Adiponectin deficiency leads to hepatic steatosis and contributes to the development of Gestational Diabetes Mellitus

by

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Abstract

Gestational diabetes mellitus (GDM) is a common complication of pregnancy, characterized by hyperglycemia and impaired glucose tolerance with first onset mid-gestation. Genetic predisposition, maternal obesity and diet contribute to development of GDM. In addition, low levels of serum adiponectin are associated with increased risk for GDM. Adiponectin is an adipose tissue derived cytokine that improves systemic insulin sensitivity. Since adiponectin has a role in hepatic lipid metabolism, we hypothesize that adiponectin deficiency leads to fatty liver during pregnancy, worsening insulin resistance and contributing to GDM development.

We compared adiponectin KO (strain B6;129-*Adipoq*^{tm1Chan}/J) mice to WT (C57/B6) controls in the third trimester of pregnancy and evaluated measures of glucose and insulin tolerance when animals were consuming LF or HFS diets in pregnancy. We performed a histological and biochemical evaluation of hepatic steatosis in pregnancy. To investigate underlying mechanisms, we used qPCR, immunoblotting, and immunofluorescence analysis of tissues from pregnant adiponectin KO and WT mice.

Adiponectin KO mice were hyperglycemic, had impaired glucose tolerance and dysregulated islet hormone secretion in the third trimester. Mice lacking adiponectin showed unchecked hepatic glucose output in the third trimester, which may contribute to hyperglycemia in pregnancy. Pregnant adiponectin KO mice had reduced mitochondrial respiration, increased lipid synthesis and hepatic steatosis. Supplementation with adiponectin improved glucose tolerance and hepatic steatosis in pregnancy.

In a cohort of First Nation's mothers and newborns, lower serum adiponectin and higher serum leptin were observed in GDM relative to T2D pregnancies and normoglycemic controls. GDM and T2D during pregnancy was associated with increased birthweight and maternal adiponectin was negatively correlated with birthweight, suggesting that adiponectin may have a role in fetal growth.

In conclusion, we observed an association between low levels of adiponectin and GDM in pregnant women. In pregnant adiponectin KO mice, lack of adiponectin leads to the development of hepatic steatosis, disruption to islet function and hyperglycemia in pregnancy. Supplementation with adiponectin to mice in the third trimester, mitigated fatty liver and glucose intolerance in the third trimester.

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LIST OF ABBREVIATIONS

ACC/Acaca– Acetyl-CoA Carboxylase

ACLY – ATP Citrate Lyase

Ad-GFP – Adenovirus containing GFP

Ad-APN – Adenovirus containing Adiponectin

AGPAT - 1-acylglycerol-3-phosphate-O-acyltransferase

α-KG – α-Ketoglutarate

AKT – Protein Kinase B

AMPK - 5' Adenosine Monophosphate-Activated Protein Kinase

ANOVA – Analysis of Variance

APGAR – Appearance, Pulse, Grimace, Activity and Respiration

ASM – Acid Soluble Metabolites

ATGL – Adipose Triglyceride Lipase

ATP – Adenosine Triphosphate

B2m*:* β-2 Microglobulin

BDH/Bdh1 – β-Hydroxybutyrate Dehydrogenase

BMI – Body Mass Index

CBD – Common Bile Duct

CIC – Citrate Carrier

cDNA – Complementary DNA

cMet – Hepatocyte Growth Factor Receptor

CMPF - 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid

CPT-1 /Cpt1a– Carnitine Palmitoyl Transferase -1

CPT-2 – Carnitine Palmitoyl Transferase -2

CRP – C-reactive Protein

CS – Citrate Synthase

DGAT/Dgat1 – Diacylglycerol – Acyltransferase DAG – Diacylglycerol

DG – Diacylglycerides

DMEM - Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleic Acid

DSM – Diagnostic Services of Manitoba

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EGTA - Ethylene Glycol-Bis [β-Aminoether] N,N,′N′,N -Tetra-Acetic Acid

ELISA – Enzyme Linked Immunosorbant Assay

ELOVL6 – Elongation of Very Long Chain Fatty Acids Protein 6

ER – Endoplasmic Reticulum

ETOX – Etomoxir

FA – Fatty Acid

FAO – Fatty Acid Oxidation

fAPN – Full Length Adiponectin

FASN/Fasn – Fatty Acid Synthase

FATP – Fatty Acid Transport Proteins

FBS – Fetal Bovine Serum

FFA – Free Fatty Acids

FoxD3 – Forkheadbox D3

FoxM1 – Forkheadbox M1

FoxO1 – Forkhead Box O1

gAPN – Globular Adiponectin

GDM – Gestational Diabetes Mellitus

GFP – Green Fluorescent Protein

GGT - γ-glutamyl transferase

GK/Gck – Glucokinase

GLP-1 – Glucagon-Like Peptide-1

GLUT2 – Glucose Transporter 2

GLUT4 – Glucose Transporter 4

G6Pase/G6pc– Glucose-6-phosphatase

Gpam - Glycerol-3-Phosphate Acyltransferase – mitochondrial

GPAT – Glycerol – 3- Phosphate Acyltransferase

GSIS – Glucose Stimulated Insulin Secretion

GSK3 – Glycogen Synthase Kinase-3

GTT – Glucose Tolerance Test

GWAT – Gonadal White Adipose Tissue

GWG – Gestational Weight Gain

HAPO - Hyperglycemia and Adverse Pregnancy Outcomes

HbA1c – Hemoglobin A1C

HDL – High Density Lipoproteins

H&E – Hematoxylin & Eosin

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFD – High Fat Diet

HFS – High Fat Sucrose

HGF – Hepatocyte Growth Factor

HMGCR - 3-Hydroxy-3-Methylglutaryl-CoA Reductase

HMGCS2 - 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2

HMW – High Molecular Weight

Hnf4a – Hepatocyte Nuclear Factor 4α

HOMA-IR – Homeostatic Model Assessment of Insulin Resistance

hPGH – Human Placental Growth Hormone

HPL – Human Placental Lactogen

Hprt1*:* Hypoxanthine Guanine Phosphoribosyl Transferase

HSL – Hormone Sensitive Lipase

5HT – 5-Hydroxytryptamine

5HTR – 5-Hydroxytryptamine Receptor

IFNγ – Interferon γ

IGF – Insulin-Like Growth Factor

IL-1β – Interleukin-1β

IP - Intraperitoneal

IRβ – Insulin Receptor-β

IRS-1 – Insulin Receptor Substrate -1

ITT – Insulin Tolerance Test

IVC – Inferior Vena Cava

KO – Knockout

LDL – Low Density Lipoproteins

LF – Low Fat

LGA – Large for Gestational Age

LMW – Low Molecular Weight

LPA – Lysophsosphatidate

MG – Monoglycerides

MGL - Monoglyceride Lipase

MiRNA – MicroRNA

MMW – Medium Molecular Weight

MODY – Maturity Onset Diabetes of the Young

mTOR – Mammalian Target of Rapamycin

MTP – Mitochondrial Trifunctional Protein

NADH – Nicatinamide Adenine Dinucleotide

NAFLD – Non-Alcoholic Fatty Liver Disease

NASH – Non-Alcoholic Steatohepatitis

OAA – Oxaloacetate

OCT – Optimal Cutting Temperature Compound PA – Phosphatidate

PAPP-A – Pregnancy Associated Protein-A

PBS – Phosphate Buffered Saline

PC/Pcx– Pyruvate Carboxylase

PC – Phosphatidylcholine

PCOS – Polycystic Ovarian Syndrome

PDC – Pyruvate Dehydrogenase Complex

PDH/Pdhe1α – Pyruvate Dehydrogenase

PE – Phosphatidylethanolamine

PEPCK/Pck1/Pck2– Phosphoenolpyruvate Carboxykinase

PEP – Phosphoenolpyruvate

PFA – Paraformaldehyde

PFK1 – Phosphofructokinase-1

PFK2 – Phosphofructokinase-2

Pgc1α – Peroxisome Proliferator-Activated Receptor γ Coactivator 1-α

PI3K – Phosphatidyl-Inositol-3 Kinase

PK/Pklr – Pyruvate Kinase

PL – Placental Lactogen

PPARα – Peroxisome Proliferater-Activated Receptor α

PPAR- γ - Peroxisome Proliferator-Activated Receptor γ

PrL – Prolactin

PrlR – Prolactin Receptor

PTT – Pyruvate Tolerance Test

PWAT – Perirenal White Adipose Tissue

Ras/ERK – Extracellular Signal-Related Kinases

RIA – Radioimmuno Assay

Rn18s*:* 18s rRNA

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RPLP-1*:* 60S Acidic Ribosomal Protein Large P1

SCD1 – Stearyol CoA Desaturase-1

SDS – Sodium Dodecylsulfate

SEM – Standard Error of the Mean

SMTNL1 – Smoothelin-like Protein-1

Srebp1C - Sterol Regulatory Element Binding Protein-1c

SVC – Superior Vena Cava

TCA CYCLE – Tricarboxcylic Acid Cycle

T2D – Type 2 Diabetes

TG – Triglycerides

TLC – Thin Layer Chromatography

TNFα – Tumor Necrosis Factor-α

UCP2 – Uncoupling Protein 2

VLDL – Very Low-Density Lipoprotein

WAT – White Adipose Tissue

WT – Wildtype

Chapter 1:

Introduction

Sections of this chapter have been published as a review in the *International Journal of Molecular Sciences*, entitled **"Maternal β-Cell Adaptations in Pregnancy and Placental Signalling: Implications for Gestational Diabetes",** Moyce Brittany L, Dolinsky, Vernon W. 2018.

Pregnancy involves several metabolic adaptations to sustain maternal health and fetal growth. In order to understand these processes, it is necessary to first review the major metabolic processes in the non-pregnant state.

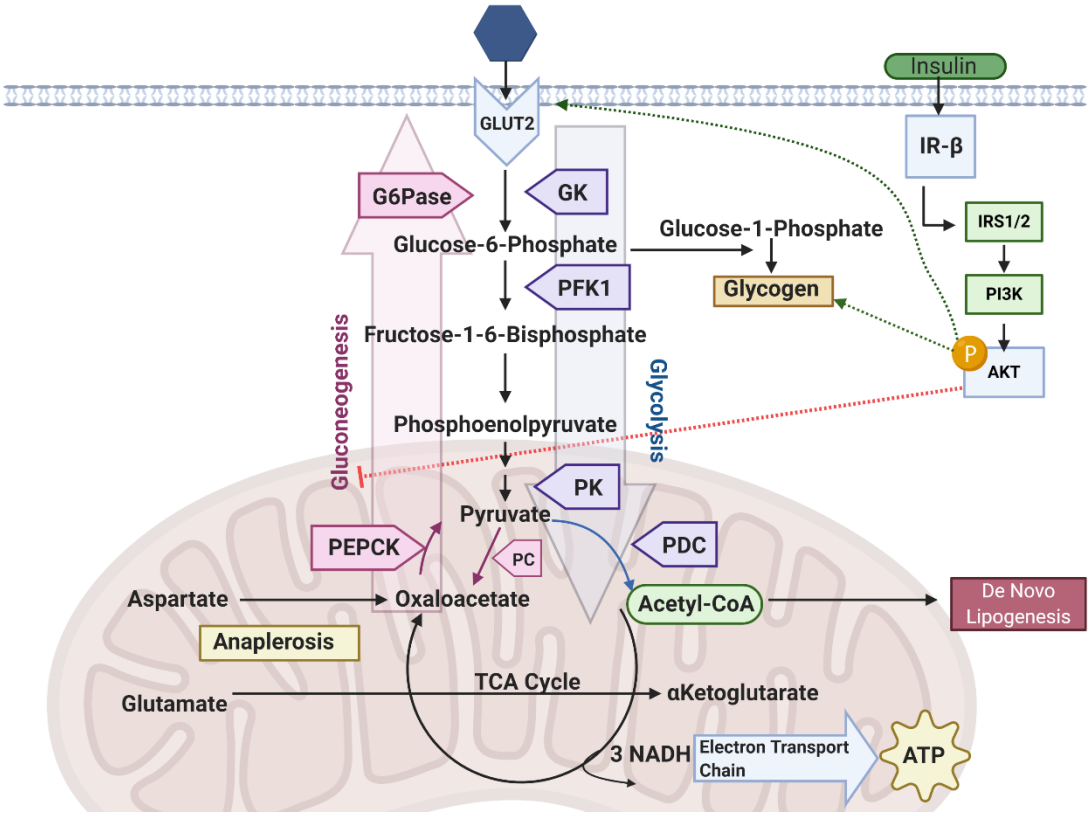
1.1 Hepatic energy metabolism

The liver is central to whole body energy metabolism and performs both anabolic and catabolic functions. It is a major target organ of insulin for the control of glucose utilization, storage and production. The liver is also central to the control of fat utilization in the body through its roles in lipogenesis, storage, ketogenesis and lipoprotein secretion. Pregnancy is a time of metabolic adaptation and the liver is central to these adaptations, utilizing and storing free fatty acids released from adipose tissue during late gestation and producing glucose by gluconeogenesis for the growing fetus (Lain & Catalano, 2007).

1.1.1 Glycolysis

In the fed condition, the liver takes up glucose from the circulation by glucose transporter 2 (GLUT2) which is non-insulin-dependent and glucose is oxidized to pyruvate by the process of glycolysis (X. Guo et al. (2012). Hepatic glycolysis is regulated by insulin signalling (Pagliassotti & Cherrington, 1992; Pagliassotti, Moore, Neal, & Cherrington, 1992), by increasing expression of glucokinase and generating glucose-6-phosphate (G6P) (reviewed in (Rui, 2014). Higher circulating insulin will increase glucose uptake and glycolysis through increased expression of GLUT2 and key genes encoding enzymes in the glycolysis pathway (glucokinase, 6-phosphofructokinase-1) (X. Guo et al., 2012; S. Y. Kim et al., 2004). In hepatic mitochondria, pyruvate can then be metabolized through the tricarboxylic acid (TCA) cycle, generating reducing equivalents in the form of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Electrons from NADH and $FADH_2$ are transferred to complex 1 (NADH dehydrogenase) and complex II (succinate dehydrogenase), respectively, and are shuttled across the electron transport chain, driving proton pumping from the mitochondrial matrix to the intermembrane space at complexes I, III and IV (Jastroch, Divakaruni, Mookerjee, Treberg, & Brand, 2010). This efflux of protons generates the proton motive force that couples energy from glucose oxidation to ATP production by ATP synthase, via a process called oxidative phosphorylation (J. Berg, Tymoczko, JL., Stryer, L.,, 2002) (Figure 1).

Figure 1: Hepatic glucose metabolism and regulation by insulin



In the liver glucose is taken up from circulation through GLUT2 and broken down through glycolysis. Key glycolytic enzymes include glucokinase (GK), phosphofructokinase-1 (PFK1), and pyruvate kinase (PK). The pyruvate dehydrogenase complex (PDC) converts pyruvate to acetyl-CoA, which can be broken down completely through the TCA cycle and oxidative phosphorylation to ATP. Gluconeogenesis is the *de novo* production of glucose from pyruvate. Key steps include conversion of pyruvate to oxaloacetate by pyruvate carboxylase (PC), oxaloacetate to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK) and the final conversion of glucose-6-phosphate to glucose by glucose-6-phosphatase (G6Pase). Insulin signalling suppresses gluconeogenesis and promotes glucose uptake and glycogen synthesis through phosphatidyl-inositol-3 kinase (PI3K)/AKT signalling. Anaplerotic reactions replenish key TCA cycle intermediates via amino acid precursors.

1.1.2 Anaplerosis

The TCA cycle is an important pathway in hepatic metabolism, linking substrate uptake to oxidative phosphorylation. Anaplerosis is defined as the non-oxidative incorporation of carbon into substrates via the TCA cycle (O. E. Owen, Kalhan, & Hanson, 2002). Increased levels of TCA cycle substrates (such as α-ketoglutarate (α-KG) or oxaloacetate (OAA)), increased precursors for TCA substrates (e.g. amino acids that can be converted to α-ketoglutarate such as glutamate), or increased expression of anaplerotic enzymes such as pyruvate carboxylase can indicate elevated anaplerosis (Cappel et al., 2019), (Sunny, Parks, Browning, & Burgess, 2011) (Figure 1). Due to the central role of the TCA cycle, imbalances in TCA intermediates (either depletion or buildup) can contribute to metabolic dysfunction. Therefore anaplerotic reactions are required to maintain metabolic homeostasis (Merritt, Harrison, Sherry, Malloy, & Burgess, 2011).

1.1.3 Gluconeogenesis and glycogen synthesis

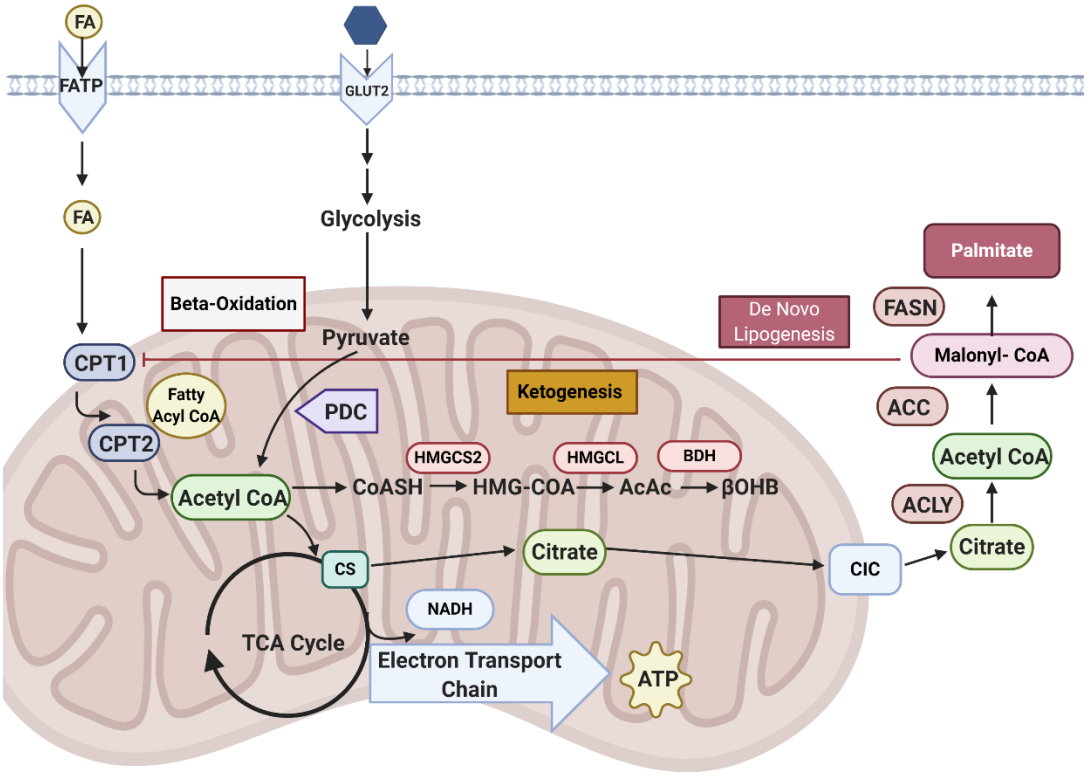
An important function of hepatic metabolism is the *de novo* production of glucose from other substrates in a process called gluconeogenesis. Gluconeogenesis is suppressed by insulin, through inhibitory phosphorylation of the transcription factor FOXO1 downstream of AKT, resulting in suppression of gluconeogenic genes (R. A. Haeusler, Kaestner, & Accili, 2010). Insulin drives energy utilization via glycolysis, and energy storage in the form of glycogen and lipids. As part of catabolism and the TCA cycle, pyruvate carboxylase converts pyruvate to OAA (Landau et al., 1996), which is an important intermediary in energy synthesis and storage. Phosphoenolpyruvate carboxykinase (PEPCK) converts oxaloacetate to phosphoenolpyruvate (PEP) in the first and rate limiting step of gluconeogenesis (Hue, 1983; Pilkis, 1992). A second important enzyme in the pathway is glucose 6-phosphatase, which converts glucose-6-phosphate to glucose for export via the bi-directional transporter GLUT2.

Under conditions of high glucose the liver can also produce and store glycogen, and this is mediated by insulin signalling (T. P. Combs & Marliss, 2014). Glucose taken up through GLUT2 is converted to glucose-6-phosphate, however instead of undergoing the remaining steps of glycolysis, glucose-6-phosphate is converted to glucose-1-phosphate and glycogen synthase produces glycogen, the storage form of glucose, which can be broken down for later use when blood glucose is low (Figure 1). Glycogenolysis and gluconeogenesis both raise blood glucose and as such are linked. Decreases in circulating insulin or insulin sensitivity can stimulate both processes and may be an important contributor to the development of hyperglycemia.

1.1.4 Lipid metabolism: β-Oxidation

In addition to glucose, the liver can also metabolize fatty acids for energy during fasting or times of increased metabolic demand (Bechmann et al., 2012). Dietary lipids or free fatty acids released from adipose tissue are taken up from circulation by the liver via fatty acid transport proteins (FATPs 2 and 5) (Doege et al., 2006; Falcon et al., 2010) or cluster of differentiation 36 (CD36) on the plasma membrane. After crossing the plasma membrane, they are activated to fatty acyl-CoAs. At this stage, fatty acids have three possible fates: utilization for oxidation or if in excess, conversion to triacylglycerol for subsequent storage or secretion. For oxidation, fatty acyl-CoA is transported across the outer mitochondrial membrane by carnitine palmitoyl transferase-1 (CPT-1) and across the inner mitochondrial membrane by CPT-2 for β-oxidation within the mitochondria. The resultant product of β-oxidation is acetyl-CoA which can be completely oxidized by the TCA cycle and electron transport chain to generate ATP (Figure 2). Acetyl-CoA can also be converted into ketone bodies via β-hydroxybutyrate dehydrogenase (BDH) in a process of incomplete oxidation. This occurs in the event of fatty acid overload as an effective method of managing excess acetyl-CoA, or under starvation conditions (Cotter et al., 2014) (Figure 2). Importantly, insulin signaling increases, fatty acid uptake, however β-oxidation is downregulated in favour of hepatic lipid storage (Bechmann et al., 2012). In the fed condition, circulating insulin strongly suppresses ketogenesis (Fukao, Lopaschuk, & Mitchell, 2004).

Figure 2: Hepatic β-oxidation and *de novo* lipogenesis



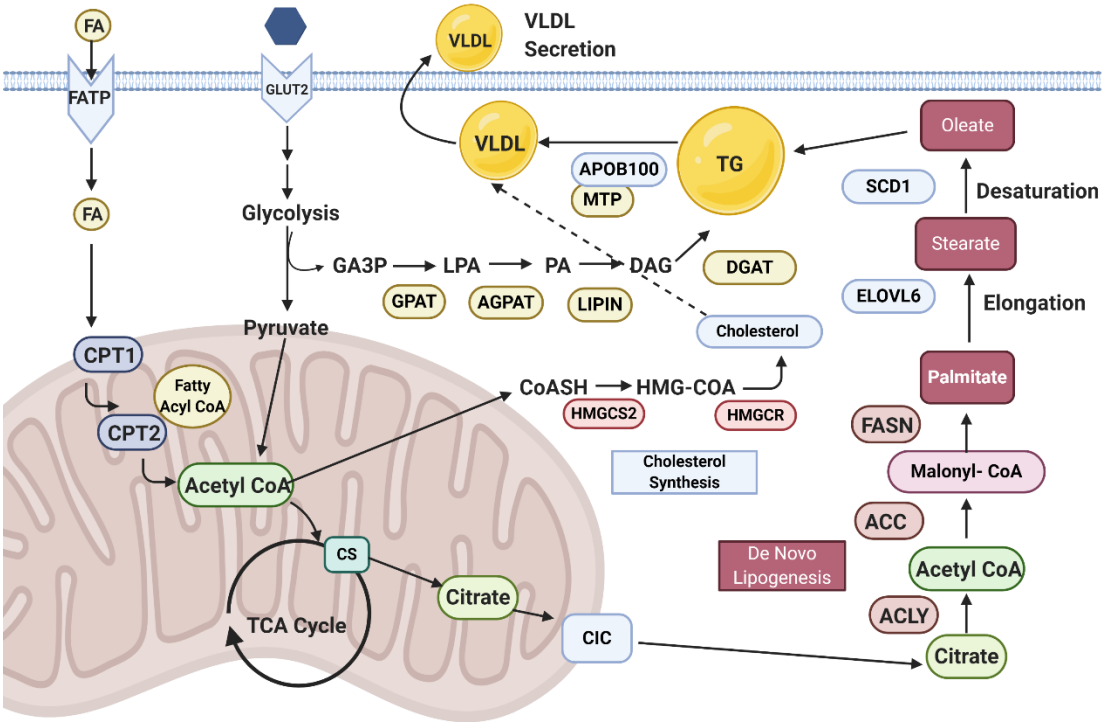
Fatty acids are taken up by the liver by fatty acid transport proteins (FATP2 and 5 in the liver, or CD36) and transported into the mitochondria by carnitine palmitoyl transferases (CPT1 and 2) and converted into acetyl-CoA and metabolized through the TCA cycle and ATP is produced by oxidative phosphorylation. Under conditions of starvation or metabolic stress, acetyl-CoA can be metabolized to ketone bodies through HMG-CoA Synthase (HMGCS2) and HMG-CoA Lyase (HMGCL) to Acetoacetate (AcAc) and further by β-hydroxybutyrate dehydrogenase (BDH) to β-hydroxybutyrate. Citrate generated in the mitochondria can be transported to the cytoplasm by the mitochondrial citrate carrier (CIC) and ATP-citrate lyase (ACLY) converts it back to acetyl-CoA for the first committed step of *de novo* lipogenesis and the resulting product, malonlyl-CoA is converted to palmitate by fatty acid synthase (FASN).

1.1.5 Lipogenesis

In the liver, glycolysis and the TCA cycle are tightly linked to the metabolism and synthesis of fatty acids. Synthesis of new fatty acids and their esterification to triglycerides occurs in the cytoplasm. Citrate produced from the TCA cycle in the mitochondria is removed to the cytosol via the mitochondrial citrate carrier where ATP citrate lyase cleaves it into OAA and acetyl-CoA, thus linking glucose metabolism and fatty acid biosynthesis (Q. Wang et al., 2010). The first committed step of fatty acid synthesis occurs with acetyl-CoA carboxylase (ACC) adding a carboxyl group to acetyl-CoA and converting it into malonyl-CoA, thus activating the fatty acid by converting it to a fatty acyl-CoA. Fatty acid synthase (FASN) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) act on malonyl-CoA to produce palmitate, a 16 carbon saturated fatty acid (S. F. Jones & Infante, 2015) (Figure 2).

De novo synthesized fatty acids as well as fatty acids taken up from the circulation can be further esterified into triglycerides for storage or export (Figure 3). The liver has a high capacity for synthesis and storage of lipids. Insulin stimulates lipogenesis through increased lipogenic gene expression (Foretz, Guichard, Ferré, & Foufelle, 1999), and lipogenesis protects against the detrimental effects of excess fatty acids. To produce triglycerides, palmitic acid, the product of lipogenesis from glucose, can be elongated and desaturated (by the enzymes elongation of very long chain fatty acids protein 6 (ELOVL6) and stearyl-CoA desaturase 1 (SCD1)) (Ntambi, Miyazaki, & Dobrzyn, 2004) for esterification by glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the first step of triglyceride synthesis (Gonzalez-Baro, Lewin, & Coleman, 2007). There are two isoforms of the acyl-CoA:diacylglycerol acyltransferase (DGAT) enzyme that are important for production of triglycerides by condensing fatty-acyl-CoAs and diacylglycerols into triglycerides. DGAT1 is active when substrates for lipogenesis are high (for instance, increased uptake of fatty acids from dietary sources) (Yen, Stone, Koliwad, Harris, & Farese, 2008). In the liver, DGAT1 is crucial for formation of lipid droplets that lead to hepatic steatosis (Villanueva et al., 2009). Hepatic DGAT2 is more active under conditions of low exogenous fatty acid provision and may serve as an important link between glucose uptake and lipogenesis (Yen et al., 2008).

Figure 3: Hepatic triglyceride and cholesterol synthesis



The further esterification of fatty acids involves elongation by ELOVL6 and desaturation by SCD1, for esterification by glycerol-3-phosphate acyltransferase (GPAT). GPAT catalyzes the first step of triglyceride synthesis. DGAT enzymes condense fatty-acyl-CoAs and diacylglycerols into triglycerides. The liver can also produce ketone bodies as an alternate source of fuel under times of nutrient stress. Hepatic ketogenesis is linked to lipid metabolism, and if acetyl-CoA is allowed to build up it can drive the production of ketone bodies through 3-hydroxy-3-methyl-glutaryl-

CoA (HMG-CoA) Synthase-2 (HMGCS2) and HMG-CoA Reductase (HMGCR) enzymes. Acetoacetate and β-hydroxybutyrate are the primary products of ketogenesis; if they are not oxidized, ketone bodies can undergo lipogenesis or be converted to cholesterol via HMGCR. Production of ketone bodies can dispose of a large proportion of hepatic lipids, and mice with ketogenic insufficiency rapidly develop fatty liver. Triglycerides and cholesterol esters can be packaged into VLDL particles for secretion into the circulation.

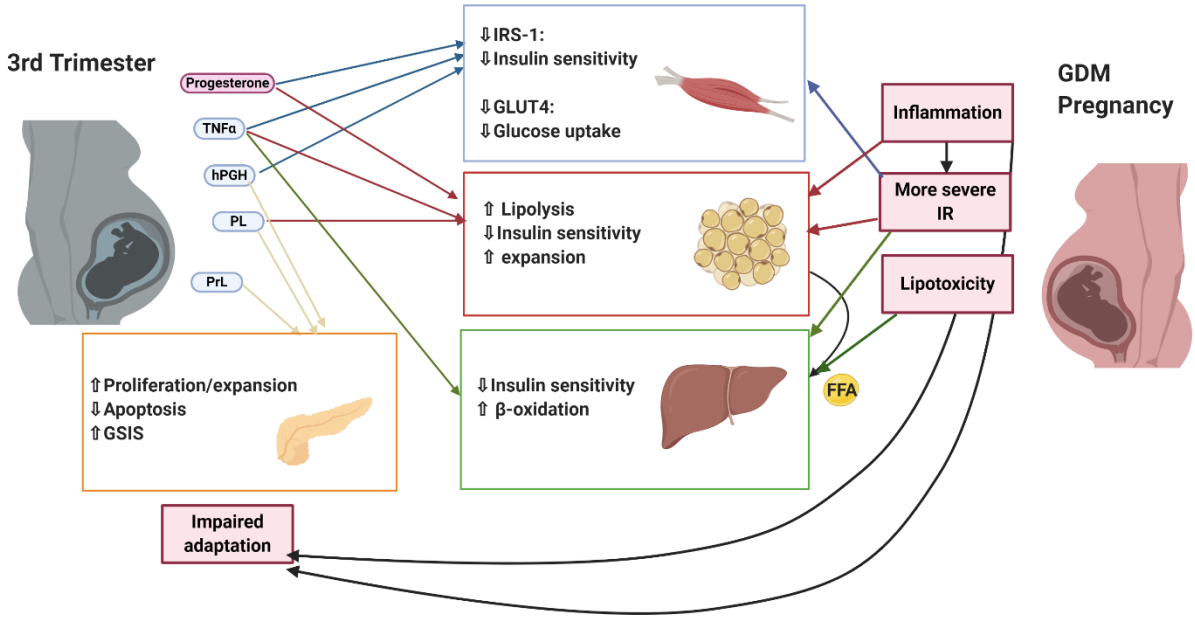
1.1.6 Ketogenesis

Under conditions of starvation, high fat consumption or low insulin, the liver is a primary producer of ketone bodies, which serve as an alternate source of energy under times of nutrient stress, allowing stored lipids to be used for energy – this becomes critically important for the brain, which uses ketones for energy during starvation (O. Owen et al., 1967). Hepatic ketogenesis is tightly linked to lipid metabolism, and therefore is regulated in part by rates of lipolysis and transport of fatty acids to hepatocytes (J. D. McGarry, Foster, D.W., 1980), as well as levels of TCA cycle intermediates. Buildup of acetyl-CoA can exceed the capacity of the TCA cycle and availability of intermediates (OAA or citrate synthase activity) and drive acetyl-CoA to production of ketone bodies (Cotter et al., 2014) (Figure 2). Acetoacetate and β-hydroxybutyrate are the primary products of ketogenesis, and while most tissues are capable of oxidizing ketones, hepatocytes are incapable of utilizing ketones for energy despite their capacity for ketogenesis. If they are not oxidized, ketone bodies can be directed to lipogenesis or be converted to cholesterol via HMGCR. Production of ketone bodies can utilize a large proportion of hepatic lipids, and mice with ketogenic insufficiency rapidly develop fatty liver (Cotter et al., 2014).

1.2 Metabolic adaptations during pregnancy

In mammals, the adaptations required to sustain pregnancy occur in two phases according to the changing metabolic demands of the mother and fetus. The first two trimesters in a human pregnancy corresponds to approximately the first 10 days in rodents; this is considered early gestation and is an overall anabolic period. During this stage, the body builds up energy stores, increasing lipid storage in tissues to prepare for increased breakdown and use later in pregnancy (Vivas et al., 2016; Wharfe, Wyrwoll, Waddell, & Mark, 2016). To achieve this, maternal energy consumption increases as does *de novo* hepatic lipogenesis. In the later stages of pregnancy, lipid deposits are preferentially broken down for fatty acid oxidation by the maternal system which allows for the critical sparing of glucose to meet the increasing nutritional needs of the fetus (R. K. Gupta et al., 2007; Vivas et al., 2016; Wharfe et al., 2016). The second half of pregnancy is also associated with increased peripheral insulin resistance. Hormones such as human placental growth hormone (hPGH) increase progressively throughout pregnancy (Handwerger & Freemark, 2000) and reduces signalling through the insulin receptor substrate – 1(IRS-1) as well as GLUT-4 mediated glucose uptake in skeletal muscle and adipose tissue. These changes return to baseline postnatally (John P Kirwan et al., 2004). Since the first half of pregnancy is anabolic, the latter half of pregnancy is considered catabolic, as energy stored during early gestation is broken down. Due to the shifting nutritional needs of mother and fetus, pregnancy is a time of metabolic plasticity, during which many adaptations are required to maintain metabolic homeostasis (Figure 4).

Figure 4: Metabolic adaptations of late gestation and gestational diabetes mellitus



Changes to circulating hormones and cytokines in pregnancy impact insulin tolerance of peripheral tissues and compensatory adaptations of pancreatic β-cells. In the third trimester, progesterone increases and may have a role in increasing adipose tissue lipolysis and decreasing insulin sensitivity and glucose uptake in skeletal muscle. Increased lipolysis from adipose tissue may lead to increased fatty acid uptake by the liver and reduced hepatic insulin sensitivity. hPGHincreases significantly throughout gestation and has been implicated in both reduced insulin signalling in skeletal muscle (by blunted insulin-receptor subtrate-1 (IRS-1) signalling and impaired GLUT4 translocation) as well as stimulating pregnancy induced β-cell adaptations. Prolactin (PrL) and placental lactogen (PL) have been implicated in pregnancy associated β-cell adaptations as well as insulin resistance and increased lipolysis. TNFα increases in circulation towards the end of pregnancy and has been shown to mediate the insulin resistance that occurs in the third trimester of pregnancy. In gestational diabetes, there is more severe insulin resistance, increased inflammation and lipotoxicity and impairments in the necessary adapations by the pancreatic β-cell.

Many hormonal, metabolic and immunological changes are set in motion to adequately adapt to maternal and fetal needs throughout gestation (Vivas et al., 2016). For instance, the maternal system will increase blood volume and cardiac output, accompanied by a corresponding increase in renal activity (Soma-Pillay et al., 2016). In addition, increased respiratory capacity and neurological changes, specifically increased neural plasticity and increased neurogenesis have also been observed during pregnancy and lactation (Hillerer, Jacobs, Fischer, & Aigner, 2014; Soma-Pillay et al., 2016). As the catabolic phase begins in late gestation, circulating lipids are transiently increased and mild hyperinsulinemia occurs. Despite these changes, in most pregnancies normoglycemia is maintained and blood glucose has even been shown to decrease, which could be due to the dilution effect as blood volume increases in the maternal system (Angueira et al., 2015). Additionally, increased insulin production and secretion by the maternal pancreas also largely contribute to the maintenance of maternal glycemia (A. E. Butler et al., 2010). In pregnancy, peripheral tissues become more insulin resistant (L. Chen, Chen, Wang, & Liang, 2015; Vivas et al., 2016) and hepatic

glucose output is not suppressed. In order to achieve glucose homeostasis in a healthy pregnancy, circulating insulin is increased to overcome insulin resistance (Wharfe et al., 2016).

1.2.1 Adaptation by the liver during pregnancy

Due to its many roles in energy metabolism, the liver plays a large role in maintaining maternal glucose homeostasis throughout gestation. Importantly, insulin signalling in the liver regulates many of these processes. In early pregnancy when the metabolic demands of fetal growth are low, hepatic insulin sensitivity remains high. Due to increased lipogenesis and lipid storage in adipose tissue, there is an increase in the amount of fatty acid taken up and metabolized by the liver, and more glucose is utilized for maternal energy (Lain & Catalano, 2007; Zeng, Liu, & Li, 2017). Consequently, insulin can still suppress hepatic gluconeogenesis (Abraham et al., 2016). In late pregnancy, maternal metabolism switches to favor fatty acid oxidation and maternal systemic insulin resistance allows glucose to be spared for fetal growth. To compensate for the insulin resistant state, the pancreatic β-cell increases insulin secretion, however energy metabolism in the liver also undergoes changes in response to decreased insulin sensitivity.

Insulin resistance in adipose tissue leads to increased lipolysis, resulting in increased free fatty acids in circulation – this period of lipid mobilization allows for uptake by the liver and increased β-oxidation. Since insulin promotes energy storage, reduced hepatic insulin sensitivity results in decreased lipogenesis (Lain & Catalano, 2007; Towler & Hardie, 2007). Hepatic glucose uptake and glycolysis are down regulated and reduced hepatic insulin signalling results in increased gluconeogenesis as glucose is shunted towards the growing metabolic demands of the fetus.

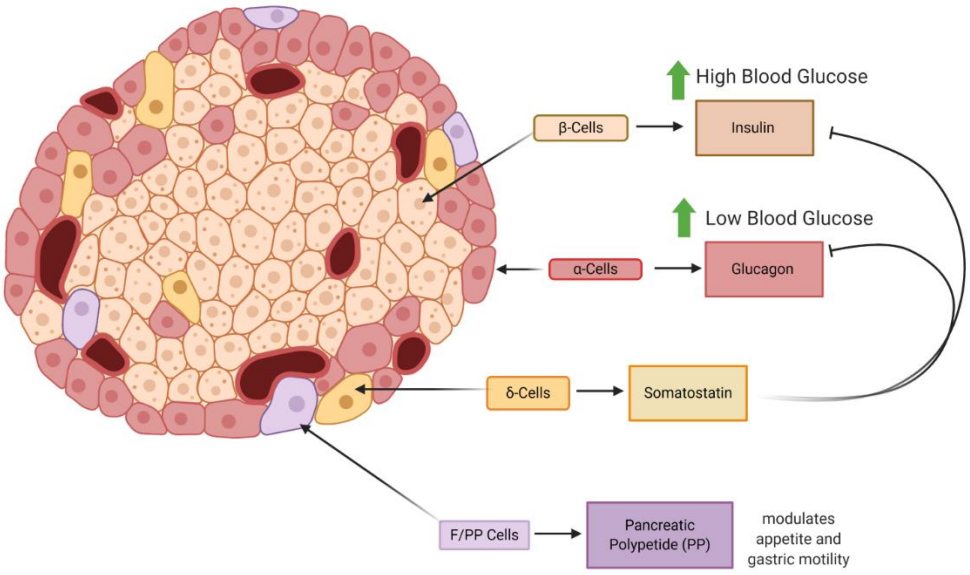
1.2.2 The endocrine pancreas and its adaptation to pregnancy

Islet architecture varies between species, but mice and rats have relatively well-defined pancreatic islets structure, with abundant β-cells (in mice, making up 60-80% of cells in the islet) and scarcer polypeptide (PP)-cells, δ-cells and α-cells (Baetens, Malaisse-Lagae, Perrelet, & Orci, 1979; A. Kim et al., 2009; Wieczorek, Pospischil, & Perentes, 1998). The δ-cells secrete somatostatin (Dubois, 1975), which in the pancreas acts as a paracrine signal to suppress endocrine secretions of insulin and glucagon from β-cells and α-cells (Dobbs et al., 1975; J. Li et al., 2015). Secretion of pancreatic polypeptide (PP) by the pancreatic PP cells has been shown to increase gastric secretions and motility, and can also inhibit the secretory function of the pancreas (Schwartz, 1983; Ueno et al., 2000) (Figure 5). Regulation of insulin and glucagon secretion by somatostatin and PP represents an important level of paracrine regulation within the pancreatic islet. Blood glucose is tightly regulated, and this is managed by the actions of insulin and glucagon under conditions of high or low blood glucose respectively and in response to a variety of other stimuli (Baeyens, Hindi, Sorenson, & German, 2016; P. C. Butler, Meier, Butler, & Bhushan, 2007).

Studies in human and animal models have provided evidence for plasticity of the pancreatic islet during pregnancy (A. E. Butler et al., 2010; A. Kim et al., 2009; Van Assche, Aerts, & Prins, 1978), which allows increases in mass and secretory capacity to compensate for the metabolic demands of pregnancy. This research has largely focused on the β-cell and to a lesser extent the α-cell due to their role in the management of blood glucose requirements during pregnancy. However, one study in pregnant and virgin rats showed that rats without diabetes had decreased

levels of PP in pregnancy relative to non-pregnant controls (Van Assche et al., 1978), indicating a level of plasticity may be present in all cells of the pancreatic islet to allow for pregnancy-induced changes.

Figure 5: Structural morphology of the pancreatic islet in mice



In rodents, the pancreatic islet has well defined structure. The most abundant cell type are insulin-secreting β-cells, which are centrally located and comprise 60-80% of the islet in mice. The next most abundant cell type in the islet are the glucagon secreting α-cells which comprise 15-20% and are located around the periphery. δ-cells are 5-10% of islet cells and secrete somatostatin. F cells or pancreatic polypeptide (PP) cells are sparse and may to be involved in gastric motility, appetite regulation, and the exocrine function of the pancreas.

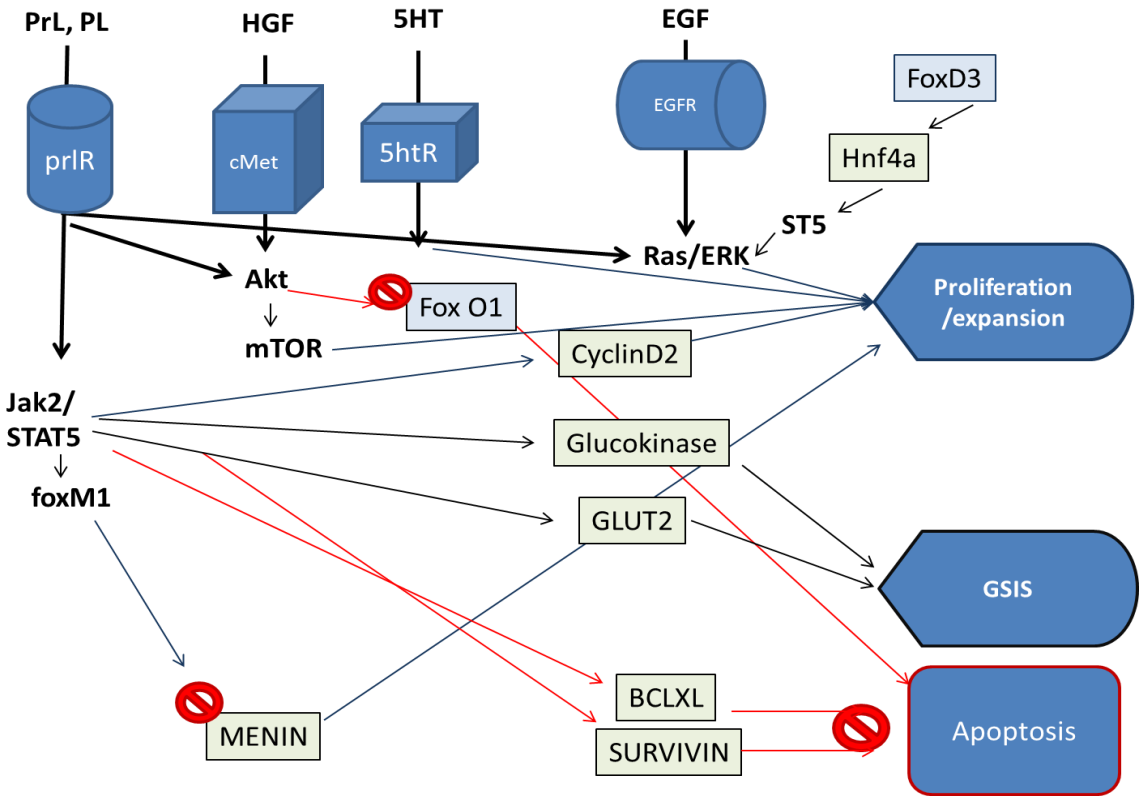
While the mechanisms continue to be investigated, it is believed that β-cell populations are managed in adulthood by control of apoptosis and proliferation (Finegood, Scaglia, & Bonner-Weir, 1995). Evidence supports the theory that β-cell populations fluctuate in order to accommodate changing metabolic demands throughout adult life (Schraenen et al., 2010; Tschen, Dhawan, Gurlo, & Bhushan, 2009), even though neogenesis of β-cells dramatically slows after the neonatal period (S. Bonner-Weir, 2000; Susan Bonner-Weir et al., 2000). As the metabolic demands increase, the hormonal changes that accompany pregnancy also act at the endocrine pancreas to promote compensatory adaptations. The goal of these expansions is to improve β-cell survival and function, and ultimately maintain glucose homeostasis even in the presence of increasing insulin resistance in late gestation (Figure 4).

