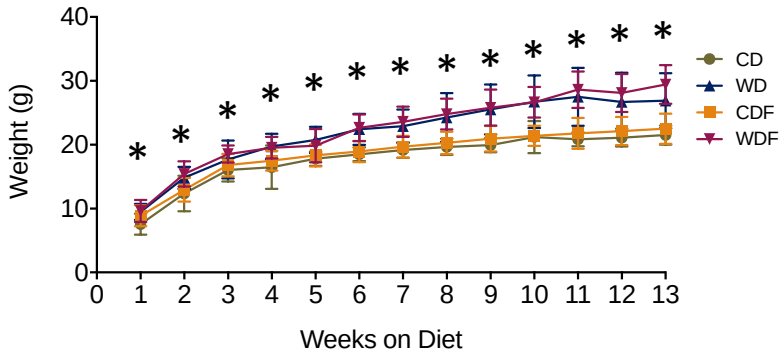


**Figure 3.1. Body Weight of Dams Throughout 13 Week Feeding Period.** Dams were weaned at age 3 weeks onto the experimental diets and fed for 13 weeks. Body weight was measured weekly. Each time point on the line graph was analyzed by two-way ANOVA. Data are presented as mean ± SD (n=11-14/group). *$p<0.05$, effect of diet.

### 3.2.2 Folic acid supplementation altered insulin sensitivity prior to pregnancy

Prior to breeding, physiological assessments of glucose homeostasis were carried out on the dams. At 11 weeks on diet, ISTs were performed on fasted dams as a physiological indicator of β cell function. Fasting insulin was higher ($p$=0.032) in western dams compared to control dams (Figure 3.2A). No difference in glucose-stimulated insulin secretion was observed following IP injection of glucose (Figure 3.2B), however IST AUC was higher ($p$=0.042) for western dams compared to control dams (Figure 3.2C); there was no effect of folic acid supplementation. At 12 weeks on diet, IPITTs were conducted on fasted dams as an indicator of insulin sensitivity. An interaction between diet and supplement ($p$<0.05) was observed in insulin clearance at the 90 minute and 120 minute time points, and for overall IPITT area over the curve (AOC) (Figure 3.2D, E). Control dams supplemented with folic acid had reduced insulin sensitivity compared to non-supplemented control dams. The opposite effect was observed in dams fed the western diet; those supplemented with folic acid had improved insulin sensitivity compared to western dams with no folic acid supplementation. At 13 weeks on diet, glucose tolerance was assessed by IPGTT. Dams fed the western diet had significantly higher fasting glucose concentrations and were glucose intolerant ($p$<0.01) compared to dams fed the control diet (Figure 3.2F-H); no effect of folic acid supplementation was observed.

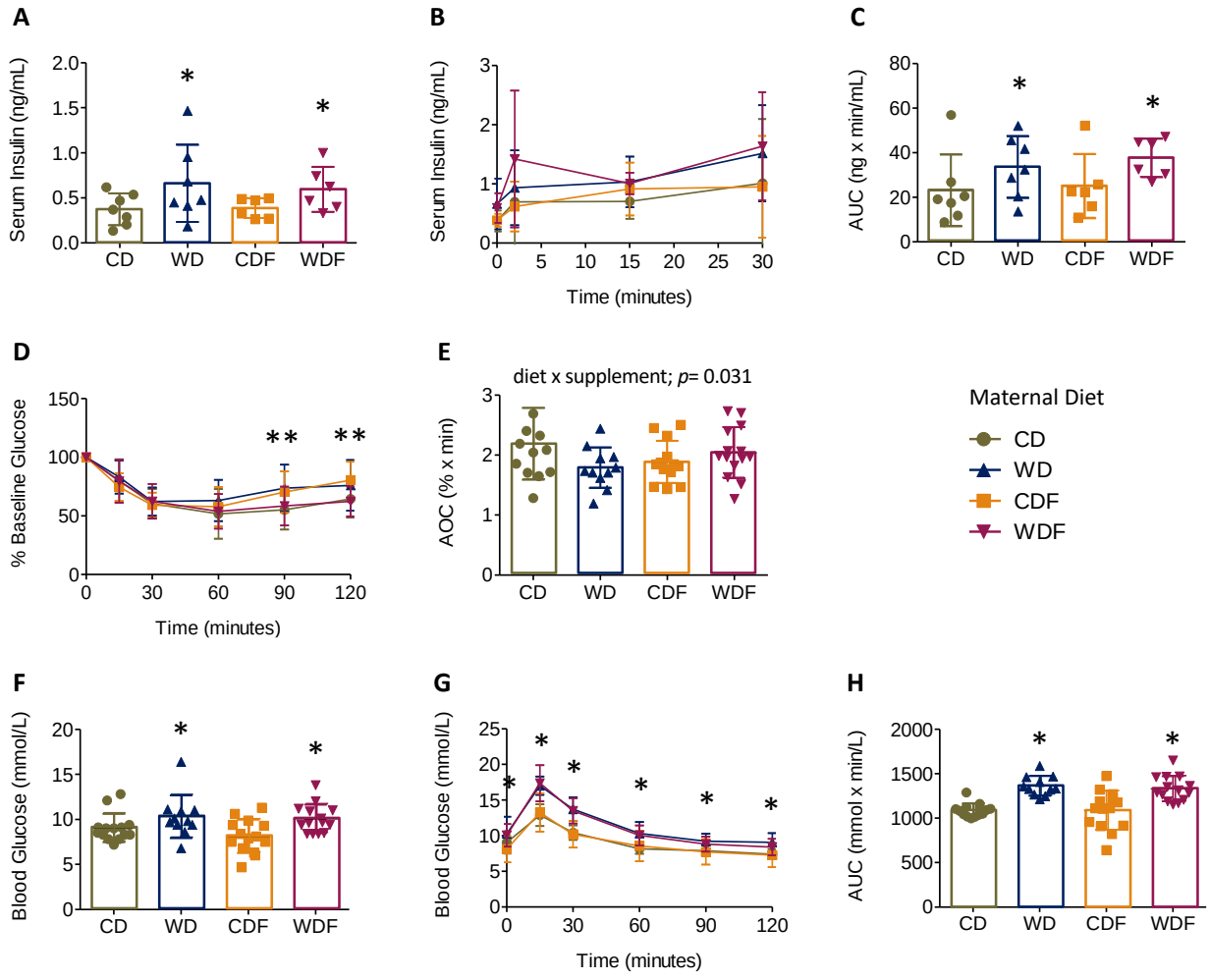


**Figure 3.2. Physiological Assessments of Glucose Homeostasis Before Breeding.** Dams were fasted for 5 hours before assessing their glucose tolerance, insulin sensitivity, and β cell function. (A) Fasting insulin concentrations before IST. (B) Serum insulin concentrations after IP injection of glucose for IST and (C) area under the curve during IST. (D) Blood glucose expressed as a percent from fasting blood glucose after IP injection of insulin for IPITT and (E) area over the curve during IPITT. (F) Fasting glucose concentrations before IPGTT. (G) Glucose excursion after IP injection of glucose for IPGTT and (H) area under the curve during IPGTT. Bar graphs and each time point on line graphs were analyzed by two-way ANOVA. Data are presented as mean ± SD (n=11-14/group). *$p$<0.05, effect of diet; **$p$<0.05, effect of supplement.

### 3.2.3 Western diet feeding increased adiposity before and during pregnancy

Body composition data were collected just before glucose homeostasis assessments were conducted. Prior to pregnancy, dams fed the western diet had greater fat mass and lean mass

($p$<0.001) compared to control diet-fed dams (Figure 3.3A, B). At E18.5, both subcutaneous and visceral fat depots, including the gonadal and retroperitoneal fat pads, were larger ($p$≤0.001) in western dams compared to control dams (Figure 3.3C-E). Folic acid supplementation did not affect adiposity during pregnancy. In addition to fat depots, organ weights differed with diet. Western dams had larger livers ($p$=0.008) and smaller pancreata ($p$=0.001) than control dams (Figure 3.4A, B). An interaction ($p$<0.05) between the diet and supplement was observed for the kidney and heart weight. Control dams supplemented with folic acid had larger kidneys and hearts ($p$<0.05) compared to non-supplemented control dams (Figure 3.4C, D). This was not observed in the western dams.