Pregnancy-induced β-cell expansion is achieved by a combination of mechanisms, such as proliferation, hypertrophic expansion, and possibly neogenesis from progenitor cells; these mechanisms are accompanied by a temporary reduction in apoptosis (Susan Bonner-Weir et al., 2012; A. E. Butler et al., 2010; Ernst, Demirci, Valle,

Velazquez-Garcia, & Garcia-Ocaña, 2011). These adaptations have also been observed in islets during human pregnancy (A. E. Butler et al., 2010). These changes may be mediated by crosstalk between increased placental signalling, the maternal pancreas and peripheral tissues, which have been illustrated by the use of animal models (Aye, Powell, & Jansson, 2013; Rawn et al., 2015; R. C. Vasavada et al., 2000). While some adaptive mechanisms are conserved between species, there are variations in the primary adaptive responses between rodent and human islets in pregnancy. For instance, in rodent models of pregnancy neogenesis may only contribute a small amount to β-cell mass, with proliferation playing a more significant role (Sebastian Rieck & Kaestner, 2010). In human pregnancy, neogenesis may exceed proliferation as an adaptive mechanism (Susan Bonner-Weir et al., 2012; Søstrup, Gaarn, Nalla, Billestrup, & Nielsen, 2014). These mechanistic differences must be kept in mind, especially when attempting to translate research from rodent models to human populations. However, human islets isolated during pregnancy are scarce, and samples that have been analyzed are heterogenous in origin (sourced from pregnancies of varying gestational ages, maternal ages, ethnicities, and causes of death) presenting additional challenges of interpretation (A. E. Butler et al., 2010).

Increases in β-cell mass by replication and expansion, and improvements to β-cell function during gestation may also be mediated in part by serotonin signalling (S. Rieck et al., 2009) (Figure 6). Placental lactogens have been postulated to promote expression of survivin, a regulator of apoptosis (X Wu et al., 2009), which down regulates β-cell apoptosis and helps maintain β-cell mass during pregnancy (Y. Xu et al., 2015). This relative plasticity of β-cell function during pregnancy is crucial to maintain glucose control and support changing maternal and fetal energetic needs. Many mechanisms of β-cell adaptation have been identified in pregnancy (as reviewed by Ernst *et al.* and Baeyens *et al*. (Baeyens et al., 2016; Ernst et al., 2011)).What is less clear, however are the reasons for β-cell dysfunction in pregnancy. As mentioned previously, variability in results from human islets isolated during pregnancy is due to differences in gestational age, maternal BMI and cause of death. In addition, these studies do not specifically examine samples from mothers with gestational diabetes mellitus (GDM). The availability of this analysis could provide mechanistic insight into compensatory failure by the β-cell associated with GDM.

Figure 6: Molecular mechanisms of β-cell adapations in pregnancy



Hormones of pregnancy such as prolactin (PrL) and placental lactogen (PL) signal through the prolactin receptor (PrlR) and through the Jak/Stat pathway. This results in improved glucose stimulated insulin secretion (GSIS), prevents apoptosis and can promote expansion and proliferation. Hepatocyte growth factor (HGF) signals downstream through AKT and mTOR via the receptor cMet, which results in improved β-cell compensatory adaptations. Epidermal growth factor (EGF) binds to epidermal growth factor receptor (EGF-R) and downstream ras/ERK signalling can mediate β-cell proliferation and expansion. The ras/ERK pathway can also be triggered by signalling from lactogens. FoxD3, Hnf4α and FoxM1 are examples of transcription factors that can modulate expression of genes that regulate these adaptations. Serotonin (5HT) signalling can affect proliferation and expansion, and may regulate its own expression in the β-cell (Moyce & Dolinsky, 2018).

Micro-RNAs (miRNAs) add an additional level of genetic regulation, which control gene expression and could affect adaptive mechanisms in pregnancy. miRNA375 has been shown to increase β-cell expansion (Poy et al., 2009). miRNA7a increases mammalian target of rapamycin (mTOR) and its downstream signals (You Wang, Liu, Liu, Naji, & Stoffers, 2013) (Figure 6). Other miRNAs can activate neurogenin-3, resulting in increased neogenesis (Plaisance, Waeber, Regazzi, & Abderrahmani, 2014), and the downregulation of miRNA338-3p (Jacovetti et al., 2012) is thought to potentiate incretin signalling, in particular glucagon-like peptide (GLP)-1, which has a stimulatory effect on insulin secretion and β-cell proliferation, and may improve insulin sensitivity of peripheral tissues (Lencioni et al., 2011).

Hormones associated with pregnancy such as placental lactogen (PL) and prolactin (PrL) can signal through the prolactin receptor (PrlR) and trigger downstream signalling through the Jak/STAT pathway to improve GSIS, inhibit apoptosis, and promote increases in β-cell mass via proliferation and expansion (Demirci et al., 2012; Lombardo et al., 2011). Additionally, hepatocyte growth factor (HGF) can signal through AKT and mTOR via cMet receptor, resulting in improved β-cell compensations in pregnancy (Johansson, Mattsson, Andersson, Jansson, & Carlsson, 2006) (Figure 6). Epidermal growth factor (EGF), when it binds to epidermal growth factor receptor (EGF-R) can activate the ras/extracellular signal-regulated kinases (ERK) cascade and mediate proliferation and expansion (Hakonen, Ustinov, Palgi, Miettinen, & Otonkoski, 2014). The ras/ERK pathway may also be activated by lactogens PrL and PL (R. K. Gupta et al., 2007). Transcription factors such as forkhead box proteins D3 or M1, or hepatocyte nuclear factor 4α, may regulate gene expression of targets that impact β-cell adaptations, and dysregulation of processes can contribute to the development of GDM (Baeyens et al., 2016; Jacovetti et al., 2012; Sebastian Rieck & Kaestner, 2010; S. Rieck et al., 2009).

1.2.3 Adaptations by peripheral tissues during pregnancy

During the first two trimesters, pregnancy is marked by anabolic adaptations. This anabolic phase involves energy storage in the form of lipid accumulation in maternal tissues, hyperphagia and weight gain (Knopp, Herrera, & Freinkel, 1970; López-Luna, Muñoz, & Herrera, 1986). Glucose is used primarily for maternal energy during this period, as the energetic demands from the fetus are relatively low and insulin sensitivity is similar to preconception (reviewed in (Lain & Catalano, 2007; Zeng et al., 2017)). At this stage when there are increases in blood volume (Angueira et al., 2015) and glucose utilization, fasting blood glucose is maintained by increases in insulin secretion (A. E. Butler et al., 2010). In the third trimester, glucose is spared for fetal growth and lipid stores are utilized for maternal energy; this metabolic switch is facilitated by increased maternal insulin resistance (Knopp et al., 1970).

1.2.4 White adipose tissue in pregnancy

Maternal white adipose tissue (WAT) undergoes expansion during early pregnancy, in order to store energy that will be required during later stages of pregnancy. There is an increase in *de novo* lipogenesis due to increased insulin sensitivity and low levels of adipose tissue lipolysis (Coltart & Williams, 1976; Zeng et al., 2017). Due to the higher sensitivity to insulin during the earlier stages of pregnancy, the phenotype of adipose tissue is lipogenic and tends towards expansion. Since maternal metabolism still relies on glucose during this stage, insulin increases glucose

transport from serum as well as increasing glycolysis. During this phase of energy storage, lipolysis is downregulated and as a result circulating free fatty acids are lower during early gestation (Pujol, Proenza, Llado, & Roca, 2005).

Adipose tissue expansion is important, especially during early gestation, and there is evidence to suggest that cross talk with the placenta in pregnancy may promote adipose tissue expansion (Jayabalan et al., 2017). Pregnancy-associated plasma protein A (PAPP-A) can increase levels of insulin-like growth factor (IGF) in adipose tissue, promoting expansion and proliferation required to allow energy storage and prevent excess ectopic lipid deposition elsewhere in the body (Rojas-Rodriguez et al., 2015). Expansion of adipose tissue can be achieved through hypertrophic adipocytes (to accommodate larger lipid droplets) or hyperplasia, which is production of new adipocytes from precursors (Rojas-Rodriguez et al., 2015). However, all forms of adipose tissue expansion are not equal – hyperplasia is linked to better glucose control whereas hypertrophy is associated with more severe insulin resistance and is more prone to inflammation (Hoffstedt et al., 2010).

As pregnancy progresses, the energetic demands of the fetus require that maternal metabolism spares glucose for fetal growth, and energy stores accumulated during the anabolic period are broken down during this catabolic phase (Knopp et al., 1970; Palacín, Lasunción, Asunción, & Herrera, 1991; Pujol et al., 2005). When peripheral tissues become more insulin resistant, whole body lipid mobilization and lipolysis in white adipose tissue is increased. Experimental evidence shows steady increases in circulating free fatty acid (FFA) and glycerol, as well as decreases in adipocyte size and WAT mass towards the end of gestation (Knopp et al., 1970). Adipose tissue triglycerides are broken down by lipases in three stages, ultimately releasing free fatty acids and glycerol. Adipose triglyceride lipase (ATGL) converts triglyceride to diglyceride (DG) and free fatty acid. DG is broken down by hormone sensitive lipase (HSL) to monoglyceride (MG) and free fatty acid for terminal breakdown to glycerol and free fatty acid by monoglyceride lipase (MGL) (Langin, 2006). At each step of catabolism FFA is released, accounting for increased circulating lipids in late gestation. During this phase of pregnancy, maternal metabolism relies more heavily on fatty acid oxidation for energetic needs, so the increased availability of lipids is an important physiological adaptation to late gestation. Indeed, there is some evidence that the hormonal changes associated with pregnancy including circulating human placental lactogen (HPL) and increased tumor necrosis factor-α (TNF-α) might increase insulin resistance and inhibit anti-lipolytic effects (J. P. Kirwan et al., 2002).

1.3 Gestational Diabetes Mellitus

While it is well established that insulin resistance builds throughout pregnancy, this process is physiological and adaptive, and compensation by the endocrine pancreas and peripheral tissues work together to maintain glucose homeostasis until parturition. When compensatory mechanisms are insufficient to overcome this insulin resistance, such as in the case of pre-existing maternal obesity or excessive gestational weight gain, the resulting hyperglycemia and metabolic stress can develop into GDM (Plows, Stanley, Baker, Reynolds, & Vickers, 2018).

GDM is defined as the new onset of hyperglycemia and glucose intolerance mid-gestation. Worldwide, as many as 1 in 7 pregnancies are affected by GDM (Guariguata, Linnenkamp, Beagley, Whiting, & Cho, 2014). Health Canada statistics from 2011 place the rate of GDM at approximately 5.4% and climbing, as more women enter their pregnancies being either obese or overweight. Like many Western nations, Canadian women are becoming pregnant

later in life, which can increase the risk for GDM development (J. Brown et al., 2017; Hui, Sevenhuysen, Harvey, & Salamon, 2014). The implications of these trends may be far-reaching. Some of the risks associated with GDM include difficulties with pregnancy (such as pre-eclampsia), complications in labor and delivery, and increased risk for maternal health complications postnatally (Buchanan, 2001; Guariguata et al., 2014; Sherifali et al., 2018). Women who have had GDM are at higher risk for developing GDM with subsequent pregnancies and are at increased risk of developing T2D later in life (Herath, Herath, & Wickremasinghe, 2017). Further, fetal exposure to GDM may be associated with increased risk for respiratory distress syndrome, pre-term birth, and increased incidence of cardiometabolic dysfunction in adulthood (P. Agarwal et al., 2018; C. Kim, 2010).

1.3.1 β-Cell Dysfunction in GDM

During pregnancy, the β-cell must implement structural and functional changes to overcome insulin resistance and maintain normoglycemia. However, in a subset of women, functional and structural adaptations are impaired, contributing to GDM development. While the specific causes of impaired β-cell compensation in GDM continue to be investigated, this adaptive failure is likely multifactorial including underlying genetic defects, nutrient overload and metabolic stress, or increased inflammation. Pancreatic β-cells have been shown to be affected by systemic inflammation (L. Chen et al., 2015). Obese individuals, and women with GDM have been found to have higher levels of circulating tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine which is linked to disrupted β-cell function and de-differentiation (Nordmann et al., 2017; Y. Yang et al., 2018). Other markers of inflammation like interleukin-1β (IL-1β) and interferon-γ (IFNγ) are reportedly increased under conditions of metabolic stress (Boyle, Newsom, Janssen, Lappas, & Friedman, 2013; Liong & Lappas, 2016), and can trigger endoplasmic reticulum (ER) stress in the β-cell, contributing to β-cell dysfunction (Ehses et al., 2007) and prevention of adaptation to insulin resistance during pregnancy.

The pancreatic β-cell is also susceptible to the effects of obesity and nutrient overload. Lipotoxicity can result from continued exposure to high levels of lipids and can lead to β-cell dysfunction (Roger H. Unger, 1995). In addition, prolonged exposure to hyperglycemia such as in GDM and T2D can lead to impairments in β-cell function, and reduced insulin secretion that may be irreversible (Moran et al., 1997). ER stress (Kitamura et al., 2005) and oxidative stress (Sharma & Alonso, 2014) are triggered when lipids build up within the pancreatic islet and can lead to impaired insulin production. Increased oxidative and ER stress can contribute to cell damage and β-cell apoptosis (Sharma & Alonso, 2014). Lipotoxicity as well as glucotoxicity are potential mechanisms involved in β-cell dysfunction in T2D and GDM (Sharma & Alonso, 2014).

In addition to nutrient overload and inflammation, impaired lipid metabolism has been shown to contribute to beta cell dysfunction in GDM. Metabolomic analyses of women with GDM who subsequently developed T2D revealed the accumulation of a furan fatty acid metabolite, 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid (CMPF) in their blood. Mechanistic studies in cells and rodents have demonstrated that CMPF triggers oxidative stress and directly impairs beta cell mitochondrial function (Prentice et al., 2014), including upregulation of uncoupling protein 2 (UCP2). In previous studies, upregulation of UCP2 has been shown to occur in the obese and T2D states, which negatively impacts insulin secretion (C. Y. Zhang et al., 2001). If increased systemic inflammation (for example due

to obesity) and nutrient-mediated toxicity continually overload the β-cell, the net result is often increased β-cell apoptosis (Bensellam, Laybutt, & Jonas, 2012) and compensatory failure.

1.3.2 Impact of GDM on peripheral tissues

Through the lens of required metabolic changes associated with gestation, GDM might be considered a failure of compensatory mechanisms in the face of increased peripheral insulin resistance of pregnancy. The added metabolic stress of maternal obesity prior to pregnancy or excessive gestational weight gain can increase this insulin resistance, and insulin secretion is insufficient to maintain glucose homeostasis. The end result is hyperglycemia and impaired glucose tolerance that characterize GDM (Boyle et al., 2014). When peripheral tissues become more insulin resistant in GDM, mitochondrial dysfunction, increased oxidative stress and ER stress have also been observed (Boyle et al., 2013; Liong & Lappas, 2016). Pregnancy has a unique inflammatory profile, but disruptions to cytokine levels or macrophage infiltration of insulin responsive tissues (for instance, white adipose tissue) can trigger inflammation, worsen insulin resistance and contribute to GDM development (Boyle et al., 2014; Y. Yang et al., 2018).

1.3.3 Impact of GDM on the liver

Hepatic insulin resistance in GDM can also contribute to more severe hyperglycemia during pregnancy, and lack of suppression of hepatic gluconeogenesis can result in ongoing endogenous glucose production (Butte, 2000). In addition, the physiological function of insulin resistance in pregnancy is to allow for increased fatty acid oxidation to support maternal metabolism. However, exposure to hyperlipidemia as a result of excessive lipolysis can overload oxidative capacity, leading to ectopic fat deposition in the liver. The resultant lipotoxic and oxidative stress can exacerbate hepatic insulin resistance (S. S. Choi & Diehl, 2008). There is a strong link between the excessive storage of lipids in the liver, hepatic steatosis (or fatty liver), and GDM. Women with a history GDM are at higher risk for development of hepatic steatosis later in life (Lavrentaki et al., 2019). The development of hepatic steatosis can cause insulin resistance, and in fact it has been shown that development of fatty liver early in gestation (first trimester) precedes hyperglycemia in GDM (S. M. Lee et al., 2019).

1.3.4 Impact of GDM on Adipose Tissue

In white adipose tissue, the severe insulin resistance that characterizes GDM can result in excessive lipolysis, low grade inflammation and dysregulated adipokine signalling (Tumurbaatar et al., 2017). Insulin positively regulates adiponectin synthesis and release *in vitro,* and inflammatory cytokines such as TNF-α (which is increased in circulation during gestation) opposes the stimulatory effects of insulin on adiponectin secretion (Hajri, Tao, Wattacheril, Marks-Shulman, & Abumrad, 2011). While some insulin resistance is a physiological adaptation in late gestation, there are significant impairments in downstream insulin signalling in white adipose tissue observed in GDM – particularly decreased insulin receptor-β (IR-β) phosphorylation, downregulation of IRS1, and signalling through PI3K (Sevillano, de Castro, Bocos, Herrera, & Ramos, 2007). Glucose uptake by adipose tissue is also blunted, with defects in GLUT4 translocation observed in GDM (Lain & Catalano, 2007).

Insulin resistance in adipose tissue also impacts lipid metabolism, and impaired post-receptor signalling has been shown to result in dramatically elevated lipolysis in women with GDM (Tumurbaatar et al., 2017). Adipose tissue expansion is important to accommodate increased lipogenesis in early pregnancy. However, impairments in this mechanism have been implicated in GDM (Rojas-Rodriguez et al., 2015). In GDM, adipocytes become larger but show limited vascularization and reduced expression of markers associated with expandability. This increases the risk for adipose tissue inflammation, more severe insulin resistance and ectopic fat deposition in peripheral tissues (Rojas-Rodriguez et al., 2015) (Figure 4).

1.4 Adipokines

Besides energy storage, white adipose tissue functions as an endocrine organ, secreting factors into circulation that help regulate metabolism. Adipokines such as adiponectin and leptin have been associated with physiological adaptations to pregnancy, and dysregulated adipokines can lead to metabolic dysfunction (Conde et al., 2011). Leptin is secreted by adipose tissue and regulates energy expenditure, energy intake (appetite), and metabolic functions like insulin secretion and glucose transport (Trayhurn, Hoggard, Mercer, & Rayner, 1999). Adiponectin is an adipokine that is secreted at levels inversely proportional to the level of an organism's adiposity (Soma-Pillay et al., 2016). Though it is secreted at most appreciable levels from adipose tissues it can also be produced by myocytes, endothelial cells (Achari & Jain, 2017) and under caloric restriction, in bone-marrow adipose tissue (Scheller, Burr, MacDougald, & Cawthorn, 2016).

1.4.1 Adiponectin

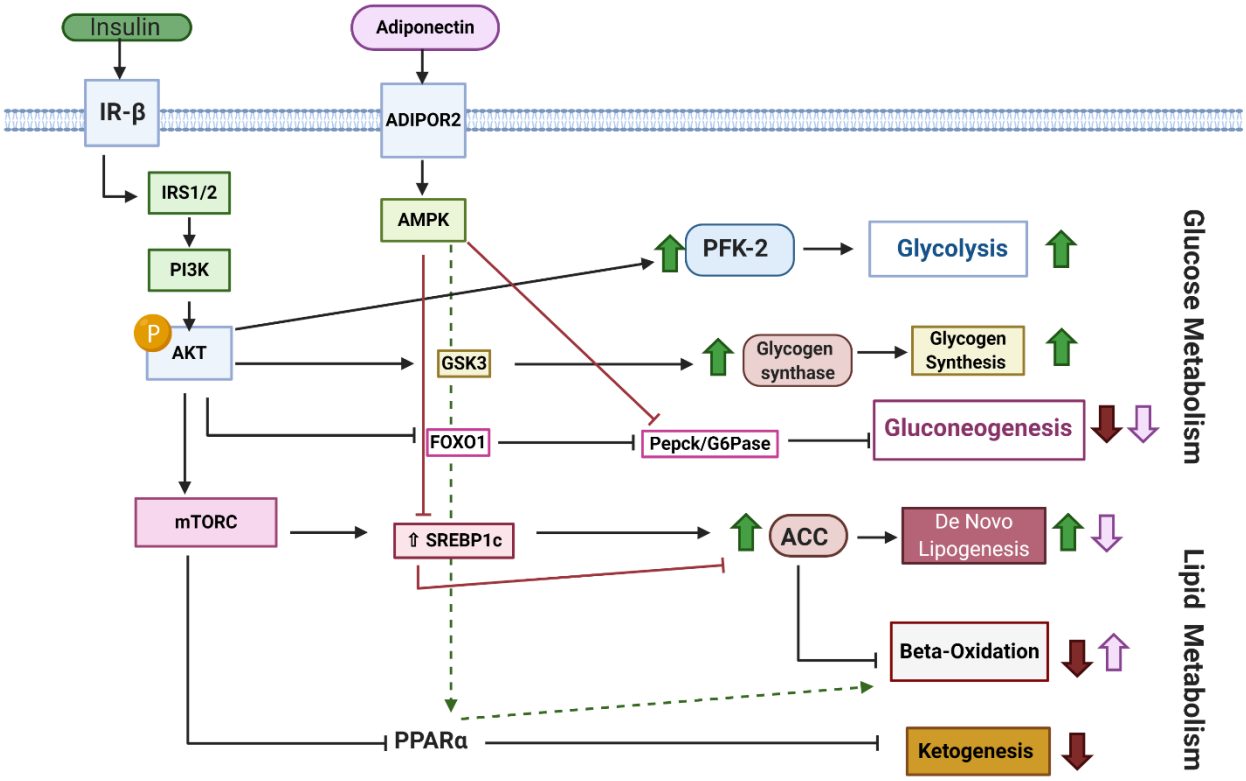
Adiponectin is the 30kD product of the *AdipoQ* gene. The adiponectin protein has an N-terminal signal domain, a variable region, collagenous domain and a globular C-terminal domain (A. H. Berg, Combs, & Scherer, 2002; Stern, Rutkowski, & Scherer, 2016). Adiponectin exists in the circulation either in a shorter globular form (gAPN), a full-length form (fAPN), or in multimeric forms of full-length adiponectin – a low molecular weight (LMW) trimer, a medium molecular weight (MMW) hexamer or a high molecular weight (HMW) oligomer (Aye, Rosario, Powell, & Jansson, 2015). There are two adiponectin receptors, ADIPOR1 and ADIPOR2. ADIPOR1 is expressed in skeletal muscle and preferentially binds gAPN over fAPN (Toshimasa Yamauchi et al., 2003). AdipoR2 is expressed in the liver (Toshimasa Yamauchi et al., 2003) and the placental trophoblast and binds both gAPN and fAPN (H. N. Jones, Jansson, & Powell, 2010). Notably, the circulating forms of adiponectin and their relative abundance have differing metabolic activities, and the ratios of HMW to total adiponectin may be a biomarker for metabolic dysfunction, wherein lower levels of HMW adiponectin may relate to higher disease risk (Aye et al., 2015).

1.4.2 Metabolic function of adiponectin

It has been postulated that the function of adiponectin is to sensitize tissues to the actions of insulin (A. H. Berg et al., 2002; Ruan & Dong, 2016; C. Wang et al., 2007). In skeletal muscle, adiponectin can stimulate glucose utilization through 5' adenosine monophosphate-activated protein kinase (AMPK) activation, stimulate glucose uptake by increasing GLUT4 translocation, and reduce lipid deposition by increasing fatty acid oxidation (via inhibitory phosphorylation of ACC downstream of AMPK) (T. Yamauchi et al., 2002) (Figure 7). In the adipose

tissue, adiponectin has been known to regulate 'healthy' adipocyte expansion and promote an anti-inflammatory phenotype (T. Yamauchi et al., 2001; Yanai & Yoshida, 2019). Additionally, adiponectin directly improves insulin sensitivity in rat primary adipocytes through AMPK signalling, partly by reducing the inhibitory effects of TNF-α on insulin signalling and increasing glucose uptake (Xiangdong Wu et al., 2003). Since impairments to PI3K mediated signalling are implicated in insulin resistance, it is interesting to note that adiponectin can also improve IRS-1 signalling downstream of the insulin receptor in one study using C2C12 myotubes (C. Wang et al., 2007).

Figure 7: Impact of adiponectin and insulin signalling on hepatic metabolism



Insulin increases glycolysis by increasing phosphofructokinase-2 (PFK2) and glycogen synthesis through GSK3 and glycogen synthase increases, mediated by AKT signalling. Insulin also suppresses gluconeogenesis by reducing gene expression of key genes (*pepck, g6pase*) through FoxO1. Insulin suppresses ketogenesis and β-oxidation through mTORC signalling. Adiponectin signals through ADIPOR2 and AMPK and suppresses genes responsible for hepatic gluconeogenesis, and in contrast to insulin, suppresses lipogenesis through srebp1c and increases β-oxidation.

Adiponectin signalling also has a role in the maintenance of β-cell mass and potentially function (Liping Qiao et al., 2017; Rakatzi, Mueller, Ritzeler, Tennagels, & Eckel, 2004; Retnakaran, 2017; Staiger et al., 2005; Wijesekara et al., 2010). Adiponectin supplementation has been shown to prevent caspase-8 mediated β-cell apoptosis in mice (Holland et al., 2011) and protect against glucotoxicity and lipotoxicity in a mouse model of diet-induced obesity (Ye, Wang, Wang, & Scherer, 2015) both of which are implicated in β-cell failure associated with GDM.

Whether or not adiponectin acts to improve glucose-stimulated insulin secretion is contested in the literature (Okamoto et al., 2008; Staiger et al., 2005).

Discrepancies in model systems (human, rodent or cell line) may partly explain the lack of consensus, as there is reason to believe adiponectin may be more important in β-cell maintenance and function under periods of stress. For instance, Okamoto and colleagues (Okamoto et al., 2008) showed increases in adiponectin-mediated insulin secretion under low-glucose conditions. In contrast to peripheral tissues where AMPK activation is implicated in mediating the effects of insulin, Wijesekara *et al* suggest that AKT signaling mediates the downstream effects of adiponectin signalling in the β-cell, independent of AMPK (Wijesekara et al., 2010).

1.4.3 Effect of adiponectin on hepatic metabolism

Adiponectin potentiates some effects of insulin in the liver but does not necessarily "mimic" them. While adiponectin augments insulin's role in suppression of gluconeogenesis (Miller et al., 2011; S. Park, Kim, Kwon, & Yang, 2011) adiponectin opposes the action of insulin in the context of lipid metabolism. Insulin promotes lipogenesis, and adiponectin has been shown to decrease lipogenesis through an AMPK mediated blockade of SREBP1c, inhibiting lipogenic gene transcription (Awazawa et al., 2011). This suppression is accompanied by increased fatty acid oxidation, mediated by upregulating inhibitory phosphorylation of ACC by AMPK which not only prevents *de novo* fatty acid synthesis but also prevents inhibition of CPT-1 and allows uptake and oxidation of fatty acids by the hepatic mitochondria (Stern et al., 2016). Adiponectin reduces endogenous glucose production through inhibition of gluconeogenic enzymes (PEPCK and G6Pase) (T. P. Combs, Berg, Obici, Scherer, & Rossetti, 2001; Miller et al., 2011) (T. Yamauchi et al., 2002) which helps to reduce blood glucose, thereby reducing the amount of insulin required by peripheral tissues (T. P. Combs & Marliss, 2014). This effect in combination with reductions in intracellular lipid content can help improve hepatic insulin sensitivity and solidify the role of adiponectin as an insulin sensitizer.

1.5 Adiponectin in pregnancy

Levels of adiponectin decrease progressively throughout pregnancy in parallel with increasing insulin resistance and gestational weight gain (Jara, Dreher, Porter, & Christian, 2020; Retnakaran et al., 2006). In healthy individuals, adiponectin is abundant in early pregnancy and, despite gestational weight gain and the steady decline in insulin sensitivity, decreases in adiponectin levels are modest (Catalano et al., 2006; Mazaki-Tovi et al., 2008). Conversely, low levels of adiponectin in all stages of gestation are associated with a higher risk of metabolic dysfunction in pregnancy, increased incidence of GDM, and increased risk of adverse outcomes for mother and baby (Bao et al., 2015; Hedderson et al., 2013; M. Lacroix et al., 2013; Retnakaran et al., 2010). In fact, low levels of adiponectin early in pregnancy (Bao et al., 2015) (M. Lacroix et al., 2013) or prior to pregnancy (Hedderson et al., 2013) are predictive of GDM development. In terms of pathophysiology, low circulating adiponectin levels are associated with impaired β-cell function (Retnakaran, 2017) and supplementation with adiponectin in mice can increase β-cell mass and circulating insulin during pregnancy (L. Qiao et al., 2017). Moreover, Qiao *et. al.* found

impairments in maternal β-cell adaptations to pregnancy in adiponectin-deficient mice, showing smaller islets and insufficient insulin secretion compared to controls (Liping Qiao et al., 2017). These studies suggest that adiponectin plays a key role in the adaptive metabolic response to pregnancy.

1.5.1 Placental Adiponectin

Adiponectin in maternal circulation does not cross the placenta, however adiponectin receptors are present on the placenta and placental signalling by adiponectin impacts nutrient transport and fetal growth (Aye et al., 2013; T. Jansson, Aye, & Goberdhan, 2012). While there are conflicting reports about whether the placenta expresses and secretes adiponectin (Caminos et al., 2005; Lappas, Yee, Permezel, & Rice, 2005). It is more likely that adiponectin exists compartmentalized in the maternal and fetal systems and signals through the placenta via adipoR2 receptors on the trophoblast (Aye et al., 2013; McDonald & Wolfe, 2009) as more sensitive techniques have determined that the placenta does not produce endogenous adiponectin (Haugen et al., 2006; Mazaki-Tovi et al., 2007; Pinar et al., 2008). Adiponectin signalling in maternal tissues appears to differ greatly from signalling in the placenta. *In vitro* studies show that both full length and globular adiponectin may suppress placental insulin signalling by reducing phosphorylation of IRS-1, AMPK and AKT through activation of PPAR-α (H. N. Jones et al., 2010). A consequence of reducing insulin sensitivity at the level of the placenta is a reduction in amino acid transport, which has implications for fetal growth (Aye et al., 2013; H. N. Jones et al., 2010).

1.5.2 Adiponectin in the fetus

As maternal adiponectin decreases throughout gestation, fetal adiponectin increases, promoting fat deposition and fetal growth (L. Qiao et al., 2012). In rodent models, administration of full-length adiponectin increased litter size and decreased neonatal size (Rosario et al., 2012). This suggests that maternal adiponectin regulates fetal growth potentially by down-regulating placental amino acid transport and insulin/mTOR signalling as mentioned above (T. Jansson et al., 2012; Rosario et al., 2012). Recent studies have clarified the fetal function of adiponectin. In contrast to maternal adiponectin, fetal adiponectin when upregulated, serves to promote fetal growth and adipose deposits, possibly even promoting fetal insulin resistance (R. C. Vasavada et al., 2000); fetal adiponectin and maternal blood glucose levels are inversely correlated in the second trimester (A. E. Butler et al., 2010; Sebastian Rieck & Kaestner, 2010). In studies performed in knockout mice, fetal fat deposition was dramatically impaired in fetuses from adiponectin-null dams, and this was rescued in heterozygous mice (L. Qiao et al., 2012). There is an inverse relationship between maternal adiponectin and fetal growth, with low circulating adiponectin associated with large for gestational age infants (Lekva et al., 2017). Studies in animal models show that supplementation with adiponectin can reduce fetal growth by impacting placental amino acid transport (Rosario et al., 2012), but also potentially by increasing IGFBP-1 which is reduced in adiponectin deficient offspring and is inversely correlated to birthweight (Liping Qiao et al., 2016).

In rodent studies, fetal adiponectin increased circulating free fatty acids and hepatic lipogenic genes (L. Qiao et al., 2012), further solidifying the contrasting activities of fetal and maternal adiponectin. While maternal obesity increases adiposity in the offspring, the expression of adiponectin is also increased in the adipose tissues of the offspring (Bayol, Simbi, Bertrand, & Stickland, 2008), suggesting that the normal regulation of adiponectin levels is

altered by the maternal environment. This presumption is strengthened by the observation that at birth, cord blood adiponectin is as much as 7 times higher than maternal levels, but decreases significantly within the first year of life (Kotani et al., 2004).

1.5.3 Adiponectin in GDM

As stated previously, due to its many roles in peripheral insulin sensitization and its potential for improving pancreatic β-cell expansion in pregnancy, adequate adiponectin in the circulation is protective against development of GDM. Since low circulating adiponectin are reported in women with GDM (Mohammadi & Paknahad, 2017; Retnakaran et al., 2006; Retnakaran et al., 2010) studies in rodent models have been able to show that supplementing pregnant mice with adiponectin reduced blood glucose and restored β-cell adaptability (L. Qiao et al., 2017). Given that hepatic steatosis is increasingly observed in women diagnosed with GDM (S. M. Lee et al., 2019), hypoadiponectinemia in pregnancy could also lead to hyperlipidemia and reduced β-oxidation of fatty acids and trigger a buildup of lipids in the liver. Without suppression of lipogenesis by adiponectin, *de novo* hepatic lipogenesis would continue in the absence of sufficient oxidation. This would lead to a greater risk of fatty liver development and exacerbation of insulin resistance.

Low circulating adiponectin would also lead to less insulin sensitization of skeletal muscle, increasing the insulin resistance that occurs in pregnancy to pathological levels. While there are known risk factors for the development of GDM such as obesity, family history, number of previous pregnancies and ethnicity, the effect of adiponectin deficiency can be isolated with the use of knockout rodent models to investigate molecular mechanisms. Low levels of adiponectin have been associated with fetal macrosomia in mice (L. Qiao et al., 2012) and clinically with large for gestational age infants (Lekva et al., 2017). From human placenta studies, cell lines and rodent models it appears that increased placental insulin sensitivity in the absence of adiponectin signalling leads to increased nutrient transport (in the form of amino acids (H. N. Jones et al., 2010; Lekva et al., 2017), glucose and potentially fatty acids (Aye et al., 2015). In a human birth cohort studying large for gestational age (LGA) infants, adiponectin was associated with LGA infants independent of gestational weight gain (Lekva et al., 2017).

Despite the body of evidence linking LGA infants and low maternal adiponectin, there are some studies that did not find an association (Ballesteros et al., 2011; Horosz, Bomba-Opon, Szymanska, & Wielgos, 2011; Jara et al., 2020). The mechanisms of fetal adiponectin in promoting fetal growth in the third trimester are not clear, hepatic lipid metabolism and overall fat storage are implicated (L. Qiao et al., 2012), but studies in monozygotic twins suggest a strong correlation for fetal adiponectin and fetal growth, as low adiponectin is linked to small for gestational age (SGA) infants (Gohlke et al., 2008). While studies in animal models are beginning to elucidate the mechanisms by which adiponectin mediates fetal growth, the way in which maternal adiponectin acts through placental signalling, and the action of fetal adiponectin have opposing effects on birth weight and fetal growth.

Current research has established the role of adiponectin in whole body energy homeostasis, including maintenance of a healthy pregnancy. Studies suggest that adequate levels of adiponectin in pregnancy may counter the effects of insulin resistance, preventing the development of hyperglycemia and GDM. While the mechanisms underlying the insulin-sensitizing properties of adiponectin have been outlined using cellular and animal models, it is

less clear whether low circulating adiponectin has a causative role in the development of GDM although studies involving supplementation suggest it is protective (Aye et al., 2015; Liping Qiao et al., 2017; Rosario et al., 2012). While studies in non-pregnant individuals show that obesity is negatively correlated with adiponectin levels, studies in pregnancy have conflicting conclusions on whether gestational weight gain is associated with cord blood adiponectin levels (Logan et al., 2017). Excessive gestational weight gain is a risk factor for development of GDM, and adipose tissue dysfunction can impact adipokine secretion. However, Retnakaran *et al*. showed that adiponectin may be more closely associated with GDM development and insulin resistance than adiposity or gestational weight gain (Retnakaran et al., 2004).

Additionally, the link between adiponectin and lipid metabolism specifically in the liver has been well established and research has drawn a connection between low adiponectin and the development of non-alcoholic fatty liver disease (NAFLD). It is well known that fatty liver development can worsen insulin resistance, and women with GDM are at higher risk for developing NAFLD later in life but it remains to be determined if the tipping point for GDM is the deposition of hepatic fat, particularly in the absence of sufficient circulating adiponectin. Evidence has shown that the liver enzyme γ-glutamyl transferase (GGT) is increased in women with GDM and is associated with both insulin resistance and intrahepatic lipids (Thamer et al., 2005; Zhu, Hedderson, Quesenberry, Feng, & Ferrara, 2018). Interestingly, increased GGT was also associated with decreased adiponectin in pregnancy. This data represents a potential link between GGT as a marker for oxidative stress and insulin resistance, intrahepatic lipids, and low adiponectin in GDM (Zhu et al., 2018). The action of adiponectin in pregnancy has been solidified with the assistance of supplementation studies in animal models, including the use of agonists that increase downstream signalling pathways implicated in adiponectin action (Liping Qiao et al., 2016). There is no published evidence for supplementation in humans, or whether direct supplementation with adiponectin is a feasible therapeutic option for GDM.

1.6 Thesis Objectives and Hypotheses

Pregnancies affected by gestational diabetes are at increased risk of complications, including risk of postnatal T2D. Studies show that offspring exposed to hyperglycemia and the gestational diabetic milieu while *in utero* are at increased risk themselves for metabolic dysfunction later in life due to adverse developmental programming. Research spanning several decades has identified risk factors for the development of GDM, including obesity, ethnicity, maternal age and genetic or familial predisposition. More recently, the identification and characterization of adipokines, particularly adiponectin, have given insight to the mechanisms behind metabolic dysfunction during pregnancy. Therefore, the overall hypothesis was that maternal adiponectin deficiency induces GDM and increases fetal growth, and replenishing adiponectin in the maternal circulation mitigates GDM development.

Objectives

- To determine how adiponectin deficiency during pregnancy contributes to the development of hyperglycemia and impaired glucose tolerance.
- To determine how adiponectin deficiency affects hepatic lipid metabolism and mitochondrial function in pregnancy.
- To determine how adiponectin deficiency affects insulin secretion and compensatory β-cell expansion during pregnancy.
- To determine whether supplementation of adiponectin to pregnant dams in the third trimester of pregnancy improves hepatic steatosis and hyperglycemia.
- To investigate the interaction between low adiponectin levels and fetal growth in a human cohort of individuals with diabetes during pregnancy.

Chapter 2:

Materials and Methods

2.1 Animal model and Experimental Procedures:

All procedures performed were approved by the Animal Welfare Committee of the University of Manitoba, in adherence with the Canadian Council on Animal Care and the Council for International Organizations of Medical Sciences. Adiponectin knockout (B6;129-Adipoq^{tm1Chan}/J) and Wildtype (C57B/6) mice were received at minimum 3 weeks of age from a breeding colony established and maintained by the University of Manitoba Central Animal Care and randomly placed on either a low fat (LF; 20% Protein, 70% Carbohydrate and 10% Fat, 3.85kcal/g, Research Diets D12450B) control diet or high fat and sucrose (HFS; 20% Protein, 35% Carbohydrate, 45% Fat 4.73 kcal/g, Research Diets D12451) obeseogenic diet (Figure 8). Mice were fed *ad libitum* for a minimum of 6 weeks prior to breeding. Female adiponectin knockout (Adiponectin KO) and wildtype (WT) mice were bred with chow-fed males of the same genotype and pregnancy was detected by ultrasound using the Vevo 2100 high frequency ultrasound system (University of Manitoba Small Animal Imaging Core) and food consumption and bodyweight of female mice was monitored weekly throughout gestation. At the end of gestation (e18.5-19) mice were sacrificed following a one hour fast and administered either saline or insulin (1mU/kg bodyweight) 10 minutes prior to sacrifice by intraperitoneal (IP) injection. Euthanasia was initiated with IP injection of an overdose of sodium pentobarbital and terminated with blood collection via cardiac puncture. Dissected tissues were snap frozen in liquid nitrogen for storage at -80°C or fixed in 10% formalin or 4% paraformaldehyde for cryosection.

2.1.1 Glucose, Pyruvate and Insulin tolerance tests:

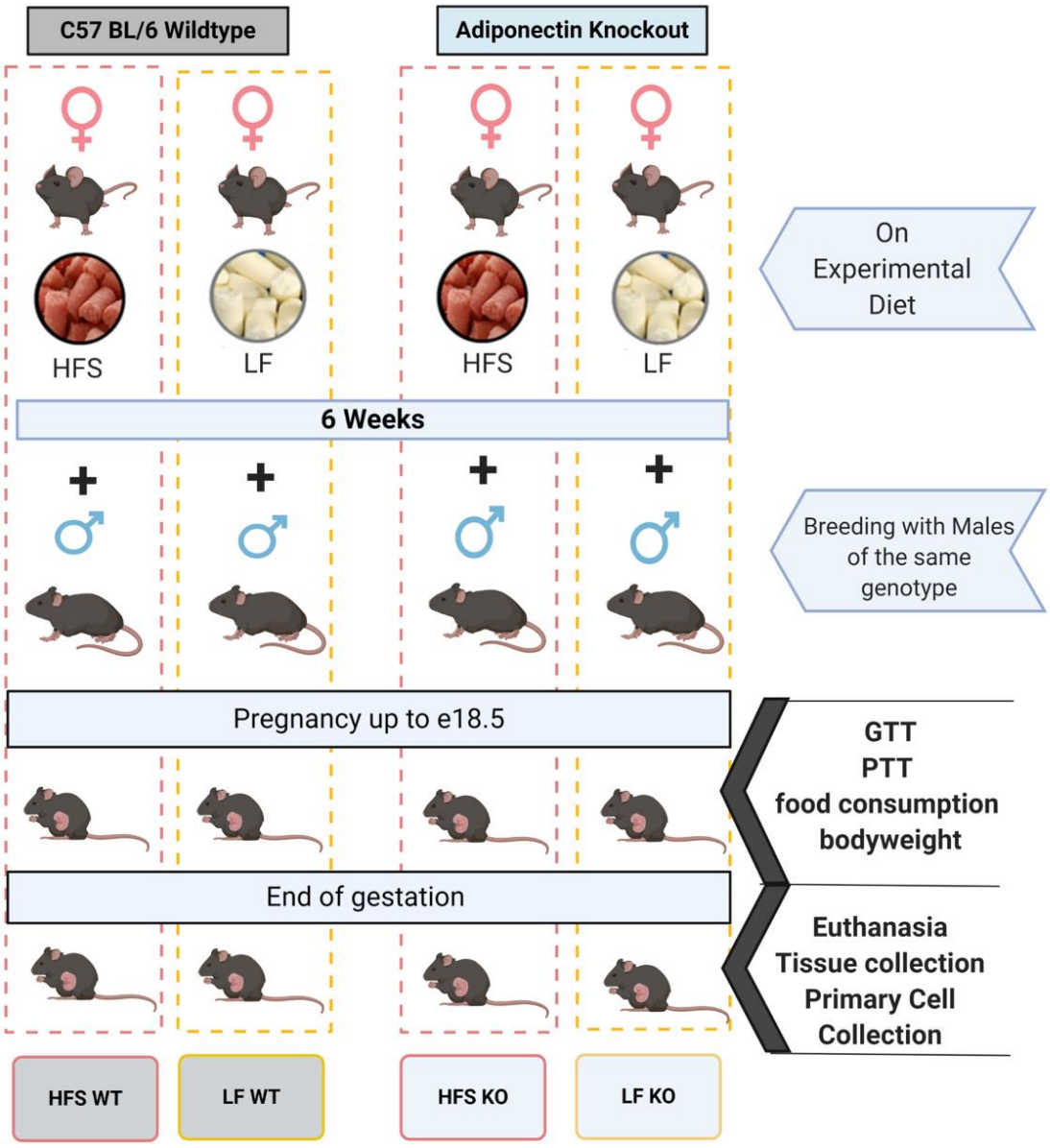
A combination of peripheral insulin resistance and dysregulated gluconeogenesis can contribute to hyperglycemia and impaired glucose tolerance. Glucose tolerance tests (GTTs) were performed on non-pregnant or third-trimester pregnant mice as previously described (Pereira et al., 2015). Non-pregnant mice were fasted overnight. However, for GTTs performed in the third trimester the period of fasting was shortened to 4 hours to prevent hypoglycemia that could become symptomatic (blood glucose below 3.0mmol/L).

A pyruvate tolerance test (PTT) measures the production of glucose in the liver from pyruvate as an indirect measure of gluconeogenesis, which is suppressed by insulin (Hers & Hue, 1983). Pyruvate tolerance tests (PTTs) were performed on non-pregnant and third-trimester pregnant mice by measuring fasting blood glucose at time 0, and then administering pyruvate intraperitoneally at a concentration 2g/kg bodyweight and subsequently measuring blood glucose using an ACCU-CHEK Advantage glucose meter (Roche Diagnostics) at 10, 20, 30, 60, 90- and 120-minutes post injection. Non-pregnant mice were fasted for 6 hours, however for pregnant mice in the third trimester the period of fasting was again shortened to 4 hours to preventing hypoglycemic episodes.

Insulin resistance can be assessed by administering exogenous insulin and monitoring the corresponding reduction in blood glucose over time. This is referred to as an insulin tolerance test (ITT). An ITT is a measure of peripheral tissue response to insulin, and the ability to increase glucose uptake and utilization in response to an insulin bolus. An insufficient reduction in blood glucose in response to insulin during an ITT is indicative of insulin resistance. Insulin tolerance tests (ITTs) were performed in the third trimester of pregnant mice. Insulin was administered by IP injection at a concentration of 1mU/kg bodyweight following an abbreviated fast of 2 hours due to observed hypoglycemic episodes in pregnant that necessitated administration of glucose. Additionally, recent studies have shown that fasting

for 2 hours prior to ITT may be optimal for assessment of insulin responsiveness while minimizing the metabolic stress and weight loss associated with longer fasts (Carper, Coué, Laurens, Langin, & Moro, 2020). Blood glucose was measured at time 0, prior to insulin injection, and subsequently at 15, 30, 45, 60, 90- and 120-minutes using an ACCU-CHEK advantage glucose meter (Roche Diagnostics, Basel, Switzerland). Experiments on third-trimester pregnant mice were performed between gestational days 15 and 18.