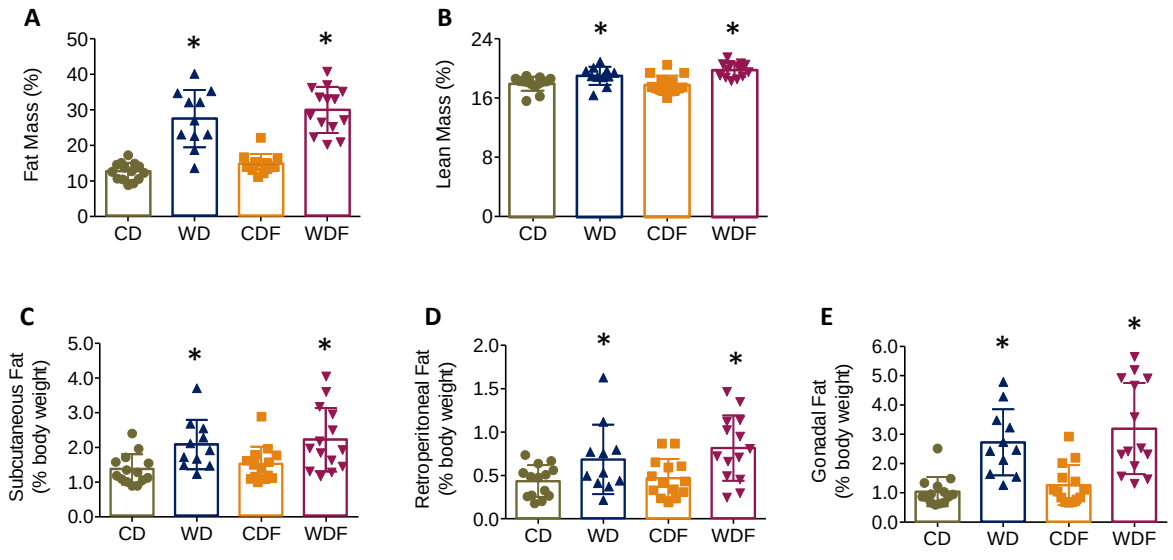


**Figure 3.3. Body Composition in Dams Before Breeding and at E18.5.** Dam's body composition was measured before breeding to calculate (A) total fat mass and (B) lean mass expressed as percent of body weight. At E18.5, fat pads were collected and weighed, including (C) subcutaneous fat, (D) retroperitoneal fat, and (E) gonadal fat. Bar graphs were analyzed by two-way ANOVA. Data are presented as mean ± SD (n=11-14/group). *$p$<0.05, effect of diet.

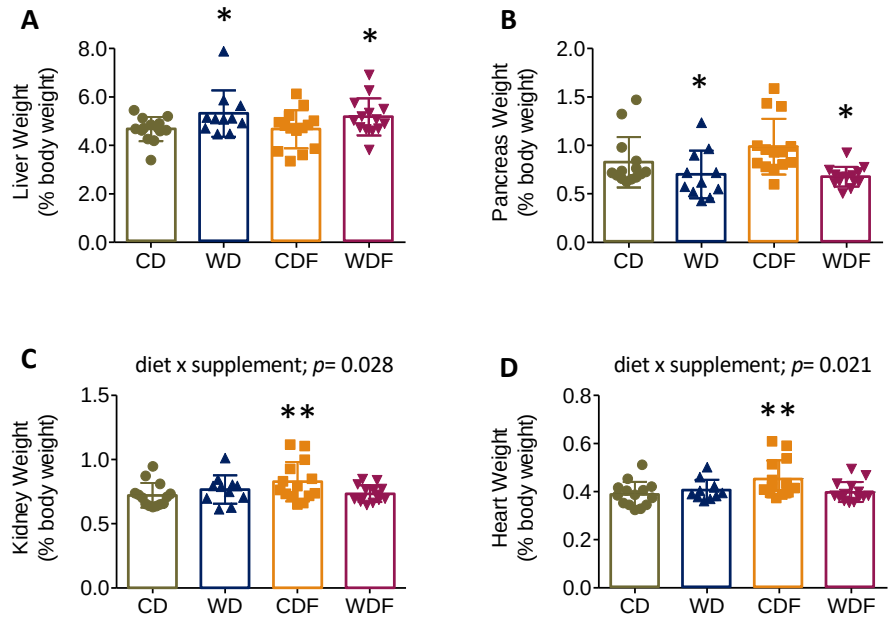


**Figure 3.4. Organ Weights in Dams at E18.5.** At tissue collection, (A) liver, (B) pancreas, (C) kidneys, and (D) heart were collected, weighed, and expressed as percent of body weight. Bar graphs were analyzed by two-way ANOVA. Data are presented as mean ± SD (n=11-14/group). *$p$<0.05, effect of diet; **$p$<0.05, effect of supplement.

### 3.2.4 Maternal diet-induced obesity and glucose intolerance affected liver one-carbon metabolism at E18.5 of pregnancy

Folate plays an essential role in the remethylation of homocysteine to methionine for the generation of key methyl donor, SAM [15,21]. For this reason, a portion of liver was prepared for quantification of SAM and SAH by HPLC. Western dams had lower liver SAM and SAH concentrations ($p$<0.05) compared to control dams (Figure 3.5A, B). No differences in SAM/SAH ratio were observed (Figure 3.5C). Folic acid supplementation had no effect on hepatic SAM and SAH concentrations at E18.5 of pregnancy.

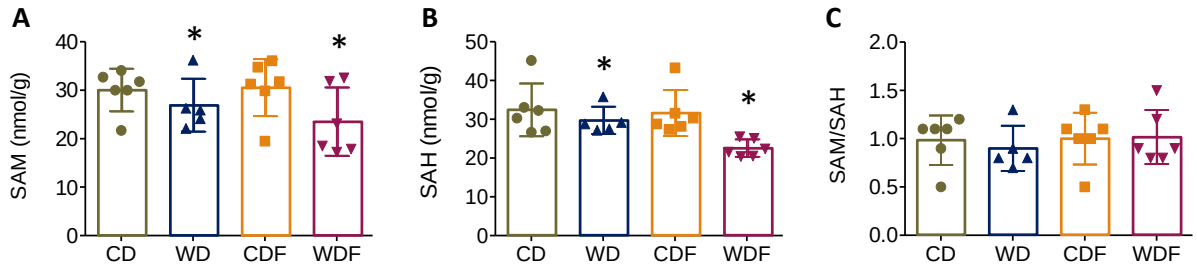


**Figure 3.5. Hepatic SAM and SAH Concentrations in Dams at E18.5.** Quantification of liver (A) SAM and (B) SAH of dams during pregnancy. (C) Ratio of SAM to SAH. Bar graphs were analyzed by two-way ANOVA. Data are presented as mean ± SD (n=5-6/group). *$p$<0.05, effect of diet.

### 3.2.5 Folic acid supplementation did not affect litter development

At E18.5, all viable pups and non-viable resorption sites were counted. There was no difference in total litter size (Figure 3.6A). Similarly, no difference in the number of resorption sites per litter, calculated as embryonic loss, was observed (Figure 3.6B). Fetal sex was determined in all pups and there was no difference in the proportion of male to female offspring (Figure 3.6C).

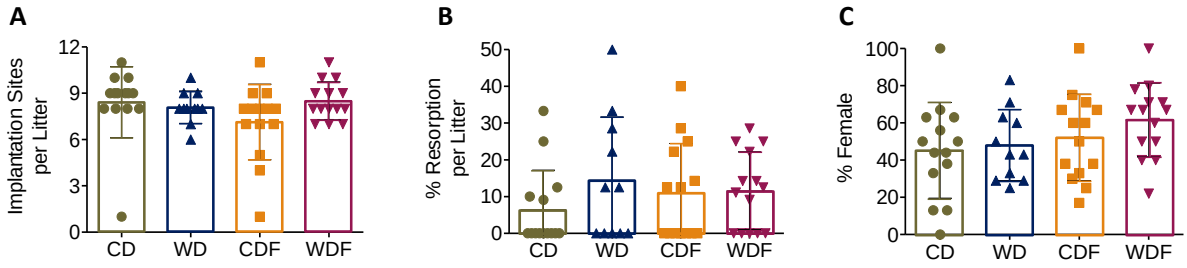


**Figure 3.6. Litter Size and Distribution at E18.5.** All viable pups and resorption sites were counted to give (A) total litter size. (B) Embryonic loss was calculated as the number of resorption sites per litter. Fetal sex was confirmed by PCR of the *Sry* gene to determine (C) percentage of female pups to male pups in each litter. Bar graphs were analyzed by two-way ANOVA. Data are presented as mean ± SD (n=11-14/group).

# Chapter 4: Effects of Maternal Diet-Induced Obesity/Glucose Intolerance and Folic Acid Supplementation on Fetal Offspring Liver One-Carbon Metabolism

This chapter presents the findings for Aim 2: To determine if maternal diet-induced obesity/glucose intolerance and folic acid supplementation affect liver one carbon metabolism in fetal offspring.

## 4.1 Rationale

The liver is a major organ involved in one-carbon metabolism. As discussed in Chapter 1, few studies have investigated the impact of maternal folic acid supplementation on fetal offspring liver methyl metabolism. In C57BL/6 mice, maternal folic acid supplementation (20 mg/kg diet; 10X AIN recommendations) reduced expression of several one-carbon enzymes including MTHFR in the E17.5 fetal liver [61]. This study additionally reported reduced concentrations of water soluble choline metabolites including betaine, choline, and phosphocholine in the E17.5 fetal liver [61]. In contrast, a study of low maternal folate intake (0.4 mg/kg diet; 0.2X AIN recommendations) reported that E17.5 fetal C57BL/6 mice had altered hepatic gene expression including many genes involved in one-carbon metabolism, adipogenesis, and fatty acid biosynthesis [116].

Maternal obesity and T2D have the potential to affect the development of the offspring and lead to long term negative consequences such as impaired glucose homeostasis and increased adiposity [76,77]. These adverse effects may manifest during fetal life. For example, female C57BL/6J mice fed a high fat diet (60% kcal fat as soybean oil + lard) for 6 weeks before breeding and during pregnancy reported that at E17.5, fetal offspring had hepatic triglyceride

accumulation and upregulated lipogenic gene expression in the liver [117]. Furthermore, fetal offspring from the high fat diet-fed dams had higher choline and lower betaine levels in the liver compared to offspring from control dams [117]. It is evident that maternal diet composition, such as folic acid supplementation or high fat intakes during pregnancy, have the potential to program fetal adiposity and one-carbon metabolism. However, little is known about the effects of maternal folic acid supplementation in pregnancies complicated with gestational obesity and prediabetes on offspring health.

## 4.2 Results

### 4.2.1 Maternal diet-induced obesity and glucose intolerance reduced fetal liver weight

Fetal offspring were collected and tissues were weighed at E18.5. The placenta and extraembryonic tissues were removed from the fetus to obtain body weight; female offspring from western dams were smaller ($p$=0.011) than those from control dams; this was not observed in male offspring (Figure 4.1A, B). There were no differences in placental weight of male or female offspring (Figure 4.1 C, D). Interestingly, male fetal offspring from western dams had smaller livers ($p$=0.033) compared to males from control dams (Figure 4.1E). A similar trend was observed in female offspring, although not significant ($p$=0.058) (Figure 4.1F). Maternal folic acid supplementation had no effects on fetal body weight, or placenta and liver size.

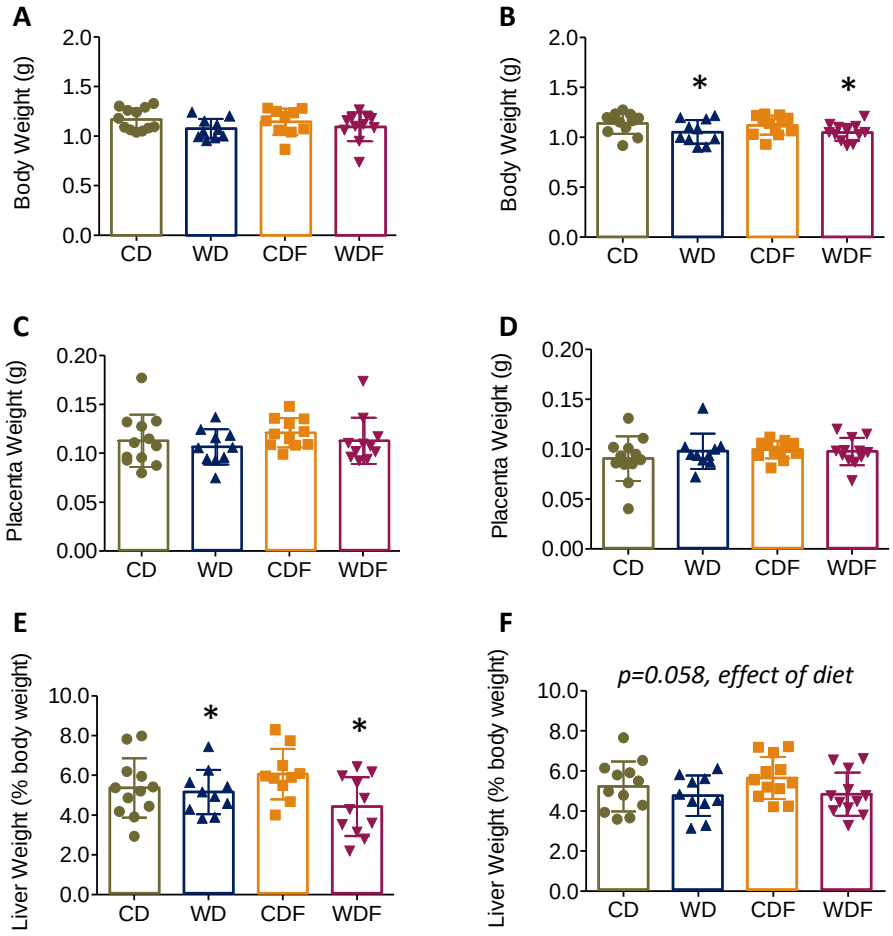


**Figure 4.1. Body, Placenta, and Liver Weights of Fetal Offspring at E18.5.** Fetal offspring were collected and tissues were weighed. Body weight of (A) male and (B) female fetuses. Placenta weight of (C) male and (D) female fetuses. Liver weight, expressed as a percentage of body weight, of (E) male and (F) female fetuses. Bar graphs were analyzed by two-way ANOVA. Each data point represents one offspring representative from each litter. Data are presented as mean ± SD (n=10-12/group). *$p$<0.05, effect of diet.

### 4.2.2 Maternal diet did not influence fetal liver triglyceride content

Given the difference in liver size observed in the E18.5 fetal offspring, hepatic triglyceride content was quantified to assess if this difference in weight was due to changes in the accumulation of triglycerides in the liver. However, I observed no differences in liver triglyceride concentrations in male or female offspring (Figure 4.2A, B).

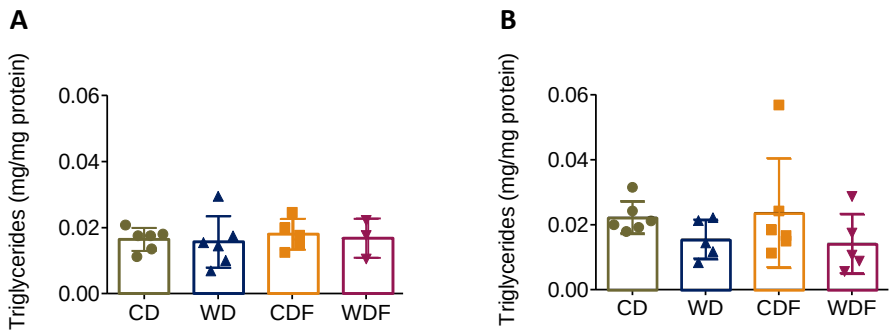


**Figure 4.2. Fetal Liver Triglyceride Concentrations at E18.5.** Hepatic triglyceride content was quantified in (A) male and (B) female fetal offspring. Bar graphs were analyzed by two-way ANOVA. Each data point represents one offspring representative from each litter. Data are presented as mean ± SD (n=3-6/group).

### 4.2.3 Maternal diet-induced obesity and glucose intolerance increased hepatic water soluble choline metabolite concentrations in fetal offspring

Sex-specific differences in hepatic water soluble choline metabolite concentrations were observed. Male offspring from western dams had higher betaine and free choline concentrations ($p$<0.05), compared to male offspring from control dams (Figure 4.3A, C). Female offspring from western dams had higher liver betaine concentrations ($p$=0.026), compared to those from control dams (Figure 4.3B). No differences in phosphocholine or glycerophosphocholine (GPC) were observed in offspring (Figure 4.3E-H). Maternal folic acid supplementation had no effect on water soluble choline metabolite concentrations in the fetal liver at E18.5.