Figure 8: Experimental animal model



Female adiponectin KO and C57/BL6 wildtype mice were placed on either LF or HFS obeseogenic diets for 6 weeks prior to breeding. Females were mated with males of the same genotype and experiments were performed in the third trimester. Four experimental groups were generated: low-fat wild-type (LF WT), high-fat wildtype (HFS WT), low-fat adiponectin KO (LF KO), and high-fat sucrose adiponectin KO (HFS KO).

2.1.2 Viral supplementation:

To determine the effect of adiponectin supplementation in pregnancy, replication-deficient adenovirus containing full length adiponectin (Applied Biological Materials, Cat# 000523A) (Ad-APN, at 1x10^6 PFU/mL) or GFP (Applied Biological Materials, Cat# 000541A) (Ad-GFP, 1x10^11 PFU/mL) was administered to pregnant mice. Mice were anesthetized with isoflurane and administered 6uL of either Ad-APN or Ad-GFP in 250uL of sterile saline by tail vein injection (approximately 6x10^3 PFU Ad-APN per mouse). Following injection, mice were individually housed and isolated for 72 hours, after which they were moved to clean cages and experiments could be performed at e17.5. After supplementation with adiponectin or GFP, pregnant mice underwent GTTs, ITTs, and PTTs, approximately 3 days post-injection. Mice were sacrificed at the end of gestation (e18.5-19.5), approximately 6 days post-injection, and tissue and serum were collected for further analysis.

2.1.3 Serum collection:

Blood collected by cardiac puncture was stored on ice and serum was separated by centrifugation at 4000 RPM for 10 minutes at 4°C. Serum was stored at -80°C for further analysis.

2.1.4 Determination of circulating factors:

Serum collected at sacrifice was analyzed for total adiponectin concentration by enzyme linked immunosorbent assay (ELISA) from ALPCO (High Molecular Weight and Total Adiponectin ELISA, ALPCO, Salem, NH, USA) according to the manufacturer's instructions. Serum insulin levels were determined by ELISA (Mouse Ultra-Sensitive Insulin Elisa, ALPCO, Salem, NH, USA).

Adiponectin and leptin concentrations in maternal and cord blood serum were determined using human adiponectin and leptin ELISAs according to the manufacturer's instructions (Human Adiponectin ELISA and Human Leptin ELISA, ALPCO, Salem, NH, USA).

Concentrations of free fatty acids were determined using a colorimetric assay (Cayman Chemical, Ann Arbor, MI, USA). Serum triglycerides were measured using an assay from WAKO diagnostics (WAKO Diagnostics, Mountain View, CA, USA) and serum cholesterol and β-hydroxybutyrate were determined from assays acquired from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA).

2.2 Assessment of Hepatic Lipids and Metabolites

In mice without adenovirus supplementation, hepatic triglycerides were determined using an assay from BioVision (BioVision, CA, USA). In adenovirus supplemented pregnant mice, hepatic triglycerides were measured using an assay from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA). Levels of hepatic α-KG and oxaloacetate were measured using assays from Sigma Aldrich (Merck, Darmstadt, Germany) according to the manufacturer's instructions.

2.2.1 Liver and Adipose Histology:

Liver sections collected at the time of sacrifice (at the end of gestation), fixed in 10% formalin and sent for histological analysis. Samples fixed in formalin were sent to the University of Manitoba Histology Core Platform and Hematoxylin and Eosin (H&E) staining. Liver samples for Oil Red O staining were fixed in 4% PFA, exposed to a sucrose gradient of 10-20% and frozen in optimal cutting temperature compound (OCT) and stored at -80°C. These blocks were sent to the University of Manitoba Histology Core and Oil Red O staining were performed according to their standard procedures, with H&E staining applied as background staining (Mehlem, Hagberg, Muhl, Eriksson, & Falkevall, 2013). On H&E stained sections, images were taken at 1x, 5x, 10x and 20x magnification and a pathologist (Dr. Julianne Klein), blinded to the experimental groups, assessed the images and scored them for steatosis. Scoring takes into account percent steatosis, macro- or micro-vesicular steatosis, degree of diffusion, zonality of steatosis and hepatocellular swelling (Brown *et al* 2010). A score of 0 corresponds with less than 5% steatotic cells, a score of 1 is 5-30% steatosis, a score of 2 is 30-50% and greater than 50% steatosis is scored as 3.

Oil Red O stained sections were imaged on Zeiss LSM700 Spectral Confocal Microscope in the CHRIM microscopy platform. Images were collected at 20X magnification and degree of Oil Red O staining was determined using ImageJ software (NIH, USA). Gonadal white adipose tissue (GWAT) was collected at the end of the third trimester as described previously and stained with H&E. Images were taken at 40x magnification and adipocyte diameters were measured using ImageJ software (NIH, USA).

2.3 Pancreatic Immunofluorescence:

Immunofluorescence experiments were performed by Mr. Mario Fonseca, a laboratory technician in the Dolinsky Lab. At the time of sacrifice, the pancreas was dissected, excess fat was removed, and the tissue was fixed in 4% paraformaldehyde (PFA) for no more than 24 hours at 4°C. Following fixation in PFA, the tissue was treated with a sucrose gradient (10%, 15% and 20%), as sucrose infiltration imparts some cryoprotection to minimize freezing artifacts (Tokuyasu, 1973). Pancreases were then frozen in blocks of OCT compound on dry ice and stored at -80°C for cryo-sectioning.

Blocks were sectioned completely using a cryostat to 10um sections and mounted on slides. Approximately 1 in 10 slides were stained with cresyl violet to determine overall islet area and content per section. Slides were evaluated and representative slides were selected for immunofluorescence. Briefly, slides were blocked for 2 hours, and then incubated with primary antibody (insulin or glucagon) overnight at 4°C. Insulin antibody was applied at a 1:100 dilution, and glucagon at a 1:200 dilution, both sourced from rabbit (Cell Signalling Technology).

Following exposure to primary antibody, slides were washed with PBS + 0.05% Triton and incubated with secondary antibody for 1-2 hours at room temperature. Goat-anti-rabbit (GAR) secondary antibodies were fluorescent-conjugated to Alexa Fluor fluorescent probes for detection; for insulin detection we used GAR conjugated to Alexa Fluor 594, and for glucagon GAR conjugated to Alexa Fluor 488, which fluoresce red and green respectively (Thermofischer Scientific, Massachusetts, USA). Slides were washed again with PBS + Triton and mounted with slide covers and Vectasheild ® (VWR, Pennsylvania, USA) containing DAPI nuclei stain. Slides were stored at 4°C until

they were imaged using the Zeiss LSM 700 spectral confocal microscope, part of the CHRIM microscopy imaging platform. β-cell and α-cell positive area was estimated by determining immunopositive area from insulin and glucagon stained slides using ImageJ software (NIH, USA).

2.4 RNA Extraction:

Approximately 30mg of frozen liver tissue was crushed and mechanically homogenized in 600μL of Lysis buffer provided in the PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA, USA) and RNA was isolated using the PureLink RNA Mini Kit according to the manufacturer's instructions. RNA integrity was assessed by running the isolated RNA on 1% agarose gel and concentrations were determined using NanoDrop 2000 (Thermo Fisher Scientific, Rockford, IL, USA).

2.4.1 Gene expression – qPCR:

cDNA was synthesized from 1μg of RNA using the LUNA Superscript cDNA synthesis kit (New England Biolabs, Ipswtich, MA, USA). The Luna Universal qPCR Mastermix (New England Biolabs, Ipswitch, MA, USA) was used to monitor amplification of cDNA using the CFX Real Time PCR (BioRad, CA, USA). An initial denaturation step at 95°C for 60 seconds was followed by 40 cycles consisting of a 15 second denaturation at 95°C and a 30 second extension step at 60°C. Reference genes β-2 microglobulin (*B2m)*, 18s rRNA (*Rn18s)*, Hypoxanthine guanine phosphoribosyl transferase (*Hprt1)*, and 60S acidic ribosomal protein large P1 (*RPLP-1*) were combined and the geometric mean (geomean) was used for normalization of experimental gene expression. All samples were plated in duplicate and relative gene expression was determined using the 2^-ΔΔCT method, where gene expression data were normalized to the LF WT group. All primers used (Table 1) were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) and validated by running qPCR products on 1.5% agarose gel, and images were taken using the FluorChem® HD2 (Alpha Innotech, Germany).

Table 1: Primer Sequences for qPCR

Gene	Forward Primer (Sense) 5'—3'	Reverse Primer (Antisense) 5'—3'
Pck1	GGCCACAGCTGCTGCAG	GGTCGCATGGCAAAGGGG
Pck2	TGAACAGCCAACATGGTCCC	ATTCTCAGGGTTGATGGCCC
G6pase	GCATTTGCCAGGAAGAGAAAG	AACTGAAGCCGGTTAGACATAG
Pcx	ACCTACGGCTTCCCTATTATCT	CGGGTGTAATTCTCTTCCAACT
Srebp1c	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
Gck	CCTGTAAGGCACGAAGACATAG	CCACGATGTTGTTCCCTTCT
Pklr	CCAGCAGCATCAGTCGTATATC	ACCCAGGAGGAATCGAATTAAC
Foxo1	CGTGCCCTACTTCAAGGATAG	GCACTCGAATAAACTTGCTGTG
Hprt1	GGCCAGACTTTGTTGGATTTG	CGCTCATCTTAGGCTTTGTATTTG
B2m	GTTCGGCTTCCCATTCTCC	GGTCTTTCTGGTGCTTGTCTCA
Rn18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Rplp0	GATGGGCAACTGTACCTGACTG	CTGGGCTCCTCTTGGAATG
Dgat1	TGCTACGACGAGTTCTTGAG	CTCTGCCACAGCATTGAGAC
Acaca	CCAGCTTAAGGACAACACCT	TTGGAGGCAAAGGACATTCT
Bhb	CACTGTTCTAGCTCCTGTCTTC	CATCCCGCTGTCAGGTAAAT
Pdha1	AGAGAGGATGGGCTCAAGTA	CAAGTGACAGAAACCACGAATG
Fasn	GCGATGAAGAGCATGGTTTAG	GGCTCAAGGGTTCCATGTT
Cpt1a	TCGAAACCCAGTGCCTTAC	AAGCAGCACCCTCACATAC
AdipoQ	GCGTCACTGTTCCCACTGTA	CCCGGAATGTTGCAGTAGAA
AdipoR1	GAAGATGGAGGAGTTCGTGTATAA	AGCAGGTAGTCGTTGTCTTTC
AdipoR2	AGGACTCCAGAGCCAGATATAC	CTCTAAGCTGGGCTCCAAATC
Gpam	TCCTTCCTTCAAGACCGAATGA	CGCAGGACTTGCTGGCGGTG
Pgc1α	CTAGCCATGGATGGCCTATTT	GTCTCGACACGGAGAGTTAAAG

Validated primer sequences for qPCR performed on liver tissue isolated in the third trimester from WT and adiponectin KO mice. *Pck1*: phosphoenolpyruvate carboxykinase-1. *Pck2*: phosphoenolpyruvate carboxykinase-2, *G6pase*: glucose-6-phosphatase, *Pcx*: pyruvate carboxylase, *srebp1c:* sterol regulatory element binding protein-1c, *Gck*: glucokinase, *Pklr*: pyruvate kinase liver isoform, *Foxo1*: Forkhead box transcription factor 1, *hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *RPLP-1:* 60S acidic ribosomal protein large P1, *Dgat1*: diacyglycerol transferase-1, *Acaca*: acetyl-CoA carboxylase, , *Bdh1*: β-hydroxybutyrate dehydrogenase -1, *Pdha1*: pyruvate dehydrogenase-a1, *Fasn*: fatty acid synthase, *Cpt1a*: carnitine palmitoyl transferase-1a, *AdipoQ:* Adiponectin, C1Q and collagen domain containing, *AdipoR1:* Adiponectin receptor protein 1; *AdipoR2:* Adiponectin receptor protein 2, *Gpam:* glycerol-3-phosphate acyltransferase – mitochondrial, *Pgc1α:* peroxisome proliferator-activated receptor gamma coactivator 1-α.

2.5 Western Blotting:

Frozen liver tissue (~30mg) was homogenized in Pro-Prep lysis buffer (Intron Biotechnology, WA, USA) using a homogenizer (ProScientific, Oxford, CT). Lysate was centrifuged for 10 mins at 4000rpm at 4°C to remove cellular debris and to allow separation of excess liver fat from protein lysate. Protein concentration was determined by use of Bradford protein assay (Bio-Rad). Samples were prepared for loading using sample loading buffer from BioRad to which we added β-mercaptoethanol, and 25µg of protein was separated using 5-12% SDS page gel. Gels were transferred to nitrocellulose membrane at 4°C in cold TRIS glycine transfer buffer containing methanol. Signal was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) and developed on film. Primary antibodies were acquired from Cell Signalling Technologies (Danvers, MA, USA) and diluted to 1:1000 or as otherwise specified (Table 2), and secondary antibodies were used at concentrations between 1:5000 and 1:20,000 unless otherwise noted. Target protein expression was normalized to tubulin or vinculin (high molecular weight targets on 5% acrylamide gels) and then to LF WT controls. Signal intensity was measured using ImageJ software (NIH, USA).

Table 2: Primary antibodies for immunoblot, source and working dilutions

Target	Supplier	Catalog Number	Working Dilution
p-AKT (Ser473) and AKT	Cell Signalling Technology ®	9271/9272S	1:1000
p-FoxO1 (Ser253) and FoxO1	Cell Signalling Technology ®	84192S/2880S	1:1000
α-Tubulin	Cell Signalling Technology ®	2144	1:1000
PCK2	Cell Signalling Technology ®	6924S	1:1000
Vinculin	Cell Signalling Technology ®	4650S	1:1000
p-ACC (Ser79) and ACC	Cell Signalling Technology ®	3661S/3676S	1:1000
G6PdeH2ase	Cell Signalling Technology ®	8866S	1:2000
FASN	Cell Signalling Technology ®	3180S	1:1000
p-ATP citrate lyase (S455) and ATP	Cell Signalling Technology ®	4331P/4332P	1:1000
p-PHDa1	Millipore	ABS194	1:1000
PDH	Cell Signalling Technology ®	3205S	1:1000

Primary antibodies, source and dilutions used for western blot analysis of liver tissue isolated at the end of the third trimester from pregnant WT and adiponectin KO mice. p-AKT/AKT: protein kinase B, FoxO1: Forkhead box transcription factor 1, PCK2: phosphoenolpyruvate carboxykinase-2, ACC: acetyl-CoA carboxylase, G6Pase: glucose-6-phosphatase, FASN: fatty acid synthase, PDH/PDHa1: pyruvate dehydrogenase subunit a1.

2.6 Pancreatic Islet Isolation:

Following euthanasia, pancreatic islets were isolated from pregnant LF and HFS-fed WT and adiponectin KO mice in the third trimester. Mice were euthanized with an overdose of sodium pentobarbital by IP injection and blood collection was performed by cardiac puncture. The heart was removed snap frozen as a method of physical euthanasia. Intestinal mass was moved to the left, and the liver was moved up against the diaphragm to expose the common bile duct (CBD) which was clamped as close to the liver as possible. The intestine was clamped just distal to the junction of the CBD and the duodenum to allow visualization and access. Using scissors, the duodenum was nicked proximal to the clamp, approximately 3mm below the ampulla of the CBD. A canula was inserted through this nick and fed up to and into the CBD for perfusion. This method allowed the most consistent cannulation of the CBD. Collagenase V solution was prepared to a total volume of 50mL with RPMI 1640 + 10% penicillin/streptomycin (P/S), 2% BSA to a final concentration of 0.8mg/mL collagenase. We perfused the pancreas with approximately 3mL of collagenase solution or until the pancreas was full, at which point we excised the pancreas, removed excess fatty tissue and placed it into a 50mL tube with an additional 5mL of collagenase solution.

The isolated pancreas was digested in a 37°C water bath for ~16 minutes, shaking once halfway through. To halt digestion, RPMI containing P/S and 10 % FBS was added up to 50mL and the digested pancreas was placed on ice until islets were hand-picked into fresh RPMI +P/S+FBS media. To identify and pick islets, 5-10mL was poured into a 10m petri dish and clean islets were picked into a second petri dish containing fresh RPMI + P/S + FBS and repeated until all islets have been picked. These islets were picked a second time into another fresh petri dish containing RPMI + P/S + FBS, ensuring clean islets and no debris. Islets were cultured overnight in RPMI + P/S + FBS at 37°C.

2.6.1 Insulin and Glucagon Secretion:

Following isolation of islets and incubation overnight, islets were picked a third time into fresh RPMI + P/S +FBS to exclude any islets that degraded overnight. Low glucose (2.8mM glucose), high glucose (16.7mM glucose) media were prepared from a 1M stock glucose solution and diluted in Krebs Ringer Buffer (KRB; 128.8mM NaCl, 4.8mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$, 5mM $NaHCO_3$, 10mM HEPES) + 0.1% BSA. A 30mM KCl solution was prepared by diluting a 3M stock solution in KRB + 0.1%BSA. Approximately 15 islets each were placed into 3 tubes (to perform assays in triplicate) and 1mL of 2.8mM glucose solution was added. Islets in low glucose were incubated at 37°C for 30mins to equilibrate. After 30 minutes, the supernatant was discarded and 150µL of 2.8mM glucose was added to the islets. Islets were incubated at 37°C for 30 mins and the supernatant was transferred to clean tubes. Procedure was repeated with 150uL of high glucose solution and KCl solution, and the supernatant was stored at -20°C for analysis. Islets were lysed in 150µL of 0.4N acid ethanol and stored at 4°C overnight. DNA content from lysed islets was measured using Nano-drop and islets were frozen at -20°C. Any islets obtained in excess of those required for GSIS were stored in buffer RLT for RNA isolation.

Secreted insulin in collected supernatant as well as insulin content from lysed islets was measured by ELISA (ALPCO) and normalized to islet DNA and expressed as hourly secretion. Islet glucagon secretion and content was measured by radio immuno-assay (RIA) (Cat# GL-32K, Millipore, Darmstadt, Germany) from the same islets and normalized to DNA. Insulin secretion index and glucagon secretion index were calculated from the difference between glucose stimulated insulin secretion under high glucose conditions and low glucose conditions (basal) (Prasoon Agarwal et al., 2019).

2.7 Primary Hepatocyte isolations:

Mice were fasted for 1 hour prior to procedure and anesthetized with isoflurane. Anesthesia was maintained throughout perfusion until sacrifice. Perfusion and isolation procedure was adapted from Klaunig *et al* and Shen *et al* (Klaunig et al., 1981; Shen, Hillebrand, Wang, & Liu, 2012). Fur was removed from the abdomen with depilatory cream, and the area was sterilized with 70% ethanol. When no pedal reflex was observed, a V-shaped incision was made in the abdomen and abdominal viscera were moved to the left to expose the hepatic portal vein and inferior vena cava (IVC). A loop of surgical suture was placed around the IVC, and the portal vein was cannulated. The heart was removed as a physical method of sacrifice, and the SVC was clamped or tied to with surgical suture. The flow of EGTA solution (50mM EGTA with 19.5mM glucose, 4.17mM NaHC03 and 25mM HEPES) was initiated and the IVC was cut distal to the loop of suture, to allow outflow. The liver was monitored for blanching. Once the liver had cleared, collagenase IV (EGTA, plus 0.7mg/mL collagenase IV) solution was initiated and the suture around the IVC was tightened to allow the liver to swell, improving digestion and yield (Shen et al., 2012). Timing of digestion varied from 3 to 5 minutes based on consistency of the lobes when pressed with cotton swab. In this study, pregnancy, adiponectin KO and consumption of HFS diet made the livers prone to over-digestion.

2.7.1 Primary Hepatocyte Culture:

When digestion was complete, the liver was excised, minced in ~1mL of the collagenase solution and cells were pipetted up and down for 1-2 minutes. The volume was brought to 25mL with Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS. Cells were filtered through a sterile strainer into a 50mL conical centrifuge tube and spun at 500rpm for 5 minutes. The supernatant was discarded and 25mL of DMEM containing 15% FBS was added to the resultant pellet. Cells were mixed by pipetting 10 times and filtered through a finer strainer and spun at 500rpm for 5 minutes, and the supernatant was aspirated and discarded; 25mL of DMEM containing 15% FBS was added to the resultant pellet and mixed by pipetting. Approximately 40μL of the suspension was removed to separate 1.5mL Eppendorf tube for counting, and stored at 37°C. These cells were mixed with 40uL of Trypan Blue stain and 10-12uL were placed in the hemocytometer (Fisher Scientific). After counting, ~1 million cells were plated into 60mm dishes and incubated for 2-3 hours in DMEM containing 10% FBS at 37°C to allow attachment for subsequent radiolabelling experiments. Cells designated for mitochondrial analysis were plated directly into Seahorse 24-well plates for analysis.

2.7.2 Mitochondrial Analysis of Hepatocytes:

Experiments were performed by members of the Hatch Laboratory, according to protocols previously established (Cole et al., 2016). Approximately 100,000 hepatocytes per well were plated in Seahorse 24-well plates (Agilent, Santa Clara, CA, USA) immediately after counting. Basal oxygen consumption was calculated after inhibition of complex III by 1mmol/L antimycin A and complex I by 1mmol/L of rotenone. 1 mmol/L of oligomycin was added to inhibit ATP synthase, and the proportional change in oxygen consumption rate (OCR) is linked to ATP production. Addition of 1 mmol/L carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) to hepatocytes results in disruption of the proton gradient, uncoupling the flow of electrons from ATP synthesis and resulting in maximal OCR. Spare capacity can also be calculated from FCCP-stimulated OCR. All calculations were normalized to cellular protein, determined by Bradford assay (Bradford, 1976).

Hepatocellular metabolism using glucose was determined using a Seahorse XF-24 Extracellular Flux analyzer and a Mito Stress Test kit (Agilent, Santa Clara, CA, USA). Fatty acid oxidation by hepatocytes was determined using the XF Palmitate-BSA FAO Substrate (Agilent, Santa Clara, CA, USA) in combination with etomoxir and BSA control according to the manufacturer's instructions. For assessment of oxygen consumption during glucose metabolism, hepatocytes were plated into media containing 1mmol/L pyruvate and 25mmol/L glucose. For assessment of fatty acid metabolism, hepatocytes were plated into media containing 0.5 mmol/L carnitine, 2.5mmol/L glucose and 0.175mmol/L palmitate-BSA.

2.7.3 Radiolabelling of Hepatocytes:

After the hepatocytes attached (approximately 2 hours), cells were washed twice with serum-free DMEM and incubated with DMEM containing 2uCi [^{14}C] oleate plus 0.4mmol/L oleate-BSA or 2uCi [^{14}C] acetate per 2ml (60mm) dish. After incubation, cells were washed with cold phosphate buffered saline (PBS) and scraped into 1mL of cold PBS and stored in Eppendorf tubes.

2.7.4 Hepatocyte lipid extraction, separation and determination of radioactivity incorporation:

Lipids were extracted to determine the extent of fatty acid uptake and what proportion of radiolabeled acetate or oleate from primary hepatocytes were synthesized and excreted as diacylglycerides, triglycerides, cholesterol esters, or phospholipids. Cells were scraped into 1mL of PBS and disrupted with a 26g needle. 5µL of the cell suspension was aliquoted for protein determination by the Bradford method (Bradford, 1976). 2µL of the cell suspension was removed and combined with 5mL of liquid scintillation fluid.

Incorporation of radioactivity was determined using a Beckman LS 6500 Multipurpose Liquid Scintillation Counter (Beckman Coulter, CA, USA). 800µL of cell suspension and 1mL of cold PBS were combined with 5mL of a 2:1 chloroform/methanol solution and mixed by vortexing for 60 seconds. Samples were centrifuged at 2000 rpm for 20 minutes at 4°C. The lower organic phase was extracted and dried down under nitrogen gas and resuspended in 70µL of chloroform. 55µL of the suspension was loaded onto silica gel G plates (Millipore, Darmstadt, Germany) and lipids

separated by thin layer chromatography (TLC). To determine the proportion of radiolabelled lipid secreted into the media, 1.5mL of cellular media was combined with 8mL of 2:1 chloroform/methanol solution and mixed by vortexing for 60 seconds. Samples were centrifuged at 2000 rpm for 20 minutes at 4°C. The lower organic phase was extracted and dried under nitrogen gas and resuspended in 70μL of chloroform. 55μL was loaded onto plates for TLC. A portion of media from the supernatant was treated with acid and BSA and centrifuged to remove precipitate. An aliquot of the supernatant was counted with scintillation fluid to quantify acid soluble metabolites (ASM) in the media.

2.7.5 Thin layer chromatography (TLC):

Lipids were separated by TLC on 20 X 20 Cm silica gel G glass TLC plates (Millipore, Darmstadt, Germany), which, in combination with choice of solvent, separates lipid species by polarity, and allows identification relative to positive controls (Deranieh, Joshi, & Greenberg, 2013). In our experiments, plates were incubated in 217mL chloroform. 130mL methanol and 35mL of acetic acid solution for 25 minutes to allow polar lipids (such as phospholipids) to migrate, and the plate was dried for 10 minutes. Following this, the plate was immersed in 235 mL heptane, 156mL isopropylether, 16mL acetic acid solvent system for 45 minutes to separate neutral lipids and allow them to migrate down the plate.

Plates were developed for 45 minutes, dried and then exposed to iodine vapor in tanks to visualize lipids. Spots corresponding to the lipid of interest were removed and combined with 5mL of scintillation fluid, vortexed well and left overnight. Radioactivity was determined by liquid scintillation counting as described above.

2.8 The Next Generation Study: Maternal and Cord Blood Adipokines

These experiments were performed in collaboration with Dr. Brandy Wicklow, a Pediatric Endocrinologist at the DER-CA and the Univerity of Manitoba, and the Next Generation Study Cohort. Maternal and cord blood serum was obtained from 64 First Nations mother and infant dyads enrolled in the Next Generation cohort in Manitoba, Canada, with ethics approval provided to the original study by the University of Manitoba Ethics Board (Jabar, Colatruglio, Sellers, Kroeker, & Wicklow, 2019; Pylypjuk, Sellers, & Wicklow, 2020). The Next Generation Study was initiated in 2003 in Winnipeg, Manitoba and consists of First Nations mothers diagnosed with pre-existing type-2 diabetes and gestational diabetes and their offspring (Pylypjuk et al., 2020). Informed consent was obtained at enrollment. Population characteristics and anthropometric values for mothers and babies were obtained from a combination of self-reported data and hospital records where available.

2.8.1 Maternal and Cord Blood Serum Collection:

Maternal serum was collected by clinical staff on site at the time of delivery for analysis of circulating factors. Levels of C-reactive protein (CRP) were measured by Diagnostic Services of Manitoba (DSM). Cord blood was collected at the time of delivery by one of four potential methods, involving blood collection from the cut umbilical cord, or the cord or placental vein or artery. Collected samples were sent to the nearest DSM lab and centrifuged within 2 hours and aliquoted for analysis within 4 hours of collection. Samples destined for adiponectin analysis were collected in

4.5 mL Plasma Separation Tubes (Vacutainer, Becton Dickson, Oakville, ON) and for leptin analysis were collected in 5mL gold-topped Serum Separation Tubes (Vacutainer, BD, Oakville, ON). Samples were centrifuged at 2600 rpm for 15 minutes at 4°C and aliquots were stored at -80°C.

Adiponectin and leptin concentrations were determined as described in section 2.1.4. Dilutions were discarded and remaining samples were returned to the Next Generation Study for storage.

2.9 Statistical Analysis:

Data are presented as mean +/- standard error of the mean (SEM), and statistical analysis was performed using GraphPad Prism Software (La Jolla, CA, USA). We used One- and Two-Way Analysis of Variance (ANOVA) to compare group means between WT and adiponectin KO mice fed LF or HFS diets in pregnancy, and Tukey post hoc test to determine differences between groups. When comparing only two groups an unpaired t-test was used. Correlations shown in Chapter 7 are represented by the Pearson correlation coefficient (r value) and p-value. For all analysis, statistical significance was defined as $p<0.05$.

Chapter 3:

The role of adiponectin in the development of hyperglycemia and impaired glucose tolerance in the third trimester

3.1 Introduction

In a healthy pregnancy, maternal insulin resistance is a biological response to spare glucose for the fetus. This insulin resistance is normally overcome without the development of hyperglycemia by compensatory increases in insulin secretion. In late gestation, hyperglycemia and severe insulin resistance can signal the development of GDM. Hyperglycemia can occur due to impaired glucose tolerance as a result of reduced uptake by peripheral tissues, and by the unchecked production of glucose by the liver as a result of hepatic insulin resistance. There is abundant evidence suggesting an association between adiponectin and the regulation of glucose homeostasis. Individuals who are overweight or obese have lower levels of circulating adiponectin (De Rosa et al., 2013; Meyer, Ciaraldi, Henry, Wittgrove, & Phillips, 2013; Ukkola & Santaniemi, 2002). Due to its role in energy homeostasis, low circulating adiponectin is implicated in the development of T2D (S. Li, H. J. Shin, E. L. Ding, & R. M. van Dam, 2009; C. S. Mantzoros, Li, Manson, Meigs, & Hu, 2005; Yeli Wang et al., 2018). Studies in porcine models illustrated that levels of adiponectin are lower in "fat type" sows relative to lean type animals (Daniele et al., 2008). Adiponectin has been shown to stimulate glucose utilization and fatty acid oxidation *in vitro* (T. Yamauchi et al., 2002). Additionally, in rodent models, consumption of a high fat and sucrose obesogenic diet is linked with low circulating adiponectin (Yamauchi, 2001, Maeda, 2002).

In pregnancy, levels of adiponectin decrease throughout gestation as insulin resistance progresses. However, women who develop GDM have significantly lower serum adiponectin than control pregnant women (Mazaki-Tovi et al., 2008; Retnakaran et al., 2004). Indeed, studies are beginning to suggest that low adiponectin early in pregnancy or prior to conception may be predictive of GDM development or risk for a GDM pregnancy (Bozkurt et al., 2018; Hedderson et al., 2013; Marilyn Lacroix et al., 2013). Diagnosis of GDM is made using a glucose tolerance test (GTT) that is administered in pregnancy. The GTT measures glucose uptake and clearance by peripheral tissues as well as the insulin secretion response to glucose administration. Insulin resistance will impair glucose uptake through reduced glucose transport, downstream glucose utilization and storage resulting in hyperglycemia (R. P. D. A. Barros, A. Morani, A. Moriscot, & U. F. Machado, 2008). Due to its role in potentiating insulin signalling (Lihn, Pedersen, & Richelsen, 2005), adiponectin is proposed to help maintain glucose tolerance particularly during pregnancy and may help to prevent development of GDM (Pala et al., 2015).

Insulin resistance that develops in late pregnancy is physiological, diverting glucose to the increasing demands of fetal growth. In late gestation there is a mild increase in hepatic glucose output (Butte, 2000). In healthy pregnancies, gluconeogenesis remains sensitive to insulin but there is some evidence that in GDM hepatic gluconeogenesis is not sufficiently suppressed, which may contribute to hyperglycemia (Butte, 2000; Harlev & Wiznitzer, 2010). In a streptozotocin-induced rat model of GDM, increased hepatic gluconeogenesis has been observed as part of the pathogenesis of hyperglycemia in pregnancy (Abraham et al., 2016). This evidence suggests there may be a role for increased hepatic gluconeogenesis as a contributing factor in the development of hyperglycemia associated with GDM.

There are conflicting reports in the literature regarding the effect of adiponectin KO on insulin sensitivity and hyperglycemia. In general, it appears that adiponectin KO mice may require a second "hit" to develop a metabolic

phenotype. In some studies, this second hit is the added insulin resistance of pregnancy (L. Qiao et al., 2017), drug-induced insulin resistance (Benrick et al., 2017), or diet induced obesity (Asano et al., 2009; Maeda et al., 2002; Nawrocki et al., 2006). In our study, we utilized both a LF and HFS diet to evaluate the impact of high-fat diet feeding and obesity with or without a background of adiponectin deficiency. LF fed WT mice were used as a control, and HFS fed WT mice represent a model of diet-induced obesity prior to pregnancy. Adiponectin KO mice fed a LF diet shows the effect of adiponectin deficiency, independent of obesity and the addition of a HFS diet to adiponectin KO mice allows us to observe the added metabolic stress of obesity to adiponectin deficiency. This model allowed us to separate the effects of obesity, pregnancy, and adiponectin deficiency on the development of GDM.

In this chapter, this model was used to test the hypothesis that adiponectin deficiency during pregnancy impairs whole body glucose homeostasis and leads to a GDM phenotype. Using an adiponectin-knockout mouse model it was determined that adiponectin deficiency alone does not cause metabolic impairments in non-pregnant female mice when consuming a low-fat diet. However, in the third trimester of pregnancy, adiponectin KO mice were hyperglycemic and had impaired glucose tolerance and dysregulation of gluconeogenesis relative to wild-type controls. These findings show that adiponectin deficiency leads to dysregulated responses to insulin in the third trimester of pregnancy and may contribute to hyperglycemia that is characteristic of GDM, independent of obesity.

3.2 Materials and Methods – refer to page 24

3.3 Results

3.3.1 Gestational Weight Gain

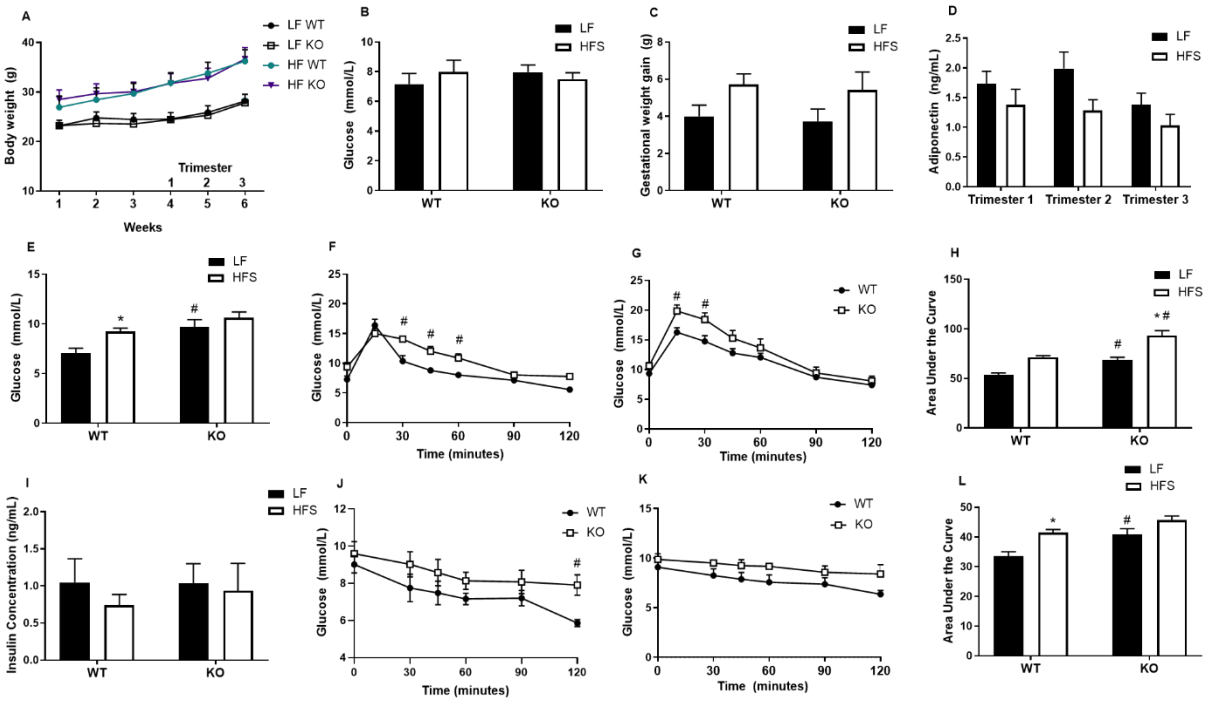
Entering pregnancy overweight or obese can increase the risk of developing GDM (Chu et al., 2007), so we aimed to determine whether adiponectin deficiency lead to increased gestational weight gain or obesity in mice. In female mice, adiponectin deficiency was not a significant determinant of bodyweight gain prior to mating, or throughout pregnancy (Figure 9A). LF fed adiponectin KO mice gained weight at the same rate as WT controls. The consumption of a HFS diet lead to a similar level of gestational weight gain in WT and adiponectin KO mice suggesting that the primary driver of gestational weight gain appears to be diet as opposed to genotype. In pregnant WT mice consumption of a HFS diet appeared to suppress the normal pattern of circulating adiponectin, reducing levels of adiponectin mid-gestation, likely due to increased adiposity (Figure 9D).

In order to rule out maternal overnutrition, food consumption was measured in both LF and HFS fed WT and adiponectin KO mice (Table 3). When mice consumed a LF diet there were no differences in food consumption between adiponectin KO and WT control animals prior to or during pregnancy. Overall, all animals consumed ~1.6 fold more food on a HFS diet, and adiponectin KO mice in the 1st and 3rd trimester consumed significantly more HFS food than adiponectin KO mice fed the LF diet (Table 3). There were no genotype specific differences in food intake regardless of diet and this corresponded with the absence of increased bodyweight or gestational weight gain. Thus, adiponectin deficiency did not lead to obesity, excess gestational weight gain, or excess food consumption when compared to WT controls (Table 3).

Prior to pregnancy, there was no significant change in visceral fat deposition as represented by perirenal white adipose tissue (PWAT) or gonadal white adipose tissue (GWAT) mass in adiponectin KO mice relative to WT controls when animals were fed a LF diet (Table 4). Notably, while HFS diet feeding lead to increases in both PWAT and GWAT mass in WT mice, adiponectin KO mice did not have marked increases in fat pad mass with HFS diet feeding relative to LF diet (Table 4). In fact, adiponectin KO mice on a HFS diet had significantly less visceral fat than WT mice. Consumption of a HFS diet in pregnancy correlated to increased bodyweight in both WT and adiponectin KO mice. It is apparent from tissue collected at sacrifice that some of this weight gain may be due to increased visceral adiposity (Table 6). Both WT and adiponectin KO mice had increased GWAT mass when fed a HFS diet, but no changes were observed in PWAT (Table 6). These results indicate that HFS diet consumption rather than genotype was the major determinant of adiposity prior to pregnancy and in the third trimester.

HFS diet feeding increased serum leptin in both WT and adiponectin KO mice relative to LF diet in the third trimester (Table 6). An adaptation of pregnancy is the ability of WAT to expand during early gestation, followed by increased lipolysis in late gestation. However, the mechanism of expansion, whether by proliferation of adipocytes or hypertrophy of existing adipocytes, may have implications for metabolic health (Rojas-Rodriguez et al., 2015). The size of adipocytes within GWAT depots from pregnant mice isolated at the end of the third trimester were examined and it was found that consumption of a HFS diet increased adipocyte size in both adiponectin KO and WT mice (Figure 10A). However, adiponectin KO mice had overall larger adipocytes even when fed a LF diet. This can be seen more clearly in Figure 10B where there were on average more, smaller adipocytes (with most in the 10-20μm range) in WT mice fed a LF diet. In addition, adiponectin KO mice had adipocytes that were larger, with the majority averaging 30-40μm in diameter. When these mice are fed a HFS diet, both adiponectin KO and WT mice have more adipocytes with larger diameters. WT mice exhibited adipocytes in the 30-40 μm range whereas adipocytes from adiponectin KO mice were 40-50 μm in diameter. This suggests that while a HFS diet in pregnancy increases GWAT mass and adipocyte size in both WT and adiponectin KO mice, adipocytes from pregnant adiponectin KO mice are more hypertrophic which could be a maladaptive mechanism.

Figure 9: Effects of pregnancy on glucose control and insulin resistance in wild type and adiponectin knockout mice.



A)Body weight (g) over time, prior to breeding and during gestation N=12-13. B) Non-pregnant fasting blood glucose prior to breeding N=8-14. C) Gestational weight gain calculated from bodyweight at the end of the third trimester and bodyweight prior to conception. N=11-15 D) Serum total adiponectin in WT mice throughout gestatation in the first, second and third trimesters N=5-7. E) Third trimester fasting blood glucose N=11-15. GTTs performed in the third trimester (e15.5-17.5) on LF (F) and HFS (G) fed mice N=8-12. H) AUC calculation for GTTs. I) Third trimester serum insulin (N=6-10). Third trimester ITTs performed on LF (J) and (HFS) fed (K) pregnant mice (e15.5-17.5) (N=5-7). L) AUC for third trimester pregnant ITTs. P-values represent significance (<0.05) after Two-Way ANOVA. # p<0.05 KO vs WT * p <0.05 LF vs HFS. LF: low fat, HFS: high fat and sucrose. KO: knockout, WT: wild type, GTT: glucose tolerance test, ITT: insulin tolerance test, AUC: area under the curve. INS: insulin treated. SAL: saline treated.

Table 3: Food consumption prior to breeding and during gestation is not significantly different between wildtype and adiponectin knockout mice.

Week	Wildtype Food Consumption		Knockout Food Consumption	
	Low Fat (kcal/kg mouse/day)	High Fat Sucrose (kcal/kg mouse/day)	Low Fat (kcal/kg mouse/day)	High Fat Sucrose (kcal/kg mouse/day)
1	0.31 +/- 0.05	0.43 +/- 0.07	0.24 +/- 0.02	0.40 +/- 0.07
2	0.28 +/- 0.04	0.40 +/- 0.06	0.25 +/- 0.01	0.46 +/- 0.06
3	0.19 +/- 0.03	0.36 +/- 0.05	0.27 +/- 0.01	0.41 +/- 0.04
Trimester 1	0.34 +/- 0.04	0.33 +/- 0.05	0.26 +/- 0.02	0.49 +/- 0.07*
Trimester 2	0.32 +/- 0.06	0.46 +/- 0.08	0.35 +/- 0.03	0.55 +/- 0.03
Trimester 3	0.29 +/- 0.03	0.42 +/- 0.08	0.28 +/- 0.03	0.58 +/- 0.05*

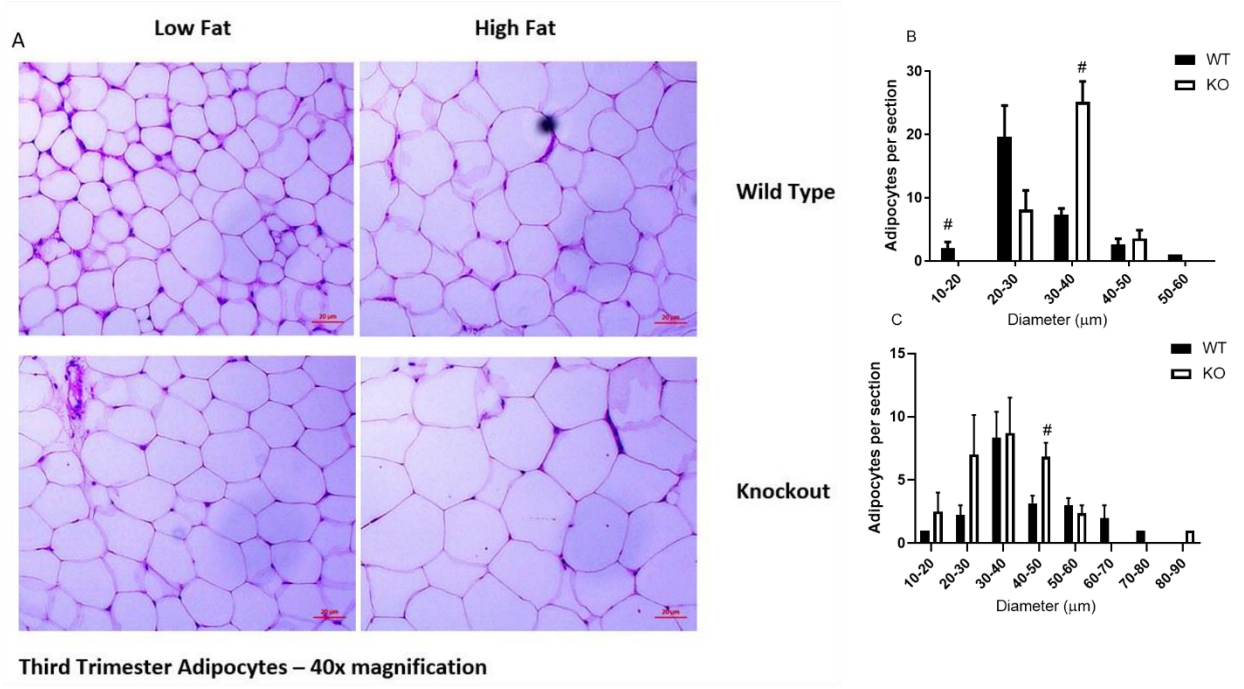
Adiponectin KO mice did not consume more food prior to pregnancy or during pregnancy relative to WT mice, however HFS diet feeding increased food consumption equally across both genotypes. P Values represent significance ($p<0.05$) after Two-way ANOVA. * $p<0.05$ between LF and HFS fed mice. N=10-12 mice per group.

Table 4: Litter characteristics from WT and adiponectin KO mice

	Wildtype	Knockout
LF Litter Size	5.64 ± 0.52	4.64 ± 0.65
HFS Litter Size	5.72 ± 0.56	7.07 ± 0.62*
LF Pup size (mg)	1288.12 ± 48.01	1167.10 ± 68.63
HFS Pup size (mg)	1070.11 ± 51.82	1113.10 ± 60.94

Effect of adiponectin deficiency and HFS diet feeding on litter sizes and pup sizes at the end of the third trimester. N=11-15 pups per group, N=14 litters per group. P values represent significance ($p<0.05$) after Two-way ANOVA. *$P<0.05$ between LF and HFS fed mice.

Figure 10: Gonadal white adipose tissue from adiponectin KO mice contains larger adipocytes relative to WT controls in pregnancy



A) Representative images of H&E stained adipocytes at 40x magnification. Adipose tissue was sampled from gonadal white adipose tissue (GWAT) at the time of sacrifice at the end of the third trimester. B) GWAT size (diameter in μm) in LF fed adiponectin KO and WT mice in the third trimester. N = 6-7. C) GWAT size (diameter in μm) in HF fed adiponectin KO and WT mice in the third trimester. N=6-7. P values represent significance ($p<0.05$) after Two-way ANOVA. # $p<0.05$ between WT and adiponectin KO. WT: Wildtype; KO: Knockout. LF: Low-Fat, HFS: High fat and sucrose.

Table 5: Visceral fat mass in non-pregnant WT and adiponectin KO mice

	Non-Pregnant Wildtype		**Non-Pregnant Adiponectin Knockout**	
	Low Fat	**High Fat Sucrose**	**Low Fat**	**High Fat Sucrose**
GWAT weight (mg)	288.77 ± 121.53	1888.18± 387.36*	131.60 ± 21.30	268.40 ± 97.20 #
PWAT weight (mg)	595.91 ± 207.81	3139.50 ± 564.10 *	155.50 ± 19.40	672.36 ± 214.76#

Visceral fat depot weights in gonadal white adipose tissue (GWAT) and perirenal white adipose tissue (PWAT) at the time of sacrifice in non-pregnant mice. P Values represent significance ($p<0.05$) after Two-way ANOVA * $p<0.05$ between LF and HFS fed mice. # $p < 0.05$ between WT and adiponectin KO mice.

Table 6: Visceral fat mass and serum leptin levels in the third trimester of pregnancy

	Wildtype		**Adiponectin Knockout**	
	Low Fat	**High Fat Sucrose**	**Low Fat**	**High Fat Sucrose**
GWAT weight (mg)	187.2 ± 21.5	418.6 ± 57.8 *	291.1 ± 57.3	566.4 ± 191.4
PWAT weight (mg)	135.5 ± 28.4	185.5 ± 42.0	212 ± 55.1	331.0 ± 91.7
Serum Leptin (pg/mL)	11.6 ± 2.5	21.2 ± 4.6 *	16.3 ± 2.7	25.8 ± 5.4 *

Visceral fat depot weight in GWAT and PWAT in pregnant mice at the time of sacrifice at the end of the third trimester. Serum leptin levels measured from blood collected at the end of the third trimester in adiponectin KO and WT mice. P Values represent significance ($p<0.05$) after Two-way ANOVA. * $p<0.05$ between LF and HFS fed mice. WT: Wildtype, KO: Knockout, LF: Low-Fat, HFS: High fat and sucrose.

3.3.2 Hyperglycemia and Glucose Tolerance

Whole body glucose homeostasis was assessed during pregnancy and blood glucose levels were measured at each trimester of pregnancy. Importantly, adiponectin KO mice did not develop fasting hyperglycemia prior to pregnancy compared to WT mice (Figure 9B). However, both LF- and HFS-fed adiponectin KO mice had higher blood glucose levels in the third trimester, relative to WT controls, consistent with the development of GDM (Figure 9E). Additionally, LF-fed adiponectin KO mice had impaired glucose tolerance relative to WT mice in the third trimester as determined by GTT (Figure 9F). While HFS-fed WT and adiponectin KO dams were hyperglycemic compared to LF-fed dams (Figure 9E, 9F) the impairment in glucose tolerance was more severe in adiponectin KO mice (Figure 9G). Although hyperinsulinemia often accompanies insulin resistance as a compensatory mechanism to overcome hyperglycemia, no increase in third trimester circulating insulin between adiponectin KO and WT mice was observed (Figure 9I).

3.3.3 Insulin Tolerance

In the third trimester of pregnancy, insulin resistance occurs in response to increased fetal nutrient demand. Insulin tolerance tests (ITT) showed that that adiponectin KO mice in the third trimester of pregnancy were more insulin resistant than WT mice, when fed either a LF (Figure 9J) or HFS (Figure 9K) diet. Additionally, WT mice fed a HFS diet had impaired insulin tolerance (Figure 9L) in the third trimester, indicating that diet induced obesity affects insulin sensitivity in WT mice but did not have a significant additive effect in adiponectin KO mice.

3.3.4 Gluconeogenesis

Insulin resistance in pregnancy may lead to worsening hyperglycemia through the inability to effectively suppress hepatic glucose output. Increasing adiponectin through supplementation or gene therapy was shown to reduce hepatic gluconeogenesis and improve insulin sensitivity. (T. P. Combs et al., 2001; Y. Ma & Liu, 2013; Shklyaev et al., 2003; T. Yamauchi et al., 2002). Using a PTT to measure of the production of *de novo* glucose from pyruvate, in non-pregnant females, adiponectin deficiency had no impact on gluconeogenesis relative to wild-type controls (Figure

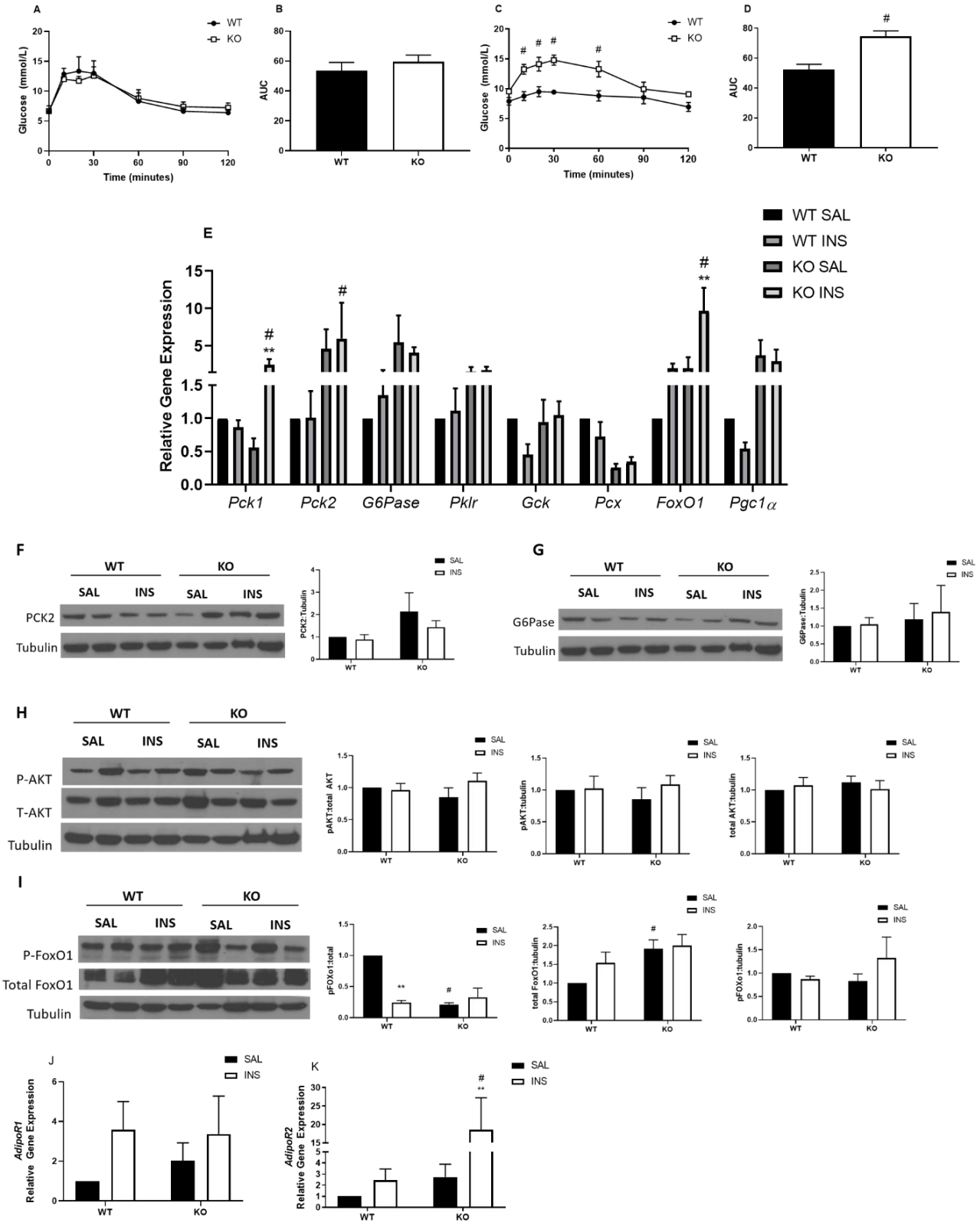
11A, B). However, in the third trimester, adiponectin KO mice had elevated glucose production from pyruvate relative to WT controls (Figure 11C, D).

In order to better understand the mechanism, qPCR was performed on livers isolated at the end of gestation to determine expression of genes responsible for gluconeogenesis and glycolysis. WT and adiponectin KO mice were injected with either saline or insulin 10 minutes prior to sacrifice and mRNA expression determined. Due to natural insulin resistance in the third trimester, there was minimal suppression of gluconeogenic gene expression (*Pck1*, *Pck2*, *G6pase*) in the WT mice administered insulin (Figure 11E). However, expression of *Pck1*, *Pck2* and *FoxO1* were upregulated in the livers of adiponectin KO mice treated with insulin compared to WT controls. Based on the impaired glucose tolerance, it was hypothesized that there may be impairments in the ability of adiponectin KO mice to perform glucose uptake and glycolysis. However, mRNA expression of pyruvate kinase (*Pklr*) as well as glucokinase (*Gk*) were unaltered in livers of third trimester pregnant mice even with insulin administration (Figure 11E). This may indicate the presence of insulin resistance, given that even under conditions of hyperglycemia in adiponectin KO mice, insulin supplementation did not increase expression of glycolytic genes.

In order to determine if changes at the level of mRNA translated to changes in protein expression, western blot analysis was performed on liver tissue for key targets. Phosphoenolpyruvate carboxykinase 2 (PCK2) is the mitochondrial isoform of the protein encoded by the *Pck2* gene. In livers of adiponectin KO mice there was no significant change in PCK2, glucose-6-phosphatase, phosphorylated S-473 AKT and total AKT protein expression relative to wildtype mice (Figure 11F-H). In addition, there was no effect of insulin administration on expression of these proteins in WT or adiponectin KO mice. Protein expression of FoxO1, a downstream target of AKT signalling, was increased in adiponectin KO mice compared to WT animals (Figure 11I). This was accompanied by a significant decrease in phosphorylated FoxO1 in adiponectin KO mice compared to WT animals. Since FoxO1 plays a key role in upregulation of gluconeogenic genes, this may explain the increased observed mRNA expression of *G6pase* and *Pck2* in adiponectin KO mice compared to WT animals.

A previous study indicated that FoxO1 increased the expression of adiponectin receptors (Tsuchida et al., 2004). In addition, insulin was shown to reduce adiponectin receptor expression in the liver (Tsuchida et al., 2004). *AdipoR1* expression was not significantly different between adiponectin KO and WT mice in the third trimester, and insulin administration resulted in a small but not significant increase (Figure 11J). *AdipoR2* is the more abundant adiponectin receptor expressed in the liver. Treatment of adiponectin KO mice with insulin significantly increased mRNA expression of *AdipoR2* compared to WT animals (Figure 11K) The higher *AdipoR2* expression may indicate a compensatory attempt to increase adiponectin signalling. Interestingly, a previous study showed that insulin resistant patients had higher hepatic expression of *AdipoR1* and *R2* relative to healthy controls, and that *AdipoR1* expression correlated with increased *FoxO1* in obese, insulin resistance subjects (Felder et al., 2010). This may be due to the fact that fasting has been shown to increase adiponectin receptor expression in the liver (Tsuchida et al., 2004), and that there may be some degree of insulin resistance at this stage of pregnancy. Combined, these results suggest that adiponectin KO mice have increased hepatic glucose output, which may contribute to hyperglycemia and GDM in the third trimester.

Figure 11: Hepatic gluconeogenesis is increased in pregnant adiponectin knockout mice.



A) PTT performed prior to conception. B) Area under the curve (AUC) for data from A. C) PTT performed on LF fed pregnant mice in the third trimester (e15.5-17.5). D) AUC for data from C. E) Hepatic mRNA expression from third trimester pregnant mice for *Pck2, G6pase, Pklr, Gck, Pdha1, Pcx* and *Foxo1*. mRNA expression is relative to geomean of *Rn18s, B2m, RPLP-1* and *hprt1* and normalized to LF WT SAL. F.-I. Hepatic protein expression from pregnant LF fed mice. F) PCK2 protein expression relative to tubulin and normalized to WT SAL, G) G6Pase protein expression relative to tubulin and normalized to WT SAL, H) P-AKT relative to total-AKT and tubulin, normalized to WT SAL, and I) p-FoxO1 relative to total FoxO1 and tubulin, normalized to WT SAL. N=4-6 by group. J) Hepatic *AdipoR1* and K) *AdipoR2* mRNA expression relative to geomean and normalized to WT SAL. N=4-6 P-values represent significance (<0.05) after Two-Way ANOVA. # p<0.05 KO vs WT **p <0.05 SAL vs INS. *B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *RPLP-1:* 60S acidic ribosomal protein large P1, *Pck1*: phosphoenolpyruvate carboxykinase-1. *Pck2*: phosphoenolpyruvate carboxykinase-2, *G6pase*: glucose-6-phosphatase, *Pklr*: pyruvate kinase liver isoform, *Gck*: glucokinase, *Pdha1*: pyruvate dehydrogenase-a1, *Pcx*: pyruvate carboxylase, *Foxo1*: Forkhead box transcription factor 1. *Pgc1α:* peroxisome proliferator-activated receptor gamma coactivator 1-α. *AdipoR1:* Adiponectin receptor protein 1; *AdipoR2:* Adiponectin receptor protein 2. WT: wildtype, KO: knockout, SAL: saline treated, INS: insulin treated. PTT: pyruvate tolerance test, LF: Low Fat

3.4 Discussion/Conclusions

The objective of this study was to determine whether adiponectin deficiency in pregnancy influences whole body glucose homeostasis during pregnancy, and whether this effect is independent of obesity and gestational weight gain. Studies have linked the consumption of a high fat and sucrose (HFS) diet, or maternal overnutrition with both low circulating adiponectin and higher risk of GDM. However, due to the nature of adiponectin expression and secretion, individuals who are overweight or obese secrete less adiponectin. Therefore, it is important to separate the role of obesity and overnutrition from the impact of adiponectin deficiency when looking at GDM. For this reason, both a HFS and LF control diet were utilized to examine the effect of obesity and excessive gestational weight gain in the context of adiponectin deficiency in pregnancy.

WT and KO mice that consumed a HFS diet prior to and during pregnancy were heavier overall (Figure 9A), and consumption of a HFS diet during pregnancy lead to ~40% more gestational weight gain in both groups (Figure 9C). However, there was no genotype specific difference in bodyweight prior to or during pregnancy in LF or HFS fed mice. This suggests that when WT and KO mice are fed the same diet (LF or HFS) rate of weight gain is diet dependent and is not mediated by adiponectin, either prior to pregnancy or during gestation. It was interesting to note that HFS diet feeding increased GWAT and PWAT mass in WT mice prior to pregnancy (Table 4) compared to LF feeding. On the contrary, non-pregnant adiponectin KO mice did not show the same increases in adipose tissue mass. In fact, they had a decrease in both depots when fed a HFS diet relative to WT controls. This may point to impairments in adipose tissue expansion which is a key component of "metabolically healthy" obesity and may be important during a normal pregnancy.

Although both adiponectin KO and WT mice had increased visceral adiposity, as measured by GWAT collected at the end of pregnancy, when consuming a HFS diet (Table 6) adiponectin KO mice had overall larger adipocytes, even on a LF diet relative to WT mice (Figure 10B). Adipose tissue expansion is an important adaptation of pregnancy, and both proliferation and hypertrophy are required to store lipids and, crucially, to prevent ectopic lipid deposition in other tissues (Rojas Rodriguez, 2015). It has also been demonstrated that both methods of adipose tissue expansion are not metabolically equal. Hyperplasia, the presence of more, smaller adipocytes is associated with better

glucose control and hypertrophy is linked to increased inflammation and worse adipose tissue insulin resistance (Hoffstedt et al., 2010). In GDM, white adipose tissue has been shown to have increased resistance to insulin (Sevillano et al., 2007) as well as reduced GLUT4 expression, decreasing capacity for glucose uptake (R. Barros, A. Morani, A. Moriscot, & U. Machado, 2008). Even though adiponectin KO mice do not have increased visceral fat mass relative to WT mice in the third trimester, the proportion of larger adipocytes is much higher (Figure 10B, 10C). This suggests that there may be a trend towards the less metabolically healthy phenotype of adipose tissue expansion in adiponectin KO mice

Adiponectin has been shown to enhance the lipid storage capacity of adipose tissue and promote healthy adipose tissue expansion (Fu, Luo, Klein, & Garvey, 2005; J. Y. Kim et al., 2007; Tao, Sifuentes, & Holland, 2014). The ability of adipose tissue to safely expand is important to prevent spill over of lipids, resulting in hyperlipidemia and ectopic lipid deposition in other peripheral tissues which can lead to worsening insulin resistance (Gray & Vidal-Puig, 2007). Increasing circulating adiponectin in leptin deficient *ob/ob* mice was shown to improve this healthy adipose tissue expansion and restored glucose and insulin levels improving the metabolic phenotype (J.-Y. Kim et al., 2007). Adiponectin also inhibits lipolysis from adipose tissue (Liping Qiao, Kinney, Schaack, & Shao, 2011), and as adiponectin levels decrease with increasing gestation, levels of adipose tissue lipolysis increase which is important in the third trimester of pregnancy. Without adiponectin, there may be a predisposition towards dysregulation of adipose tissue expansion especially when adiponectin KO mice are exposed to pregnancy or a HFS diet. This may explain some of the increased peripheral insulin resistance and glucose intolerance observed in adiponectin KO mice in the third trimester.

Obesity (due to the consumption of a HFS diet) as well as pregnancy both increase serum levels of leptin (Tessier, Ferraro, & Gruslin, 2013). Leptin is involved in regulating energy homeostasis by increasing energy expenditure and regulating appetite and satiety (Brunton & Russell, 2008; Trayhurn et al., 1999). As pregnancy progresses through the first and second trimester, leptin levels increase and are maintained throughout the third trimester (Hardie, Trayhurn, Abramovich, & Fowler, 1997). Leptin resistance is observed in pregnancy and lactation, where an increased appetite is required to consume adequate nutrition for fetal demands (Johnstone & Higuchi, 2001). However, hyperleptinemia is also associated with obesity and metabolic dysfunction (Leon-Cabrera et al., 2013).

Overall, all animals consumed ~1.6 fold more food on a HFS diet, and adiponectin KO mice in the 1st and 3rd trimester consumed significantly more HFS diet than adiponectin KO mice fed LF food (Table 1). This may be due to increased palatability of the diet (higher sugar and fat content) which has been associated with hyperphagia in other studies (la Fleur, van Rozen, Luijendijk, Groeneweg, & Adan, 2010; Welch, Kim, Grace, Billington, & Levine, 1996), or satiety changes associated with HFS diet induced obesity (Covasa & Ritter, 1999). White adipose tissue mass was increased in both adiponectin KO and WT mice, as well as increases in serum leptin, when mice were fed a HFS diet. Thus, leptin resistance resulting from hyperleptinemia due to obesity in pregnancy (Ladyman, 2008) may play a role in the observed hyperphagia on a HFS diet. There were no genotype specific differences in food intake regardless of diet, which corresponds with the absence of increased bodyweight or gestational weight gain. This

suggests that even in the absence of adiponectin, bodyweight, gestational weight gain and food consumption were largely diet dependent.

It is well established in human and rodent models that consumption of an obeseogenic diet is associated with decreases in circulating adiponectin (Cano et al., 2009; Pereira et al., 2015), and that the natural insulin resistance of late pregnancy is also accompanied by decreases in serum adiponectin. We measured circulating adiponectin in pregnant WT mice in early, middle and late gestation in order to determine the effect of HFS diet consumption on the normal pattern of adiponectin secretion (Figure 9D). In WT mice fed a LF diet, adiponectin remained high and decreased in the third trimester. However, when mice consumed a HFS diet, serum adiponectin was lower throughout gestation. This corresponds with data from human pregnancies, in which adiponectin naturally decreases towards the end of pregnancy. Thus, determination of adiponectin levels earlier in pregnancy (such as in women with pre-existing obesity) may be a biomarker for development of GDM later in pregnancy (Marilyn Lacroix et al., 2013).

Since GDM is diagnosed in pregnancy, it is important to differentiate between pre-existing hyperglycemia, or T2D that may exist prior to gestation. In order to ascertain that our model was appropriate for GDM fasting blood glucose was measured prior to breeding (Figure 9B). Adiponectin KO mice were not hyperglycemic prior to pregnancy relative to WT controls when fed either a LF or a HFS diet. This confirms that adiponectin deficiency was not causing hyperglycemia prior to gestation. During late pregnancy (third trimester), insulin resistance combined with obesity lead to hyperglycemia in WT mice fed a HFS diet (Figure 9E). Interestingly, in the third trimester adiponectin KO mice had higher fasting blood glucose levels than WT mice even when fed a LF diet (Figure 9E), and this increase in blood glucose was barely exacerbated by the addition of HFS diet feeding. This suggests that adiponectin deficiency is sufficient to cause hyperglycemia in pregnancy, even in the absence of obesity. In order to further characterize the metabolic phenotype of the pregnant adiponectin KO mice, glucose tolerance by GTT was assessed. Marked impairments in glucose tolerance were observed in adiponectin KO mice in the third trimester relative to WT controls (Figure 9F, 6G). Once again, although HFS fed animals had impaired glucose tolerance (Figure 9G) what was striking was that these impairments were evident in adiponectin KO animals fed a LF diet (Figure 9F), further emphasizing the role of adiponectin deficiency in glucose control in pregnancy.

Peripheral insulin resistance is a feature of late gestation, and a common compensatory mechanism of pregnancy is increased insulin secretion in order to maintain maternal metabolic homeostasis. When fasted serum insulin levels were measured in the third trimester, no differences between adiponectin KO and WT controls, and no appreciable effect of HFS diet feeding on either group was observed (Figure 9I). While hyperinsulinemia is a common phenomenon in T2D, individuals with GDM have been found to have impairments in insulin secretory capacity (Bowes et al., 1996; A. Kautzky-Willer et al., 1997) and lower serum insulin than healthy counterparts (C. Homko, E. Sivan, X. Chen, E. A. Reece, & G. Boden, 2001). While no effect of adiponectin deficiency on third trimester serum insulin in the fasted state was observed, other studies using adiponectin KO mice have shown reduced levels of circulating insulin in the fed state during pregnancy (L. Qiao et al., 2017). In the current study, serum was collected at the time of sacrifice and not collected at the same time of day. Although animals were fasted prior to serum collection, insulin shows circadian regulation, thus the diurnal expression pattern of insulin may contribute to

variability in serum insulin levels (Kalsbeek & Strubbe, 1998). When comparing results pertaining to insulin resistance and hyperinsulinemia in pregnancy from this study to those found in the literature, it is important to consider these experimental differences.

Impaired insulin tolerance in pregnant adiponectin KO mice relative to WT controls when fed a LF diet was also observed (Figure 9J) and we saw a similar impairment when both groups were fed a HFS diet (Figure 9K). Although adiponectin is widely considered to be involved in potentiating insulin signalling, there are conflicting results on whether adiponectin deficiency in pregnancy results in insulin resistance. Qiao and colleagues found that in an adiponectin knockout mouse model, there was no effect on glucose disposal or downstream insulin signalling as evidenced by a lack of any alteration to AKT phosphorylation in pregnant adiponectin KO (Liping Qiao et al., 2017). Insulin resistance in this study was measured by glucose disposal using the hyperinsulinimic-euglycemic clamp assay. Other studies using non-pregnant adiponectin knockout mice have observed impairments to peripheral insulin sensitivity using ITT (Kubota et al., 2002; Nawrocki et al., 2006) and the euglycemic clamp method (Yano et al., 2008). The hyperinsulinemic-euglycemic clamp method is widely considered a gold standard for assessing insulin sensitivity (J. K. Kim, 2009), preventing hypoglycemia that can occur during ITT and therefor may be a more accurate picture of whole-body insulin sensitivity without the interference of counter-regulatory hormones (Patarrão, Wayne Lautt, & Paula Macedo, 2014) in studies where this method was utilized (L. Qiao et al., 2017; Yano et al., 2008).

It is possible that the discrepancies observed regarding insulin sensitivity in adiponectin KO mice are due to differences in methods of assessment, as mechanistic studies implicate adiponectin in whole body insulin sensitivity as well as during adaptation to pregnancy (Awazawa et al., 2009; Awazawa et al., 2011; L. Qiao et al., 2017). Additionally, it is important to note that studies showing changes in insulin sensitivity without the added metabolic challenge of high fat feeding or pregnancy were performed in different adiponectin knockout mouse lines than those used in the present study or by Qiao *et al* (Kubota et al., 2002; K. Ma et al., 2002; Nawrocki et al., 2006; Liping Qiao et al., 2017). Nawrocki *et al* and Kubota *et al* generated their own adiponectin KO mice, rather than the strain available from The Jaxon Laboratory, originally generated by Chan *et al* ((K. Ma et al., 2002). Taken together, these differences in experimental procedures and animal models may help explain the discrepancies between the results observed in this study and the literature with regard to the level of insulin resistance and hyperinsulinemia in pregnant adiponectin KO mice.

An important role of insulin signalling is control of hepatic glucose output. Pregnant women with GDM had elevated hepatic glucose output relative to metabolically healthy women (Patrick M. Catalano, Larraine Huston, Saeid B. Amini, & Satish C. Kalhan, 1999). Qiao and colleagues observed increased hepatic glucose output at baseline, and impaired insulin-inhibited suppression in adiponectin KO mice in late gestation, indicating dysregulated gluconeogenesis (Liping Qiao et al., 2017). Pyruvate tolerance tests measure the production of endogenous glucose by the liver after an injected bolus of pyruvate. There were no differences in hepatic glucose production as measured by PTT in LF fed adiponectin KO mice relative to WT controls prior to pregnancy (Figure 11A). In the third trimester, however, adiponectin KO mice had significantly higher rates of hepatic glucose production from pyruvate compared to WT mice (Figure 11C). Adiponectin is thought to contribute to suppression of gluconeogenesis independent of

insulin (Zhou et al., 2005) and consequently the combination of adiponectin deficiency and insulin resistance in late gestation dysregulates hepatic gluconeogenesis and contributes to hyperglycemia during pregnancy.

Pregnant adiponectin KO mice appeared to have increased hepatic glucose output in the third trimester in combination with some degree of insulin resistance. Previous studies have shown that adiponectin regulates hepatic glucose output by reducing expression of genes responsible for gluconeogenesis (Zhou et al., 2005). Consistent with this, we showed that *Pck1* and *2* were upregulated in livers of adiponectin KO mice and were not suppressed in response to insulin administration. In *Pck1,* this was significant in comparison to saline treated samples. The PEPCK enzyme has a cytosolic and a mitochondrial variant, *Pck1* and *Pck2* respectively. In the rodent liver the cytosolic isoform appears to be responsible for the majority of the activity in rats (Wiese, Lambeth, & Ray, 1991). Additionally, overexpression of non-insulin responsive *Pck1* has been shown to result in resistance to suppression of gluconeogenesis (Rosella, Zajac, Kaczmarczyk, Andrikopoulos, & Proietto, 1993), and deletion of *Pck1* has been shown experimentally to improve glycemic control and hepatic dyslipidemia in the *db/db* mouse (Gómez-Valadés et al., 2008), implicating gluconeogenic control by PEPCK in hepatic metabolism. Increased *Pck1* and *Pck2* expression may suggest a role for elevated PEPCK expression in increased gluconeogenesis in pregnant adiponectin KO mice.

Gene expression can be modulated by transcription factors, and *Foxo1* is implicated in the control of gluconeogenic genes. Higher levels of FoxO1 increase insulin resistance and gluconeogenic gene expression (Y. Wang, Zhou, & Graves, 2014), often in response to exercise or fasting (Liping Qiao & Shao, 2006). Additionally, research done in other rodent models has shown that inhibition of FoxO1 improves metabolic homeostasis and glucose control, particularly in diabetic animals (Xie et al., 2018). In the liver, downstream signalling from insulin leads to AKT phosphorylation; AKT phosphorylates FoxO1 leading to exclusion from the nucleus and inhibition of activity (Y. Wu et al., 2018). In adiponectin KO mice, supplementation with insulin did not suppress *Foxo1* expression. In fact, there was a significant increase in *Foxo1* gene expression in the livers of pregnant KO mice relative to WT mice (Figure 11E). This is paired with the fact that we did not observe any changes in AKT phosphorylation in KO mice (Figure 11H) even in response to insulin administration. Correspondingly, there was a decrease in phosphorylation of FoxO1in adiponectin KO mice. Since insulin-induced AKT phosphorylation appears to be impaired in the third trimester in both groups it seems as though the downstream phosphorylation of FoxO1 is also inhibited. In combination with adiponectin deficiency in the third trimester, this may contribute to excessive gluconeogenesis and hyperglycemia in adiponectin knockout mice.

Collectively, these findings show that adiponectin KO mice develop hyperglycemia, impaired glucose and insulin tolerance and excessive hepatic glucose output in the third trimester of pregnancy that are characteristic GDM. Further, these disruptions occur in the absence of obesity, increased food consumption, excessive gestational weight gain or HFS diet feeding. Increases in hepatic gluconeogenic gene expression in adiponectin KO mice in the third trimester are observed. These elevations were persistent even in the presence of endogenous insulin administration, suggesting insulin resistance in adiponectin KO mice may contribute to unchecked gluconeogenesis. Insulin administration did not result in changes to AKT phosphorylation in WT or adiponectin KO mice. This could result in decreased phosphorylation of downstream targets of AKT, the net result of which is increased gluconeogenic gene

expression and hepatic glucose output. Overall, these results suggest that more severe insulin resistance in adiponectin KO mice in the third trimester leads to increased hepatic glucose output, hyperglycemia and impaired glucose tolerance that is characteristic of GDM and that this occurs independent of obesity.

While it is clear that adiponectin KO mice have impaired glucose tolerance and insulin sensitivity in pregnancy, it is less clear what the mechanisms are that lead to this dysregulation. Due to central role of the liver in glucose homeostasis, it has been postulated that increased hepatic fat deposition may increase insulin resistance and worsen metabolic adaptation in pregnancy. It is known that both T2D and GDM increase the risk for fatty liver development (Foghsgaard et al., 2017; Forbes et al., 2011; Prikoszovich et al., 2011). Additionally, since adiponectin has been shown to influence lipid metabolism as well as peripheral insulin sensitivity (Towler & Hardie, 2007; Viollet & Andreelli, 2011) it is possible that dysregulation of lipid metabolism or fatty liver development could be a key player in the development of GDM in adiponectin KO mice.

Chapter 4:

Lack of adiponectin leads to hepatic steatosis in pregnancy

4.1 Introduction

We are only beginning to understand the pathophysiological processes leading to the development of GDM. Advanced maternal age, pre-pregnancy overweight/obesity and insulin resistance are recognized risk factors for GDM. It is also increasingly recognized that hepatic fat deposition and abdominal adiposity in early pregnancy predict glucose intolerance in mid-pregnancy (De Souza et al., 2016). Prospective cohort studies have shown that the presence of elevated visceral adipose tissue together with sonographically detectable hepatic fat predicted GDM independent of maternal age, ethnicity and BMI (De Souza et al., 2016; S. Lee et al., 2019). The prevalence of NAFLD among women of childbearing age is estimated to be 10% (Hershman, Mei, & Kushner, 2019). While GDM has been identified as a risk factor for the development of NAFLD (Ajmera et al., 2016), the mechanisms linking NAFLD to the development of GDM remain to be determined.

Adipokines are bioactive hormones that are secreted primarily from adipocytes. As outlined in Chapter 1, adiponectin is one of the best characterized adipokines and sensitizes tissues to the actions of insulin (A. H. Berg et al., 2002). In the liver, adiponectin stimulates fatty acid oxidation and decreases *de novo* lipogenesis (Awazawa et al., 2009; G. Chen, Liang, Ou, Goldstein, & Brown, 2004). The degree of adiponectin secretion is reduced with elevated adiposity and even further following a diagnosis of dysglycemia (Yeli Wang et al., 2018). In human studies, low circulating adiponectin is associated with NAFLD (Bugianesi et al., 2005; Pagano et al., 2005), and rodent studies show that adiponectin knockout mice develop more severe high fat diet-induced hepatic steatosis (Asano et al., 2009). Increasing circulating adiponectin levels in rodent models of obesity attenuates hepatic steatosis (A. Xu et al., 2003). Clinical data suggests reduced adiponectin levels in the first trimester of pregnancy are an independent predictor for the development of GDM (Williams et al., 2004). Interestingly, maternal plasma adiponectin correlated with both the severity of fatty liver disease and the risk of developing GDM (S. Lee et al., 2019). While these associations are compelling, it has yet to be determined if fatty liver is a determining factor in the development of GDM, and additionally if adiponectin deficiency plays a mechanistic role.

We hypothesized that adiponectin deficiency can contribute to increased hepatic lipid deposition, more severe hepatic insulin resistance and ultimately higher risk for GDM. In this chapter we analyzed liver tissue from adiponectin KO and WT mice to determine whether adiponectin deficiency contributes to the development of fatty liver in the third trimester. Mechanisms that can lead to the buildup of lipids in the liver can include increased lipid uptake, decreased lipid oxidation and increased *de novo* lipogenesis, many of which are influenced by adiponectin signalling. We performed experiments on isolated primary hepatocytes to measure oxidative capacity of hepatic mitochondria utilizing both glucose and fatty acids, as well as the rate of fatty acid uptake and triglyceride synthesis by radiolabeled precursors, [^{14}C] acetate and [^{14}C] oleate. Additionally, we performed gene expression analysis on liver tissue to compare levels of lipogenic genes between adiponectin KO and WT control mice in the third trimester.

We determined that adiponectin KO mice develop hepatic steatosis in the third trimester, even when fed a LF diet. These mice have marked reductions in mitochondrial metabolism, specifically β-oxidation. Primary hepatocytes from adiponectin KO mice show increased triglyceride synthesis and secretion in the third trimester, and

hepatic gene expression analysis shows increased lipogenic gene expression relative to WT controls. These findings highlight the links between adiponectin levels, hepatic steatosis during pregnancy and the development of GDM.

4.2 Materials and Methods – Refer to Page 24

4.3. Results

4.3.1 Development of fatty liver

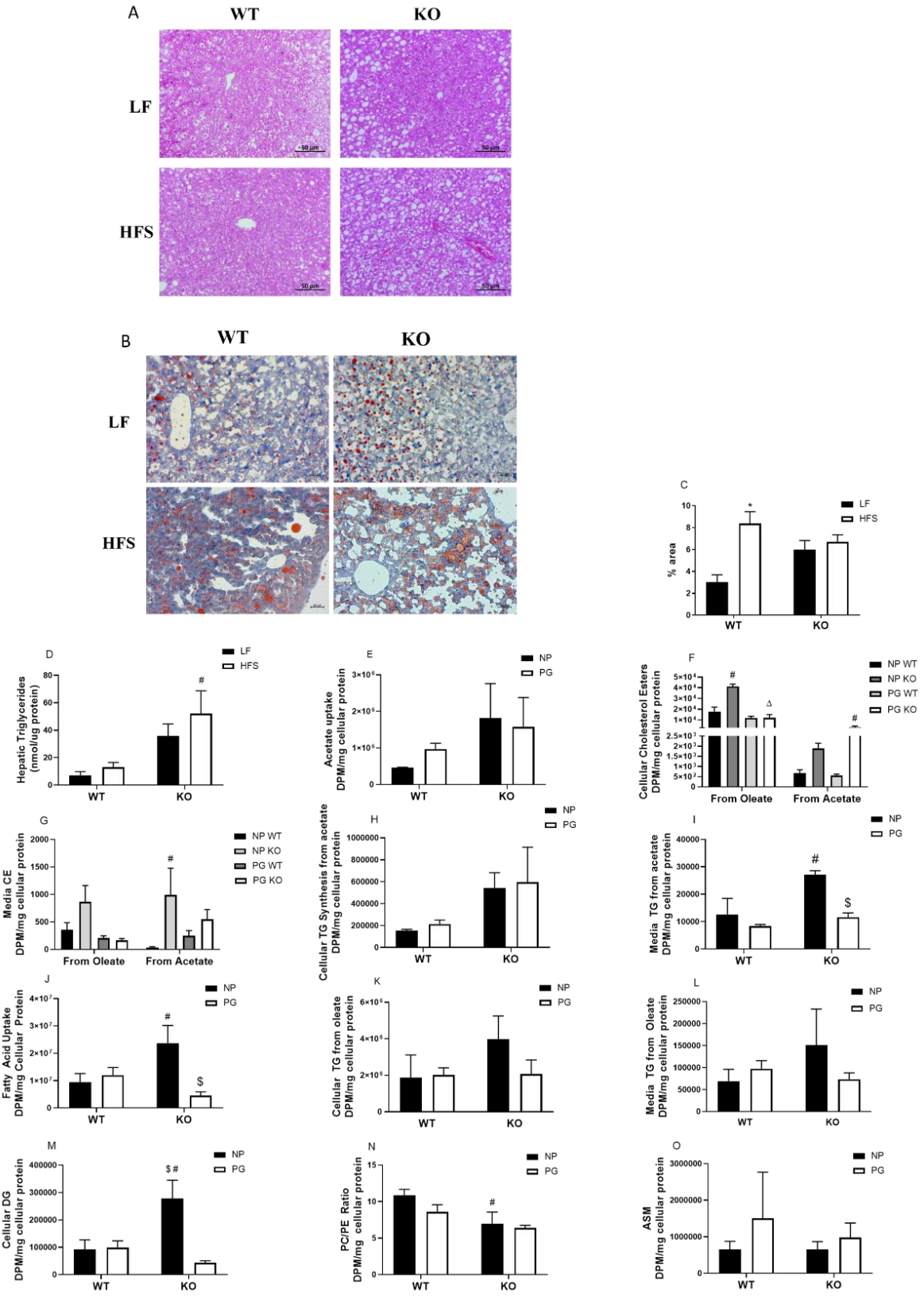
Increased lipid utilization is a hallmark of late gestation. Since adiponectin is involved in the control of lipogenesis and fatty acid oxidation, we investigated the impact of adiponectin deficiency on hepatic lipid content during pregnancy. To this end we performed a histological and biochemical analysis of liver tissues isolated at the end of the third trimester. Consumption of a HFS diet increased hepatic fat deposition in pregnant WT mice (Figure 12A, B, C). HFS diet feeding in the third trimester lead to significantly increased steatosis score relative to LF diet in WT mice (Table 7). Notably, adiponectin KO mice developed significant hepatic steatosis even on a LF diet in the third trimester, relative to WT mice (Table 7). Steatosis was accompanied by a corresponding increase in hepatic TG content (Figure 12D). LF-fed Adiponectin KO mice also showed a trend towards increased triglyceride content, with roughly 2-3 fold higher hepatic TG relative to WT (Figure 12D), and HFS diet consumption lead to significant increase in hepatic TG content relative to WT. These results indicate that while HFS diet feeding predictably increased hepatic fat deposition in WT pregnant mice, adiponectin KO mice were sensitive to the development of histologically detectable hepatic steatosis during pregnancy, even without HFS feeding.

Table 7: Liver weight and steatosis score in third trimester pregnant mice.

	Wildtype		**Knockout**	
Diet	**LF**	**HFS**	**LF**	**HFS**
Liver Weight (mg)	1747.90 ± 112.00	1524.3 ± 127.40	1687.30 ± 139.10	1551.10 ± 55.80
Steatosis Score	0.60 ± 0.20	2.30 ± 0.42*	1.65 ±. 0.26#	2.46 ± 0.18

Consumption of a HFS diet increased hepatic steatosis in adiponectin KO and WT mice in the third trimester, and pregnant adiponectin KO mice developed hepatic steatosis on a LF diet. p values represent significance (<0.05) after Two-way ANOVA. *p<0.05 LF vs HFS; #p<0.05 WT vs KO. WT: Wildtype, KO: Knockout, LF: Low fat, HFS: High Fat-Sucrose

Figure 12: Adiponectin KO mice develop hepatic steatosis accompanied by increased *de novo* lipogenesis in the third trimester of pregnancy



A) Representative images of H&E stained liver sections from third trimester pregnant adiponectin KO and WT mice, 20X magnification. B) Representative images of Oil Red O stained liver sections from third trimester pregnant adiponectin KO and WT mice C) percent area stained with Oil Red O, 40X magnification. N=5-7 D) hepatic triglyceride content from LF and HFS fed adiponectin KO and WT mice at the end of the third trimester. N=5-7 E) [^{14}C] acetate uptake by primary hepatocytes isolated from LF fed adiponectin KO and WT mice prior to pregnancy (NP) in the third trimester (PG). Cellular cholesterol esters synthesized from [^{14}C] acetate and [^{14}C] oleate(F) and secreted into the media (G) from [^{14}C] acetate (H) and [^{14}C] oleate by primary hepatocytes isolated from LF fed NP and PG adiponectin KO and WT mice. H) Cellular triglyceride synthesis and secretion into media(I) from [^{14}C] acetate by primary hepatocytes from LF fed NP and PG adiponectin KO and WT mice. J) Fatty acid uptake as measured by [^{14}C] oleate incorporation into primary hepatocytes isolated from LF fed NP and PG adiponectin KO and WT mice. Cellular triglyceride synthesis (K) from [^{14}C] oleate and secretion into media (L) by primary hepatocytes isolated from LF fed NP and PG adiponectin KO and WT mice. M) Cellular diacylglycerol synthesis from [^{14}C] oleate by primary hepatocytes isolated from LF fed NP and PG adiponectin KO and WT mice. N) Ratio of synthesized cellular phosphatidylcholine to phosphatidylethanolamine (PC/PE) from [^{14}C] oleate by primary hepatocytes isolated from LF fed NP and PG adiponectin KO and WT mice. O) Acid soluble metabolites (ASM) synthesized from [^{14}C] oleate and secreted into the media by primary hepatocytes isolated LF fed NP and PG adiponectin KO and WT mice. Primary hepatocyte experiments N=2-7 by group. p values represent significance (<0.05) after Two-way ANOVA. *p<0.05 LF vs HFS; #p<0.05 WT vs KO; $p<0.05 NP vs PG. WT: Wildtype, KO: Knockout, LF: Low Fat, HFS: High fat sucrose, NP: non-pregnant, PG: pregnant.

4.3.2 Assessment of fatty acid uptake and metabolism

In order to determine the underlying cause for the buildup of lipids in the liver, primary hepatocytes were isolated from LF fed WT and adiponectin KO mice both prior to pregnancy and in the third trimester. Acetate is metabolized into acetyl-CoA which is crucial for biosynthetic pathways including lipid synthesis. In addition, increased uptake of acetate into hepatocytes is associated with the development of NAFLD (Nejabat et al., 2018). Measuring the uptake of oleate, a long chain fatty acid, will allow for the determination of the capacity of primary hepatocytes to take up fatty acids from the periphery as well as package them for storage and export, or oxidation (Wasfi, Weinstein, & Heimberg, 1980). Hepatocytes from adiponectin KO mice fasted for 1 hour showed a trend towards higher rates of [^{14}C] acetate uptake prior to and during pregnancy compared to WT controls (Figure 12E). The cellular synthesis of cholesterol esters (Figure 12F) from both acetate and oleate was significantly increased in non-pregnant adiponectin KO mice relative to WT. In contrast, in pregnant adiponectin KO mice cholesterol ester synthesis was decreased to levels similar to that of WT mice.

In non-pregnant adiponectin KO mice, there was an increase in the secretion of cholesterol esters synthesized from acetate compared to non-pregnant WT mice (Figure 12D). However, cholesterol ester secretion was suppressed in hepatocytes from pregnant adiponectin KO mice (Figure 12G). Despite apparent changes in rates of synthesis, the secretion of cholesterol esters synthesized from oleate was unaltered between pregnant adiponectin KO and WT mice (Figure 12I). It is worth noting that there were no significant differences in circulating cholesterol esters between WT and adiponectin KO mice (Table 8). In addition, circulating free fatty acids were reduced in pregnant adiponectin KO mice on a LF diet compared to WT controls (Table 8). Further, while insulin administration lead to reduced circulating free fatty acids (due to suppression of lipolysis) in WT mice, this was not observed in adiponectin KO mice indicating increased insulin resistance in pregnant adiponectin KO mice.

Table 8: Third trimester circulating lipids

	Wildtype			**Adiponectin Knockout**		
	Low Fat	Low Fat + insulin	High Fat Sucrose	Low Fat	Low Fat +insulin	High Fat Sucrose
Cholesterol (mg/dL)	51.1± 7.3		81.5 ±13.1	54.4 ± 5.4		75.1 ± 15.4
Free Fatty Acid (nmol/L)	33.0± 6.0	12.0±3.0 **	19.0 ± 3.0	16.0±3.0#	24.0±8.0	23.0 ± 5.0
Triacylglycerol (mg/dL)	24.3± 6.0		23.7 ± 6.1	36.4±8.4		28.5 ± 4.9

Adiponectin KO mice were not significantly hyperlipidemic in the third trimester. However, HFS diet feeding increased serum cholesterol in both adiponectin KO and WT mice and adiponectin KO mice have impaired insulin sensitivity due to impaired suppression of lipolysis after insulin administration resulting in increased serum free fatty acids. P Values represent significance ($p<0.05$) after Two-way ANOVA. #$p < 0.05$ between WT and adiponectin KO mice, ** $p<0.05$ between saline and insulin treated mice.

Due to the observed increases in triglyceride content in livers of adiponectin KO mice in pregnancy, triglyceride synthesis in primary hepatocytes was examined. Adiponectin KO mice exhibited elevated cellular synthesis of triglycerides from acetate in the third trimester relative to WT mice, but this elevation was not observed in non-pregnant adiponectin KO mice (Figure 12H). Secretion of TG into the media followed a similar pattern with increased TG secretion observed from pregnant adiponectin KO mice relative to WT mice and to non-pregnant adiponectin KO mice (Figure 12I). In hepatocytes isolated from non-pregnant mice, [^{14}C] oleate uptake was unchanged from WT mice and was significantly reduced relative to adiponectin KO mice in pregnancy (Figure 12J). Cellular synthesis of triglycerides from oleate followed a similar pattern as oleate uptake, but these changes were not significant (Figure 12K). Interestingly, secretion of triglycerides synthesized from oleate and secreted into the media showed a downward trend during pregnancy in both adiponectin KO and WT mice relative to hepatocytes from non-pregnant mice (Figure 12L). Diacylglycerol (DG) synthesis from oleate was increased in hepatocytes from non-pregnant adiponectin KO mice compared to non-pregnant WT mice and was unaltered in pregnancy (Figure 12M). Overall, adiponectin KO mice fed a LF diet had increased hepatic *de novo* lipid synthesis from acetate in the third trimester, and lipid synthesis from oleate was increased in non-pregnant animals relative to WT controls.