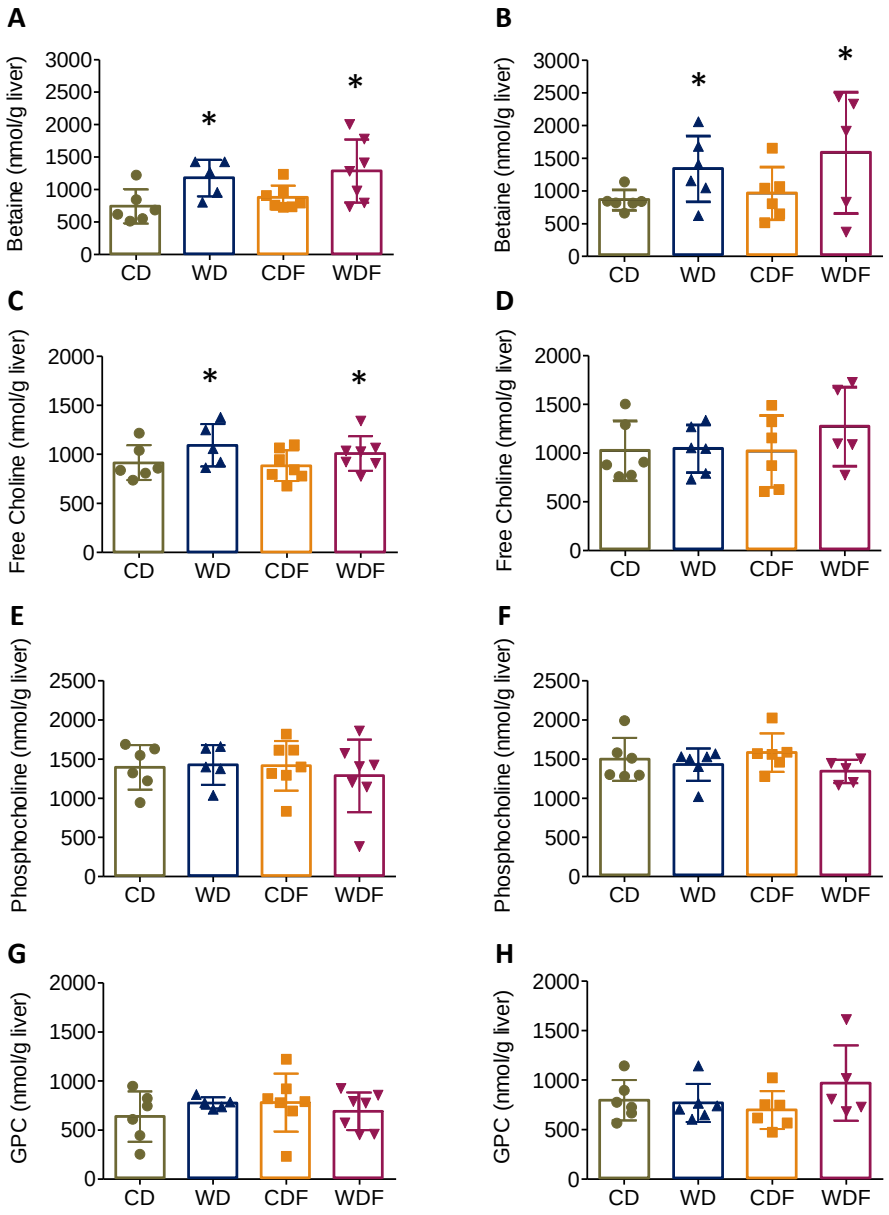


**Figure 4.3. Fetal Liver Water Soluble Choline Metabolite Concentrations at E18.5.** Hepatic water soluble choline metabolites were quantified in fetal offspring. Betaine in (A) male and (B) female fetuses. Free choline in (C) male and (D) female fetuses. Phosphocholine in (E) male and (F) female fetuses. Glycerophosphocholine (GPC) in (G) male and (H) female fetuses. Bar graphs were analyzed by two-way ANOVA. Each data point represents one offspring representative from each litter. Data are presented as mean ± SD (n=5-7/group). *$p$<0.05, effect of diet.

### 4.2.4 Maternal folic acid supplementation increased hepatic SAM in female offspring

Female offspring from control and western dams supplemented with folic acid had higher liver SAM concentrations ($p$=0.04; Figure 4.4B) and SAM/SAH ratios ($p$=0.046; Figure 4.4F) compared to female offspring from dams not supplemented with folic acid. No effect of maternal folic acid supplementation on fetal liver SAH concentrations were observed (Figure 4.4D). Maternal diet had no effect on hepatic SAM and SAH concentrations in E18.5 male offspring (Figure 4.4A, C, E).

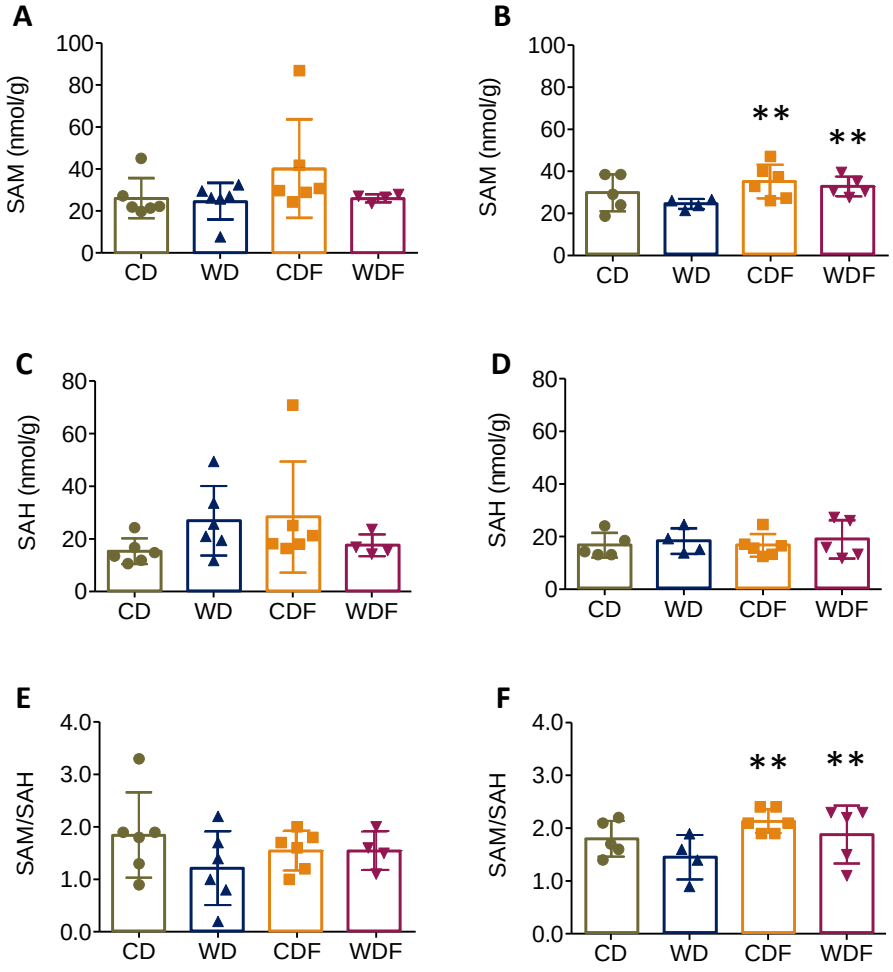


**Figure 4.4. Hepatic SAM and SAH Concentrations in E18.5 Fetal Offspring.** At E18.5, fetal offspring liver was prepared to quantify SAM in (A) male and (B) female offspring, and SAH in (C) male and (D) female offspring. The SAM/SAH ratio was calculated for (E) male and (F) female offspring. Bar graphs were analyzed by two-way ANOVA. Each data point represents one offspring representative from each litter. Data are presented as mean ± SD (n=4-6/group). ***p*<0.05, effect of supplement.

# Chapter 5: Effects of Maternal Diet-Induced Obesity/Glucose Intolerance and Folic Acid Supplementation on Fetal Offspring Islet Morphology

This chapter presents the findings for Aim 3: To determine if maternal diet-induced obesity/ glucose intolerance and folic acid supplementation affect β and α cell mass in fetal offspring.

## 5.1 Rationale

Proper development of the pancreas is critical for the regulation of glucose homeostasis. As discussed in Chapter 1, population-based studies and rodent studies have provided evidence to suggest that exposure to a perturbed maternal environment, such as obesity or diabetes during pregnancy can result in impaired glucose homeostasis and increased adiposity in the offspring later in life [77,94,95]. This manifestation may be due to improper development of β cells in the offspring that begins during fetal development. In rodent models of maternal diet-induced obesity (high fat diet feeding), both neonatal and adult offspring display alterations in α and β cell mass, as well as altered glucose tolerance [79,118,119]. Elevated blood glucose, reduced β cell mass, and increased α cell mass were reported in 1 day old neonatal Wistar rat offspring from dams fed a high fat diet (40% kcal fat; fat source not specified) [118]. Furthermore, impaired glucose tolerance and elevated β and α cell mass were reported in adult male C57BL/6 mouse offspring from dams fed a high fat diet (49% kcal fat from soybean oil + lard) [79].

Parallel to maternal obesity, *in utero* exposure to maternal hyperglycemia, as in uncontrolled GDM, results in negative consequences for the offspring [92,93]. Interestingly, a recent study in China reported that folic acid supplementation (≥ 0.8 mg/day) before and during pregnancy is associated with an increased risk of developing GDM [112]. However, the effect of

folic acid supplementation in pregnancies complicated by obesity and GDM, on fetal offspring α and β cell mass is unknown.

## 5.2 Results

### 5.2.1 Maternal folic acid supplementation increased fetal β cell mass in male offspring

To assess if maternal folic acid supplementation in combination with maternal obesity and glucose intolerance conferred any changes in fetal islet morphology, fetal β and α cell mass were quantified. No effects of maternal diet or folic acid supplementation on pancreas weight were observed in male and female offspring (Figure 5.1A, B). A trend towards greater β cell mass ($p$=0.058) was observed in male offspring from control and western dams supplemented with folic acid (Figure 5.1C). No differences in β cell mass were observed in female offspring (Figure 5.1D). Additionally, no difference in α cell mass was observed in male or female offspring (Figure 5.1E, F). The increase in β cell mass in male fetal offspring from control and western dams supplemented with folic acid was accompanied by greater β cell density ($p$=0.039) (Figure 5.2A). No differences in β cell density were observed in female offspring (Figure 5.2B). Further, no differences in the size distribution of islets were observed in male or female offspring (Figure 5.2C-H).