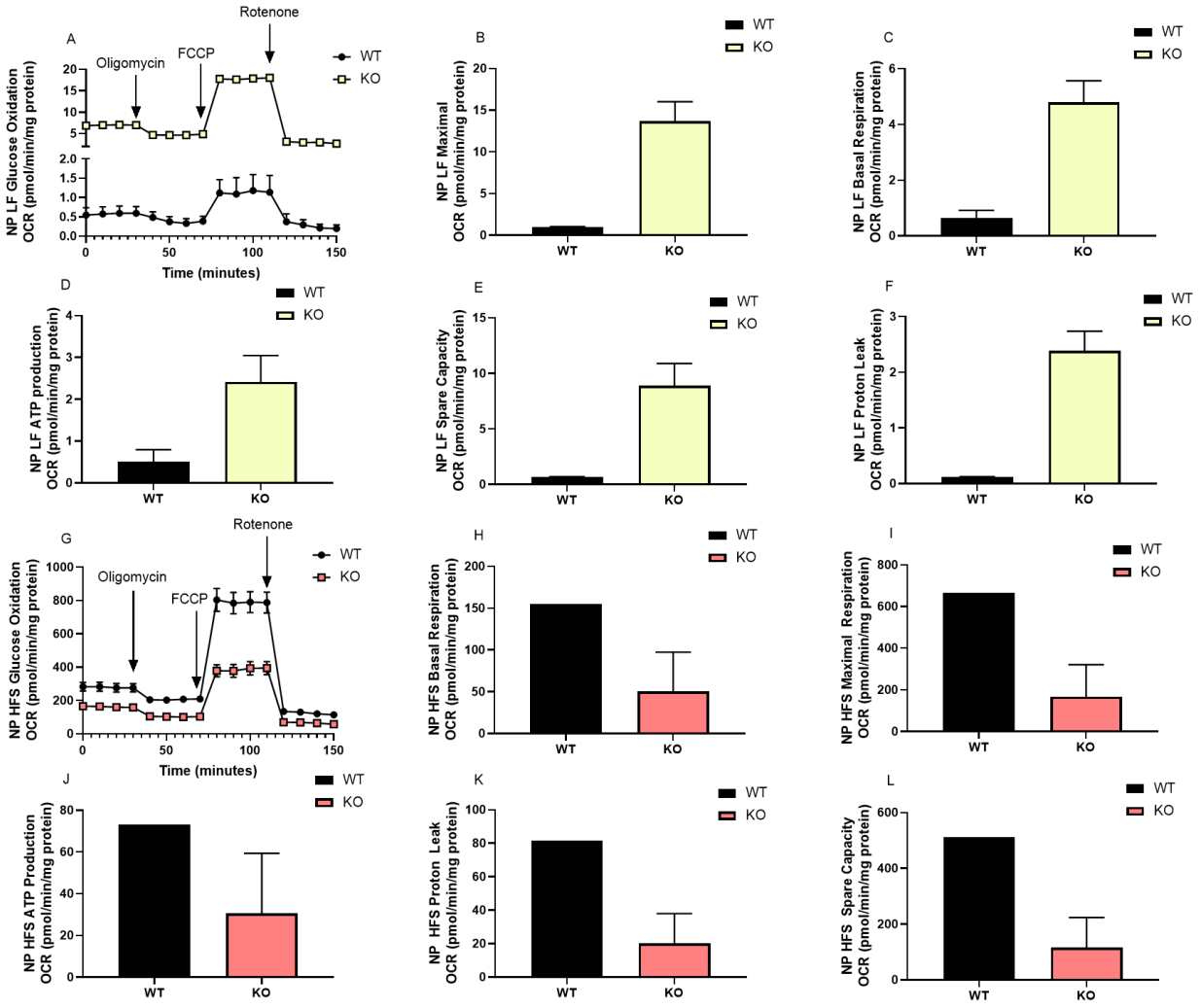
Phospholipids are crucial to cellular integrity, and levels of the major cellular membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and the ratio of PC to PE are associated with the development of NAFLD and progression to steatohepatitis and vulnerability to injury or cell death (Z. Li et al., 2006). Studies in human patients with NAFLD found reduced hepatic PC/PE ratio relative to controls (Arendt et al., 2013). Similarly, adiponectin KO mice had a reduced PC/PE ratio in primary hepatocytes relative to WT controls (Figure 12N). Labelling with [^{14}C] oleate allows for the detection of acid soluble metabolites (ASM), a by-product of fatty-acid oxidation. Prior to pregnancy there were no differences in levels of ASM between adiponectin KO and WT controls (Figure 12Q). However, in the third trimester there is a trend towards decreased ASM in adiponectin KO

mice relative to WT (Figure 12Q). This may correspond to reduced fatty acid oxidation in the third trimester of pregnancy, when fatty acids are the primary source of maternal energy as glucose is spared for fetal growth.

4.3.3 Mitochondrial energy metabolism

Given the evident hepatic steatosis in combination with increased incorporation of fatty acids into triglycerides, the impact of adiponectin deficiency in pregnancy on hepatocyte glucose and fatty acid oxidation was examined. Prior to pregnancy, there was a trend towards increased uptake of [^{14}C] acetate (Figure 12E) and [^{14}C] oleate (Figure 12J) by primary hepatocytes from adiponectin KO mice. In concert with increased acetate uptake and lipid synthesis, primary hepatocytes isolated from non-pregnant adiponectin KO mice fed a LF diet had elevated oxygen consumption rate (OCR) relative to WT controls (Figure 13A). This included a marked increase in maximal (Figure 13B) and basal respiration (Figure 13C) when utilizing glucose. ATP production from glucose (Figure 13D) and spare capacity (Figure 13E) were unaltered. In contrast, proton leak (Figure 13F) was increased relative to WT controls. However, when non-pregnant adiponectin KO mice were fed a HFS diet, their overall mitochondrial respiratory capacity was inhibited relative to WT controls (Figure 13G), although these results are not conclusive due to low sample number. Consumption of a HFS diet did not markedly affect basal respiration (Figure 13H), maximal respiration (Figure 13I), ATP production (Figure 13J), spare capacity (Figure 13K) and proton leak (Figure 13L) relative to WT mice. These data suggest that the addition of a metabolic stress adds strain to the respiratory capacity of adiponectin KO mice, and that pregnancy may induce similar deficits.

Figure 13: Adiponectin KO mice have elevated hepatic mitochondrial respiration prior to pregnancy, which is impaired by the addition of a HFS diet.

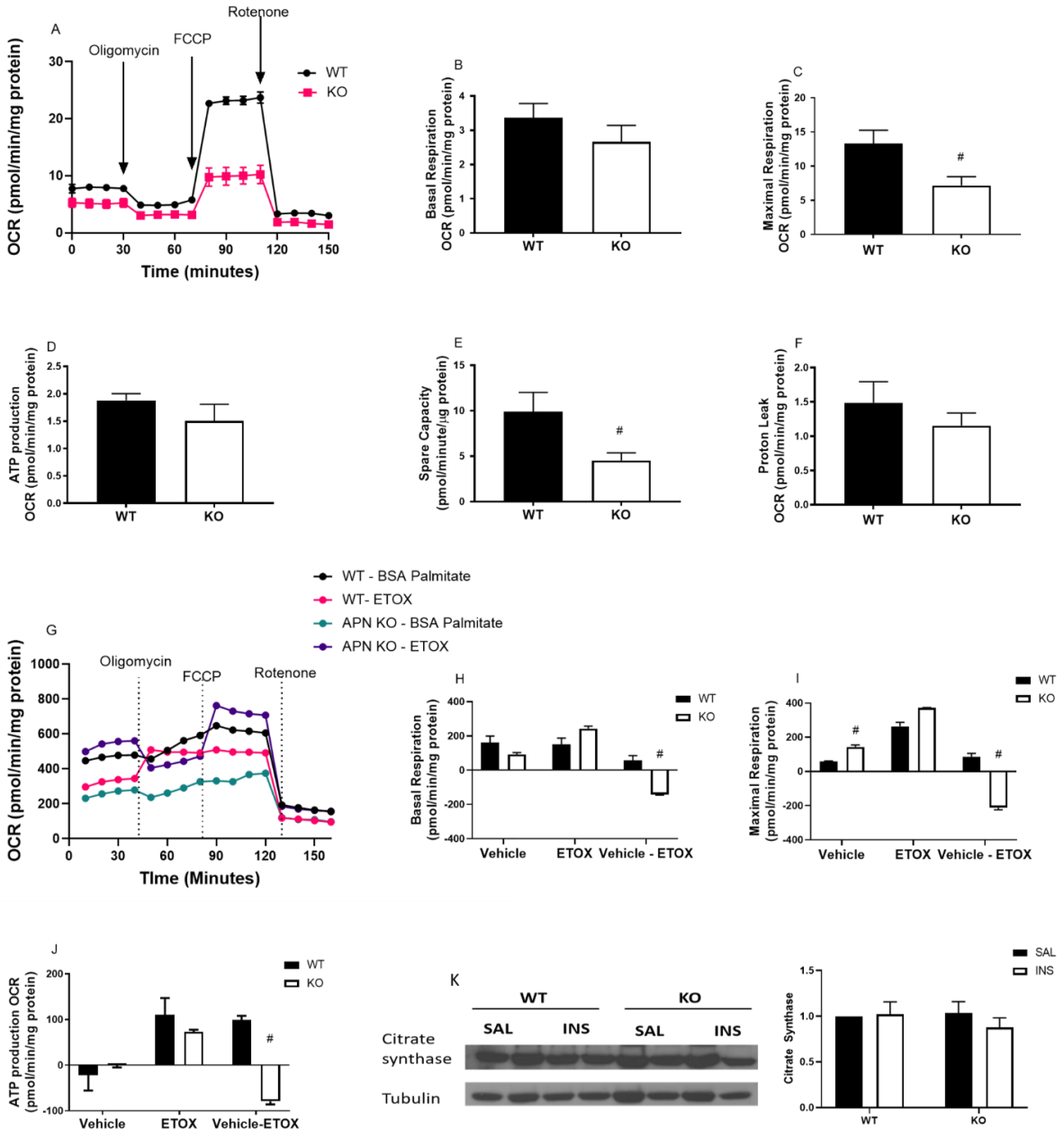


Hepatocytes were isolated from LF fed adiponectin KO and WT mice older than 12 weeks, prior to pregnancy. A) Representative trace of oxygen consumption rate (OCR) over time by primary hepatocytes utilizing glucose, normalized to cellular protein. N=4-11 wells per group. B) Basal respiration C) Maximal (FCCP uncoupled) respiration D) oligomycin-sensitive OCR (ATP production) E) Spare Capacity and F) Proton leak by primary hepatocytes utilizing glucose. N= 2-5 per group. Hepatocytes were isolated from HFS fed adiponectin KO and WT mice older than 12 weeks, prior to pregnancy. G) Representative trace of oxygen consumption rate (OCR) over time by primary hepatocytes utilizing glucose, normalized to cellular protein. N=11 wells per group. H) Basal respiration I) Maximal (FCCP uncoupled) respiration J) oligomycin-sensitive OCR (ATP production) K) Proton Leak and L) Spare Capacity in primary hepatocytes utilizing glucose. N=1-2 per group.

Hepatocytes isolated from adiponectin KO mice had reduced OCR relative to WT controls in the third trimester when using glucose as primary energy source (Figure 14A). There was no difference in basal respiration in hepatocytes from adiponectin KO and WT mice in the third trimester (Figure 14B). In contrast, hepatocytes from pregnant adiponectin KO mice showed significant impairments in maximal respiratory capacity (Figure 14C) and

spare capacity (Figure 14E) relative to WT controls. ATP production (Figure 14D) and proton leak (Figure 14F) were unaltered. These findings suggest there may be an impaired capacity of primary hepatocytes to handle increased metabolic stress.

Figure 14: Hepatic fatty acid mitochondrial respiration is impaired in adiponectin KO mice in the third trimester.



A) Representative trace of oxygen consumption rate (OCR) over time by primary hepatocytes utilizing glucose, normalized to cellular protein. N=10-11 wells per group. B) Basal respiration C) Maximal (FCCP uncoupled) respiration D) ATP production (oligomycin sensitive) E) Spare Capacity and F) Proton Leak by primary hepatocytes utilizing glucose. N=4-6. p values represent significance ($p<0.05$) as determined by unpaired t-test. G) Representative trace of OCR over time (vehicle-etomoxir) by primary hepatocytes utilizing fatty acids, normalized to cellular protein. N=6 wells per group. H) Basal respiration I) Maximal (FCCP uncoupled) respiration and J) oligomycin-sensitive

OCR (ATP production) by primary hepatocytes when respiring utilizing all substrates available (vehicle), all substrates with fatty acid oxidation inhibited (ETOX), and fatty acid oxidation only (vehicle-ETOX) N=4-6. p values represent significance ($p<0.05$) as determined by unpaired t-test K) Hepatic citrate synthase protein expression normalized to tubulin from livers of adiponectin KO and WT mice N=5-6. p values represent significance ($p<0.05$) as determined by Two-way ANOVA. #$p<0.05$ WT vs KO. SAL = saline; INS = insulin. VEH = vehicle, ETOX = etomoxir. WT: Wildtype, KO: Knockout.

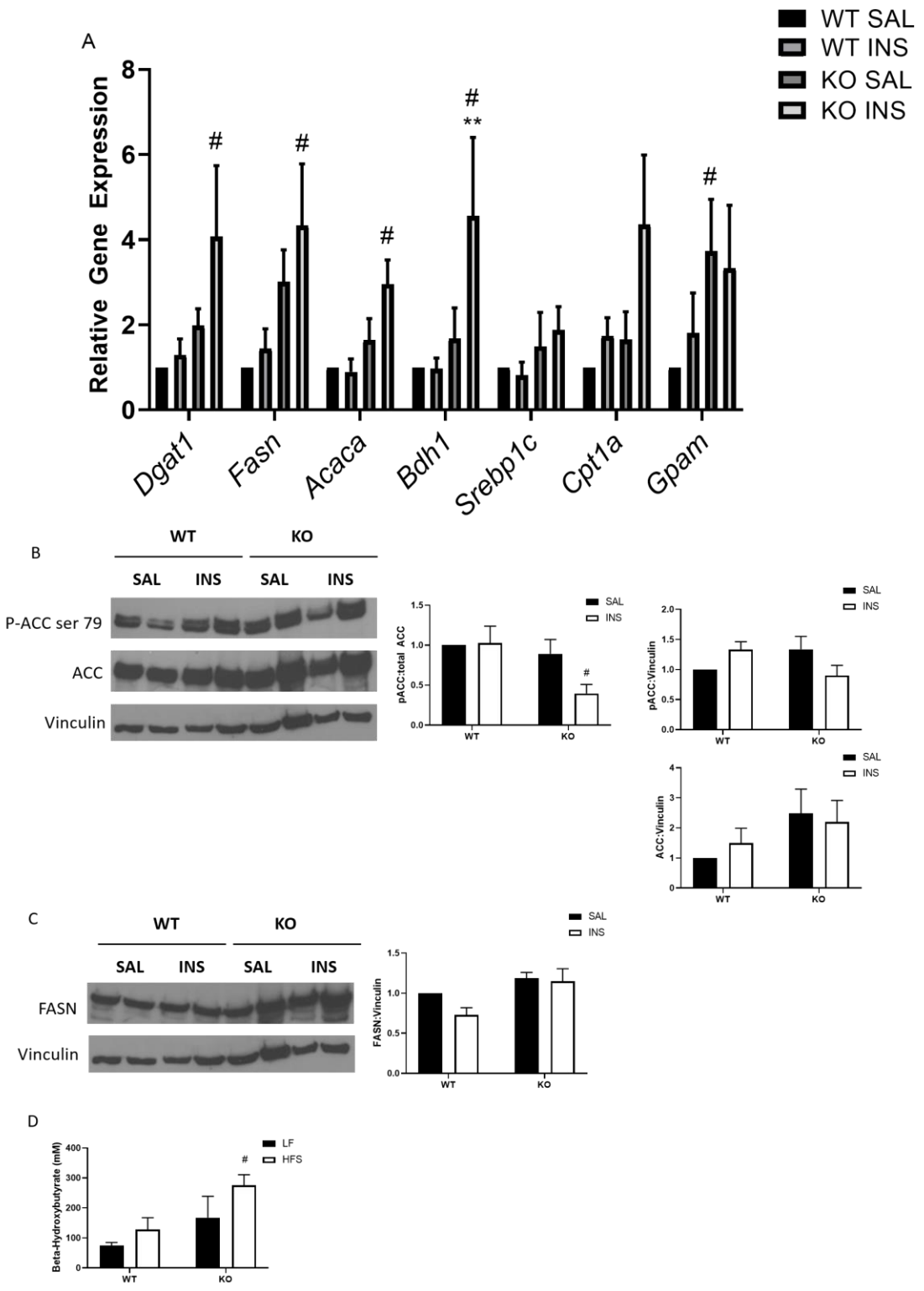
It was next determined whether adiponectin deficiency altered hepatocyte fatty acid oxidation in the third trimester. Primary hepatocytes were isolated from LF fed adiponectin KO and WT mice in the third trimester of pregnancy. OCR was examined in the presence of etomoxir, an inhibitor of mitochondrial CPT-1, and media containing BSA-palmitate as fatty acid substrate with only low levels of other energetic substrates. Etomoxir inhibits mitochondrial uptake of fatty acids, and changes in oxygen consumption related to inhibition of β-oxidation provide information regarding oxidative capacity. When utilizing fatty acids as a substrate (represented as VEH-ETOX), hepatocytes from pregnant adiponectin KO mice showed marked impairments in basal (Figure 14H) and maximal (Figure 14I) respiration, and their ability to produce ATP was blunted dramatically (Figure 14J). These impairments were more severe than those observed when hepatocytes were respiring using all available substrates (Figure 14A-12F). These findings suggest that adiponectin deficiency in pregnancy has a negative impact on hepatic lipid metabolism, including fatty acid oxidation. These deficits do not appear to be a consequence of alterations of mitochondrial content since changes in levels of citrate synthase were not observed between WT and adiponectin KO mice (Figure 14K).

4.3.4 Lipogenesis and Ketogenesis

Having observed hepatic steatosis and increased *de novo* lipogenesis from acetate in the hepatocytes of pregnant adiponectin KO mice, the expression of genes involved in key steps of lipid synthesis were examined in the liver of these animals. These included fatty acid synthase (*Fasn),* glycerol-3-phosphate acyltransferase - mitochondrial (*Gpam),* diacylglycerol transferase-1 (*Dgat1),* acetyl-CoA carboxylase (*Acaca)* and sterol regulatory element binding protein-1c (*Srebp1c)* (Figure 15A). *Gpam* expression was elevated in the livers of pregnant adiponectin KO mice compared to WT animals (Figure 15A). Adiponectin KO mice given insulin prior to sacrifice had elevated levels of lipogenic gene expression (*Fasn, Acaca, Dgat1)* relative to WT animals given insulin prior to sacrifice (Figure 15A).

Since experiments in primary hepatocytes showed impaired fatty acid oxidation in pregnant adiponectin KO mice, the expression of *Cpt1a* was measured in livers of pregnant WT and adiponectin KO mice (Figure 15A). Interestingly, insulin administration increased expression of *Cpt1a* in pregnant adiponectin KO mice but not WT controls (Figure 15A), although this was not significant. β-hydroxybutyrate dehydrogenase (*Bdh1*) expression in the livers of pregnant adiponectin KO mice was increased in pregnant adiponectin KO mice administered insulin prior to sacrifice compared to WT controls, implicating elevated ketogenesis (Figure 15A). The levels of circulating β-hydroxybutyrate in the serum were then determined. Serum β-hydroxybutyrate was increased in pregnant adiponectin KO mice that consumed a HFS diet compared to WT controls (Figure 15D). These data suggest that insulin suppression of ketogenesis in adiponectin KO mice may be impaired, and this corresponded with the increased *Bdh1* gene expression observed in pregnant adiponectin KO mice treated with insulin.

Figure 15: Adiponectin deficiency is associated with increased liver lipogenic gene expression and ketogenesis in the third trimester of pregnancy.



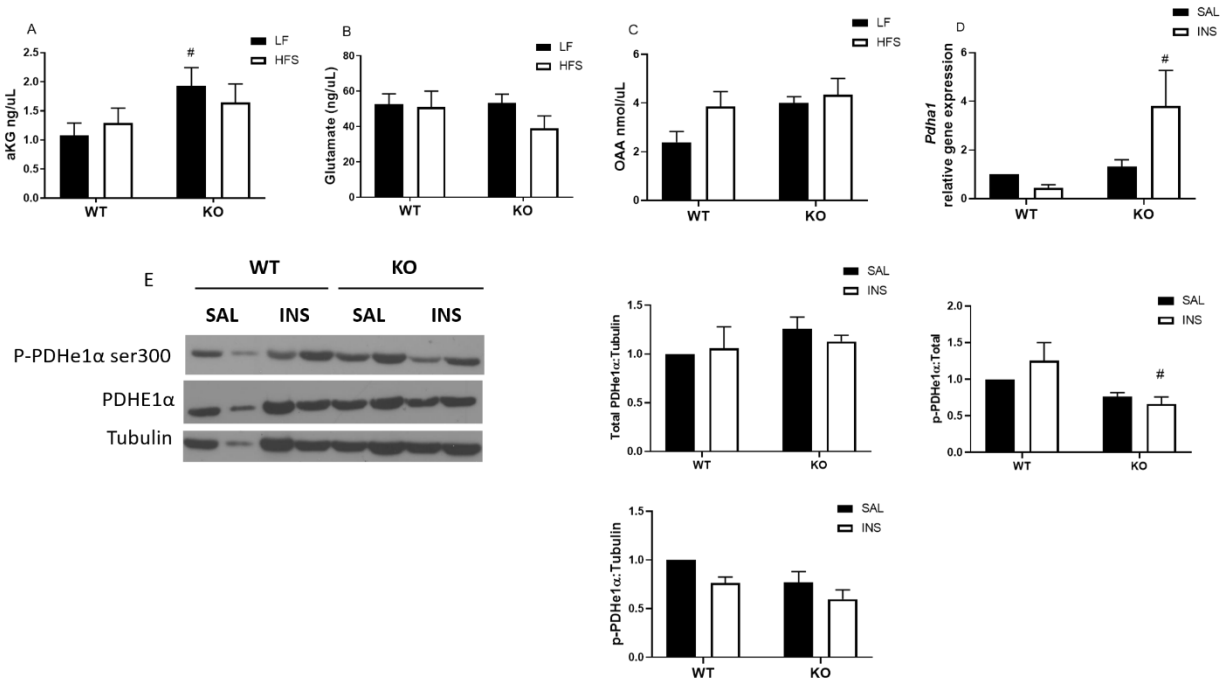
A) Gene expression analysis from liver tissue collected at sacrifice from LF fed adiponectin KO and WT mice at the end of the third trimester. Gene expression is relative to geomean of *Rn18s, B2m, RPLP-1* and *hprt1* and normalized to LF WT SAL. N=5-8 per group. B) Protein expression of phosphorylated ACC serine 79 to total ACC relative to vinculin and normalized to WT SAL, from liver tissue of LF fed adiponectin KO and WT mice collected at the end of the third trimester N=6 per group. C) Protein expression of FASN relative to vinculin and normalized to WT SAL, from liver tissue of LF fed adiponectin KO and WT mice collected at the end of the third trimester. N=6 per group. D) Serum β-hydroxybuyrate from pregnant adiponectin KO and WT mice in the third trimester. N=6 per group. *B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *Hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *RPLP-1:* 60S acidic ribosomal protein large P1, *Dgat1*: diacyglycerol transferase-1, *Fasn*: fatty acid synthase, *Acaca*: acetyl-CoA carboxylase, *Bdh1*: βhydroxybutyrate dehydrogenase -1, *Srebp1c:* sterol regulatory element binding protein-1c, *Cpt1a*: carnitine palmitoyl transferase-1a, *Gpam:* glycerol-3-phosphate acyltransferase – mitochondrial. SAL = saline; INS = insulin, WT: Wildtype, KO: Knockout p values represent significance ($p<0.05$) as determined by Two-way ANOVA; #$p<0.05$ WT vs KO. **$p<0.05$ SAL vs INS. LF = low fat

Next, the protein expression of a selected lipogenic genes was examined. Since post-translational modifications can impact protein activity, the levels of the inhibitory phosphorylation of ACC1 at Serine 79 in liver tissue was determined. Phosphorylation of ACC1 at Serine 79 was reduced in pregnant adiponectin KO mice administered insulin prior to sacrifice compared to WT controls (Figure 15B). In addition, total levels of hepatic ACC (Figure 15B) and expression of FASN appeared to be unaltered with insulin administration, although FASN expression shows a trend towards increased expression in adiponectin KO mice relative to WT controls (Figure 15C). These results suggest that pathways involved in lipogenesis and fatty acid synthesis are upregulated in adiponectin KO mice relative to WT controls during pregnancy. These changes are observed at the level of gene expression and to a lesser extent in altered protein synthesis. Increases to hepatic lipogenesis were accompanied by a decrease in fatty acid oxidation. These factors combined could lead to the increased hepatic fat deposition observed in adiponectin KO mice in the third trimester.

4.3.5 TCA cycle

Complete oxidation of fatty acids to ATP requires cycling through the TCA cycle. Since adiponectin KO mice have significant impairments in β-oxidation in the third trimester, as well as increased lipogenesis and ketogenesis, it is possible that fatty acids are incompletely or inefficiently oxidized, and this may be accompanied by TCA cycle dysfunction. The levels of hepatic α-ketoglutarate (α-KG), a key TCA cycle intermediate which has been shown to be upregulated in human models of NAFLD (Aragonès et al., 2016; Clarke et al., 2014) were examined. Hepatic α-KG content from LF fed third trimester adiponectin KO mice was elevated relative to WT controls (Figure 16A). HFS diet consumption did not affect hepatic α-KG content in either WT or adiponectin KO animals (Figure 16A). In the liver, α-KG can be produced from glutamate by glutamate dehydrogenase, combining amino acid metabolism and gluconeogenesis (Karaca et al., 2018). Adiponectin KO mice did not have altered hepatic glutamate relative to WT mice in the third trimester (Figure 16B). The levels of OAA determine the rate at which the TCA cycle functions (Kuang et al., 2018). Hepatic OAA levels were unaltered in all groups of animals (Figure 16C).

Figure 16: Adiponectin KO mice have reduced activity of hepatic TCA cycle in the third trimester relative to WT controls.



A) Hepatic α-ketoglutarate content from LF and HFS fed adiponectin KO and WT mice, livers tissue collected at sacrifice at the end of the third trimester. N= 6-9 per group. B) Hepatic glutamate content from LF and HFS fed adiponectin KO and WT mice, livers tissue collected at sacrifice at the end of the third trimester. N=5-7 per group. C) Hepatic oxaloacetate (OAA) content from LF and HFS fed adiponectin KO and WT mice, livers tissue collected at sacrifice at the end of the third trimester. N=5-6 per group. D) Hepatic gene expression of *Pdha1* relative to geomean of *Rn18s, B2m, RPLP-1* and *Hprt1* and normalized to LF WT SAL. E) Protein expression of phosphorylated PDHE1α serine 300 to total PDHE1α, relative to tubulin and normalized to WT SAL, from liver tissue of LF fed adiponectin KO and WT mice collected at the end of the third trimester. N=4-6 per group. *B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *Hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *RPLP-1:* 60S acidic ribosomal protein large P1, *Pdha1*: pyruvate dehydrogenase a1. SAL = saline; INS = insulin, WT: Wildtype, KO: Knockout p values represent significance ($p<0.05$) as determined by Two-way ANOVA. #$p<0.05$ WT vs KO.

The pyruvate dehydrogenase (PDH) complex regulates conversion of pyruvate into acetyl CoA as part of glucose catabolism, linking glycolysis to the TCA cycle (Holness & Sugden, 2003). Hepatic gene expression of *Pdha1* in livers isolated from all groups of mice in the third trimester of pregnancy was determined. Adiponectin KO mice had elevated *Pdha1* gene expression compared to WT mice after insulin administration (Figure 16D). Regulation of PDH e1α by inhibitory phosphorylation at serine 300 can impact how substrates are utilized in the hepatic mitochondria (Sungmi Park et al., 2018). In correlation with the increased gene expression, inhibitory phosphorylation of PDH is decreased in the livers of pregnant adiponectin KO mice relative to WT controls after insulin administration (Figure 16E). These data suggest that both expression and activity of PDH are increased in adiponectin KO mice in the third trimester of pregnancy, which is consistent with high glucose conditions (Holness & Sugden, 2003). Taken together these data demonstrate of buildup of some key TCA cycle intermediates which accompany a reduced β-

oxidation in pregnant adiponectin KO mice. This may indicate that reduced TCA cycle function contributes to a potential bottleneck in hepatic mitochondrial energy metabolism, with a subsequent development of hepatic steatosis in the third trimester of pregnant adiponectin KO mice.

4.4 Discussion/Conclusions

The aim of this chapter was to determine whether the development of fatty liver contributed to more severe insulin resistance in pregnancy and lead to hyperglycemia in adiponectin KO mice. There is evidence to implicate the buildup of intracellular lipids in ectopic tissues in the pathogenesis of T2D and insulin resistance (Bachmann et al., 2001; Oakes, Cooney, Camilleri, Chisholm, & Kraegen, 1997; Roden, 2006). Compared to other metabolic tissues, the excess accumulation of hepatic lipids appears to have greater consequences for increased risk of GDM (S. Lee et al., 2019). In line with its role in regulating insulin sensitivity, low adiponectin levels are observed in women diagnosed with GDM, independent of pre-pregnancy BMI or insulin sensitivity (Retnakaran et al., 2004). Recently, Qiao *et al* showed that adiponectin null mice developed glucose intolerance and hyperlipidemia in late pregnancy (Liping Qiao et al., 2017), although hepatic steatosis and its contribution to the dysregulation of glucose homeostasis during pregnancy was not evaluated. In studies of human pregnancy NAFLD is often detected sonographically using ultrasound (De Souza et al., 2016; S. Lee et al., 2019). Nonetheless histological analysis remains the most accurate methodology for NAFLD diagnosis and provides insight into the degree and severity of steatosis, including hepatocellular damage, inflammation and immune cell infiltration that can lead to more severe disease (G. T. Brown & Kleiner, 2016). In this study, histopathology was used to examine the degree of fatty liver present in mice lacking adiponectin in the third trimester, and subsequent mechanisms that contribute to hepatic steatosis were examined.

It is known that the consumption of a high-fat diet can contribute to the development of NAFLD (Oakes et al., 1997; Roden, 2006). Unsurprisingly, WT mice fed a HFS diet developed detectable NALFD in the third trimester (Figure 12A). However, pregnant adiponectin KO mice fed a LF diet developed marked hepatic steatosis (Figure 12A, Table 7), demonstrating a central role for sufficient adiponectin in preventing steatosis in the absence of a HFS diet. Adipokine levels including adiponectin have been correlated with the development of insulin resistance and NAFLD (Bugianesi et al., 2005). A separate study showed circulating adiponectin to be inversely correlated with increasing severity of NAFLD in pregnant women (S. Lee et al., 2019). Since adiponectin has important roles in lipid metabolism and potentiating insulin signalling, low circulating adiponectin may create an environment in which hepatic insulin resistance and impaired lipid metabolism can develop. Based on these results, a lack of adiponectin in pregnancy is associated with the development of NAFLD in the third trimester, and this is independent of the consumption of a HFS diet.

Mice lacking adiponectin develop hepatic steatosis more readily than WT mice, as adiponectin is known to decrease lipogenesis (Awazawa et al., 2011) and increase β-oxidation in the liver (Stern et al., 2016). Additionally, mouse models have shown that administration of adiponectin can improve NAFLD (A. Xu et al., 2003). Indeed, there is some evidence that adiponectin signalling may play a key role in therapeutic strategies using PPAR-γ agonists for NAFLD (Belfort et al., 2006; Bouskila, Pajvani, & Scherer, 2005) due to the fact that PPAR- γ also upregulates

adiponectin gene expression (Iwaki et al., 2003), and adiponectin is able to exert insulin sensitizing effects, and oppose the development of NAFLD (Bouskila et al., 2005). Interventional studies on NAFLD using resveratrol showed improvements mediated by decreased inflammation (Bujanda et al., 2008) and other studies have shown that resveratrol can increase circulating adiponectin (Jimoh, Tanko, Ayo, Ahmed, & Mohammed, 2018). Treatment with anti-inflammatory compounds have shown to reduce improve fatty liver in rodent models of HFD induced NAFLD (Barbuio, Milanski, Bertolo, Saad, & Velloso, 2007). Similar to obesity, pregnancy is a condition with an increased inflammatory profile (Christian & Porter, 2014; Shoelson, Herrero, & Naaz, 2007) and may provide a background for development of NAFLD. With increased intracellular lipid deposition, the resulting lipotoxicity or increased ROS production can worsen insulin resistance, leading to increased hepatic glucose output (Malaguarnera, Di Rosa, Nicoletti, & Malaguarnera, 2009). This might perpetuate a cycle that leads to metabolic distress in pregnancy and could potentially lead to GDM.

In the previous chapter, impaired glucose tolerance and dysregulation of gluconeogenesis in pregnant adiponectin KO mice was observed. Notably, these pathological changes in glucose homeostasis correlated with the development of hepatic steatosis in the third trimester. NAFLD can result from one or more impairments in lipid handling, including increased lipid uptake, altered synthesis or secretion of hepatic triglycerides, and disruptions to β-oxidation. First, lipid synthesis from acetyl CoA was evaluated since it is utilized for fatty acid synthesis, cholesterol synthesis and triglyceride synthesis (Lyssiotis & Cantley, 2014). The increased acetate uptake observed in adiponectin KO mice relative to WT controls agrees with previous reports that individuals with NAFLD have increased uptake of labelled acetate relative to healthy controls (Nejabat et al., 2018). Consistent with lipid accumulation in the liver, acetate incorporated into triglycerides was increased in hepatocytes from pregnant adiponectin KO mice. Interestingly, synthesis and secretion of cholesterol esters (Figure 12F, 9G) was increased in adiponectin KO mice prior to pregnancy and decreased in the third trimester. To a lesser extent this was also observed in WT mice. While cholesterol is necessary for fetal development, in late gestation there is minimal requirement for maternal cholesterol by the fetus (Herrera 2002). It is worth noting that the incorporation of labeled acetate into cholesterol can be limited by incorporation into ketone bodies (Dietschy & McGarry, 1974), and given the observed increase in β-hydroxybutyrate dehydrogenase gene expression in adiponectin KO mice in the third trimester, and serum β-hydroxybutyrate in adiponectin KO mice this may be a limitation of this methodology. Thus, in adiponectin KO mice acetate appears to be primarily utilized for cholesterol synthesis prior to pregnancy, but triglyceride synthesis during pregnancy.

Primary hepatocytes were exposed to [^{14}C] oleate to measure uptake and incorporation of long-chain fatty acids. Contrary to acetate, uptake of [^{14}C] oleate by adiponectin KO mice, while elevated compared to WT mice prior to pregnancy, decreased in the third trimester. Oleate was incorporated into cholesterol esters in a similar pattern as acetate, with an ~2 fold increase in synthesis and ~1.5 fold increase in secretion of cholesterol esters by adiponectin KO mice prior to pregnancy and an observed decrease in both synthesis and secretion in the third trimester (Figure 12H, 9I). However, there was no increase in triglyceride synthesis from oleate or triglyceride secretion in adiponectin KO mice (Figure 12M, 9N). While the results of oleate incorporation contrasts with the results from acetate incorporation, Rohwedder *et al* found similar results from [^{14}C] oleate pulse-chase and mass spectroscopy

experiments, in which there was no increase in incorporation of radiolabeled oleate into triglycerides or cholesterol esters after short term pulses, despite increases of oleate incorporation in lipid droplets (Rohwedder, Zhang, Rudge, & Wakelam, 2014). In addition, long chain fatty acids like oleate increase lipid droplet formation (Fujimoto, 2006). As mentioned above, Rohwedder et al proposed a mechanism by which oleate uptake by hepatocytes increases lipid droplet formation, and formation of lipid droplets presents an opportunity for structured storage of cellular lipids that may be protective against cellular damage (Rohwedder et al., 2014). However, the formation of very large lipid droplets like those that occur in obesity or NAFLD can lead to ER stress and worsening insulin resistance (Özcan et al., 2004), and thus a compensatory mechanism can become a pathological adaptation.

In the presence of such obvious fatty liver, it is somewhat surprising that adiponectin KO mice had reduced incorporation of oleate into cholesterol esters (Figure 12H, 9I), triglycerides and diacylglycerides (Figure 12M, 9N) in the third trimester in comparison to non-pregnant animals. This may be due to the overall reduced uptake of fatty acids (Figure 12L) by pregnant adiponectin KO mice. Long-chain fatty acids such as oleate require an esterification step, and fatty acid binding proteins to facilitate uptake; this leads to additional steps that may be altered in the uptake process (Stremmel, Strohmeyer, & Berk, 1986). Additionally, with impairments to mitochondrial β-oxidation it is possible that the oleate that is being taken up is oxidized inefficiently, resulting in incomplete oxidation (and the resulting by-products: ASM, ketone bodies) rather than being converted to other lipids. There is also evidence showing that if rats are already adapted to increased lipids, such as through high-fat feeding, oleate oxidation and esterification can be altered relative to control fed rats (Malewiak, Griglio, Kalopissis, & Le Liepvre, 1983; Triscari, Greenwood, & Sullivan, 1982). Results from the acetate uptake studies, as well as examination of serum cholesterol levels showed that adiponectin KO mice have reduced synthesis and secretion of cholesterol esters in pregnancy. It has been postulated that cellular cholesterol content can impact uptake of long chain fatty acids (Ehehalt et al., 2008), so potentially the reduction in cellular cholesterol in adiponectin KO mice in pregnancy may feedback and impact uptake of oleate.

While there were no marked increase in fatty acid uptake in pregnancy in our model, it has been shown that the capacity for hepatic lipid transport increases during gestation and returns to normal post-lactation in rodents (Y. Zhang, Kallenberg, Hyatt, Kavazis, & Hood, 2017). Therefore, it is possible that impairments in these pathways could represent disruptions in important metabolic adaptions to pregnancy. Additionally, the decrease in circulating free fatty acids in serum after insulin administration to adiponectin KO mice in the third trimester may indicate impairments to insulin-inhibited lipolysis (Langin, 2006) although there were no increases in circulating triglycerides or cholesterol esters (Table 8). Taken together, the results from the oleate uptake experiments point to impaired uptake in pregnant adiponectin KO mice (possibly due to impaired cholesterol synthesis and secretion). In addition, oleate may be metabolized into lipid droplets, as demonstrated by Rohwedder *et al*, or incompletely oxidized, rather than incorporated into triglycerides as is the fate of radiolabelled acetate.

Although hepatic triglyceride synthesis was increased, adiponectin KO mice did not have higher levels of circulating triglycerides relative to WT mice in the third trimester (Table 8). In the absence of hyperlipidemia, the synthesized lipids may be stored as indicated by the increased hepatic steatosis observed in Figure 12A. WT dams fed

a HFS diet showed a trend towards increased serum cholesterol in the third trimester (Table 8), however there was no increase in serum cholesterol in adiponectin KO mice. The UK Pregnancies Better Eating and Activity Trial (UPBEAT) detailed metabolic profiling of obese women with and without GDM showed overall *decreases* in total cholesterol in late gestation relative to obese women without GDM. Closer analysis showed increased VLDL and reduced LDL, HDL and esterified cholesterol (S. L. White et al., 2017). It is important to note that serum cholesterol measured included all particles (LDL, HDL and VLDL) and may be masking alterations in lipid ratios that could be significant, particularly if there are impairments in VLDL synthesis and secretion due to disrupted phospholipid synthesis.

Phospholipids serve as molecular signalling molecules and essential membrane components. Changes in phospholipid composition can provide information regarding the health and integrity of the cell. In patients with NAFLD or NASH the levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are altered (Puri et al., 2009). PC levels are required for VLDL production and secretion and low levels of PC can lead to hepatic steatosis (Zeisel, 2006). Additionally, low levels of one or both PC and PE can impact membrane integrity that damages the cell (Z. Li et al., 2006). The UPBEAT study showed moderately reduced PC levels in obese women with GDM in late gestation (S. L. White et al., 2017). Adiponectin KO mice have a reduced PC/PE ratio in the liver prior to pregnancy as well as in the third trimester of pregnancy, similar to WT mice (Figure 12P), and this could contribute to more severe hepatic steatosis even on a LF diet.

Another way in which lipids can accumulate in the liver resulting in NAFLD is the dysregulation in β-oxidation of fatty acids. Disruptions to β-oxidation, for example if the pathway is saturated, can lead to lipid peroxidation, ROS generation, inflammation and insulin resistance (Verna,2008). This can affect AKT signalling and gluconeogenesis (Malaguarnera et al., 2009). There is a high degree of overlap between these mechanisms and the pathways that are affected by adiponectin deficiency. Thus, we aimed to determine how mitochondrial β-oxidation during pregnancy changed in adiponectin KO mice. Using glucose as a substrate, primary hepatocytes from pregnant adiponectin KO mice fed a LF diet had impairments in maximal respiration and spare capacity, without defects in basal respiration (Figure 14B), ATP production (Figure 14D) or proton leak (Figure 14F).

Spare capacity is the ability to respond to increased metabolic demand (Burkart, 2016) and mitochondrial spare capacity is reduced in the insulin resistant state. Since pregnancy is a period of both insulin resistance and increased metabolic demand, these impairments in spare capacity and maximal respiration, especially in the third trimester, may indicate limitations to required metabolic flexibility. While the changes in mitochondrial respiration using glucose were relatively modest, hepatocytes from adiponectin KO mice had marked impairments in fatty acid oxidation. The inhibitor etomoxir blocks uptake of fatty acids into the mitochondria through CPT-1a (Schlaepfer & Joshi, 2020). Therefore, calculating the differences in oxidative capacity with and without addition of etomoxir can indicate the ability of a cell to utilize fatty acid oxidation for energy relative to substrates other than fatty acids. In the third trimester, LF-fed adiponectin KO mice showed more profound defects in mitochondrial metabolism when using fatty acid oxidation, including basal and maximal respiration and complete oxidation to ATP relative to WT controls.

Adiponectin increases hepatic β-oxidation through an AMPK mediated pathway (reviewed in (Stern et al., 2016)). Lack of adiponectin has been implicated in mediating the metabolic adaptations to pregnancy (Liping Qiao et al., 2017). With this in mind, it is not surprising that mice lacking adiponectin had marked disruptions to mitochondrial fatty acid oxidation in the third trimester, and this aspect of reduced lipid utilization could contribute to the development of NAFLD in pregnancy. Insulin resistance and NAFLD can impact hepatic mitochondrial content as well as function (Shannon et al., 2020). However, protein levels of citrate synthase were unaltered between adiponectin KO and WT mice. This suggests that the dysfunction in mitochondrial metabolism is likely due to functional impairments in the third trimester, rather than alterations in mitochondrial content. The lack of alteration in citrate synthase does not rule out reduced mitochondrial content or altered structure, as other measures may be more precise including visual assessment using microscopy. However, citrate synthase is a good surrogate marker (Larsen et al., 2012) and served as a better marker than members of the OXPHOS family which may be altered in NAFLD (K. Lee et al., 2018).

In the liver, adiponectin signals through AMPK to decrease lipogenesis by suppression of Srebp-1c and increase fatty acid oxidation through increases in PPAR-α (Awazawa et al., 2011; T. Yamauchi et al., 2002). Insulin is known to induce expression of Srebp-1c and stimulate lipogenesis in the liver (G. Chen et al., 2004). In order to separate the effect of insulin resistance from the genotype effect in adiponectin KO mice, liver gene expression analysis was performed in livers from insulin and saline treated animals in the third trimester of pregnancy. Adiponectin KO mice exhibited increased expression in lipogenic genes *Dgat1*, *Acaca* and *Fasn* (Figure 15A). Consistent with the hepatic fat accumulation, adiponectin KO mice had higher lipogenic gene expression relative to WT mice, even without exogenous insulin administration, and in the absence of adiponectin, upstream *Srebp1*-c also showed a trend towards elevated expression in adiponectin KO mice.

Interestingly, hepatic expression of C*pt1a* appears to be increase in pregnant adiponectin KO mice after insulin administration (Figure 15A). This is somewhat surprising in light of the observed impairments in mitochondrial fatty acid oxidation in adiponectin KO mice, particularly since insulin typically suppresses fatty acid oxidation (Hamel, Bennett, Upward, & Duckworth, 2001). In pregnant adiponectin KO mice, hepatic gene expression of *Acaca*, while not statistically significant, was increased approximately 2-fold over WT mice in the presence of insulin (Figure 15A) and a reduction in inhibitory phosphorylation of ACC was also observed (Figure 15B). It is possible that in the absence of adiponectin, increased lipogenesis via ACC results in production of malonyl CoA (Figure 2). Malonyl CoA inhibits CPT1a (Hamel et al., 2001; J. D. McGarry, Leatherman, & Foster, 1978) and increased gene expression of *Cpt1a* could be a compensatory action to dispose of increased intracellular lipids and overcome increased inhibition. Indeed, results from primary hepatocytes showed that inhibition of CPT1 with etomoxir did not reduce mitochondrial respiration (Figure 14H-J). This suggests that in the third trimester, fatty acid oxidation is not contributing significantly to overall hepatic respiration in adiponectin KO mice. In this case, gene expression of *Cpt1a* could likely be increased as a compensatory mechanism.

Cpt1a is the rate limiting enzyme for fatty acid oxidation in the liver (Drynan, Quant, & Zammit, 1996), and *Cpt2*, located on the inner mitochondrial membrane, converts acyl-carnitines back to fatty acyl-CoA once they have

crossed into the mitochondria (Drynan et al., 1996). Since adiponectin KO mice exhibited increased *Cpt1a* expression but impairments to complete fatty acid oxidation, it is possible that incomplete oxidization through β-oxidation, the TCA cycle and the electron transport chain occurred. Rather, this acyl-CoA could be oxidized to acetyl-CoA and converted to ketone bodies or new lipids through lipogenesis. Indeed, a study of hepatic CPT enzymes found that *Cpt1* closely regulates ketogenesis in the liver of rats, regardless of nutritional status (Drynan et al., 1996). The potential increased gene expression of *Cpt1a* combined with elevated serum levels of ASM may indicate increased ketogenesis in pregnant adiponectin KO mice. It was possible that the compensatory increase in *Cpt1* increase the pool of acetyl-CoA but that due to downstream defects, the fatty acids are misdirected away from complete oxidation and towards lipogenesis and ketogenesis.

Gene expression of *Dgat1* was increased in adiponectin KO mice relative to WT mice in the third trimester, even without administration of insulin (Figure 15A). While results from oleate uptake experiments in primary hepatocytes showed dramatically elevated diacylglycerol in adiponectin KO mice prior to pregnancy (Figure 12O), there was a significant reduction by late gestation. This could be due to the fact that DG was more quickly converted to triglycerides (Figure 3), given the increase in synthesis and secretion of triglycerides in the third trimester in adiponectin KO mice (Figure 12K, 9L). Increased expression of *Dgat1* supports this hypothesis, and knockout and over-expression mouse models have linked increased DGAT1 with NAFLD and hepatic steatosis (Monetti et al., 2007; Villanueva et al., 2009). *Gpam* is a component of the lipogenic pathway and increased expression was observed in adiponectin KO mice, in the absence of insulin administration. Elevated *Gpam* expression has been implicated in the development of hepatic steatosis (Kodama et al., 2018), through increasing buildup of triglycerides and diacylglycerides and potentially impairing β-oxidation (Lindén et al., 2006; Nishikawa, Doi, Nakayama, & Uetsuka, 2008).

Adiponectin KO mice exhibited increased lipogenesis and decreased β-oxidation in pregnancy. However, incomplete oxidation can also lead to the production of ketone bodies (Huynh, Green, Koves, & Hirschey, 2014). Even in a normal pregnancy, increased ketogenesis is not uncommon due to the hormonal milieu of pregnancy and increased lipolysis (Bronisz, Ozorowski, & Hagner-Derengowska, 2018; Frise, Mackillop, Joash, & Williamson, 2013; Knopp, Montes, Childs, Li, & Mabuchi, 1981; Spanou et al., 2015). This also results in a predisposition towards ketoacidosis in pregnancies affected by GDM.

The growing fetus can also use ketones for growth (Herrera, 2002), and large amounts of acetyl-CoA can drive ketogenesis. Additionally, the UPBEAT trial showed increased ketogenesis in obese women with GDM (S. L. White et al., 2017). Primary hepatocytes from adiponectin KO mice showed increased acetate uptake in pregnancy (Figure 12F). However, when combined with reduced β-oxidation, it is possible that acetyl-CoA is being redirected towards ketogenesis. Consistent with this, β-hydroxybutyrate dehydrogenase (*Bdh1)* gene expression was significantly increased (Figure 15A) and serum β-hydroxybutyrate elevated in adiponectin KO mice (Figure 15D). Interestingly, gene expression of *Bdh1* was highly increased after insulin administration. Insulin administration should reduce ketone synthesis (Rui, 2014) Thus there may be more severe insulin resistance in pregnancy in adiponectin KO mice. Additionally, adiponectin KO mice had elevated serum β-hydroxybutyrate with the consumption of a high fat diet,

suggesting that adiponectin deficiency on top of the pregnancy induced changes to lipid metabolism that may predispose adiponectin KO mice to increased ketogenesis.

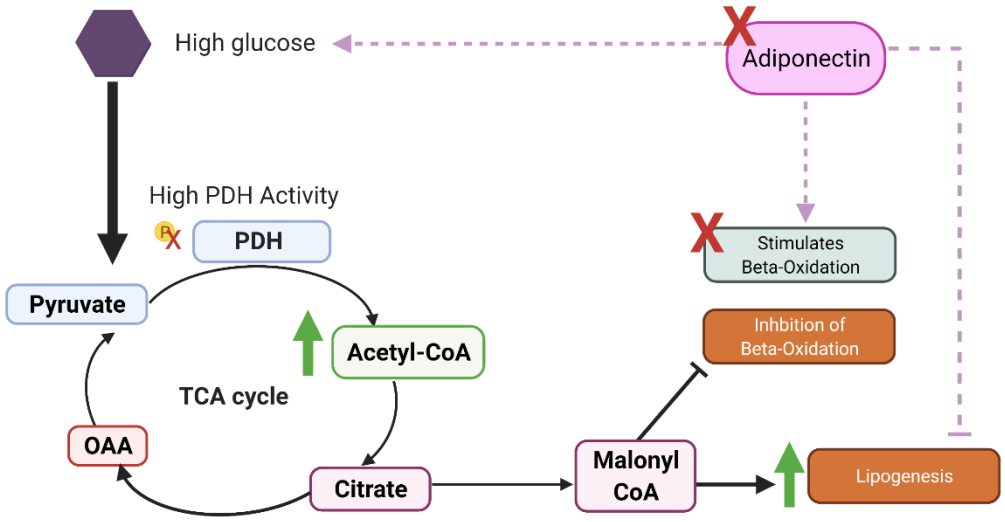
In order to completely oxidize fatty acids, acetyl-CoA must enter into the TCA cycle. Adiponectin KO mice had reduced production of ATP from fatty acids in the third trimester (Figure 14J), with approximately 2-fold increased ASM relative to WT (Figure 14Q) which are markers of ketogenesis, indicating potential for incomplete oxidation as well as inefficient or reduced β-oxidation. Incomplete or inefficient fatty acid oxidation can be accompanied by TCA cycle dysfunction (Satapati et al., 2012). Consistent with this, α-KG, a TCA cycle metabolite at a key regulatory point, was elevated in the third trimester in adiponectin KO mice (Figure 15A). Moreover, increased α-KG in human plasma is associated with NAFLD (Aragonès et al., 2016; Rodríguez-Gallego et al., 2015). While this build-up may represent a metabolic bottleneck, a recent study suggested that increased αKG may be a protective mechanism in NAFLD (Nagaoka et al., 2020). In that study, supplementation with αKG reduced progression of disease (Nagaoka, 2020). Other studies have shown an anti-oxidant role for αKG (S. Liu, He, & Yao, 2018). Given this, increased hepatic αKG could potentially be a relevant compensatory mechanism, since overloading of mitochondrial β-oxidation can lead to increases in peroxisomal lipid oxidation, which produces much higher levels of ROS, increasing the risk of oxidative damage (Rao & Reddy, 2001).

OAA is another key regulatory TCA cycle metabolite (White, 2015). Levels of OAA were somewhat but not significantly elevated in pregnant adiponectin KO mice relative to WT controls. While this may argue against TCA cycle impairment, as gluconeogenesis draws on OAA and in Chapter 3 we reported increased gluconeogenesis in pregnant adiponectin KO mice. If the amount of acetyl-CoA is higher than the TCA cycle capacity, it may lead to acetyl-CoA being diverted to incomplete oxidation and lipogenesis (H. M. White, 2015). Anaplerosis is required to maintain effective TCA cycle function, and experiments have shown that impaired anaplerosis can lead to blunting of TCA cycling and compensatory increases in ketogenesis (Cappel et al., 2019). Increased fatty acid exposure can also result in a constitutively active but an inefficient respiratory chain, leading to cycling of the TCA cycle but inefficient electron use (Satapati et al., 2012) which may also lead to increased oxidative stress.

Pyruvate dehydrogenase (PDH) is an important enzyme that acts at the interface of glycolysis and the TCA cycle, converting pyruvate to acetyl-CoA. Under high glucose conditions, PDH is more active, promoting complete oxidation of glucose (Sungmi Park et al., 2018). Phosphorylation of PDH is inhibitory, resulting in reduced acetyl-CoA production (Figure 17). In other tissues such as skeletal muscle, PDH has been proven to be involved in metabolic flexibility and fuel switching (M. Zhang, Zhao, Li, & Wang, 2018), which is of increased importance in the liver in the third trimester of pregnancy. Insulin activates PDC, and we observed increased *Pdha1* gene expression in adiponectin KO mice relative to WT when exposed to insulin (Figure 16C). Post-translational modifications affect the activity of PDC. We observed increased total expression of PDH protein as well as reduced inhibitory phosphorylation in adiponectin KO mice (Figure 16D). Combined with an apparent decrease in mitochondrial oxidation of both glucose

and fatty acids, this increase in PDH activity may result in increased acetyl-CoA content leading to increased lipogenesis and incomplete oxidation to ketones.

Figure 17: Regulation of PDH



Under high glucose conditions (such as in pregnant adiponectin KO mice) PDH remains active and dephosphorylated, producing acetyl-CoA. Acetyl-CoA can accumulate at a rate faster than it can be metabolized through the TCA cycle, and can lead to lipogenesis, incomplete oxidation to ketone bodies, and the buildup of malonyl-CoA can inhibit CPT1 leading to reduced β-oxidation, especially in the absence of adiponectin.

Overall, adiponectin KO mice readily develop histologically detectable hepatic steatosis in the third trimester which is profound even when fed a LF diet. Primary hepatocytes isolated from these mice showed a trend towards increased acetate uptake, the fate of which appears to be the synthesis and secretion of triglycerides. Interestingly, primary hepatocytes from adiponectin KO mice showed no differences uptake of labelled oleate relative to WT mice in the third trimester, however oleate uptake was reduced relative to non-pregnant KO mice. Consequently, there was less observable incorporation of oleate into TG, cholesterol esters and DG in adiponectin KO mice in pregnancy. These cells also showed potential impairments to phospholipid synthesis, which can increase susceptibility to fatty liver, and more severe disease progression. Impaired lipid synthesis and secretion by primary hepatocytes in pregnant histologically detectable hepatic steatosis in the third trimester which is profound even when fed a LF diet. Primary hepatocytes isolated from these mice show increased uptake of labelled acetate, the fate of which appears to be the synthesis and secretion of triglycerides. Interestingly, primary hepatocytes from adiponectin KO mice had reduced uptake of adiponectin KO mice may therefore impact not just metabolic function but cellular structure and integrity.

Primary hepatocytes of adiponectin KO mice showed decreased maximal and spare capacity when using glucose as a primary energy source. However, the most notable impairments were observed when isolated hepatocytes from these mice were forced to respire using fatty acids. Basal and maximal respiration were significantly impaired, and ATP production was reduced relative to WT controls. These impairments were evident regardless of no apparent change to mitochondrial content. Gene expression and protein analysis showed significant increases in lipogenic genes in liver tissue isolated from adiponectin KO mice. Increased lipogenesis in combination with decreased fatty acid oxidation can lead to the development of fatty liver. The development of NAFLD is associated with insulin resistance, and it may prove to be a tipping point of metabolic inflexibility in the third trimester that leads to the development of GDM in adiponectin KO mice.

Chapter 5:

Impact of Adiponectin Supplementation on GDM Development in the Third Trimester

5.1 Introduction

As reviewed in Chapter 1, adiponectin exists in circulation as full length (fAPN) or the globular cleavage product (gAPN). The dominant circulating form of adiponectin is fAPN, and it is found in multimers of varying size from low (LMW) trimer to high (HMW) oligomers (Aye 2015). fAPN and its proteolytic cleavage product, gAPN have different specificities for adiponectin receptors. For example, AdipoR2, found in the liver, binds fAPN with higher specificity (Toshimasa Yamauchi et al., 2003). This leads to tissue specific responses to the different forms of adiponectin based on receptor composition. HMW adiponectin has an increased half-life in circulation compared to other isoforms (Halberg et al., 2009), and the ratio of HMW to total serum adiponectin may be a potential biomarker for metabolic dysregulation. Levels of HMW adiponectin have been shown to be significantly decreased, even relative to total adiponectin in patients with insulin resistance, in T2D and in childhood obesity (Araki, Dobashi, Kubo, Asayama, & Shirahata, 2006; R. Basu, Pajvani, Rizza, & Scherer, 2007). There are also sex differences in both humans and rodent adiponectin isoforms (U. Pajvani et al., 2003; U. B. Pajvani et al., 2004; Waki et al., 2003), with females having higher levels of HMW adiponectin relative to lower molecular weight isoforms.

Experiments using cell-based techniques and animal models have determined that adiponectin is important for maintaining peripheral insulin sensitivity. Administration of globular adiponectin to mice suppressed lipid accumulation in high fat diet-fed mice, as well as the db/db and KKA obese mouse models (T. Yamauchi et al., 2001). Adiponectin administration also reduced hepatic gluconeogenesis (Miller et al., 2011; S. Park et al., 2011), increased peripheral insulin sensitivity (T. Yamauchi et al., 2002) and increased hepatic β-oxidation (Stern et al., 2016), which in turn improved hepatic insulin sensitivity. The effects of adiponectin on the liver were shown to be mediated by full-length high molecular weight adiponectin (T. Yamauchi et al., 2002). In previous chapters it was established that adiponectin KO mice have impaired glucose and insulin tolerance and increased fasting blood glucose in the third trimester of pregnancy. It also appears that adiponectin KO mice are more sensitive to the effects of diet-induced obesity, and readily develop hepatic steatosis in the third trimester, however it has not been determined if supplementation with adiponectin can rescue this metabolic decompensation.

Observations on the metabolic effects of adiponectin in pregnancy have been validated in rodent studies wherein levels of circulating adiponectin are increased, and markers of metabolic syndrome are improved. Previously it was shown that infusion of fAPN improved maternal insulin sensitivity and reduced circulating cholesterol in pregnant mice (Aye et al., 2015). Reconstitution of adiponectin in pregnant adiponectin KO mice using gene transduction showed similar effects, with improvements to maternal blood glucose and reductions in circulating lipids (Liping Qiao et al., 2017). From these studies it appears that increasing circulating adiponectin in pregnancy appears to improve symptoms of GDM, whether it is brought on by diet induced obesity or complete lack of adiponectin in an adiponectin KO mouse model.

These results have underlined the link between adiponectin and insulin sensitivity in pregnancy, as well as some effect on lipid metabolism in the form of circulating lipids. Adiponectin supplementation to pregnant mice in late gestation can prevent fatty liver development in the offspring of obese dams (Paulsen, Rosario, Wesolowski, Powell, & Jansson, 2019). In non-pregnant mice, over-expressing adiponectin reduced hepatic fat deposition in

response to a HFD (Asterholm & Scherer, 2010). However, there are no studies that have examined the effect of adiponectin supplementation on fatty liver development in pregnancy and its impact on GDM. Fatty liver development may occur in conjunction with GDM (S. Lee et al., 2019) and increasing hepatic fat content can create a negative-feedback effect on insulin sensitivity, increasing risk for GDM. Additionally, low circulating adiponectin has been implicated in the development of both fatty liver and GDM.

We hypothesized that increasing circulating adiponectin in the third trimester could protect against the development of GDM in adiponectin KO mice. In this chapter, we examined whether supplementation with adiponectin in the third trimester improved glucose tolerance and hyperglycemia in pregnant adiponectin KO mice, and whether increasing levels of circulating adiponectin protect against the development of hepatic steatosis observed in adiponectin KO mice in late gestation.

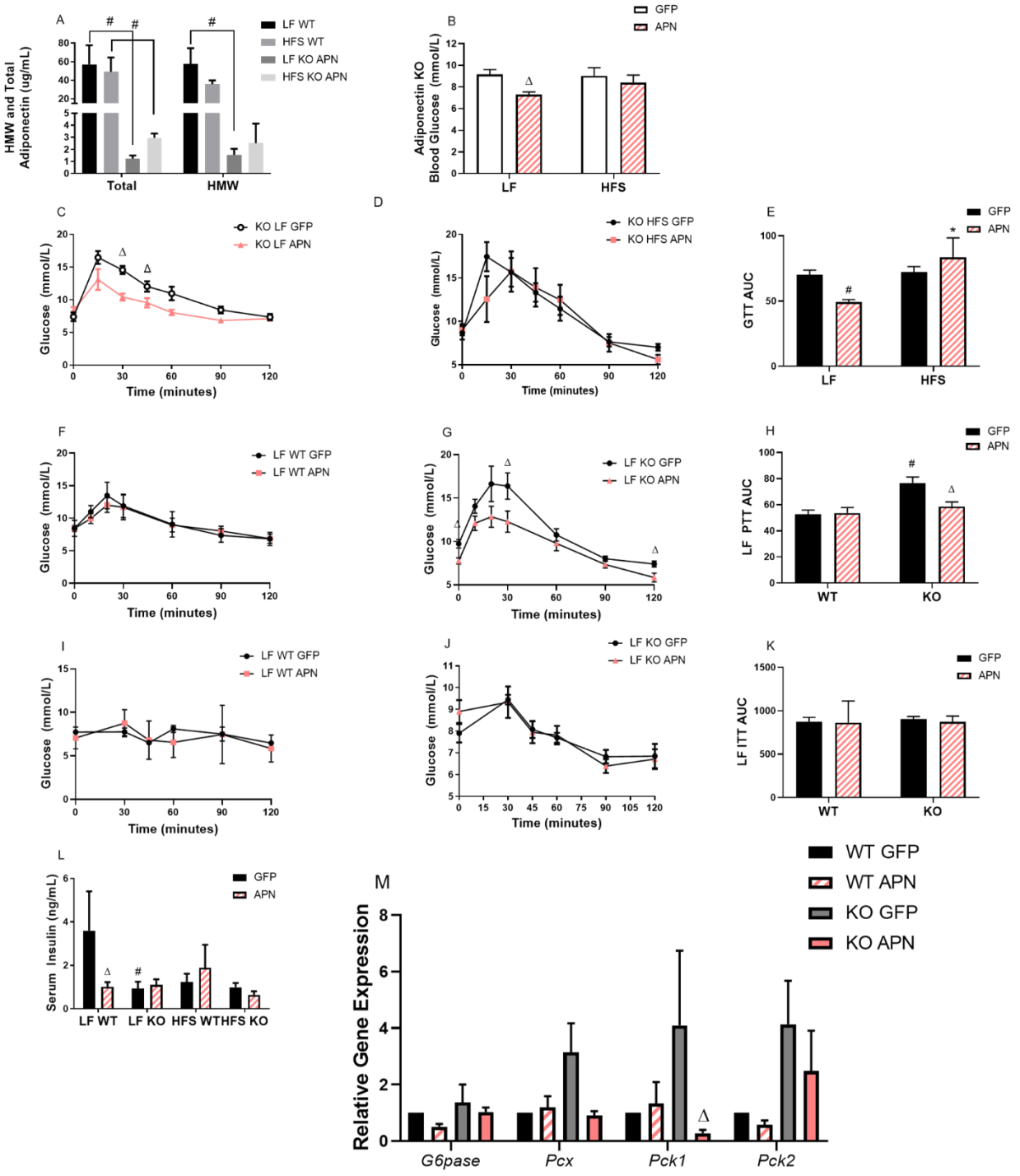
5.2 Materials and Methods – Refer to Page 24

5.3 Results:

5.3.1 Glucose control

After confirming pregnancy, mice were supplemented with adenovirus containing GFP or full-length adiponectin at the end of the second trimester (~e14). To determine the effect on circulating adiponectin, and whether this was sustained through to the end of gestation, serum adiponectin was measured at the end of pregnancy from blood collected at the time of sacrifice, approximately 7-days post-injection. Since the HMW isoform of adiponectin is thought to be more metabolically active, both total and HMW adiponectin were measured. Similar to results shown in Chapter 3, HFS diet consumption did not alter circulating total or HMW adiponectin in WT mice in the third trimester compared to LF controls (Figure 18A). Total and HMW adiponectin were lower in both LF- and HFS-fed adiponectin KO mice given APN compared to LF WT and HFS WT mice, respectively. Pregnant adiponectin KO mice who received GFP control supplementation had undetectable levels of adiponectin.

Figure 18: Adiponectin supplementation improved glucose homeostasis and reduced hepatic glucose output in pregnant adiponectin KO mice.



A) Total and high molecular weight (HMW) adiponectin in serum isolated from pregnant WT and adiponectin KO mice fed either LF or HFS diet. Serum was isolated at the time of sacrifice at the end of the third trimester. N=4-6 per group. B) Effect of adenovirus mediated GFP or APN supplementation to pregnant adiponectin KO mice in the third trimester on blood glucose levels n=6-13 per group. GTT on LF fed C) and HFS fed D) pregnant adiponectin KO mice in the third trimester, after supplementation with adenovirus mediated GFP or APN. N=4-5 per group. E) Area under

the curve (AUC) for GTTs performed on adiponectin KO mice supplemented with GFP or APN in the third trimester. PTT performed on LF fed WT (F) and adiponectin KO (G) mice in the third trimester after supplementation with adenovirus mediated GFP or APN. N= 5-6 per group. H) AUC for PTT performed on LF fed WT and adiponectin KO mice supplemented with GFP and APN in the third trimester. ITT performed on pregnant LF fed WT (I) and adiponectin KO (J) mice in the third trimester after supplementation with adenovirus mediated GFP or APN. N=3-6 per group. K) AUC for ITT performed in the third trimester on GFP or APN supplemented WT and adiponectin KO mice. L) Third trimester serum insulin levels after supplementation with GFP or APN. N=4-8 per group. M) Hepatic gene expression of *G6pase, Pcx, Pck1 and. Pck2.* Gene expression is relative to geomean of *Rn18s, B2m, RPLP-1* and *Hprt1* and normalized to LF WT GFP. *Pck1:* phosphoenolpyruvate carboxykinase-1; *G6pase:* glucose-6-phosphatase. *Pcx:* Pyruvate carboxylase. *Pck1:* phosphoenolpyruvate carboxykinase-1, *Pck2:* phosphoenolpyruvate carboxykinase-2. *B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *Hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *RPLP-1:* 60S acidic ribosomal protein large P1. P values represent significance ($p<0.05$) as determined by Two-way ANOVA. # $p<0.05$ WT vs KO Δ $p<0.05$ GFP vs APN injection * $p<0.05$ LF vs HFS diet fed mice. LF: Low fat, HFS: High fat-sucrose, WT: Wildtype, KO: Knockout, GFP: green fluorescent protein, APN: adiponectin

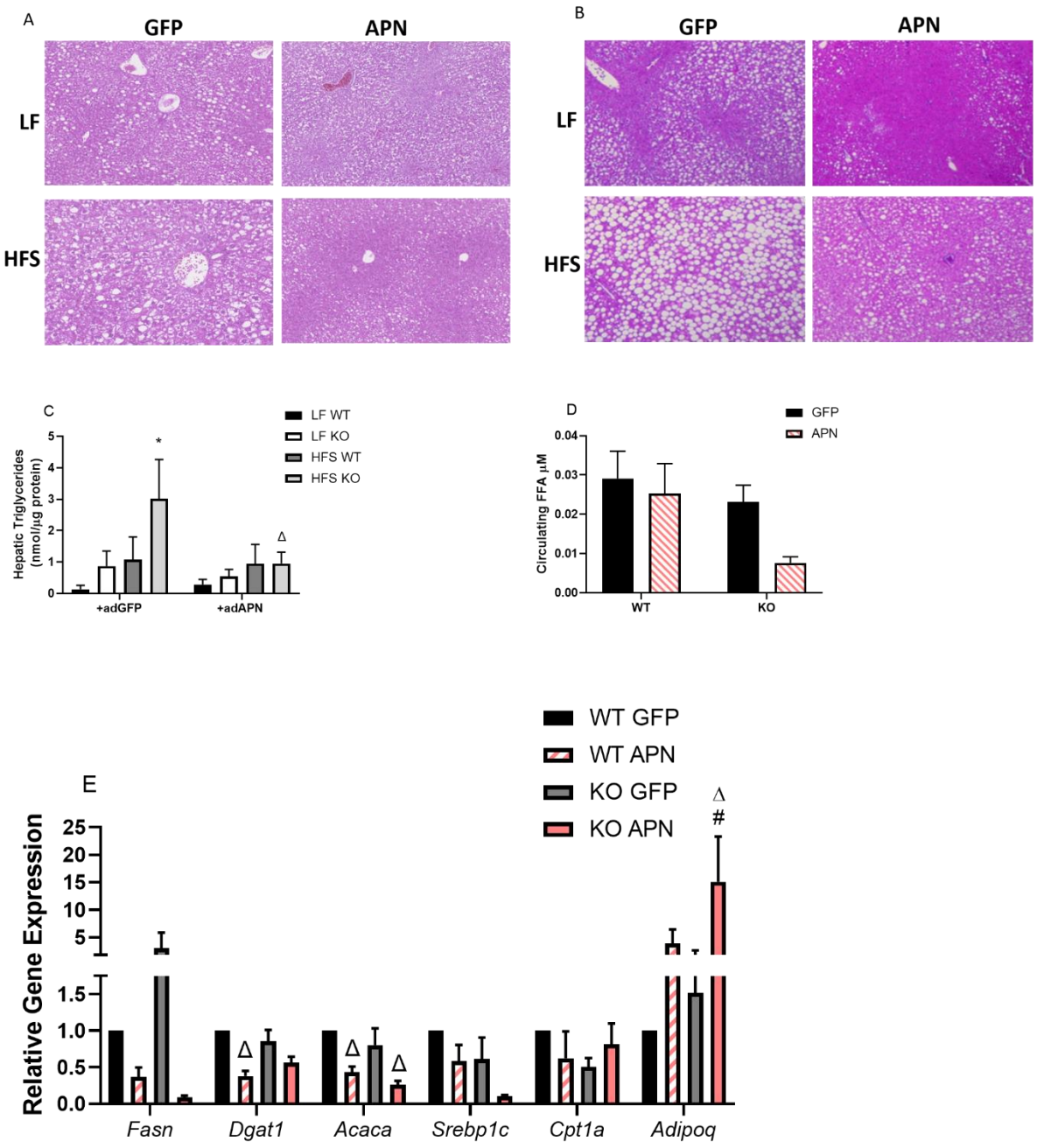
Supplementation of adiponectin to pregnant adiponectin KO mice reduced fasting blood glucose levels in LF- but not HFS-fed mice in the third trimester compared to GFP controls (Figure 18B). Adiponectin supplementation of LF fed pregnant adiponectin KO mice improved glucose tolerance compared to GFP control (Figure 18C, E) but not glucose tolerance in HFS fed adiponectin KO mice (Figure 18D, E). One mechanism for adiponectin induced improvement in glucose homeostasis is reduced hepatic glucose output. Thus, the amount of *de novo* glucose production from pyruvate was measured using a PTT. There was no difference in hepatic glucose output with adiponectin supplementation in the third trimester of LF-fed WT mice (Figure 18F, H). In contrast, a reduction in glucose production from pyruvate was observed in LF fed adiponectin supplemented adiponectin KO mice compared to GFP control (Figure 18G, H). Supplementation of LF adiponectin KO mice with adiponectin did not have an appreciable effect on insulin tolerance in the third trimester compared to GFP controls (Figure 18 I-K). Supplementation with adiponectin reduced circulating fasted insulin in LF fed WT mice relative to GFP but did not affect circulating insulin levels in LF-fed adiponectin KO mice in the third trimester compared to GFP controls (Figure 18L).

In Chapter 3 we observed alterations in expression of hepatic gene genes associated with gluconeogenesis in adiponectin KO mice in the third trimester of pregnancy. Adiponectin supplementation reduced the expression of *Pck1* in adiponectin KO mice on a LF diet in the third trimester (Figure 18M) relative to GFP. While adiponectin supplementation had no effect on hepatic pyruvate carboxylase (*Pcx*) expression in pregnant WT mice, adiponectin supplementation reduced *Pcx* expression in adiponectin KO mice relative to GFP (Figure 18M). Similar to results shown in Chapter 3, *Pck2* expression was elevated in adiponectin KO mice relative to WT mice although this was not statistically significant. Expression of *Pck2* was not affected by supplementation with adiponectin in either WT or adiponectin KO mice in the third trimester. Taken together these results suggest that adiponectin supplementation in the third trimester improves glucose tolerance and may contribute to suppression of gluconeogenesis in adiponectin KO mice.

5.3.2 Fatty liver development

We observed that adiponectin KO mice developed hepatic steatosis in the third trimester, even on a LF diet. In order to determine whether adiponectin supplementation attenuated fatty liver during pregnancy, histological analysis was performed on liver tissue isolated from LF- or HFS-fed pregnant adiponectin KO and WT mice at sacrifice at the end of the third trimester. Supplementation with adiponectin reduced hepatic fat deposition in both WT and adiponectin KO mice fed a LF diet, and this effect was more pronounced in animals that consumed a HFS diet (Figure 19A, 17B). Adiponectin supplementation significantly reduced steatosis score in WT mice fed a HFS diet in the third trimester (Table 9). Adiponectin supplementation lead to trend in reduced steatosis score in adiponectin KO mice, however low sample size prevents these results from being conclusive (Table 9). As previously observed in Chapter 3, hepatic TG content trended upward with HFS diet consumption in both WT and adiponectin KO mice, and in adiponectin KO mice given GFP, this was significant (Figure 19C). Adiponectin KO mice appeared to have more hepatic TG compared to WT mice (Figure 19C). Adiponectin supplementation reduced hepatic triglycerides in HFS-fed adiponectin KO mice, but this was not statistically significant (Figure 19C). Supplementation with adiponectin significantly reduced hepatic TG content in adiponectin KO mice fed a HFS diet in the third trimester relative to GFP control (Figure 19C). Since, hepatic TG levels are dependent on circulating free fatty acids (FFA), we examined circulating non-esterified FFA in LF fed adiponectin KO and WT mice from serum collected at the end of the third trimester. Circulating FFA were unchanged between WT and adiponectin KO mice (Figure 19D). Adiponectin supplementation appeared to reduce circulating FFA in LF fed adiponectin KO mice, although this was not statistically significant.

Figure 19: Adiponectin supplementation improves markers of hepatic steatosis in LF fed adiponectin KO mice in the third trimester of pregnancy.



Representative images of H&E stained liver sections from (A) WT and (B) adiponectin KO mice supplemented with adenovirus mediated adiponectin (APN) or GFP. Livers were collected at the end of the third trimester at the time of sacrifice. WT n= 2-5, adiponectin KO n=3-6 per group. C) Hepatic triglyceride content from LF and HFS fed WT and adiponectin KO mice supplemented with adenovirus mediated APN or GFP in the third trimester. D) Circulating non-esterified free fatty acids (FFA) in LF fed adiponectin KO and WT mice from serum collected at the end of the third trimester. E) Hepatic gene expression of *Fasn, Dgat1, Acaca, Srebp1c, Cpt1a, and Adipoq* from LF fed adiponectin KO and WT mice supplemented with adenovirus mediated APN or GFP in the third trimester. Gene expression is relative to geomean of *Rn18s, B2m, RPLP-1* and *hprt1* and normalized to LF WT GFP. *Dgat1*: Diacyglycerol transferase-1. *Acaca*: Acetyl-CoA Carboxylase. *Fasn*: Fatty acid synthase. *AdipoQ:* Adiponectin, C1Q and collagen

domain containing. *Cpt1a: Carnitine palmitoyl transferase 1-α, B2m:* β-2 microglobulin, *Rn18s:* 18s rRNA, *Hprt1:* Hypoxanthine guanine phosphoribosyl transferase, *RPLP-1:* 60S acidic ribosomal protein large P1. P values represent significance ($p<0.05$) as determined by Two-way ANOVA. * $p<0.05$ LF vs HFS # $p<0.05$ WT vs KO Δ $p<0.05$ GFP vs APN injection. LF: Low fat, HFS: High fat-sucrose, WT: Wildtype, KO: Knockout, GFP: green fluorescent protein, APN: adiponectin

Table 9: Effect of adiponectin supplementation on liver weight and steatosis score in LF and HFS fed WT and adiponectin KO mice in the third trimester.

	Wildtype		Knockout	
Injection	**GFP**	**APN**	**GFP**	**APN**
LF Liver weight (mg)	1616.40 ± 74.50	1834.00 ± 76.00	1970.30 ± 82.20	1938.40 ± 73.30
HFS Liver Weight (mg)	1862.20 ± 123.80	1862.90 ± 247.60	1842.70 ± 135.70	1828.70 ± 85.70
LF Steatosis Score	1.30 ± 0.33	0.60 ± 0.40	1.80 ± 0.47	1.00 ± 0.70
HFS Steatosis Score	2.80 ± 0.20	1.00 ± 0.00 Δ	2.80 ± 0.20	2.00 ± 0.45

Restoring circulating adiponectin improves hepatic steatosis in WT and adiponectin KO mice in the third trimester but had no significant effect on liver weight. Liver weight N = 5-11 per group. Steatosis scores N = 4-6 adiponectin KO livers and N=2-5 WT livers per group. P Values represent significance ($p<0.05$) after Two-way ANOVA. Δ=$p<0.05$ between GFP and APN injected mice. LF: Low fat, HFS: High fat-sucrose, GFP: green fluorescent protein, APN: adiponectin

We also noted changes in gene expression associated with hepatic lipogenesis. Supplementation of adiponectin KO mice with adiponectin lead to a trend in reduced hepatic expression of *Fasn*, and *Acaca* relative to GFP (Figure 19E). Part of the mechanism by which adiponectin may suppress lipogenesis is through suppression of Srebp1-c in the liver (Yamauchi, 2002). Although adiponectin supplementation appeared to contribute to reduced *Srebp1c* expression in adiponectin KO mice this was not statistically significant. Adiponectin KO mice supplemented with adiponectin also showed significantly increased gene expression of *AdipoQ* relative to GFP controls (Figure 19E). As seen in Chapter 3, HFS diet consumption in pregnancy was the main driver of increased adiposity in WT mice. Adiponectin supplementation to LF-fed or HFS-fed WT or adiponectin KO mice did not significantly impact visceral adiposity in the third trimester (Table 10).

Table 10: Effect of adiponectin supplementation on visceral adiposity in adiponectin KO and WT mice in the third trimester

	Wildtype		Adiponectin Knockout	
	GFP	APN	GFP	APN
LF GWAT weight (mg)	228.8 ± 41.1	190.3 ± 49.9	164.3 ± 18.0	146.4 ± 25.0
HFS GWAT weight (mg)	1357.4 ± 254.8 *	1420.9 ± 650.7 *	625.4 ± 152.2	562.5 ± 135.5#
LF PWAT weight (mg)	196.8 ± 20.9	150.4 ± 39.0	138.8 ± 27.7	168.7 ± 22.7
HFS PWAT weight (mg)	1129.2 ± 193.2*	970.5 ± 376.0*	234.4 ± 42.9#	306.7 ± 38.3#

Adiponectin supplementation did not have a significant impact on visceral adiposity in the third trimester. P Values represent significance ($p<0.05$) after Two-way ANOVA. N=5-10 per group. * $p<0.05$ between LF and HFS fed mice. # $p < 0.05$ between WT and adiponectin KO mice. LF: Low fat, HFS: High fat-sucrose, GWAT: gonadal white adipose tissue, PWAT: perirenal white adipose tissue, GFP: green fluorescent protein, APN: adiponectin

Low circulating adiponectin has been implicated in both low and high birthweights. Thus, litter sizes and fetal weights at the time of sacrifice in WT and adiponectin KO mice administered either GFP or adiponectin prior to the third trimester were examined. While there was no effect of adiponectin supplementation or genotype on litter size (Table 11), supplementation with adiponectin significantly reduced pup size in LF fed WT mice compared to controls but had no effect on adiponectin KO mice.

Table 11: Effect of adiponectin supplementation on litter size and pup weight in adiponectin KO and WT mice in the third trimester.

	Wildtype		Adiponectin Knockout	
	GFP	APN	GFP	APN
LF Litter size	6.3 ± 0.6	6.6 ± 1.2	6.7 ± 0.5	6.8 ± 0.7
HFS Litter size	6.3 ± 0.9	6.8 ± 1.2	6.3 ± 0.5	5.9 ± 0.9
LF pup size (mg)	1396.1 ± 73.0	1145.9 ± 66.4Δ	1236.8 ± 13.9	1197.4 ± 50.4
HFS pup size (mg)	944.8 ± 91.2 *	1106.7 ± 41.5	1161.3 ± 87.6	1275.8 ± 46.1

Supplementation with adiponectin reduced pup size in LF fed WT mice but had no effect on litter size in either WT or adiponectin KO mice. N= 4-9 per group. P Values represent significance ($p<0.05$) after Two-way ANOVA. * $p<0.05$ between LF and HFS fed mice. Δ $p < 0.05$ between GFP and APN supplemented mice. LF: low fat, HFS: high fat-sucrose, GFP: green fluorescent protein, APN: adiponectin

5.4 Discussion/Conclusions

Full length adiponectin exists in the circulation in multiple isoforms including low, medium and high molecular weight isoforms. Variability in the abundance of these isoforms is associated with sex as well as different states of metabolic health. The HMW isoform of adiponectin has been associated with better metabolic flexibility, and HMW adiponectin may be necessary isoform for the metabolic effects of adiponectin on the liver (U. Pajvani et al., 2003; T. Yamauchi et al., 2002). Additionally, mutations associated with diabetes have been shown to impair the disulphide bonding required for HMW adiponectin complex formation *in vitro* (Waki et al., 2003). A lower ratio of HMW to total adiponectin may be predictive of metabolic dysfunction, as shown by a relationship with glucose intolerance or response to thiazolidinediones (Fisher et al., 2005; Hara et al., 2006; U. B. Pajvani et al., 2004) .

Multimerization increases the half-life of adiponectin (Halberg et al., 2009), and in pregnancy higher levels of HMW adiponectin are found in normal weight subjects compared to obese or overweight women (Mazaki-Tovi et al., 2008). We used adenovirus vectors containing full length adiponectin to establish that supplementation increased circulating adiponectin in our KO mice and that the increase was sustained throughout gestation. Additionally, we determined whether supplementation of full-length adiponectin resulted in differences in the ratio of HMW to total adiponectin. We found that the levels of HMW and total adiponectin were lower in adiponectin KO mice compared to WT mice and unaltered in adiponectin supplemented WT mice or adiponectin KO mice.

We did not observe any significant differences between the levels of total adiponectin and HMW adiponectin in any experimental groups, which agrees with findings from the literature. Other studies using constant infusion of full-length adiponectin to obese dams did not show differences in the ratio of HMW to total adiponectin resulting from supplementation (Aye et al., 2015). Indeed, most studies using supplementation use full length adiponectin (Aye et al., 2015; Fukushima et al., 2007; J. H. Park, Lee, & Kim, 2006; Paulsen et al., 2019). Of the studies that reported levels of HMW adiponectin after supplementation, only Park *et al* found that mice had higher levels of HMW adiponectin in the circulation. The authors attributed much of the metabolic improvements observed after adiponectin gene therapy to the HMW adiponectin. However, these mice were male and it has been established that multimerization of adiponectin is subject to sexual dimorphism (U. Pajvani et al., 2003).

It was notable that even with adiponectin supplementation, adiponectin KO mice still had significantly less circulating adiponectin than WT mice, whether they were fed a LF or HFS diet (Figure 18A). Thus, it appears that an increase of adiponectin through supplementation is sufficient to elicit a metabolic improvement in pregnant adiponectin KO mice. Adiponectin supplementation improved fasting hyperglycemia (Figure 18B) and glucose tolerance (Figure 18C) in LF fed adiponectin KO mice relative to GFP control, but did not have a significant effect on fasting blood glucose (Figure 18B) and glucose tolerance (Figure 18D) in HFS fed KO mice in the third trimester. Given the modest increase in circulating adiponectin achieved in both LF and HFS fed mice, it is possible that circulating adiponectin was not sufficiently increased to overcome the insulin resistance that developed due to the combination of diet induced obesity, pregnancy, and adiponectin deficiency in HFS fed adiponectin KO mice in the third trimester.

Since we did not use a perfusion system, but a single injection of viral vector, production of adiponectin may have decreased between the time of injection at the end of the second trimester and the time of serum collection at the end of gestation (approximately 7 days). Indeed, one study using plasmid vectors to deliver adiponectin gene therapy to HF-fed non-pregnant mice showed that both transcript and protein levels decreased significantly between 2 hours and 7 days post-delivery (Y. Ma & Liu, 2013). Despite this, the authors ultimately observed an approximate 2-fold increase in adiponectin protein relative to baseline levels 7-days after supplementation (Y. Ma & Liu, 2013). Given that our adiponectin KO mice had no detectable adiponectin in serum prior to supplementation, the levels we observed 7 days after adenovirus-mediated gene therapy are on par with those observed by Ma *et al.*

Insulin resistance and adiponectin resistance have been shown to go hand-in-hand (Lin, 2007), with more severe insulin resistance associated with hyperadiponectinemia and resistance to the glucose-lowering effects of adiponectin. Given that both obesity and pregnancy can lead to insulin resistance, and HF diet feeding is linked to the development of peripheral adiponectin resistance in mice (Mullen et al., 2009), it is not surprising that the elevated levels of adiponectin in the circulation were largely insufficient to overcome the metabolic dysfunction in HF fed mice.

A number of studies have shown that adiponectin suppresses gluconeogenic gene expression (Miller et al., 2011; Zhou et al., 2005). We did not observe any changes to hepatic glucose output with adiponectin supplementation in WT mice (Figure 18F, H) relative to GFP control. However, these data suggest a reduction in glucose production from pyruvate when LF fed adiponectin KO mice were supplemented with APN, compared to GFP controls (Figure 18G, H). These data suggest that increasing circulating adiponectin may reduce *de novo* gluconeogenesis in these mice. This may also explain the reduction in fasting blood glucose observed in adiponectin KO mice that received adiponectin supplementation. This observation further supports the theory that ongoing gluconeogenesis contributes to fasting hyperglycemia in adiponectin KO mice in pregnancy.

In addition to altered insulin tolerance, adiponectin KO mice in the third trimester had impairments to insulin-mediated suppression of hepatic gluconeogenic genes relative to WT controls. We observed that adiponectin supplementation resulted in some reduction of hepatic glucose output in adiponectin KO mice as measured by PTT and decreased hepatic gluconeogenic gene expression. This data suggests that adiponectin supplementation may help to reduce hepatic gluconeogenesis that is elevated in adiponectin KO mice in the third trimester. While some changes to gene expression were not statistically significant, this could be accounted for by the fact that we did not administer insulin or saline at the time of sacrifice to separate the effects of insulin on gene expression. Our samples were collected at the time of sacrifice and although animals are fasted prior to sacrifice, circulating insulin can differ significantly. Thus, the expression level of genes that are sensitive to insulin may vary under these conditions. Therefore, natural variations in insulin levels could mask changes in gene expression that were unmasked in Chapter 3 with the addition of insulin.

We did not observe any improvements in peripheral insulin sensitivity as measured by insulin tolerance test in LF-fed WT (Figure 18I) or adiponectin KO (Figure 18J) mice supplemented with adiponectin, relative to GFP. Chapter 3, we observed impairments in insulin tolerance in pregnant WT mice when fed a HFS diet, and in LF fed

adiponectin KO mice, relative to LF fed WT mice in the third trimester. Since LF-fed WT mice did not have impairments in insulin tolerance, it is not surprising that administration of adiponectin did not show any improvement relative to GFP (Figure 18I). Aye *et al* found that supplementation of obese pregnant mice with adiponectin normalized previously elevated insulin levels, suggesting improvements to insulin sensitivity (Aye et al., 2015).

Supplementation with adiponectin reduced serum insulin levels in LF WT mice in the third trimester relative to GFP (Figure 18L). Although LF fed adiponectin KO mice had lower serum insulin than WT mice when both were given GFP, adiponectin supplementation did not appear to impact serum insulin levels in adiponectin KO mice. It is important to consider the fact that some studies have not found adiponectin KO mice to develop insulin resistance when fed a chow diet, either prior to pregnancy (Maeda et al., 2002) or in the third trimester (Liping Qiao et al., 2017). In contrast to the results of Aye *et al* (Aye et al., 2015), Qiao and colleagues found that adiponectin supplementation increased serum insulin in pregnant adiponectin KO mice (Liping Qiao et al., 2017), suggesting that adiponectin supplementation may impact insulin differently in the presence or absence of obesity or pregnancy. In our study, although adiponectin KO mice have some impairment to insulin tolerance relative to WT controls, supplementation did not affect insulin tolerance relative to GFP control within the same genotype in the third trimester.

In Chapter 4, we established that adiponectin KO mice readily developed hepatic steatosis in the third trimester of pregnancy, even when fed a LF diet. A previous study showed that supplementation with adiponectin reduced markers of fatty liver in a mouse model of alcoholic fatty liver, and *ob/ob* mice with NAFLD (A. Xu et al., 2003). Adiponectin supplementation consistently improved hepatic fat deposition in both WT (Figure 19A) and adiponectin KO (Figure 19B) mice. HFS-fed WT mice had a significant improvement to steatosis score with adiponectin supplementation (Table 9). Given the great difference between natural circulating adiponectin in WT mice and the amount of circulating adiponectin remaining by the end of the third trimester (Figure 18A), it was surprising that even marginally improving serum adiponectin levels in WT mice with diet induced NAFLD can improve the phenotype. While we did not observe statistically significant changes in steatosis score in adiponectin KO mice, our statistical power was limited by low n-numbers in this group. However, TG content in adiponectin KO mice was reduced by supplementation with adiponectin.