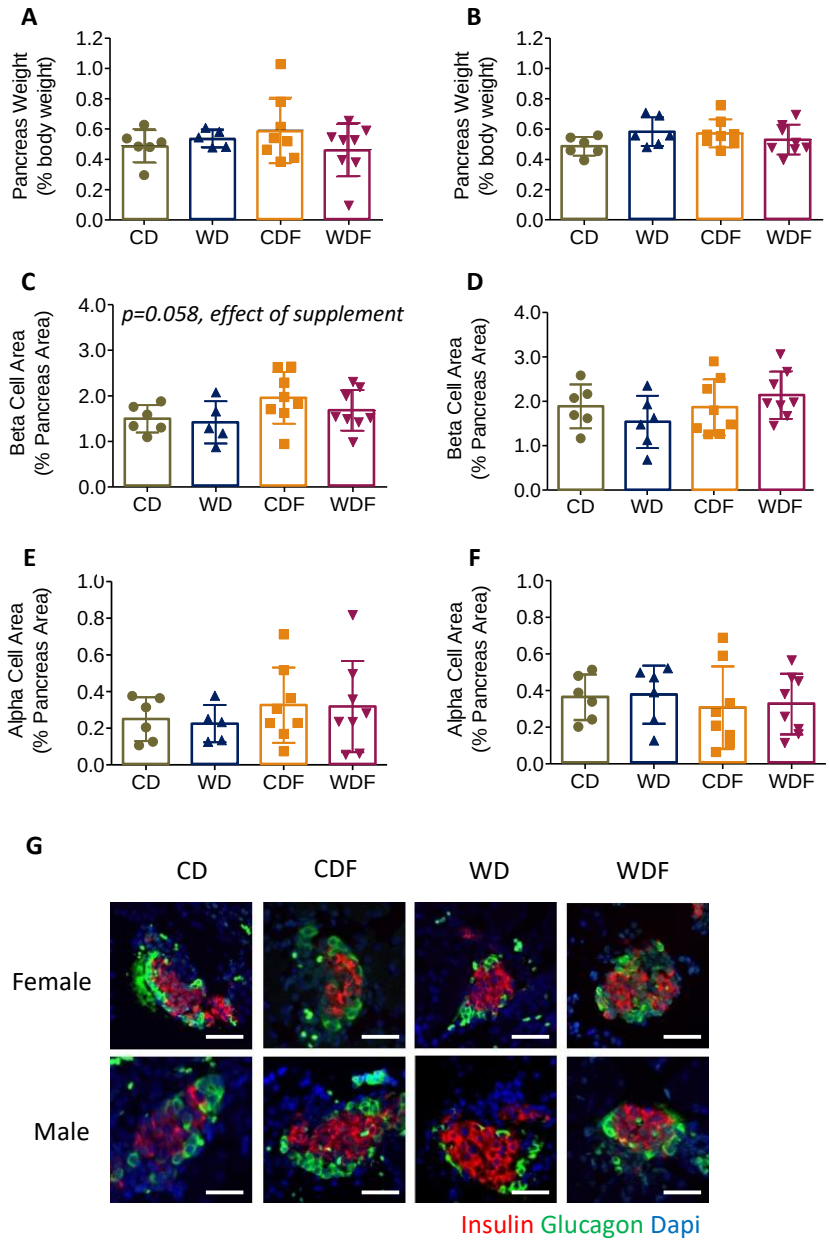


**Figure 5.1. Fetal β and α cell mass at E18.5.** Fetal pancreas expressed as a percentage of body weight for (A) male and (B) female pancreata. Beta cell area for (C) male and (D) female offspring represents the insulin-positive area expressed as a percentage of the whole pancreas area. Alpha cell area for (E) male and (F) female offspring represents the glucagon-positive area expressed as a percentage of the whole pancreas area. (G) Representative immunofluorescence images of islets stained with insulin, glucagon, and Dapi; scale bar= 50 μm. Bar graphs were analyzed by two-way ANOVA. Each data point represents an average of three sections per offspring representative from each litter. Data are presented as mean ± SD (n=5-8/group). ***p*<0.05, effect of supplement.

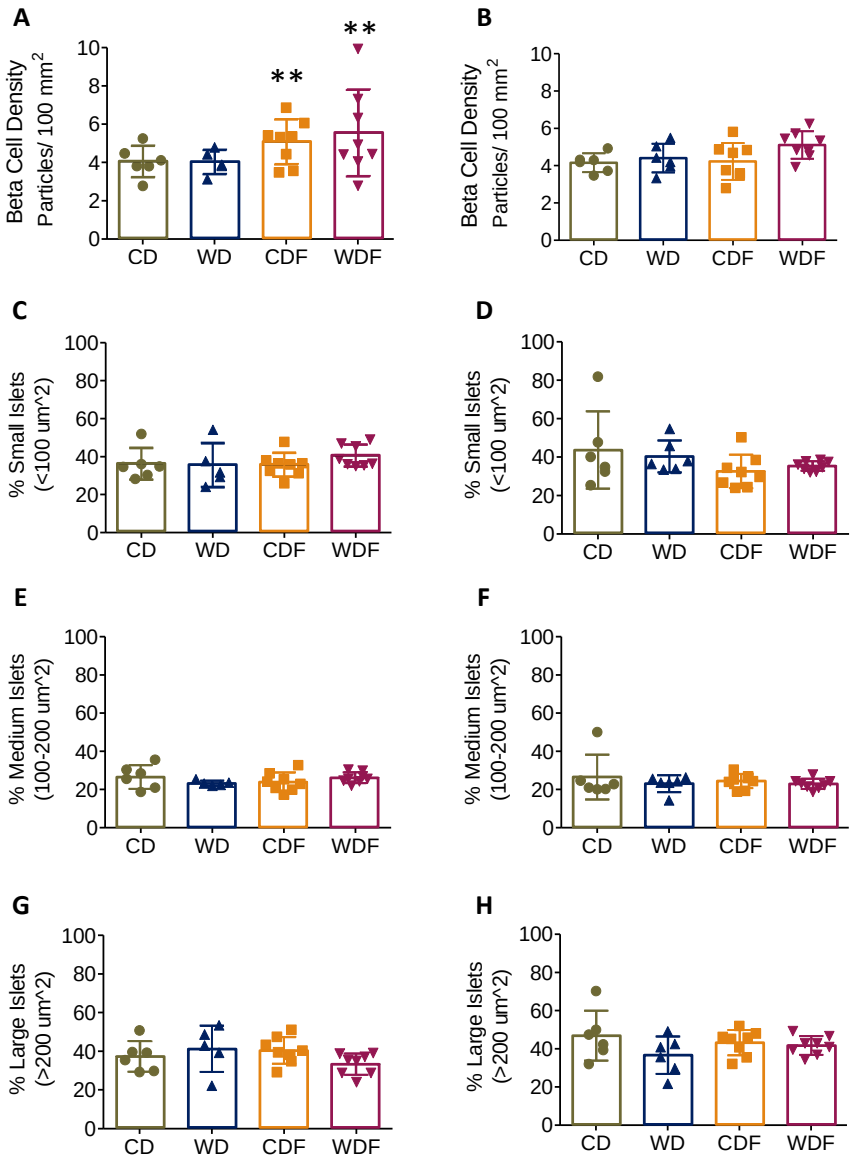


**Figure 5.2. Fetal Islet Density and Distribution at E18.5.** Beta cell density for (A) male and (B) female offspring expressed as number of particles per 100 mm$^2$ of whole pancreas area. Small islets for (C) male and (D) female offspring are particles <100 μm$^2$. Medium islets for (E) male and (F) female offspring are 100-200 μm$^2$. Large islets for (G) male and (H) female offspring are >200 μm$^2$. All islet sizes are expressed as percent of islet particles. Bar graphs were analyzed by two-way ANOVA. Each data point represents an average of three sections per offspring representative from each litter. Data are presented as mean ± SD (n=5-8/group). **$p$<0.05, effect of supplement.

# Chapter 6: Discussion and Conclusions

## 6.1 Discussion

For my Master's thesis research, I utilized a mouse model to evaluate the effects of maternal folic acid supplementation and diet-induced obesity/ glucose intolerance on the health of the mother and fetal offspring. I assessed adiposity and physiological indicators of glucose tolerance, insulin sensitivity, and β cell function in dams before pregnancy, quantified one-carbon metabolites in maternal and fetal liver, and assessed pancreatic β and α cell mass in fetal islets. My research provides insight into the health of mothers with pregnancies complicated by gestational obesity and prediabetes who are recommended to supplement with high doses of folic acid, and how this affects the development of the fetal offspring.

### 6.1.1 Folic acid supplementation before and during pregnancy does not affect adiposity, glucose tolerance, or hepatic one-carbon metabolism in dams with or without diet-induced obesity/glucose intolerance

In Chapter 3, I studied the effect of folic acid supplementation on adiposity and glucose homeostasis in the dams before and during pregnancy. Folic acid supplementation altered insulin sensitivity, but this was dependent on the dam's diet. When supplemented with folic acid, western dams had improved insulin sensitivity and control dams had reduced insulin sensitivity. The greater adiposity and glucose intolerance associated with western-diet feeding resulted in dams with larger livers accompanied by reduced hepatic SAM and SAH during pregnancy, indicating that gestational obesity and prediabetes may have consequences for liver one-carbon metabolism in the dams.

The dams were fed the western diet as a model of diet-induced obesity and glucose intolerance. I confirmed the model, and showed that western dams had greater body weight and

adiposity accompanied by glucose intolerance and impaired β cell function. My findings are consistent with other studies that have used a comparable model of high fat feeding to induce excess adiposity and glucose intolerance [120–122]. For example, female C57BL/6 dams fed obesogenic diets ranging in fat content (30- 60% kcal fat from soybean oil + lard) prior to pregnancy are more adipose [120,121] and glucose intolerant before [120] and during breeding [121,122].

Interestingly, I found that folic acid supplementation before and during pregnancy increased the size of the heart and kidney in the dams at E18.5. During pregnancy, the maternal cardiovascular system adapts to the physiological changes of pregnancy and this includes increased maternal blood pressure, blood volume, and cardiac output to support the development of the fetus [123]. Furthermore, erythropoietin, a hormone produced primarily by the kidneys for the production of red blood cells, is stimulated by placental lactogen during pregnancy for increased red blood cell production [124,125]. In order to accommodate these changes during a healthy pregnancy, the heart and kidney undergo structural changes, including enlargement [126]. The reason why folic acid supplementation would augment increases in heart and kidney size during pregnancy is not understood but may involve direct effects of folic acid or circulating folate on heart and kidney metabolism and its structure and function.

Contrary to my hypothesis, I did not find that folic acid supplementation exacerbated the effects of maternal obesity on glucose tolerance or β cell function. However, I did observe improved insulin sensitivity in western dams supplemented with folic acid. The effect of supplemental folic acid on insulin sensitivity in the context of diet-induced obesity has been previously explored, but only in adult male rats; little is known about this during pregnancy although physiological changes associated with pregnancy contribute greatly to alterations in insulin sensitivity in females. Buettner *et al.* reported that adult male Wistar rats fed a high fat

diet with supplemental folic acid (25% kcal fat from lard; 40 mg/kg diet, 20X AIN recommendations) had improved insulin sensitivity [127]. The findings from my study and that of Buettner *et al.* suggest a beneficial effect of folic acid supplementation on insulin sensitivity in the context of diet-induced obesity in male and female rodents.

Interestingly, the difference I observed in dams was only after 90 minutes following an insulin bolus. As insulin has a short half-life of approximately 10 minutes [128], all exogenous insulin should be cleared following the first 30 minutes of an IPITT [129]. Therefore, later differences following insulin administration, particularly after the initial fall at 30 minutes post-insulin injection, may not reflect a direct effect on insulin action but may be due to secondary effects such as counterregulatory hormone responses like changes in endogenous insulin production [129,130]. This suggests that the effect of folic acid supplementation with or without gestational obesity that I observed may not have a big impact on whole body insulin action despite an overall difference in IPITT AOC. Further investigation into the role of folic acid supplementation on insulin regulation is warranted.

I found that dams fed the western diet had larger livers accompanied by lower hepatic SAM and SAH compared to control dams. These changes in hepatic SAM and SAH during pregnancy complicated by diet-induced obesity and glucose intolerance are novel and have not been previously reported. Diet-induced obesity is associated with the development of nonalcoholic fatty liver disease (NAFLD) and is characterized by hepatic accumulation of fat from increased fatty acid uptake, *de novo* fatty acid synthesis, impaired fatty acid oxidation, or reduced triglyceride export by very-low-density-lipoprotein (VLDL) particles into circulation. Assembly of VLDL particles is dependent on the presence of hepatic PC, which is derived from PEMT-mediated methylation of PE using SAM as a methyl donor [23]. Further, SAM can be

synthesized through betaine-dependent remethylathion of homocysteine to methionine via BHMT. The lower SAM that I observed could potentially be related to impaired BHMT activity. Lower availability of SAM may affect PEMT enzymatic activity, and thus, lower the production of PC from PE for VLDL assembly and export of triglycerides. It would be intriguing to quantify the hepatic triglyceride content in the dams of my study, as well as histologically assess the liver for signs of NAFLD. Furthermore, investigating choline metabolism in the liver would provide additional insight into the regulation of one-carbon metabolism in dams with diet-induced obesity and glucose intolerance.

A link between glucose and lipid metabolism is well known and involves insulin signalling and regulatory factors such as, sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) [131]; these transcription factors are stimulated by insulin and glucose, respectively. The first steps of hepatic *de novo* fatty acid synthesis from acetyl-CoA are catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [131]. Insulin stimulates FAS expression via the PI3K pathway, and both ACC and FAS are transcriptionally activated by SREBP-1c and ChREBP [131]. The western dams in my study had hyperglycemia and hyperinsulinemia, suggesting that higher levels of glucose and insulin may have promoted SREBP-1c and ChREBP-mediated hepatic lipid accumulation and subsequently resulted in larger livers.

Interestingly, I found no effect of folic acid supplementation on liver SAM and SAH which contradicts what I predicted based on reports by others. Christensen *et al.* reported that female BALB/cAnN mice supplemented with folic acid (10 mg/kg diet; 5X AIN recommendations) before and during pregnancy had lower hepatic SAM, higher SAH, and reduced SAM/SAH ratio compared to control dams at E10.5 [132]. The reason for the lack of

change in liver SAM and SAH with folic acid supplementation in my study is not understood but may be due to differences in rodent species, or the gestational time point at which these metabolites were quantified.

### 6.1.2 Maternal diet-induced obesity/glucose intolerance and folic acid supplementation affect liver one-carbon metabolism in fetal offspring

In Chapter 4, I investigated the effects of maternal folic acid supplementation and diet-induced obesity/glucose intolerance on fetal offspring liver one-carbon metabolism and observed disturbances in body weight, as well as methionine and choline metabolism. Female fetuses from western dams were smaller in body weight but this was not observed in male fetuses. Fetal offspring from western dams also had higher liver choline metabolites and smaller livers without changes in liver triglyceride concentrations. Maternal folic acid supplementation increased liver SAM in female, but not in male fetal offspring.

Interestingly, female fetuses from dams with diet-induced obesity/glucose intolerance had lower body weight. However, the difference in body weight compared to fetal offspring from control dams is very subtle and the biological significance of such a small difference seems minimal. Nevertheless, maternal gestational obesity is associated with fetal growth restriction in both humans and rodents [133–135]. Furthermore, maternal gestational obesity is associated with impaired structure and vascular function in the placenta, therefore impairing blood flow to the fetus and contributing to the development of fetal growth restriction [136,137]. In a study of C57BL/6J mice fed an obesogenic diet and sweetened condensed milk (20% kcal fat from lard + 8% kcal fat from milk fat), E13 and E19 fetal male and female offspring had lower body weight and smaller placentae with impaired structural and vascular development [133]. Placenta weight was not affected by maternal diet in my study; vascular function was not assessed. Alterations in

placental structure and function due to maternal diet-induced obesity/glucose intolerance may be the reason for lower body weight in the female offspring from western dams.

Additionally, I observed that male and female fetal offspring from dams with diet-induced obesity/glucose intolerance had smaller livers accompanied by higher levels of water-soluble choline metabolites but no differences in liver triglyceride concentrations. Male offspring from western dams had higher liver free choline and betaine, whereas female offspring from western dams had higher liver betaine. As discussed in the previous section, a western diet in adult mice is associated with hepatic triglyceride accumulation and characteristic of NAFLD [138,139]. Furthermore, fetal offspring from dams with diet-induced obesity have hepatic triglyceride accumulation [117,140]. Supplementation with either choline (25mM choline chloride) or betaine (1% betaine anhydrous) in the drinking water of dams fed a high fat-diet (60% kcal fat as soybean oil + lard) resulted in lower liver triglycerides in the liver of fetal offspring at E17.5 [117,140]. These studies and others who have supplemented rodents with betaine and reported lower hepatic triglycerides, [138,141] suggest that choline and betaine lower hepatic triglyceride accumulation by potentially upregulating BHMT-mediated production of SAM, which can be used for the methylation of PE to PC for VLDL assembly and export of triglycerides. As such, the higher levels of liver choline metabolites I observed in offspring from western dams may protect them from accumulating triglycerides in the liver.

Interestingly, I found no effect of maternal folic acid supplementation on fetal liver choline metabolites, which is contrary to reports by others [61]. Lower liver choline and betaine were reported in E17.5 fetal C57BL/6 offspring from folic acid supplemented dams (20 mg/kg diet; 10X AIN recommendations) [61]. Folate and choline metabolism are interrelated and both contribute to the synthesis of SAM. Methionine is the precursor of SAM and can be synthesized

by the MTR-catalyzed remethylation of homocysteine using 5-MTHF as a methyl donor, or by BMHT using betaine as a methyl donor. In the rat liver, both MTR and BHMT pathways contribute equally under control conditions [142]. When disturbances in one of these pathways occur, compensatory changes in the other pathway may follow. For example, MTHFR deficiency is associated with greater demand for betaine-dependent remethylation of homocysteine via BHMT [141]. In contrast, BALB/cAnN dams supplemented with folic acid (10 mg/kg diet; 5X AIN recommendations) have reduced MTHFR protein at E10.5, but no changes in hepatic choline or betaine [132]. This suggests that folic acid supplementation can lead to changes in folate metabolism without compensation by, or alterations in choline metabolism. Additional experiments on fetal liver are necessary to assess disturbances in folate metabolism, such as changes in MTHFR expression and activity. This would further our understanding of the effect of maternal folic acid supplementation on alterations in fetal choline metabolism and the interrelationship between choline, betaine, and folate in fetal one-carbon metabolism.