It is worth noting that viral gene therapy delivered hydrodynamically through tail vein injection is targeted to and primarily sequestered in the liver (Shayakhmetov, Li, Ni, & Lieber, 2004; Turner, Brabb, Pekow, & Vasbinder, 2011). Full-length adiponectin (which we are using in our adenovirus vector) binds with highest specificity to adipoR2 which is the isoform of the adiponectin receptor found in the liver (Toshimasa Yamauchi et al., 2003). This corresponds with our results that showed increased levels of hepatic *AdipoQ* gene expression following adiponectin supplementation to adiponectin KO mice relative to GFP (Figure 19E). Hydrodynamic delivery of target DNA via tail vein injection without viral vectors has reportedly resulted in approximated 40% of hepatocytes expressing reporter genes in rodents (Sawyer et al., 2009; Turner et al., 2011). Given that we measured marginal increases in circulating adiponectin post adiponectin-adenoviral administration in the adiponectin KO mice, but marked improvements in hepatic steatosis even in mice with WT levels of adiponectin, the effects of adiponectin on hepatic steatosis could be a consequence of paracrine signalling of adiponectin within the liver following injection.

A number of studies have shown that adiponectin reduces hepatic lipogenesis (Awazawa et al., 2009; Stern et al., 2016). In Chapter 4, we showed that adiponectin KO mice exhibited hepatic steatosis associated with increased hepatic expression of lipogenic genes relative to WT mice. We found that with supplementation of adiponectin in the third trimester lead to decreased *Dgat1* expression in WT mice, reduced *Acaca* expression in WT and adiponectin KO mice, and a trend towards decreased expression of *Fasn* in adiponectin KO mice relative to GFP (Figure 19E), although these results are not statistically significant. This data suggests that part of the mechanism behind the reduction in hepatic steatosis with adiponectin supplementation may be a reduction in hepatic lipogenesis. Another study using transgenic adiponectin overexpression in mice showed reductions in hepatic lipogenic gene expression (Shetty, 2012). It is important to note that in adiponectin supplemented mice we did not separate out the effect of insulin on insulin sensitive genes by adding insulin or saline prior to sacrifice. The varying levels of insulin due to insulin resistance in the third trimester of pregnancy, combined with the fact that WT mice have higher baseline levels of adiponectin may contribute to variability in gene expression. Nevertheless, adiponectin supplementation appears to have an effect on lipogenic gene expression in the third trimester.

In addition to lipogenesis, fatty acid uptake from circulation can contribute to increased hepatic fat deposition, particularly if combined with inefficient β-oxidation. Adiponectin has been shown to increase β-oxidation (T. Yamauchi et al., 2001) and in Chapter 3 we found evidence for impaired fatty acid oxidation in pregnant adiponectin KO mice. Adiponectin KO mice had increased circulating FFA relative to WT mice in the third trimester, and we found that supplementation with adiponectin appeared to reduce serum FFA relative to that of GFP controls (Figure 19D). This coincides with results from Yamauchi *et al* who showed that adiponectin treatment reduced serum FFA, which were elevated in a lipoatrophic model of diabetes (T. Yamauchi et al., 2001). Similarly, in cell culture experiments with adipocytes, adiponectin inhibited lipolysis from adipocytes, (Liping Qiao et al., 2011) which could improve markers of hyperlipidemia. While we did not observe an effect of adiponectin supplementation on visceral adiposity, as measured by PWAT and GWAT mass at the end of the third trimester (Table 10), the length of time between adenoviral administration and sacrifice of the pregnant was likely too short for adiponectin-induced metabolic alterations to have an effect on visceral fat in pregnant mice.

An important consideration of interventions during pregnancy is to characterize the effect on fetal development (Brawerman & Dolinsky, 2018). Low levels of adiponectin in pregnancy have been linked to large for gestational age infants in humans (Lekva et al., 2017). Supplementation of adiponectin to pregnant mice reduced fetal size, by reducing placental insulin and mTORC signalling, as well as amino acid transporters that impact fetal growth (Rosario et al., 2012). Interestingly, adiponectin supplementation reduced fetal size in WT mice on a LF diet but did not have an effect on the size of neonates from adiponectin KO mice. This could be due to the fact that in other studies of adiponectin supplementation and fetal growth, supplementation was performed by chronic infusion, ensuring sustained supplementation by osmotic pump (Aye et al., 2015; Rosario et al., 2012). In contrast, we injected adenovirus containing adiponectin at the end of the second trimester and collected tissue and fetal data at the end of gestation, approximately 7 days later. It was possible that we did not achieve the same sustained levels of adiponectin supplementation and delivery that are accomplished with osmotic pump infusion. Additionally, adiponectin KO mice did not have significantly larger fetuses relative to WT mice in our study when given GFP. We sacrificed mice prior

to delivery and variation by even one gestational day may contribute to differences in size. Therefore, it is possible that given the chance to give birth, neonates may have shown differences in size between genotypes.

Overall, we found that adiponectin supplementation in the third trimester improved fasting blood glucose and reduced hepatic glucose output in adiponectin KO mice. Further, increasing circulating adiponectin appeared to dramatically improve hepatic steatosis in both WT mice and adiponectin KO mice in the third trimester, and that hepatic lipogenic gene expression was decreased. The impact of supplementation appeared greatest on fatty liver development, further implicating the role of adiponectin deficiency and hepatic steatosis in the development of GDM.

Chapter 6:

Effect of adiponectin KO on the adaptation of the pancreatic islet in the third trimester

6.1 Introduction

As described in Section 1.2.1, the pancreatic islet contains 5 different cell types with individual secretory functions. In healthy adults, the population of cells in the pancreatic islet remain relatively constant after the postnatal period (Finegood et al., 1995; Kushner, 2013), with growth being slow but sufficient to maintain β-cell numbers. While the β-cell can undergo adaptations in response to increased demand for insulin (due to hyperglycemia, for example) (C. Chen, Hosokawa, Bumbalo, & Leahy, 1994), there is a growing body of evidence implicating β-cell dysfunction and compensatory failure of islet adaptation in the development of T2D and GDM (Buchanan et al., 2002; A. E. Butler et al., 2003; Kahn, 2003; Prentki & Nolan, 2006; Rahier, Guiot, Goebbels, Sempoux, & Henquin, 2008). The presence of obesity increases the risk of insulin resistance (Olefsky, Farquhar, & Reaven, 1973), and some individuals may be predisposed towards β-cell dysfunction or damage (Prentki & Nolan, 2006). Additionally, *in utero* exposure to GDM may also predispose offspring to β-cell dysfunction and contribute to T2D risk later in life (Prasoon Agarwal et al., 2019; P. Agarwal et al., 2018).

In addition to GDM exposure, high-fat diets and obesity act synergistically to induce β-cell dysfunction (Prasoon Agarwal et al., 2019). Pancreatic islets are susceptible to damage by the lipotoxicity (Unger, 1995) and glucotoxicity (Unger, 1985) of obesity. HFD feeding induces compensatory β-cell hyperplasia, but also causes defects in insulin secretory capacity (Hull et al., 2005; Peyot et al., 2010). In extreme situations, lipid overload of islets can lead to steatosis and reduce the ability of islets to overcome insulin resistance, and can increase apoptosis (S. C. Lee, Robson-Doucette, & Wheeler, 2009; Y. Q. Liu, Jetton, & Leahy, 2002; Roger H. Unger, 1995). It is believed that the progression to overt diabetes involves insulin resistance and failure of the β-cell to increase insulin secretion to overcome the peripheral insulin resistance (Bergman, 2005)

Within the pancreatic islet, β-cells are the most abundant cell type, and the next most abundant are α-cells which secrete glucagon. Increased glucagon from α-cells is observed in T2D and may contribute to hyperglycemia due to its ability to increase hepatic glucose output (P. C. Butler & Rizza, 1991; Müller, Faloona, Aguilar-Parada, & Unger, 1970; Stumvoll, Meyer, Kreider, Perriello, & Gerich, 1998). Mouse models with impaired glucagon receptors are protected against the effects of diet induced obesity (Conarello et al., 2007). A recent single-cell RNAseq study showed that β-cells were more dramatically affected by high-fat diet (HFD) consumption than α-cells (Dusaulcy et al., 2019). Interestingly, α-cells showed increased expression of genes associated with protection against apoptosis, but fewer altered genes overall with HFD feeding, leading the authors to suggest that α-cells may be more resistant to the effects of HFD consumption (Dusaulcy et al., 2019). β-cells on the other hand, showed changes in genes for ion channels and exocytosis, important for insulin secretion, as well as genes related to hyperplasia, which is a compensatory mechanism by β-cells for insulin resistance (Dusaulcy et al., 2019). These findings suggest that HFD feeding may impair the processes responsible for insulin secretion and hyperplasia, with secretory function from α-cells remaining unchanged, potentially contributing to hyperglycemia.

Pregnancy requires significant adaptive changes in the cells of maternal pancreatic islets. In order to overcome the physiological insulin resistance of pregnancy that occurs to spare glucose for the fetus, β-cell expansion occurs, particularly in late gestation (J. A. Parsons, Bartke, & Sorenson, 1995; J. A. Parsons, Brelje, & Sorenson,

1992; Sebastian Rieck & Kaestner, 2010). In pregnant mice, α-cell content has also been shown to increase, largely due to increased proliferation at e18.5 (Quesada-Candela et al., 2020), and glucagon secretion increases mid-gestation in human pregnancy (Luyckx, Gerard, Gaspard, & Lefebvre, 1975). While many previous studies have used rodent models, few have confirmed similar findings in islets from human pregnancy (A. E. Butler et al., 2010; Van Assche et al., 1978). As reviewed in Chapter 1, mechanistic studies have implicated pregnancy as a driving force behind these adaptations (Figure 6). In the β-cell, hormonal changes and placental lactogens signal to induce structural and functional changes even before the onset of insulin resistance (Demirci et al., 2012; Ernst et al., 2011).

At a functional level, pregnancy increases glucose stimulated insulin secretion (Costrini & Kalkhoff, 1971; Janah et al., 2019; Sorenson & Brelje, 1997). Human islets have shown increases in β-cell content in pregnancy which appears to return to baseline postpartum (A. E. Butler et al., 2010). Rodent studies have shown β-cell expansion in response to the demands of pregnancy and to a lesser degree, neogenesis (Ernst et al., 2011; J. A. Parsons et al., 1995; S. Rieck et al., 2009; H. Zhang et al., 2010). β-cell dysfunction may contribute to the development of GDM if compensatory β cell expansion is insufficient to maintain normoglycemia (Buchanan & Xiang, 2005).

As discussed in Chapter 1, adiponectin may be involved in the adaptive response of the pancreatic islet to pregnancy (L. Qiao et al., 2017), and adiponectin deficiency may lead to impairments in the ability of the islet to adapt to metabolic stress. Previously it was reported that chow-fed male adiponectin KO mice were not hyperglycemic, but showed increased serum insulin only when fed a HFS diet (Maeda et al., 2002). However, another study showed impaired insulin secretion by adiponectin KO mice following a glucose load (Kubota et al., 2002; Maeda et al., 2002). To assess the role of adiponectin in glucose homeostasis during pregnancy, we showed in Chapter 3 that in the third trimester LF- as well as HFS-fed adiponectin KO mice had similar serum insulin levels as WT controls despite increased fasting blood glucose and impaired glucose tolerance. In line with these observations, Qiao *et al* showed that chow fed pregnant adiponectin KO mice had lower serum insulin relative to WT mice (Liping Qiao et al., 2017). It has been suggested that adiponectin plays a role in the adaptation of the endocrine pancreas to pregnancy (Liping Qiao et al., 2017; Retnakaran et al., 2005).

Adiponectin receptors have been identified on the β-cell, and may have a role in protection against lipotoxicity (Kharroubi, Rasschaert, Eizirik, & Cnop, 2003; Ye et al., 2015). However, it is not yet clear what role adiponectin plays in the adaptation of the islet to pregnancy or whether there is any impact of adiponectin on glucagon secretion. We hypothesized that adiponectin KO mice have impaired pancreatic islet adaptation, marked by suppressed insulin secretion in the third trimester of pregnancy.

6.2 Materials and Methods – Refer to Page 24

6.3 Results:

6.3.1 Insulin Secretion

T2D, obesity and GDM are associated with impairments of glucose-stimulated insulin secretion (GSIS), however the role of adiponectin in β-cell adaptation to pregnancy and insulin resistance has not been firmly established. We hypothesize that adiponectin deficiency could impair the necessary increases in GSIS during

pregnancy, contributing to hyperglycemia and the development of GDM. In order to determine the effect of adiponectin deficiency on pancreatic islet function in pregnancy, we isolated islets from LF- and HFS-fed WT and adiponectin KO mice at the end of gestation (e18.5), as well as adult non-pregnant mice older than 6 weeks of age, and assessed the insulin secretion capacity using static GSIS assays.

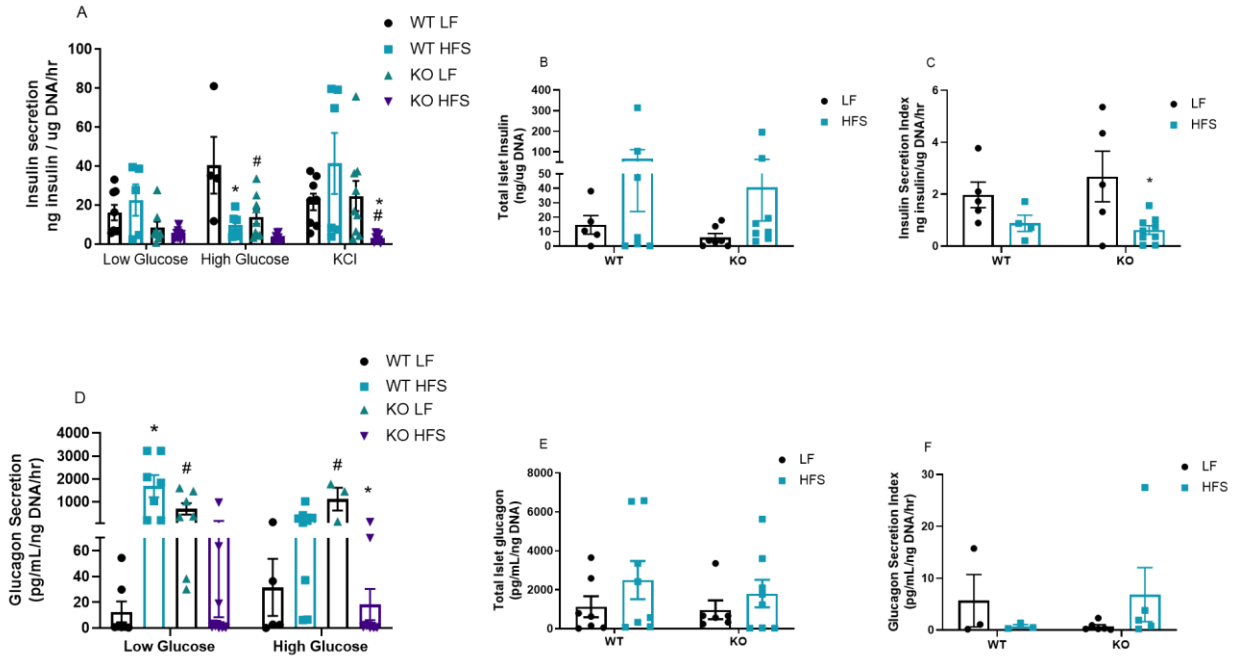
We found that WT mice fed a HFS diet had similar levels of insulin secretion as LF fed animals under low glucose (2.8mM) conditions (Figure 20A), but insulin secretion was impaired under high glucose (16.7mM) conditions. We also exposed the islets to KCl, which directly depolarizes the β-cell, bypassing glucose metabolism, and allowing us to determine if there are defects in the glucose metabolism portion of the insulin secretory pathway or downstream in the exocytotic response (Dawson, Atwater, & Rojas, 1982). Notably, diet did not affect KCl-mediated insulin secretion in WT islets, suggesting that the exocytotic pathway remained functional after HFS feeding and that HFS diet disrupts β-cell glucose metabolism, which is consistent with previous studies (É. Pepin et al., 2016; Peyot et al., 2010).

In adiponectin KO mice, consumption of a HFS diet had no effect on insulin secretion under low glucose conditions but lead to reduced insulin secretion relative to LF-fed mice under high glucose conditions (Figure 20A). KCl-mediated insulin secretion was markedly reduced in adiponectin KO mice on a HFS diet suggesting defective insulin exocytosis in these mice. When compared to WT mice, LF-fed adiponectin KO mice had significantly reduced insulin secretion with exposure to high glucose in the third trimester, and HFS-fed adiponectin KO mice had significantly impaired maximal insulin secretion compared to WT mice on a HFS diet (Figure 20A). When we measured total islet insulin content, HFS diet consumption appeared to increase total islet insulin content in both WT and adiponectin KO mice, but this was not statistically significant (Figure 20B). These results suggest that islets from adiponectin KO mice in the third trimester have reduced glucose-stimulated insulin secretion capacity, including reductions in insulin exocytosis when consuming a HFS diet. These impairments may indicate a defect in the adaptive response to pregnancy and hyperglycemia and contribute to the development of GDM.

6.3.2 Glucagon Secretion

Glucagon acts in opposition of insulin and is secreted in response to hypoglycemia; hyperglycemia suppresses glucagon secretion (Iben Rix, 2019). Since relatively little is known about the effects of adiponectin on α-cells and glucagon secretion, we performed glucagon assays on islets isolated from the same mice. We found that under low-glucose conditions WT mice fed a HFS-diet showed reduced glucose inhibited glucagon secretion relative to LF-fed animals in late gestation (Figure 20D), but under high-glucose conditions this defect was normalized and suppression was achieved. Interestingly, adiponectin KO mice fed a HFS diet had significantly reduced glucagon secretion relative to LF fed mice under both low and high glucose conditions (Figure 20D). In comparison to WT mice, adiponectin KO mice fed a LF diet had dramatically elevated glucagon secretion under low and high glucose conditions; in contrast, islets from HFS fed adiponectin KO mice had reduced glucagon secretion compared to HFS-fed WT mice when exposed to low-glucose (Figure 20D). Total islet glucagon content showed a similar pattern to insulin content, with HFS feeding increasing total content in both WT and adiponectin KO mice (Figure 20E).

Figure 20: Adiponectin KO mice show dysregulation to islet secretory capacity in the third trimester relative to WT controls



A) Glucose stimulated insulin secretion from islets isolated at the end of the third trimester. N=5-8 animals per group. B) total islet insulin content N=5-8 animals per group. C) Insulin secretion index of isolated islets expressed relative to baseline. N=4-10 animals per group. D) glucose inhibited glucagon secretion from islets isolated at the end of the third trimester N=4-10 animals per group E) total islet glucagon content N=6-8 animals per group. F) Glucagon secretion index of isolated islets expressed relative to baseline. N=3-6 animals per group. P values represent statistical significance ($p<0.05$) after Two-way ANOVA. * = $p<0.05$ between LF and HFS. # = $p<0.05$ between WT and adiponectin KO. WT: Wildtype, KO: knockout, LF: Low fat, HFS: High fat and sucrose.

6.3.3 Non-pregnant Islet Morphology

Hyperglycemia and obesity have been shown to induce compensatory changes in islets (Bock, Pakkenberg, & Buschard, 2003). In isolated mouse islets, adiponectin has been shown to improve β-cell survival and function (Wijesekara et al., 2010; Ye et al., 2014; Ye et al., 2015), and may counteract the effects of diet induced obesity on the islet in cell lines (Rakatzi et al., 2004). We analyzed pancreatic cryosections from non-pregnant adiponectin KO and WT mice to compare islet content, morphology and relative β-cell and α-cell make-up. Mice were on the diet for a minimum of 6 weeks and were older than 12 weeks of age. Prior to pregnancy, we found that consumption of a HFS diet led to a trend of increased total islet number and islets per mm^2 in WT mice (Table 12). A similar trend was observed in mean islet area and islet area relative to overall pancreas area, but this was not statistically significant (Table 12). These findings are in line with results from previous studies, which show compensatory changes in islet morphology with HF diet feeding in rodents(D. Gupta et al., 2017; Roat et al., 2014; Woodland et al., 2016).

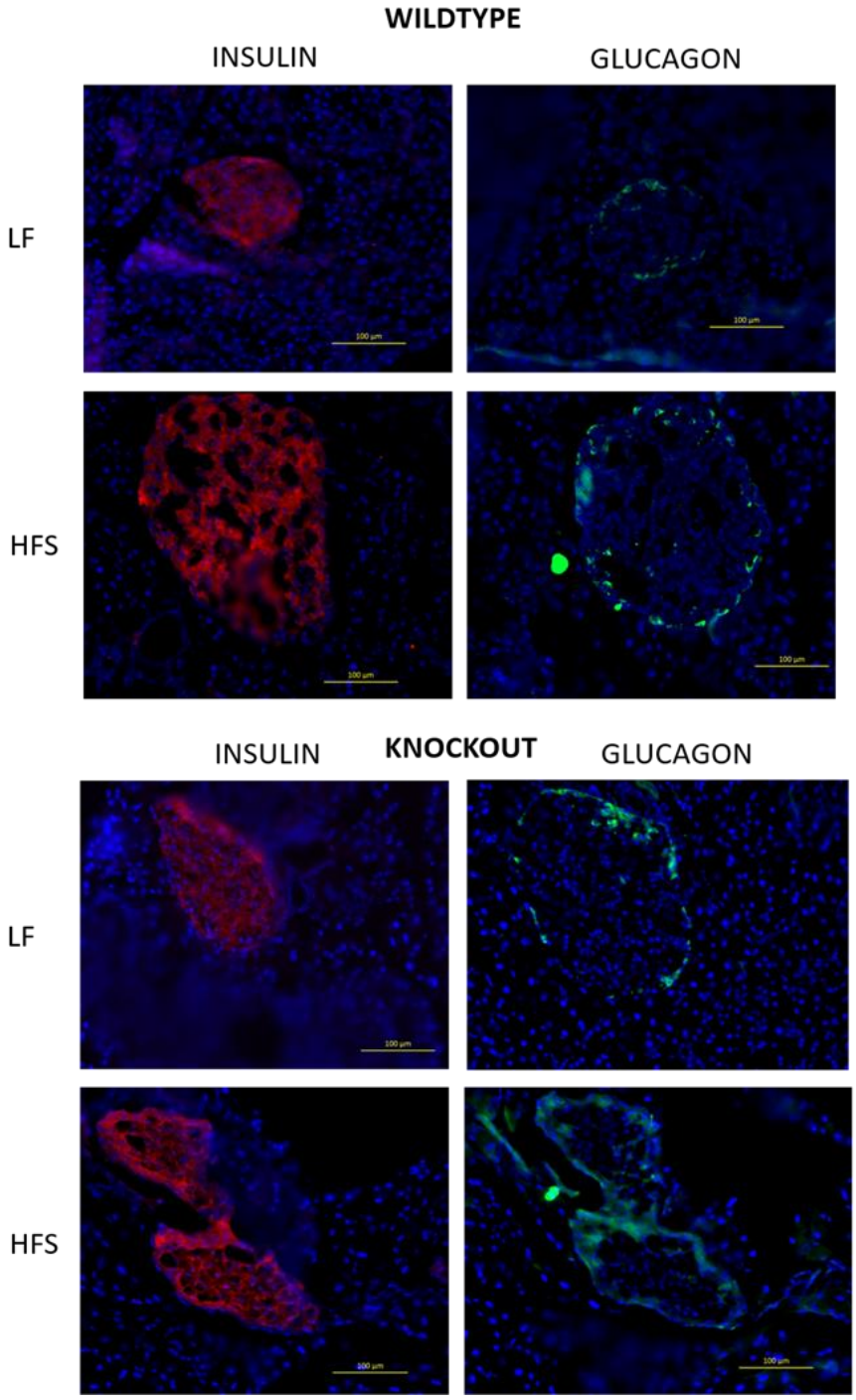
Table 12: Islet morphology in non-pregnant adiponectin KO and WT mice

***Genotype*:**	**WT**	**WT**	**APN$^{-/-}$**	**APN$^{-/-}$**
***Diet*:**	**LF**	**HFS**	**LF**	**HFS**
Number of Islets	5.1	15.9	10.2	12.7
Number of Islets/ Pancreas Area(mm^2)	0.37	0.80	0.61	0.68
Mean Islet Area(μm^2)	25184	47905	31002	29865
Islet Area/Pancreas Tissue Area(μm^2)	0.09	0.160	0.120	0.100
β-*cell parameters*				
β-cell area(μm^2)	15555	29225	22168	16143
β-cell numbers	124	239	108	113
β-cell numbers/Islet Area(μm^2) X1000	4.8	4.9	4.2	5.1
β-cell fraction	0.65	0.79	0.65	0.82
α-*cell parameters*				
α-cell area(μm^2)	822	2518	2494	4044
α-cell numbers	21	36	24	45
α-cell numbers/Islet Area(μm^2) X1000	0.94	0.96	0.73	1.39
α-cell fraction	0.14	0.08	0.12	0.23

Structural parameters of pancreatic islets in WT and adiponectin KO mice when consuming either a LF or HFS diet prior to pregnancy. N=1 mouse per group. WT: Wildtype, KO: knockout, LF: Low fat, HFS: High fat and sucrose.

Interestingly, LF-fed adiponectin KO mice had an approximately 20% increase to overall islet area, and nearly twice as many total islets and islets per pancreas area relative to LF fed WT mice. However, unlike WT mice, consumption of a HFS diet did not increase islet number or islet area in adiponectin KO mice. With regards to β-cell area, consumption of a HFS diet increase total β-cell area by approximately 2-fold in WT mice, but this effect was not observed in adiponectin KO mice and there was no effect on the fraction of β-cells in the islet (Table 12, Figure 21). In WT mice, HFS diet consumption appeared to increase the overall α-cell area and α-cell numbers, but not α-cell numbers per islet area or α-cell fraction (Table 12). Notably, LF-fed adiponectin KO mice had greater α-cell area relative to WT mice and this appeared to increase with HFS diet consumption. While the effect of HFS diet on α-cell numbers was roughly the same in adiponectin KO mice relative to WT mice, adiponectin KO mice had increased α-cells per islet area, and increased α-cell fraction with HFS diet consumption relative to WT (Table 12, Figure 21).

Figure 21: Consumption of a HFS diet impacts pancreatic islet morphology in WT and adiponectin KO mice prior to pregnancy



Representative immunofluorescence images of insulin (red) and glucagon (green) staining of endocrine pancreas from non-pregnant WT or adiponectin KO mice fed either LF or HFS diet (images 20X magnification). WT: Wildtype, KO: knockout, LF: Low fat, HFS: High fat and sucrose.

6.3.4 Pregnant Islet Morphology

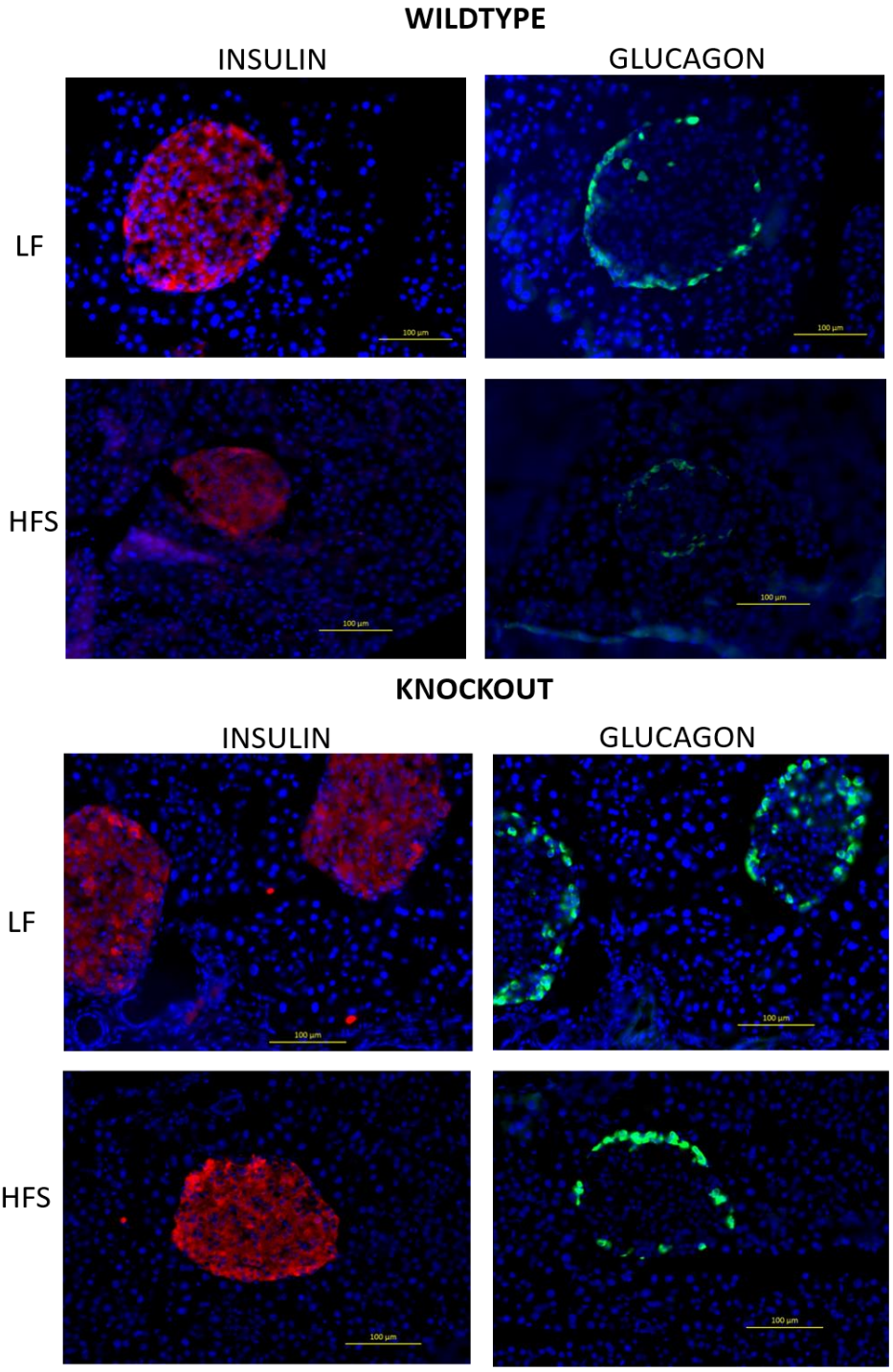
Studies have shown that pregnancy induces changes in islet morphology such as β-cell expansion and neogenesis (Sorenson & Brelje, 1997). Defects in these mechanisms can contribute to the development of GDM. In contrast to non-pregnant mice, we found that HFS diet consumption in pregnancy significantly decreased total number of islets and mean islet area in WT mice (Table 13, Figure 22). This was also true in adiponectin KO mice, however the decrease in total islet number was more drastic with HFS diet relative to HFS fed WT mice (Table 13). Despite changes to secretory capacity, neither HFS diet consumption nor adiponectin KO genotype had a significant impact on β-cell mass or ratio. However, α-cell area showed a trend towards decreasing with HFS diet consumption in pregnant WT mice and was unchanged in HFS fed adiponectin KO mice relative to LF-fed adiponectin KO mice (Table 13). HFS-fed adiponectin KO mice appear to have higher α-cell positive area per islet relative to HFS-fed WT mice, although these differences were not statistically significant (Table 13, Figure 22). β-cell area of LF-fed WT mice showed an upward trend in pregnancy compared to the non-pregnant group (Tables 10, 11). Interestingly, LF adiponectin KO mice did not increase β-cell area in pregnancy (Tables 10, 11). These results suggest that adiponectin KO mice may have impairments to structural adaptation of the pancreatic islet in the third trimester, particularly with HFS diet consumption.

Table 13: Islet morphology in the third trimester of WT and adiponectin KO mice

***Genotype*:**	**WT**	**WT**	**APN$^{-/-}$**	**APN$^{-/-}$**
***Diet*:**	**LF**	**HFS**	**LF**	**HFS**
Number of Islets	13.40 ± 0.60	9.90 ± 0.70*	13.10 ± 0.70	7.50 ± 0.50$^{*\#}$
Number of Islets/ Pancreas Area(mm^2)	0.63 ± 0.04	0.62 ± 0.13	0.72 ± 0.08	0.62 ± 0.02
Mean Islet Area(µm^2)	36151±1289	30149±376*	33764±2498	35210±1975
Islet Area/Pancreas Tissue Area(µm^2)	0.128 ± 0.005	0.103 ± 0.002	0.112 ± 0.008	0.122 ± 0.008
β-*cell parameters*				
β-cell area(µm^2)	27419± 1209	21970 ± 327	22493 ± 2091	26905 ± 1634
β-cell numbers	186.00 ± 8.00	134.00 ± 7.00	197 ± 10.00	180.00 ± 11.00
β-cell numbers/Islet Area(µm^2) X1000	5.24 ± 0.06	5.11 ± 0.09	5.58 ± 0.15	5.47 ± 0.10
β-cell fraction	0.80 ± 0.01	0.82 ± 0.03	0.83 ± 0.01	0.85 ± 0.01
α-*cell parameters*				
α-cell area(µm^2)	5989 ± 582.00	2200 ± 400	5764 ± 798	3383.00 ± 92.00
α-cell numbers	55.00 ± 5.00	32.00 ± 3.00	64.00 ± 8.00	41.00 ± 2.00
α-cell numbers/Islet Area(µm^2) X1000	1.44 ± 0.10	0.95 ± 0.11	1.79 ± 0.20	1.36 ± 0.05
α-cell fraction	0.23 ± 0.01	0.15 ± 0.02	0.21 ± 0.01	0.23 ± 0.01

Structural parameters of pancreatic islets from third trimester pregnant adiponectin KO and WT mice fed either a LF or HFS diet. (N=3-6 mice per group). *P<0.05 LF vs HFS, $^{\#}$P<0.05 WT vs. KO as determined by Two-Way ANOVA WT: Wildtype, KO: knockout, LF: Low fat, HFS: High fat and sucrose.

Figure 22: Impact of HFS diet consumption on islet morphology in WT and adiponectin KO mice in the third trimester



Representative immunofluorescence images of insulin (red) and glucagon (green) staining of endocrine pancreas from third trimester pregnant WT or adiponectin KO mice fed either LF or HFS diet (images 20X magnification). WT: Wildtype, KO: knockout, LF: Low fat, HFS: High fat and sucrose.

6.4 Discussion/Conclusions

Pregnancy is associated with several maternal metabolic adaptations that are necessary to provide energy for fetal growth (reviewed in (Lain & Catalano, 2007). In the islet, these include functional and morphological changes that act to increase the capacity for insulin secretion (Bock et al., 2003; Gonzalez et al., 2013; Hull et al., 2005; Rahier et al., 2008). Weight gain and insulin resistance also increase the need for insulin in order to maintain euglycemia (Bergman, 2005; Kahn, 2003). When obesity or insulin resistance precede pregnancy, or where there is excessive gestational weight gain, the metabolic compensation to pregnancy can become maladaptive. In the preceding chapters, we showed that HFS diets and adiponectin deficiency contributed to glucose intolerance and insulin resistance during pregnancy that is characteristic of GDM.

In this chapter, we evaluated how the HFS diet and adiponectin deficiency affected islet morphology and function in order to understand whether compensatory failure is a mechanism underlying the development of GDM in our model. We showed that prior to pregnancy, the HFS diet may increase total islet content in both WT and adiponectin KO mice (Table 12) in a representative sample. This may indicate compensatory β cell expansion since HFS diet consumption was associated with increased weight gain in non-pregnant mice in Chapter 3, however low sample number limits the ability to draw conclusions. Studies in rodents have shown that obesity, even in the absence of diabetes, is associated with increased β-cell mass (Hull et al., 2005; Klöppel, Löhr, Habich, Oberholzer, & Heitz, 1985).

The HFS-fed WT mice were not hyperglycemic prior to pregnancy and we observed a nearly 2-fold increase in β-cell area in HFS fed non-pregnant WT mice (Table 12). Overall β-cell fractions per total islet area were not increased. Interestingly, while the HFS diet trended towards adaptive increases in overall islet mass and β-cell mass in the WT mice, this effect was blunted in adiponectin KO mice prior to pregnancy (Table 12), indicating a pre-existing failure to adapt to HFS-feeding in KO mice, which may make them more susceptible to adaptation failure during pregnancy. In HFS fed adiponectin KO mice, overall islet content was lower than HFS fed WT mice, suggesting some degree of impairment in the ability of adiponectin KO mice to compensate for obesity during pregnancy (Bock et al., 2003). Overall β-cell area may also be reduced relative to WT mice when adiponectin KO mice were fed a HFS diet. This would indicate a reduced capacity for elevated insulin secretion in these mice. Although the proportion of β-cells per islet area and β-cell fraction appeared increased relative to WT mice this may have been due to the relative reduction in overall islet area in this mouse, and low sample number (n=1) analyzed.

Other studies in adiponectin KO mice have shown mixed results regarding insulin resistance in non-pregnant mice. One study reported marked insulin resistance and impaired insulin secretion following glucose load in non-pregnant KO mice fed a standard diet (Kubota et al., 2002). In agreement with our findings, it was reported that male adiponectin KO mice were not insulin resistant on a chow diet and only developed insulin resistance and hyperglycemia after 2 weeks of HFS diet consumption. (Maeda et al., 2002). Another study by Qiao *et al* did not report insulin resistance in pregnant adiponectin KO mice on a chow diet but marked decreases in β-cell mass and islet area in pregnant adiponectin KO mice relative to WT animals (Liping Qiao et al., 2017). These data implicate

adiponectin in structural β-cell adaptation to increased metabolic demand. In a drug-induced polycystic ovary syndrome (PCOS)- model of insulin resistance, islet expansion and increased insulin gene expression in response to in insulin resistance was observed in adiponectin KO mice, suggesting that in the absence of a second metabolic hit (such as diet or pregnancy) the pancreatic islet of adiponectin KO mice can compensate adequately (Benrick et al., 2017). We observed an increase in islet number in LF fed adiponectin KO mice prior to pregnancy that would agree with these findings and could represent an early compensatory mechanism to overcome the increased insulin resistance conferred by a lack of adiponectin.

Dysregulation at the level of the α-cell is also implicated in metabolic dysfunction. Increased pancreatic glucagon has been observed in obesity (Starke, Erhardt, Berger, & Zimmermann, 1984; Stern et al., 2016; Stern et al., 2019). In addition, hyperglucagonemia may be associated with the development of T2D (Dunning & Gerich, 2007). To our knowledge, this is the first study to examine α-cell morphology and function in adiponectin KO mice. While our sample is too small to be conclusive, we observed increased α-cell area in HFS-fed WT mice, but not relative α-cell to islet area or α-cell fraction. These data suggest that in non-pregnant mice the increase in α-cell area does not account for the increased islet area. As introduced in Chapter 1, insufficient adaptation of islets to the metabolic demands of pregnancy can lead to GDM (H. Zhang et al., 2010). In contrast to non-pregnant animals, consumption of a HFS diet in pregnancy resulted in a significant decrease to total islet number in both WT and adiponectin KO mice in the third trimester (Table 13). Total islet area is also markedly decreased in HFS fed WT mice relative to LF mice in the third trimester, suggesting a potential impairment of islet expansion and islet neogenesis due to obesity in pregnancy.

In adiponectin KO mice fed a HFS diet, the total number of islets was significantly reduced relative to LF fed adiponectin KO mice as well as HFS fed WT mice (Table 13). This indicates that pregnant adiponectin KO mice could be more susceptible than WT mice to the deleterious effects of a HFS diet on β-cell proliferation. Some studies show that adiponectin may increase β-cell mass, potentially through increased proliferation (Chetboun et al., 2012; Liping Qiao et al., 2017). Adiponectin addition was shown to protect INS-1 cells against lipid-induced apoptosis (Rakatzi et al., 2004). In contrast, data from human islets suggests that adiponectin may not play a major role in the protection against apoptosis (Staiger et al., 2005). Interestingly, we observed that pregnant adiponectin KO mice did not exhibit the HFS-diet induced impairments of islet expansion that were observed in non-pregnant adiponectin KO mice. However, this could be due to a greater variability in islet size in adiponectin KO mice relative to WT mice. The absence of significant alterations of β-cell morphology in adiponectin KO mice consuming a LF diet may imply that adiponectin KO mice are unable to compensate for the added metabolic stress of a HFS diet in pregnancy.

Expansion of the islet in pregnancy has been demonstrated in rodent models (Kalkhoff & Kim, 1978) and transgenic studies have shown that islet mass increases with increasing placental lactogen (R. Vasavada et al., 2000; Rupangi C. Vasavada et al., 2000), as outlined in Figure 5. Consistent with this, both WT and adiponectin KO mice appeared to have increased islet area in pregnancy relative to non-pregnant animals. Unfortunately, statistical significance due to diet or genotype specific differences could not be determined due to the low representative

sampling of non-pregnant animals. Nonetheless, the trends we observed are consistent with other studies on the endocrine pancreas in pregnancy (S. Bonner-Weir, 2000; A. E. Butler et al., 2010; Kalkhoff & Kim, 1978; Sebastian Rieck & Kaestner, 2010; R. Vasavada et al., 2000).

As shown in Chapter 3, we observed some insulin resistance in pregnant adiponectin KO mice relative to WT controls. However, we did not observe accompanying hyperinsulinemia that is often associated with insulin resistance (S. H. Kim & Reaven, 2008). Adiponectin KO mice fed a LF diet showed a relatively normal pattern of insulin secretion with exposure to high glucose and KCl. However, the level of insulin secretion was decreased relative to WT mice on a LF diet, most notably under high glucose conditions (Figure 20A). Although there were no notable changes to β-cell content in adiponectin KO mice relative to WT, HFS diet consumption appeared to further impair glucose stimulated insulin secretion, even relative to LF fed adiponectin KO mice. Interestingly, the secretion of insulin on exposure to KCl was significantly decreased, which may indicate impairments in insulin secretion independent of glucose uptake and metabolism.

Other experiments in pregnant adiponectin KO mice showed decreased serum insulin compared to WT that was improved with adiponectin supplementation, without changes in GSIS from isolated islets (Liping Qiao et al., 2017). GSIS experiments in that study confirmed other findings from non-pregnant mice that adiponectin potentiates glucose stimulated insulin secretion (Bratanova-Tochkova et al., 2002; Straub & Sharp, 2004; Wijesekara et al., 2010). In islets isolated from insulin resistant C57 female mice fed a HF diet for 8 weeks, *in vitro* treatment with adiponectin suppressed insulin secretion under low glucose conditions and increased insulin secretion under high glucose conditions (Winzell, Nogueiras, Dieguez, & Ahrén, 2004). Although Winzell *et al* noted increased glucose oxidation in islets from HF fed mice, they paradoxically did not note any alterations to ATP/ADP ratio (Winzell et al., 2004). While Okamoto *et al* did determine that adiponectin can increase insulin secretion in an ATP independent mechanism (Okamoto et al., 2008), more research is necessary to elucidate these mechanisms and determine whether they persist in pregnancy.

Our results showed that the HFS diet in pregnancy did not change basal insulin secretion in WT mice, but HFS diet consumption impaired GSIS (Figure 20A). Insulin secretion is biphasic and requires glucose sensing, uptake and metabolism to ATP (Bratanova-Tochkova et al., 2002; Rorsman et al., 2000). ATP binding to K-ATP channels result in membrane depolarization and calcium uptake, which triggers exocytosis of docked vesicles containing insulin (Ashcroft, Harrison, & Ashcroft, 1984; Cook & Hales, 1984; Kasai, Takahashi, & Tokumaru, 2012). Exposing islets to KCl result in rapid membrane depolarization and exocytosis of insulin, bypassing ATP synthesis and the rapid secretion of readily available insulin. Defects to KCl-depolarized insulin secretion may indicate impairments in first-phase insulin secretion capacity (Henquin, 2000; Seino, Takahashi, Fujimoto, & Shibasaki, 2009; Suriben et al., 2015). First-phase insulin release occurs rapidly, within 2-5 minutes (Curry, Bennett, & Grodsky, 1968; Gerich, 2002). Due to this quick release, defects are often missed during other analysis such as GTT, however impairments to first phase insulin secretion may be an early indicator of β-cell dysfunction (Gerich, 2002). In human studies, pregnant women have shown increased first-phase insulin response in the third trimester relative to non-pregnant women (Buchanan, Metzger et al. 1990, Powe, Huston Presley et al. 2019) and women with GDM had reduced first phase insulin secretion

compared to those with normal glucose tolerance (Buchanan, Metzger et al. 1990). Adiponectin KO mice fed a HFS diet had significantly reduced KCl-stimulated insulin secretion relative to WT mice, which may indicate a defect in first-phase insulin secretion in pregnancy, contributing to the development of GDM. In order to isolate the location of the defect in insulin secretion, it would be helpful to measure the ability of pancreatic islets to take up glucose and synthesize ATP. Additionally, perifusion studies provide the best picture of secretory capacity of islet cells in response to stimuli in real time.

In INS-1 cells, Wang *et al* determined that adiponectin protects against apoptosis and defective insulin secretion under high glucose, and the combination of high glucose and high lipid conditions in an AMPK-dependent fashion (Yan Wang, Li, Qiao, Li, & Qiao, 2019). Interestingly, Okamoto *et al* determined that adiponectin can increase insulin secretion independent of AMPK under low glucose conditions suggesting that adiponectin may exert its protective effects on the β-cell in different ways, depending on nutrient status (Okamoto et al., 2008). APPL-1, an adaptive protein downstream of adiponectin, is known to potentiate adiponectin signaling (Mao et al., 2006), and experiments using APPL-null β-cells showed defects in insulin secretion and exocytotic machinery, which was rescued with APPL-1 overexpression and downstream AKT signaling (Cheng et al., 2012). Similar to results seen by Okamoto *et al* with adiponectin, upregulating APPL-1 also resulted in increased docking of exocytotic vesicles (Cheng et al., 2012). While Cheng *et al* did not explicitly implicate adiponectin in their defined mechanism, this could represent a viable downstream mechanism independent of AMPK by which adiponectin increases insulin exocytosis. Additionally, pregnancy-specific changes may alter Ca+ dependent exocytosis (Horn et al., 2016). Okamoto *et al* determined that adiponectin can trigger insulin secretion through a Ca+ independent pathway, potentially indicating a unique role for adiponectin in the β-cell under varying nutritional and metabolic stress, potentially those involved in the development of GDM (Okamoto et al., 2008).