I reported that female E18.5 offspring from control and western dams supplemented with folic acid had higher hepatic SAM and SAM/SAH ratio compared to those from non-supplemented dams. My findings are contrary to reports by others [61,132]. In the study by Christensen *et al.*, E10.5 BALB/cAnN fetuses from folic acid supplemented dams (10 mg/kg diet; 5X AIN recommendations) had no differences in liver SAM or SAH compared to control fetuses [132]. Similarly, Bahous *et al.* reported that E17.5 fetuses from dams supplemented with folic acid (20 mg/kg diet; 10X AIN recommendations) had no differences in hepatic SAM and SAH concentrations compared to control offspring [61]. SAM is a key methyl donor and is central for many biological processes. The SAM/SAH ratio is frequently used as an indicator of cellular methylation potential, such that a lower ratio is indicative of lower methyl group donor

availability [143]. However, studies have reported that changes in SAH are more strongly associated with methylation potential than changes in SAM alone because SAH is an inhibitor of methyltransferases [20]. Further, several studies have investigated relationships between tissue SAM and SAH concentrations and global and gene-specific DNA methylation [143–145]. For example, C57BL/6J male mice with higher liver SAH concentrations and lower SAM/SAH had global DNA hypomethylation in the liver as well as other tissues including the kidney, brain, and testes [143]. Similarly, Glier *et al.* reported that male mice with diet-induced hyperhomocysteinemia had higher liver SAH and lower SAM/SAH and this was accompanied by lower gene-specific DNA methylation in the liver [145]. This study also found higher gene-specific DNA methylation in the brain with no differences in SAM and SAH, suggesting that the relationship between SAM and SAH to gene-specific DNA methylation is tissue specific and that changes in DNA methylation can occur without changes in these methyl nutrients. I did not observe changes in SAH in the fetal female offspring, however, the Devlin Lab has previously reported increased hepatic SAH in adult female offspring from folic acid supplemented dams [56]. This suggests that hepatic SAM and SAH levels are dynamic and can change throughout life. It is not understood why SAM is higher in female livers from folic acid-supplemented dams during fetal life, or if higher SAM-mediated SAM/SAH ratio can improve methylation potential in the fetus. Further investigation of global and gene-specific DNA methylation in the fetal liver would provide insight on the effects of elevated SAM in the fetus.

### 6.1.3 Maternal folic acid supplementation affects islet morphology in male fetal offspring

In Chapter 5, I identified greater β cell mass and density in male fetal offspring from control and western dams supplemented with folic acid; this effect was not observed in female offspring. Alterations in β cell mass are an indicator of impairments in insulin secretion and

glucose tolerance. Increased β cell mass has been observed in E18.5 fetal and 1 day old postnatal pups from C57BL/6J dams fed a high fat diet before pregnancy (60% kcal fat from soybean oil + lard) [119]. Increased β cell mass was associated with fetal and neonatal hyperinsulinemia and consequently lead to hypoglycemia in the fetus and neonate. Untreated and recurrent severe hypoglycemia in early life is associated with several negative consequences, including neurological impairments [146]. This study is one of many examples of how the maternal environment can influence fetal β cell mass and lead to adverse consequences. It further implies that other maternal environments, such as one with folic acid supplementation, may have consequences for offspring health. Typically, a reduction in β cell mass in combination with impaired glucose-stimulated insulin secretion is indicative of increased T2D susceptibility [77,79,147]. This suggests that maternal folic acid supplementation may protect against T2D susceptibility by increasing β cell mass in male fetal offspring. This notion compliments previous findings in the Devlin Lab in which maternal folic acid supplementation had no effect on glucose tolerance in adult male offspring, but worsened glucose tolerance in female offspring [55,56]. Furthermore, adult female offspring from this previous study did not exhibit any changes in β cell mass [56], which is consistent with my findings in fetal female β cell mass.

Others have investigated the effects of maternal folic acid supplementation on β cell mass in older offspring. Seven week old male and female C57BL/6J offspring from folic acid supplemented dams (40 mg/kg diet; 20X AIN recommendations) had lower β cell mass (assessed as insulin-positive cell diameter) compared to offspring from control dams [148]. Additionally, both offspring had lower fasting insulin levels and females were glucose intolerant following an oral GTT. Several reports on adult male and female offspring from folic acid supplemented dams

have observed glucose intolerance [56,59,60]. Changes during fetal life may program glucose tolerance in adulthood. However, the murine pancreas continues to develop in the early postnatal period, undergoing a wave of β cell apoptosis and neogenesis over the first 20 days of postnatal life until weaning [149,150]. This β cell turnover is required for the functional maturation of immature "fetal type" β cells, to fully glucose sensitive adult β cells [150,151]. It is therefore possible that the alterations in fetal β cell mass that I observed may differ from the health of the β cells and glucose regulation of these offspring later in life; further studies to follow these offspring into adulthood are necessary.

It is reasonable to consider that the effect of maternal folic acid supplementation on fetal β cell mass may not be direct and may occur as a consequence of supplementation in the dams. There have been conflicting reports on the association between folic acid supplementation and risk for GDM [111,112]. Diabetes during pregnancy has several negative consequences for the mother and the offspring, including obesity and glucose intolerance later in life [92,93]. In my study, folic acid supplementation had no effect on glucose tolerance or β cell function in the dams, suggesting that the effect of maternal folic acid supplementation on male fetal β cell mass is not because of effects of folic acid supplementation on glucose homeostasis in the dams. This implies that either maternal folic acid supplementation has direct effects on male fetal offspring β cell mass, or that there are other unidentified effects in the dams supplemented with folic acid which in turn influence male offspring β cell mass. Further studies are required to elucidate the mechanisms underlying my observations.

Surprisingly, maternal obesity did not influence fetal β and α cell mass, contrary to several other reports of alterations in β and α cell mass from fetal and adult offspring of dams

fed high fat diets [79,118,119]. Elevated blood glucose, reduced β cell mass, and increased α cell mass has been reported in 1 day old neonatal Wistar rat offspring from dams fed a high fat diet (40% kcal fat; fat source not specified) [118]. Likewise in adult offspring, impaired glucose tolerance and elevated β and α cell mass has been reported in male C57BL/6 offspring from dams fed a high fat diet (49% kcal fat from soybean oil + lard) [79]. However, similar to my findings, a study of C57BL/6J dams fed a high fat diet and sweetened condensed milk (20% kcal fat from lard + 16% kcal fat from milk fat) prior to pregnancy, reported no effects on β and α cell mass in adult male and female offspring [152]. Additionally, Cerf *et al*., reported reduced β cell mass in one day old neonates but no effect in adult offspring from the same female Wistar rat dams fed a high fat diet (40% kcal fat from lard) before and during pregnancy [153]. These findings provide evidence for the dynamic nature of β cell mass that change with age. Considering the β cell turnover that occurs during the neonatal period, it is likely that the β cell mass observed in fetal life will be different from the β cell mass present in adulthood.

Finally, the greater β cell mass that I observed in male fetal offspring from folic acid supplemented dams may be due to the increased β cell density, which I also found. However, the greater β cell density was not accompanied by differences in islet size distribution. The number and size of islets contribute to β cell mass [154]. In a study of non-diabetic human islets (n=72 samples), authors reported that islet density, rather than islet size, was more predictive of β cell mass [154]. This suggests that although the increase in β cell mass in male offspring from folic acid supplemented dams in my study was not statistically significant ($p$=0.058), the β cell density was significantly greater and provides some confidence that the change in β cell mass is biologically relevant.

## 6.2 Summary

In summary, my thesis research demonstrates that maternal folic acid supplementation induced changes in fetal offspring one-carbon metabolism and pancreatic islet morphology. Dams were supplemented with folic acid before and during pregnancy to investigate if this exacerbates the adverse effects associated with gestational obesity and prediabetes on the mother and fetal offspring. Contrary to my hypothesis, folic acid supplementation did not affect maternal adiposity, glucose tolerance, β cell function, or hepatic one-carbon metabolism before or during pregnancy. Folic acid supplementation did however, mildly alter insulin sensitivity in dams, dependent on the dietary fat content before pregnancy. In the fetal offspring, maternal folic acid supplementation was associated with sex-specific alterations in hepatic one-carbon metabolism and pancreatic β cell mass. Although supplementation did not affect maternal one-carbon metabolism, female offspring had higher liver SAM and SAM/SAH ratio. Also, despite no effect of folic acid supplementation on β cell function and glucose tolerance in dams, male offspring from folic acid supplemented dams had elevated β cell mass likely due to an increase in β cell density.

Interestingly, gestational obesity and prediabetes altered liver size in dams and their offspring, possibly due to disturbances in liver one-carbon metabolism. Dams with diet-induced obesity and glucose intolerance had larger livers with reduced hepatic SAM and SAH levels. In the offspring, maternal diet-induced obesity and glucose intolerance altered hepatic choline metabolism. Male and female offspring from western dams also had smaller livers which I infer may be due to the protective effects of higher choline and betaine concentrations in the liver that lead to the prevention of triglyceride accumulation. Taken together, gestational obesity/

prediabetes and folic acid supplementation before and during pregnancy affect maternal and fetal health. An overarching summary of my findings is depicted in Figure 6.1.

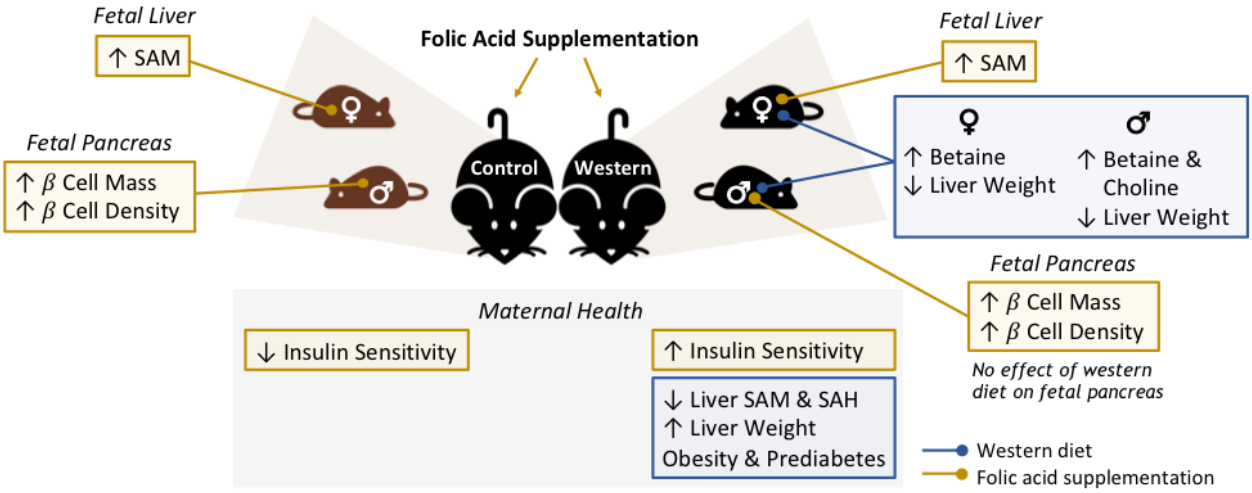


**Figure 6.1. Overarching Summary of Findings.** Effects of maternal western diet are highlighted in blue and effects of maternal folic acid supplementation are highlighted in gold. "Mouse" icons by Darrin Higgins and zidney, from thenounproject.com. Licensed under Creative Commons Attribution 3.0.

## 6.3 Strengths and Limitations

My thesis research has several strengths. Analyses in fetal offspring were able to be done separately in male and female fetuses. It is well known that offspring exhibit sexually dimorphic phenotypes in regards to maternal obesity and folic acid supplementation [55,56,59,77,78]. Many studies conducted in fetal offspring often do not genotype the fetuses for analysis, grouping both together when making conclusions. For example, studies by Christensen *et al.* and Bahous *et al.* provide compelling data with regards to fetal one-carbon metabolism [61,132]. However, the offspring in these studies were not genotyped for sex, making it difficult to conclude if alterations from maternal folic acid supplementation were related to male and/or female fetal mice. By analyzing the offspring separately in my study, I was able to tease out sex-specific

effects of one-carbon metabolism and islet morphometrics in the fetal offspring that may not have been possible, had the fetal offspring been analyzed as one group. This furthers our understanding of how males and females differentially adapt to the maternal environment, allowing for better identification of potential risk factors for male and female offspring health.

Another strength of my study is the assessment of β cell density and islet size distribution to compliment assessment of β cell mass. Mass may be altered through changes in the size or number of β cells as well as changes in vascularization or sympathetic innervation [155–157]. Addition of these assessments enhanced the findings and provided further insight into the changes seen in β cell mass.

My study is not without limitations. There is no doubt that studies of maternal folic acid supplementation in the literature have been variable in terms of dose and duration of supplementation and the outcomes on offspring health. The dose of folic acid supplementation in the control diet I used in my study met the AIN-93 recommendations of 2 mg/kg diet of folic acid for mice and rats [101,102]. This amount provided 0.5 μg of folic acid/kcal (the control diet is 3800 kcal/kg diet, see Table 2.1), which is approximately equivalent to a woman consuming 1 mg of folic acid in a 2000 kcal/day diet. The folic acid supplemented diets of my study contained 10 mg/kg diet of folic acid which is 2.5 μg of folic acid/kcal, and is equivalent to 5 mg/day of folic acid in humans. This amount is recommended for women at high risk of having a NTD pregnancy [35]. However, it should be noted that there are differences between mice and humans in relation to the metabolism of folic acid by DHFR whereby enzyme activity is higher in the liver of rodents compared to humans [158,159]. This suggests that mice and humans may have different capacities for handling large doses of folic acid and the supplemental dose that we provided the

animals may not be considered “high” intake for a mouse. In addition, the timing and duration of folic acid supplementation may have differential effects on offspring [160], which is similar to findings from the Dutch Famine Birth Cohort where exposure to famine at different points of gestation lead to varying adverse outcomes for offspring [6–11]. These differences in study design make it challenging to compare data across studies and formulate solid overarching conclusions.

I found that diet-induced obesity and folic acid supplementation did not impact β cell function, as assessed by IST. However, this physiological assessment is only one indicator of β cell function and does not provide a direct functional measure. Further studies should include direct measures of β cell function including islet secretory function and insulin content in the β cell [161]. To obtain a full understanding and confirm that β cell function is in fact not impaired would require functional experiments using isolated islets for *ex-vivo* glucose-stimulated insulin secretion to assess the influence of folic acid supplementation and diet-induced obesity/glucose intolerance on the secretory function of β cells.

### 6.4 Future Directions

The data presented in this thesis provide a starting point for future research to further elucidate the phenotypes observed and understand the underlying mechanisms of maternal folic acid supplementation on maternal and offspring health. The Devlin lab has previously reported that adult offspring from folic acid supplemented dams display alterations in glucose homeostasis [55,56]. I found that at E18.5, fetal β cell mass was altered in male offspring, but not in female offspring. The pancreas continues to develop in the early postnatal period until weaning to become mature adult β cells [149,150]. Investigating the effect of maternal folic acid supplementation on neonatal β cell maturation and function, including detection of apoptosis,

expression of transcription factors crucial for maintaining β cell identity and functional maturation such as *Pdx1* and *Mafa* [162], and glucose-stimulated insulin secretion of isolated islets, would provide insight on how the β cells develop for future adult physiologic handling of glucose.

Given that folic acid is an oxidized synthetic form of folate, it may have different metabolic effects compared to other folate forms. Differential effects may be due to accumulation of unmetabolized folic acid, which has been implicated in adverse health consequences associated with folic acid supplementation in both humans and animals [163–166]. In a study of folic acid supplemented male BALB/c mice (20 mg/kg diet; 10X AIN recommendations), unmetabolized folic acid was detected in control mice fed the recommended 2 mg folic acid/kg diet, and was increased by 60% in mice fed the folic acid supplemented diet [166]. Folic acid supplementation in these males lead to reduced methylation potential, indicated by reduced SAM/SAH ratio. The metabolically useful form of folic acid, 5-MTHF, is as effective as folic acid in raising red blood cell folate levels in humans without the accumulation of unmetabolized folic acid [167–169]. It is therefore of interest to test the different forms of folate to investigate if the phenotypes observed in fetal offspring of dams supplemented with folic acid are different with alternate folate forms. Additionally, exploring if differences observed due to exposure to folic acid are elicited by elevated maternal folate status or accumulating unmetabolized folic acid would be of interest.

The placenta plays a critical role in the developing fetus. When pregnancy is accompanied by obesity, placental function may be impaired through structural and functional changes [170,171]. Vascular remodeling of the placenta involves the invasion into, and the remodeling of spiral arteries for proper hemochorial blood circulation between mother and fetus

[172]. This process can be impaired by obesity [120,136,137,173]. Maternal folic acid supplementation may also impair placental development. For example, in the study by Christensen *et al.*, BALB/cAnN female mice supplemented with folic acid (10 mg/kg diet; 5X AIN recommendations) had abnormal placental development, including the presence/ absence of, and alterations in thickness of the labyrinth layer [132]. This study suggested that changes in SAM/SAH ratio observed in the fetal offspring at E10.5 may have been affected by an abnormal placenta. This insinuates that changes observed in my study may have also been affected by alterations in the placenta at E18.5. Given the high doses of folic acid being recommended to women with obesity during pregnancy, it is of interest to understand how nutrient transport across the placenta is impacted by folic acid supplementation, particularly in combination with maternal obesity and glucose intolerance.

In conclusion, maternal environmental exposures during fetal and early postnatal life are influential on offspring development and on the programming of long-term offspring health. The maternal environment may evoke an influence on offspring health through direct changes to fetal development, or through changes in placental development that subsequently affect the growth and development of the fetus. It is well accepted that maternal folic acid supplementation prevents NTDs during pregnancy. However, it is critical to continue to understand the impact of increased folic acid and folate on the metabolic health of the mother and offspring, particularly for pregnancies complicated with gestational obesity and prediabetes. This will provide further knowledge and understanding to be considered when developing and implementing policies for the recommendation of folic acid supplementation before and during pregnancy.

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