Aberrant glucagon signaling and secretion is noted in obesity and diabetes (Conarello et al., 2007; Luyckx et al., 1975; Müller et al., 1970; Starke et al., 1984). Glucagon signaling in the liver increases hepatic glucose output, and decreases lipogenesis, instead increasing fatty acid oxidation and ketogenesis (A. Young, 2005). Glucagon secretion by the α-cell is increased under conditions of hypoglycemia and suppressed by glucose (Palmer, Benson, Walter, & Ensinck, 1976) and insulin (Vergari et al., 2019). In pregnancy, plasma glucagon levels gradually increase and peak in mid gestation but are indistinguishable from postnatal levels in late gestation in a healthy pregnancy (Luyckx et al., 1975). A recent study showed that in pregnancy, impaired early phase glucagon suppression (within the first 20 minutes) after oral glucose challenge is associated with increased need for insulin therapy in women with GDM (Horie et al., 2019). Quesada-Candela *et al* did not observe *in vitro* alterations in glucagon secretion by islets from pregnant mice and in fact reported reduced circulating glucagon which may be due to an increase in compensatory insulin secretion in the third trimester (Quesada-Candela et al., 2020).

While we did not measure circulating levels of glucagon in our study, we did measure glucagon secretion by isolated islets. Under low glucose conditions when glucagon secretion is generally increased, we observed that HFS diet feeding significantly increased glucagon secretion in WT mice in the third trimester relative to LF fed animals.

However, high glucose conditions still suppressed glucagon secretion in WT mice fed a HFS diet in pregnancy. One study showed that 12 weeks of HFD feeding of non-pregnant female mice increased α-cell apoptosis and decreased proliferation and basal glucagon secretion, but glucagon secretion was not suppressed under high glucose conditions (Merino et al., 2015). When HFD feeding was extended to 24 weeks, the *in vitro* glucagon secretion and α-cell morphology reverted to control, and function began to deteriorate (Merino et al., 2015). Kim *et al* reported dysregulation to the structural makeup of pancreatic islets in *ob/ob* mice during pregnancy, frequently recording centrally-located α-cells, an observation we also noted in adiponectin KO mice (Figure 22) (A. Kim et al., 2009). However, information is lacking regarding the impact of HFD feeding during pregnancy on maternal α-cell compensation in WT animals. Thus, our observations in HFS fed pregnant WT mice may represent the first report of the interplay between pregnancy and response of the α-cell to HFS diet.

Both LF fed adiponectin KO mice showed significantly increased basal glucagon secretion relative to WT mice in the third trimester. Considering the role of glucagon signaling in increasing hepatic glucose output, and the fact that LF fed adiponectin KO mice showed increased hepatic glucose output during fasted pyruvate tolerance tests, it is possible that increased glucagon output may play a role in increased gluconeogenesis during third trimester in LF fed adiponectin KO mice. Under high glucose conditions, when glucagon secretion should be suppressed, LF fed adiponectin KO mice showed increased glucagon secretion relative to WT mice. The total glucagon content was also increased in islets from HFS fed dams relative to LF controls, but there was no genotype effect observed. These data suggest that the effect of adiponectin KO in pregnancy on α-cells may be associated with altered glucagon secretion rather than content.

There is currently no strong evidence for adiponectin signaling in α-cell function, but it may represent an area for investigation (Stern et al., 2016). While it has been determined that adiponectin receptors are present on the β-cell (Kharroubi et al., 2003; Staiger et al., 2005), it is unclear whether adipoR1 or R2 are present on the α-cell. Adiponectin could also impact glucagon signaling via other α-cell secretion products such as GLP-1. Although often considered to exert effects by secretion from intestinal L-cells, GLP-1 has recently been shown to have an impact when secreted from α-cells, exerting paracrine effects on insulin and glucagon secretion (Chambers et al., 2017). GLP-1 receptor agonists increase adiponectin expression in cell culture (F. Wang et al., 2015) and adiponectin increased GLP-1 secretion from L-cells *in vitro* (Kihira et al., 2014). Thus, there may be an interplay between adipokine signaling and GLP-1 which is missing in adiponectin KO mice. While this was not an area of investigation in the present study, it may represent an additional level of regulation, and a missing piece in the compensation by pancreatic islets in adiponectin KO mice.

It is important to consider that the adaptations of the pancreatic islets to pregnancy in the rodent differ from humans in scope and mechanism. In humans, β-cell neogenesis is considered to be a more important mechanism than in rodents (Susan Bonner-Weir et al., 2012; Søstrup et al., 2014). From our results it appears that adiponectin KO mice have an impaired adaptive response to pregnancy (particularly with HFS diet consumption), however additional non-

pregnant sample analysis is required to be conclusive. Future work using additional samples that have already been collected will provide conclusive insights into the effect of adiponectin KO on pancreatic islets prior to pregnancy.

We did not perform experiments to determine the level of proliferation or apoptosis in β-cells or α-cells, which may provide more information on the mechanisms behind adaptations (or lack thereof) in HFS fed animals, or in adiponectin KO mice in pregnancy. Sectioned pancreas from pregnant and non-pregnant mice could be stained with Ki-67, a marker of proliferation, or caspase-3 for apoptosis which would provide more information about morphological adaptations of pancreatic islets to pregnancy. In addition, gene expression data for pancreatic islets in pregnancy in the context of adiponectin KO and LF versus HFS feeding would provide a deeper mechanistic understanding. Currently we are in the processes of collecting RNA for RNAseq to profile the effects of adiponectin KO on pancreatic islet gene expression in the third trimester.

Our model of adiponectin KO is a whole-body knockout, which may add a layer of complexity when studying the pancreatic islet, due to the effect of adiponectin KO on peripheral tissues. Other studies have shown that APPL-1 null β-cells have impaired insulin secretion. Thus, it would be relevant to study β-cell specific impairments to adiponectin signaling by knocking down the adiponectin receptor in a tissue specific manner. Although targeted over-expression of AdipoR1 in the β-cell of Akita mice did not improve islet morphology or insulin secretion relative to WT, adiponectin signaling through AdipoR1 increased signaling through AMPK and AKT in the β-cell (J. Choi et al., 2018) . While no protective or adaptive improvements were seen with β-cell over-expression of AdipoR1, this study was not performed in pregnant or HFS fed mice, during which time adiponectin signaling has been shown to be protective and this may present an opportunity for additional study.

It has been suggested that diabetes is a “bi-hormonal disorder” (RH Unger & Grundy, 1985; RogerH Unger & Orci, 1975; Roger H. Unger & Cherrington, 2012). HFS diet feeding in pregnancy impacts the response of both α- and β-cells in the islets, and that adiponectin KO mice show disruptions in islet hormone secretion during pregnancy, even in the absence of obesity. The combination of dysregulated glucagon secretion and the finding of dysregulated hepatic glucose output reported in Chapter 3 suggest that this could be a central mechanism affecting whole body glucose homeostasis in adiponectin KO mice in pregnancy. Morphologically, pancreatic islets in adiponectin KO mice may have a reduced ability to adapt to a HFS diet prior to pregnancy, but due to low sample size these results require future experiments to be conclusive. Despite the absence of marked impairments in β-cell fraction or islet area in pregnancy, adiponectin KO mice showed impairments to insulin secretion, which became more profound with the consumption of a HFS diet. This suggests that adiponectin may play a role in insulin secretory adaptations in the third trimester. While more research is required, these findings collectively suggest that adiponectin may play a role in the adaptation of the pancreatic islet to HFS diet feeding, or pregnancy and that adiponectin deficiency contributes to β cell dysfunction in the islet.

Chapter 7:

Association between Maternal Diabetes and Serum and Cord Blood Adipokines

7.1 Introduction

Mean birthweight has been increasing across the western world in parallel with rates of obesity and diabetes in pregnancy (Ferrara, Kahn, Quesenberry, Riley, & Hedderson, 2004; Kramer et al., 2002; Surkan, Hsieh, Johansson, Dickman, & Cnattingius, 2004). Pregnancies affected by maternal obesity and diabetes are considered to be at higher risk for fetal macrosomia and pregnancy complications such as preeclampsia, pre-term birth (<37 weeks), and NICU admission (Baeten, Bukusi, & Lambe, 2001; Buchanan, Xiang, & Page, 2012). Following delivery, offspring born to mothers with diabetes in pregnancy are more likely to develop obesity or metabolic syndrome later in life (Group, 2008; B. E. Metzger et al., 2008; Lucilla Poston, Harthoorn, van der Beek, & On Behalf of Contributors To The, 2011).

Beginning pregnancy obese or overweight increases the risk of GDM as well as other complications such as preterm labour, large for gestational age (LGA) infants, and gestational hypertension (McDowell, Cain, & Brumley, 2019; Stubert, Reister, Hartmann, & Janni, 2018). Being born LGA is associated with the risk of delivery complications such as traumatic birth injuries and low APGAR score (Wikström, Axelsson, Bergström, & Meirik, 1988), and an increased risk of emergency caesarean delivery (Boriboonhirunsarn & Waiyanikorn, 2016; Kwon, Kwon, Park, & Kim, 2016). There is also evidence that the combination of high BMI and GDM can have an additive effect, worsening outcomes in all BMI classes (Whiteman et al., 2015).

While T2D and GDM share similar risk factors, some research shows that populations of women with T2D versus those with GDM are not always identical. For instance, Xiang *et al* showed that women diagnosed with GDM were more likely than women with T2D to be primiparous (a finding also seen by Westgate *et al*, (Westgate et al., 2006)), and women with T2D had higher risk of preeclampsia and congenital anomalies (Xiang et al., 2018). One study comparing outcomes of diabetes in pregnancy (T1D, T2D, and GDM with and without pharmacological intervention) found that maternal and neonatal morbidities were more likely to occur in mothers with pre-existing T2D, relative to mothers with GDM that could be managed with diet and lifestyle intervention (O'Neil Dudley, Jenner, Mendez-Figueroa, Ellis, & Chauhan, 2017).

The duration of exposure to diabetes in pregnancy may also increase the risk for adverse offspring health outcomes. Mothers with pre-pregnancy T2D had infants with a higher incidence of congenital anomalies and mean birthweight relative to GDM (Xiang et al., 2018). In First Nations populations there is evidence to suggest that exposure to T2D *in utero* has a more profound effect on the early development of metabolic syndrome, even in comparison to *in utero* GDM exposure (Wicklow et al., 2018; A. Young, 2005). This suggests that GDM and T2D have different outcomes for both maternal and infant health, indicating a need to investigate the underlying mechanisms separately.

Obesity reduces maternal insulin sensitivity (reviewed in (Patrick M Catalano, 2010) which leads to increased blood glucose levels. This increase translates into elevated fetal glucose levels resulting in accelerated intrauterine growth (Kc, Shakya, & Zhang, 2015; Simmons, 1995). In addition, hyperinsulinemia, as a consequence of increased glucose levels *in utero*, also contribute to fetal macrosomia and may cause long term alterations in metabolic programming (Boney, Verma, Tucker, & Vohr, 2005; Plagemann & Harder, 2011). Thus, exposure to maternal

obesity, GDM and T2D may influence fetal insulin signalling and adiposity increasing the risk for macrosomia (Milner & Hill, 1984; Ruiz-Palacios, Ruiz-Alcaraz, Sanchez-Campillo, & Larqué, 2017). This developmental programming may disrupt metabolism potentially leading to obesity later in life. For example, offspring born to mothers with GDM were found to be twice as likely to develop obesity later in life (Symonds, Sebert, Hyatt, & Budge, 2009).

Experiments in rhesus monkeys showed that elevating fetal, but not maternal, insulin increased fetal growth and adiposity without increasing fetal plasma amino acids, glucose or lipid levels (Susa et al., 1979). According to Pedersen's Hypothesis, increasing maternal glycemia leads to increased production of fetal insulin later in gestation and since insulin is anabolic the combination of fetal hyperinsulinemia and increased substrate availability from the mother contributes to increased fetal growth (Kc et al., 2015; Macfarlane & Tsakalakos, 1988). While maternal insulin does not cross the placenta, it does signal to increase nutrient transport across the placenta and this is associated with increased fetal growth, particularly in diabetic pregnancies (Ruiz-Palacios et al., 2017).

As outlined in Chapter 1, adipokines are adipose tissue derived hormones that have been implicated in regulation of insulin sensitivity and metabolism. In a healthy pregnancy, adiponectin is abundant in serum and decreases modestly towards the end of gestation in parallel with increasing gestational weight gain and physiological insulin resistance (Fuglsang, Skjaerbaek, Frystyk, Flyvbjerg, & Ovesen, 2006; Jara et al., 2020; Retnakaran et al., 2006). It has been shown that the presence of obesity or diabetes during pregnancy is associated with decreased circulating adiponectin (Mazaki-Tovi et al., 2009; Mohammadi & Paknahad, 2017; Retnakaran et al., 2004).

In contrast to adiponectin, levels of leptin increase throughout gestation. Obese women have higher serum levels of leptin compared to women with normal BMI in pregnancy (Misra, Straughen, & Trudeau, 2013). Women with GDM also have increased levels of serum leptin relative to controls and there is a positive association between serum leptin, HOMA IR and serum insulin in pregnancy suggesting a link between hyperleptinemia and insulin resistance in pregnant women (Soheilykhah, Mojibian, Rahimi-Saghand, Rashidi, & Hadinedoushan, 2011).

Levels of adipokines in the maternal and fetal system are also implicated in fetal growth and metabolism. As discussed in Chapter 1, maternal adiponectin generally shows an inverse relationship with birthweight (Lekva et al., 2017). This potentially occurs due to signalling through placental receptors that impact nutrient transport to the fetus (T. Jansson et al., 2012; H. N. Jones et al., 2010). In contrast, there is a positive association between fetal growth and adiposity with fetal adiponectin and an inverse relationship to maternal glycemia (Luo et al., 2013a; L. Qiao et al., 2012; Sivan et al., 2003).

While there are conflicting reports, the placenta is generally not considered a significant source of adiponectin. However, the placenta does produce leptin. Placental leptin may contribute to maternal hyperleptinemia (Masuzaki et al., 1997). One study found as much as 95% of leptin derived from the placenta was destined for the maternal circulation (Lepercq et al., 2001). Similar to levels of maternal leptin, fetal leptin also increases throughout gestation and fetal leptin produced by fetal adipose tissue (Lepercq et al., 2001) is closely linked to birthweight (Sindiani, Obeidat, Jbarah, & Hazaimeh, 2019). Based on the distribution of fetal leptin receptors evidence suggests that leptin may regulate fetal growth (Sandra G. Hassink et al., 1997; Javaid et al., 2005; Valūniene et al., 2007). Additionally, a study using twins showed that small for gestational age (SGA) or growth-restricted twins had lower

cord blood and placental leptin relative to the normal sized twin (S. M. Lee et al., 2016). In addition, macrosomic infants born to mothers with diabetes in pregnancy had significantly higher cord-blood leptin levels (Lea et al., 2000).

In adults, leptin regulates appetite via the hypothalamus reducing energy intake. Individuals with obesity have demonstrated leptin resistance (Myers, Leibel, Seeley, & Schwartz, 2010). In fact, it has been suggested that hyperleptinemia early in pregnancy may indicate a higher risk for GDM development (Qiu, Williams, Vadachkoria, Frederick, & Luthy, 2004). While there is a positive association between cord-blood leptin and birthweight, cord blood leptin may be negatively associated with growth up to 3 years of age (Ong et al., 1999). It is clear that both *in utero* and postnatal leptin levels play a role in neonatal growth and metabolism and are linked to risk for obesity and metabolic syndrome in childhood and in later life (Ong et al., 1999; Valūniene et al., 2007).

Since dysregulation of maternal and fetal adipokines are linked to fetal growth and adiposity, we aimed to determine whether the duration of diabetes during pregnancy (GDM diagnosis versus pre-existing T2D) was associated with altered maternal serum or cord blood adiponectin and leptin levels. Moreover, we examined whether adipokine levels in the maternal as well as fetal systems were associated with additional neonatal outcomes including birthweight.

7.2 Materials and Methods – Refer to Page 24

7.3 Results:

7.3.1 Next Generation Cohort

In this study, serum from 64 mother-infant pairs from Indigenous mothers in Manitoba, a subpopulation previously identified to be at increased risk of developing GDM (Aljohani et al., 2008), was used. Maternal serum was collected in the third trimester and cord-blood was collected at delivery. Pairs were categorized as control (no diabetes in pregnancy), GDM (diabetes newly diagnosed in pregnancy) or pre-existing T2D. There were no significant differences between groups in average age at delivery (Table 14). Interestingly, mothers with GDM had approximately 20% higher pre-pregnancy bodyweight and BMI, and higher BMI at delivery relative to both control and T2D mothers. However, control mothers gained the most weight during pregnancy and T2D mothers gained the least during pregnancy (Table 14). In addition, mothers with T2D had the most previous pregnancies on average compared to mothers with GDM or control mothers.

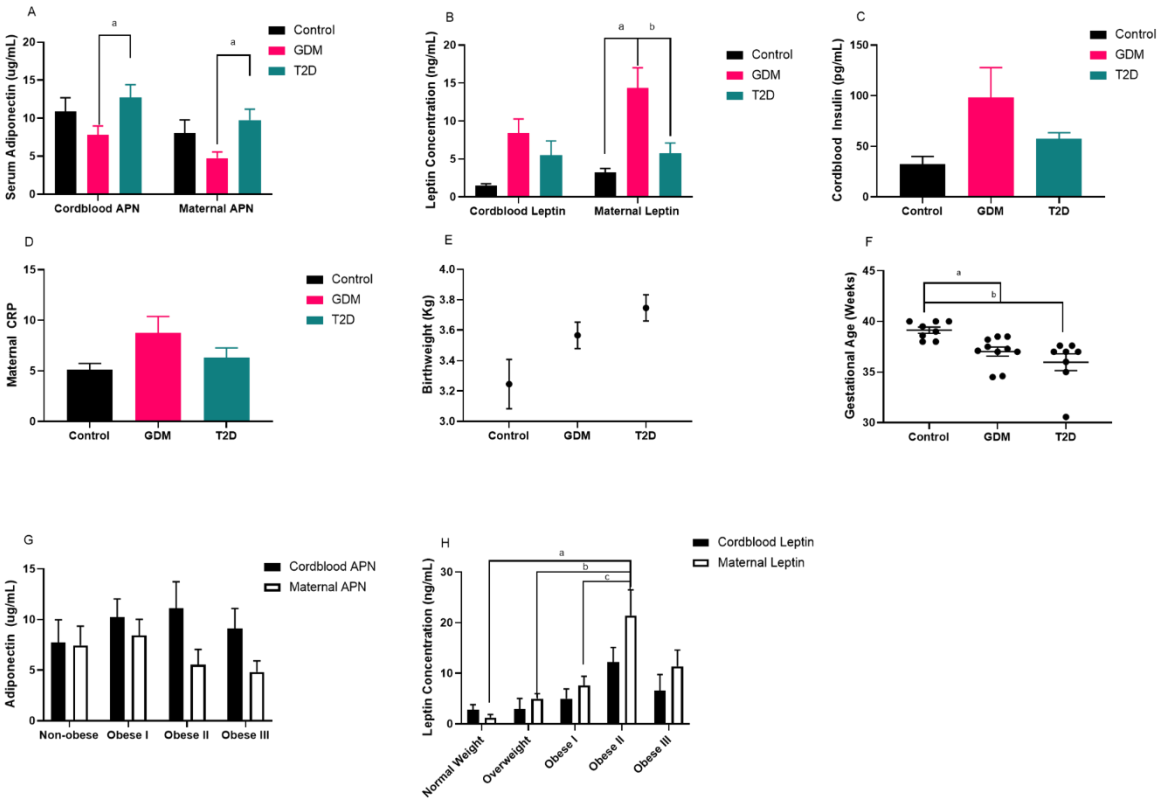
Table 14: Characteristics of the Next Gen study population and neonates at delivery

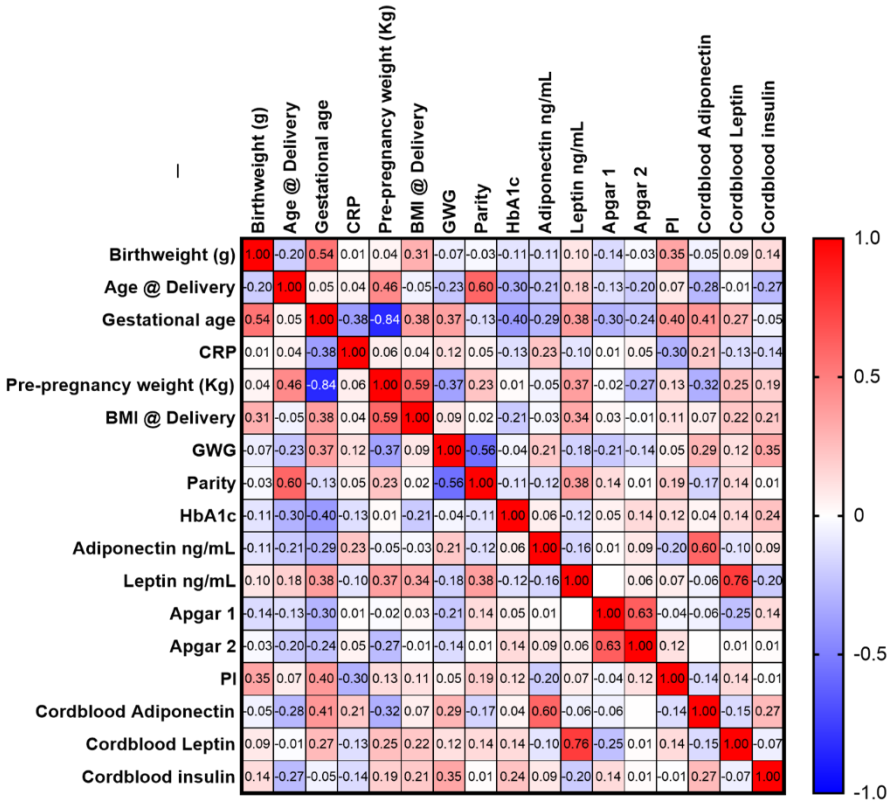
	Control			**GDM**			**T2D**		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Age @ Delivery (years)	29.26	2.10	13	28.36	1.35	22	26.29	1.50	24
Maternal age >35 (%)	36%			27%			15%		
Gestational age (weeks)	39.10	0.30	7.00	36.99	0.44	10.00	36.27	0.69	10
CRP	7.44	1.71	9.00	10.00	2.90	22.00	13.00	4.86	22.00
Pre-pregnancy weight (Kg)	73.65	8.50	5.00	91.21	4.60	10.00	81.45	5.96	10.00
Pre-pregnancy BMI	28.51	3.31	4	33.81	1.28	9	28.74	1.66	10
BMI @ Delivery (kg/m^2)	35.08	1.78	9.00	36.43	1.40	21.00	32.33	1.14	24
GWG (Kg)	18.25	1.54	5.00	6.62	2.10	9.00	12.21	1.64	9.00
Ponderal Index (g/cm^3)	2.59	0.06	8.00	2.68	0.07	21.00	2.78	0.09	21.00
Parity	1.00	0.44	9.00	1.82	0.40	22.00	3.23	0.60	22.00
HbA1c (%)	5.29	0.13	10	6.46	0.33	22	7.58	0.37	23
HbA1c ≥6%	10%			45%			88%		
Length (cm)	50.50	0.77	9.00	49.84	0.46	15.00	47.40	2.35	9.00
Birthweight (g)	3378.9	548.1	8	3536.7	437.5	20	3535.1	545.9	23
Proportion LGA (>90^{th} percentile)	~50%			33-88%			~75%		
Birthweight for gestational age: (percentile)	60.67			15.74			6.00		

Maternal and neonatal characteristics in the subset of the Next Generation Cohort used for adiponectin and leptin analysis. Data represents mean ± Standard error of the mean (SEM), N represents maternal-infant pairs, CRP: C-reactive protein, GWG: Gestational weight gain, HbA1c: Hemoglobin A1c (%), LGA: large for gestational age.

Since levels of maternal and cord blood adipokines are associated with metabolic function and fetal growth, we measured third trimester maternal and cord blood adiponectin and leptin in our mother-infant dyads. e found that cord blood adiponectin was between 30 and 60% higher than maternal serum adiponectin, with the exception of T2D pairs, while mothers with GDM had lower serum adiponectin overall and significantly lower adiponectin relative to mothers with T2D (Figure 23A). Cord-blood leptin levels were elevated in samples from GDM and T2D infants (Figure 23B). Interestingly, mothers with GDM had significantly elevated serum leptin relative to both control and T2D mothers (Figure 23B). Cord-blood insulin levels were measured at delivery, and newborns from GDM mothers had increased cord-blood insulin relative to controls, although this was not statistically significant (Figure 23C). Maternal C-reactive protein (CRP) is a marker of inflammation and high levels of CRP in pregnancy have been associated with adverse outcomes including preeclampsia and preterm birth (Vecchié et al., 2018). Serum adipokines have been implicated in management of inflammation; adiponectin has been associated with decreased inflammation (C. S. Mantzoros et al., 2005) and evidence suggests that leptin may play a pro-inflammatory role (Leon-Cabrera). We observed a trend towards elevated maternal CRP in mothers with GDM relative to control, with GDM mothers having approximately 2-fold higher serum CRP (Figure 23D).

Figure 23: Maternal and cord blood adipokines are impacted by GDM, T2D, and BMI in pregnancy





A) Circulating cord blood and maternal adiponectin at delivery. Values are mean ± SEM. a, $p<0.05$ between GDM and control, b, $p<0.05$ between GDM and T2D by Two-way ANOVA. B) Leptin levels in cord blood and third trimester maternal serum at delivery. Values are mean ± SEM a, $p<0.05$ between control and GDM; b, $p<0.05$ between GDM and T2D by Two-way ANOVA C) Cord blood insulin levels. Values are mean ± SEM. $p<0.05$ between control and GDM by Two-way ANOVA. D) Maternal C-reactive protein. Values are mean ± SEM. E) Mean birthweight at delivery. Values are mean ± SEM. F) Gestational age at delivery. Individual values are plotted. a, $p<0.05$ between control and GDM; b, $p<0.05$ between control and T2D by Two-way ANOVA. G) Maternal and cord blood adiponectin stratified by maternal BMI classifications. Values are mean ± SEM. H) Maternal and cord blood leptin stratified by maternal BMI classifications. Values are mean ± SEM. a, $p<0.05$ between normal weight and obese class II; b, $p<0.05$ between overweight and obese class II; c, $p<0.05$ between obese class I and obese class II; d, $p<0.05$ between obese class II and obese class III by Two-way ANOVA. I) Heat map showing Pearson's correlation (r) value between observations in all mother-infant pairs in the cohort, reflecting general associations. GDM: gestational diabetes mellitus; T2D: Type 2 diabetes mellitus; CRP: C-reactive protein; GWG: Gestational weight gain; HbA1c: Hemoglobin A1c; APGAR: Appearance, Pulse, Grimace, Activity, Respiration. APGAR 1: assessment 1 minute after delivery APGAR 2: assessment 5 minutes after delivery.

Diagnosis of diabetes in pregnancy is associated with fetal macrosomia and in our study birthweight showed an upward trend in mothers with GDM or T2D (Figure 23E). Low levels of adiponectin and high levels of maternal leptin are both associated with increased birthweight. When all groups were combined, maternal adiponectin showed a small negative correlation with birthweight and maternal leptin a small positive correlation (Figure 23I). These correlations held when we separated mother-infant pairs by diabetes status, with the exception of GDM pregnancies, wherein maternal adiponectin showed a positive correlation with birthweight (Table 16). In GDM pregnancies, low levels of adiponectin in the circulation were associated with preterm birth (Mazaki-Tovi et al., 2009). In our study, mothers in the control group tended to deliver at a later gestational age (~39 weeks) relative to mothers with GDM or T2D (~37 weeks) (Figure 23F). Thus, diabetes in pregnancy (GDM or T2D) may reduce average gestational age at delivery.

7.3.2 Maternal Obesity and Adipokines

Maternal obesity is associated with adverse outcomes for mother and baby and is a risk factor for the development of GDM (L. Poston et al., 2016). The majority of participants included in our mother-infant pairs were overweight or obese. Of the 51 pairs for which BMI at delivery data was available, 5 had a BMI $<30 kg/m^2$ (9.8%) and only 4 had a BMI $<25 kg/m^2$. (7.8%). The remaining 82.4% of mothers were class I, II or III obese. According to Health Canada, the classes are defined as follows: class I (BMI 30– 34.9), class II (BMI 35– 39.9) and class III (BMI ≥ 40) (Katmarzyk). Since both a healthy pregnancy and obesity are associated with decreasing levels of serum adiponectin (Jara et al., 2020; Retnakaran et al., 2006), we stratified maternal and cord blood adiponectin levels by maternal BMI class to determine the impact of maternal obesity on adiponectin levels. We observed that maternal adiponectin levels showed a decreasing trend with increasing obesity. Mothers in the class III obese BMI category had a ~40% reduction in serum adiponectin compared to mothers in the class I obese category (Figure 23G). Interestingly, cord blood adiponectin did not appear to be dramatically impacted by maternal BMI. As mentioned in Chapter 1, fetal adiponectin

Maternal serum leptin increases with increasing maternal BMI (Figure 23H). Serum leptin was highest in mothers who are class II obese, with a BMI between 35.0 – 39.9 kg/m^2 and was significantly higher than serum leptin in mothers with class I obesity, overweight, or normal BMI (Figure 23H). Interestingly, cord blood leptin follows a similar pattern, being highest in cord blood when mothers were class II obese, although these increases were not significantly different between BMI classes (Figure 23H). These results suggest that the degree of maternal obesity may influence serum adipokine levels, and that maternal adiposity has a significant effect on serum leptin levels in the third trimester.

When we examined the associations between maternal and cord blood adipokines and the clinical characteristics of the study participants, we found several significant associations. In this cohort, maternal adiponectin showed a positive correlation with gestational weight gain (GWG) in control (Table 15) pregnancies and no correlation with GWG was observed in T2D or GDM pregnancies. This finding was surprising as adiponectin usually decreases with weight gain (Arita et al., 1999; Jara et al., 2020). It is important to note that obesity was prevalent in all groups, which can impact gestational weight gain, and in turn may also affect associations with maternal adipokines.

Table 15: Correlation between maternal and cord blood adipokines and maternal and fetal characteristics at delivery in control pregnancies.

Control Pregnancies	**Maternal Adiponectin**			**Maternal Leptin**			**Cord-blood Adiponectin**			**Cord-blood Leptin**			**Cord-blood Insulin**		
	r	p	XY pairs	r	p	XY pairs	r	P	XY pairs	r	p	XY pairs	r	p	XY pairs
Birthweight (g)	-0.22	0.63	7.00	0.72	0.17	5.00	-0.20	0.66	7.00	0.77	0.13	5.00	0.35	0.50	6.00
Age @ Delivery	-0.19	0.56	12.00	-0.26	0.47	10.00	-0.23	0.47	12.00	0.36	0.31	10.00	-0.10	0.83	7.00
Gestational age	-0.50	0.31	6.00	-0.93	0.07	4.00	0.62	0.19	6.00	-0.12	0.88	4.00	0.30	0.56	6.00
CRP	-0.15	0.73	8.00	0.31	0.55	6.00	-0.27	0.51	8.00	0.74	0.09	6.00	0.53	0.36	5.00
Pre-pregnancy weight	-0.30	0.70	4.00				-0.57	0.43	4.00						
BMI @ Delivery	0.55	0.20	7.00	0.86	0.06	5.00	-0.17	0.72	7.00	0.63	0.26	5.00	0.74	0.09	6.00
GWG	0.82	0.18	4.00				0.41	0.59	4.00						
Parity	-0.16	0.74	7.00	0.68	0.20	5.00	-0.49	0.26	7.00	***0.94***	***0.02***	***5.00***	0.80	0.06	6.00
HBA1c	-0.13	0.75	9.00	0.69	0.08	7.00	-0.48	0.19	9.00	0.57	0.18	7.00	0.74	0.09	6.00
Maternal Adiponectin	1.00		12.00	-0.14	0.70	10.00	0.43	0.16	12.00	-0.24	0.51	10.00	0.00	1.00	6.00
Maternal Leptin	-0.14	0.70	10.00	1.00		10.00	-0.48	0.16	10.00	0.24	0.50	10.00	0.61	0.28	5.00
Ponderal Index	-0.06	0.89	7.00	0.73	0.16	5.00	-0.54	0.21	7.00	***0.96***	***0.01***	***5.00***	***0.90***	***0.04***	***5.00***
Cord-blood Adiponectin	0.43	0.16	12.00	-0.48	0.16	10.00	1.00		12.00	-0.21	0.56	10.00	-0.54	0.26	6.00
Cord-blood Leptin	-0.24	0.51	10.00	0.24	0.50	10.00	-0.21	0.56	10.00	1.00		10.00	0.69	0.20	5.00
Cord-blood insulin	-0.001	1.00	6.00	0.61	0.28	5.00	-0.54	0.26	6.00	0.69	0.20	5.00	1.00		7.00

Pearson's correlation coefficient (r value) from regressions performed on maternal and cord blood adipokine and serum measures against maternal and fetal anthropometric values. Bold and italic font indicates significance ($p<0.05$).

Table 16: Correlation between maternal and cord blood adipokines and maternal and fetal characteristics in GDM pregnancies

GDM Pregnancies	**Maternal Adiponectin**			**Maternal Leptin**			**Cord-blood Adiponectin**			**Cord-blood Leptin**			**Cord-blood Insulin**		
	r	p	XY pairs	r	p	XY pairs	r	P	XY pairs	r	p	XY pairs	r	p	XY pairs
Birthweight (g)	0.19	0.44	18	0.26	0.30	18	0.14	0.57	18	0.32	0.20	18	0.57	0.07	11
Age @ Delivery	0.12	0.61	19	0.21	0.38	19	0.17	0.48	19	-0.23	0.37	18	-0.52	0.10	11
Gestational age	0.14	0.74	8	0.44	0.28	8	0.38	0.35	8	0.35	0.44	7	0.11	0.82	7
CRP	-0.15	0.55	19	-0.20	0.42	19	-0.14	0.57	19	-0.17	0.49	18	-0.25	0.46	11
Pre-pregnancy weight (Kg)	0.07	0.86	9	-0.09	0.82	9	-0.08	0.83	9	0.56	0.15	8	0.46	0.54	4
BMI @ Delivery	-0.05	0.84	18	0.21	0.40	18	0.37	0.14	18	0.23	0.38	17	0.10	0.77	10
GWG	-0.03	0.95	9	0.54	0.13	9	0.18	0.65	9	0.69	0.06	8	0.86	0.14	4
Parity	0.44	0.06	19	0.35	0.14	19	0.32	0.18	19	0.10	0.70	18	-0.20	0.55	11
HbA1c	0.16	0.50	19	-0.24	0.31	19	-0.08	0.73	19	-0.01	0.96	18	0.42	0.19	11
Maternal Adiponectin	1.00		19	***0.51***	***0.02***	***19***	***0.61***	***0.01***	***19***	***0.55***	***0.02***	***18***	0.15	0.68	10
Maternal Leptin	***0.51***	***0.02***	***19***	1.00		19	***0.58***	***0.01***	***19***	***0.78***	***0.00***	***18***	-0.38	0.28	10
Ponderal Index	0.22	0.37	19	0.22	0.37	19	0.35	0.14	19	0.19	0.45	18	0.26	0.44	11
Cord-blood Adiponectin	***0.61***	***0.01***	***19***	***0.58***	***0.01***	***19***	1.00		19	0.44	0.07	18	-0.31	0.38	10
Cord-blood Leptin	***0.55***	***0.02***	***18***	***0.78***	***0.00***	***18***	0.44	0.07	18	1.00		18	0.36	0.30	10
Cord-blood insulin	0.15	0.68	10	-0.38	0.28	10	-0.31	0.38	10	0.36	0.30	10	1.00		11

Pearson's correlation coefficient (r value) from regressions performed on maternal and cord blood adipokine and serum measures against maternal and fetal anthropometric values. Bold and italic font indicates significance ($p<0.05$).

Table 17: Correlation between maternal and cord blood adipokines and maternal and fetal characteristics in T2D pregnancies

T2D Pregnancies	**Maternal Adiponectin**			**Maternal Leptin**			**Cord-blood Adiponectin**			**Cord-blood Leptin**			**Cord-blood Insulin**		
	r	p	XY pairs	r	p	XY pairs	r	P	XY pairs	r	p	XY pairs	r	p	XY pairs
Birthweight	-0.10	0.65	18	0.20	0.41	18	-0.05	0.83	22	0.07	0.77	19	0.148	0.56	19
Age @ Delivery	-0.26	0.21	19	0.08	0.71	19	***-0.49***	***0.02***	***24***	0.03	0.89	22	-0.28	0.22	21
Gestational age	-0.71	0.07	8	0.62	0.19	8	-0.30	0.56	6	0.55	0.34	5	***0.72***	***0.04***	***8***
CRP	0.20	0.37	19	-0.15	0.52	19	0.22	0.34	21	-0.19	0.44	19	-0.10	0.71	18
Pre-pregnancy weight	0.22	0.52	9	0.25	0.45	9	-0.34	0.30	11	-0.01	0.98	11	-0.33	0.47	7
BMI @ Delivery	0.06	0.79	18	***0.47***	***0.04***	***18***	0.08	0.73	21	0.26	0.27	20	0.34	0.13	21
GWG	0.13	0.73	9	-0.03	0.95	9	0.34	0.37	9	0.17	0.67	9	0.55	0.21	7
Parity	-0.28	0.21	19	0.20	0.40	19	-0.36	0.11	21	0.02	0.93	20	-0.05	0.84	19
HbA1c	-0.07	0.74	19	0.13	0.59	19	0.07	0.76	22	0.21	0.36	20	0.10	0.68	19
Maternal Adiponectin	1.00		19	-0.22	0.31	19	***0.65***	***0.00***	***25***	-0.15	0.50	22	0.32	0.18	19
Maternal Leptin	-0.22	0.31	19	1.00		19	-0.32	0.13	23	***0.86***	***0.00***	***22***	-0.20	0.44	17
Ponderal Index	-0.30	0.21	19	0.16	0.53	19	-0.31	0.21	18	0.12	0.65	17	-0.15	0.55	18
Cord-blood Adiponectin	***0.65***	***3.86E-04***	***19***	-0.32	0.13	19	1.00		25	-0.27	0.22	22	***0.57***	***0.02***	***17***
Cord-blood Leptin	-0.15	0.50	18	***0.86***	***3.03E-07***	***18***	-0.27	0.22	22	1.00		23	-0.17	0.51	17
Cord-blood insulin	0.32	0.18	10	-0.20	0.44	10	***0.57***	***0.02***	***17***	-0.17	0.51	17	1.00		21

Pearson's correlation coefficient (r value) from regressions performed on maternal and cord blood adipokine and serum measures against maternal and fetal anthropometric values. Bold and italic font indicates significance ($p<0.05$).

7.3.3 Cord Blood Adipokines and Maternal Diabetes

When compared to cord blood adipokines, maternal adiponectin showed a positive association with cord blood adiponectin in all groups (Table 15-15). Maternal adiponectin was negatively correlated with serum leptin in control (Table 15) and T2D mothers (Table 17) which is expected in the third trimester of pregnancy. However, we observed a significant positive correlation in mothers with GDM (Table 16). A similar pattern was also observed when comparing maternal adiponectin to cord blood leptin, with an inverse correlation observed in control (Table 15) mothers, and a strong positive correlation in GDM mothers (Table 16). There was no relationship between cord blood leptin and maternal adiponectin in mothers with T2D (Table 17). Maternal leptin showed a positive relationship with pre-pregnancy bodyweight and BMI at delivery, and the relationship between maternal leptin and BMI at delivery was significant in T2D mothers (Table 17). However, in mothers with GDM there was no association between pre-pregnancy bodyweight and maternal leptin (Table 16). Additionally, a moderate positive association was observed between increased maternal leptin and parity, but this was most pronounced among mothers with normoglycemia in pregnancy (Table 15). Both maternal and cord blood adiponectin showed a trend towards decreasing with increasing maternal age in control (Table 15) and T2D mothers (Table 17), but neither maternal or cord blood adiponectin showed an association with increased maternal age in GDM mothers (Table 16). Cord blood adiponectin also showed a positive correlation with gestational age in control pregnancies (Table 17), but this increase became less pronounced with GDM (Table 16) and was negatively associated with gestational age in T2D pregnancies (Table 17).

While fetal adiponectin decreased somewhat with increasing pre-pregnancy maternal weight in control (Table 15) and T2D (Table 17) pregnancies, this association was lost in mothers with GDM (Table 16). In contrast, cord blood leptin appeared to increase in parallel with maternal weight prior to pregnancy in mothers with GDM (Table 16) but not those with T2D (Table 17). Additionally, cord blood leptin was strongly associated with GWG in mothers with GDM (Table 16). With the exception of mothers with GDM, the number of previous pregnancies had an inverse relationship with cord blood adiponectin (Table 15-16). Cord blood leptin showed a significant positive correlation with parity in control mothers (Table 15), however showed no relationship with parity in mothers with GDM (Table 16) or T2D (Table 17) in pregnancy. Cord blood and maternal leptin are attributed to different physiological sources. In our study, cord blood leptin and maternal leptin showed a strong positive correlation across all groups (Table 15-16). Much like maternal leptin, cord blood leptin showed a small negative association with maternal adiponectin in control mothers (Table 15) and mothers with T2D (Table 17). However, we observed a significant positive association between maternal adiponectin and cord blood leptin in mothers with GDM (Table 16).

7.3.4 Cord Blood Insulin, Maternal Glycemia and Birthweight

Since cord blood insulin levels are associated with cord blood leptin as well as infant size at birth (Ong et al., 1999), we performed similar comparisons in our study population. Cord blood insulin showed a moderate negative relationship with maternal age at delivery in mothers with diabetes in pregnancy (Table 16, Table 17) but no relationship was observed in control mothers (Table 15).Increasing HbA1c was associated with increased cord blood insulin in control and GDM mothers, although this association was lost in mothers with T2D. Cord blood insulin showed a positive correlation to birthweight in babies from control pregnancies (Table 15), and a positive relationship

was observed in GDM pregnancies (Table 16) however this association was not observed in T2D pregnancies (Table 17). Cord blood insulin also showed a significantly positive association with ponderal index in infants not exposed to diabetes in pregnancy (Table 15), but the strength of this association became weaker in GDM (Table 16) and was lost in T2D (Table 17).

7.4 Discussion/Conclusions

In this chapter, we aimed to distinguish the association between GDM and T2D in pregnancy and maternal or cord blood adipokines (leptin and adiponectin). As discussed in previous chapters, pregnancy may represent a unique "metabolic stress test" (Patrick M Catalano, 2010). While GDM and T2D overlap in etiology and risk factors, studies have shown that women with GDM and women with T2D in pregnancy differ in several ways. For example, since age and previous GDM are risk factors for the development of T2D, women with GDM may be younger and have fewer previous pregnancies than women with T2D. Additionally women with pre-existing T2D are likely to be monitoring and managing their hyperglycemia with a health care professional (Feig & Moses, 2011) (Diabetes Canada). However, in the case of pre-pregnancy diabetes, fetal exposure to hyperglycemia may encompass a longer period of organogenesis (Lawrence, Contreras, Chen, & Sacks, 2008). It has also been suggested that in First Nations populations, exposure to T2D confers a higher risk of metabolic dysfunction in offspring, even relative to GDM (Wicklow et al., 2018; T. K. Young, Reading, Elias, & O'Neil, 2000).

While advanced maternal age has been implicated as a risk factor for the development of GDM (Feig & Moses, 2011; Lao, Ho, Chan, & Leung, 2006), in this cohort, age was not a factor since maternal age was similar across the control, GDM or T2D groups, with the average age at delivery ranging between 26 and 30 years old (Table 14). This is similar to the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study and the ACHOIS studies in which correlation between maternal age and GDM was not observed (P. M. Catalano, 2010; Boyd E Metzger et al., 2008; Pirc et al., 2007). We noted a positive association between maternal age and the number of previous pregnancies. Parity likely plays a role because a woman who has had a previous pregnancy affected by GDM is more likely to have GDM with subsequent pregnancies (Herath et al., 2017). Mothers who have had a previous GDM pregnancy are also at increased risk for developing T2D later in life (Bellamy, Casas, Hingorani, & Williams, 2009; Herath et al., 2017). This may possibly help explain the high number of previous pregnancies in mothers with T2D compared to those without diabetes in pregnancy. While we do not currently have information regarding the GDM status of previous pregnancies, it is a possible confounding factor influencing the associations between parity and incidence of T2D, age and decreased serum adiponectin.

Leptin is generally considered to regulate appetite (McDuffie et al., 2004) and mice lacking the ability to produce leptin due to a mutation in the *Ob* gene (the *ob/ob* mouse) display insulin resistance and hyperglycemia (Dubuc, 1976). In humans, leptin levels are somewhat paradoxical, as obese individuals have higher levels of serum leptin (Considine et al., 1996). Hyperleptinemia in obesity may be due to the development of leptin resistance, during which leptin increases in parallel with adiposity but it is not sufficient to decrease appetite (Frederich et al., 1995; Szczesna & Zieba, 2015). While it is not entirely clear if leptin resistance is a cause or consequence of diet induced obesity, rodent models have shown that leptin resistance can predispose animals to hyperphagia and obesity (Levin,

Dunn-Meynell, & Banks, 2004). In pregnancy, levels of leptin increase throughout gestation and then decrease postpartum (Schubring et al., 1998). In addition, leptin resistance has been reported in rodent models of pregnancy (Ladyman, 2008). In a healthy pregnancy, leptin levels may increase as a means to mobilize fat stores for fetal growth (Hauguel-de Mouzon, Lepercq, & Catalano, 2006; Misra et al., 2013). Women with GDM had significantly higher serum leptin and leptin was positively correlated with insulin resistance, particularly in GDM (Z. Liu et al., 2003; Soheilykhah et al., 2011). In line with this, we also observed increased serum leptin in HFS fed mice in Chapter 3, regardless of genotype in the third trimester.

Studies in rodents suggest that fetal or placental leptin signalling is involved in the induction of leptin resistance, which allows for increased food consumption in pregnancy (Grattan, Ladyman, & Augustine, 2007). It is also believed that leptin has an important role in establishing and maintaining pregnancy in mice (Kawamura et al., 2002; Pérez-Pérez et al., 2008). The placenta secretes leptin throughout gestation, contributing to the rise in maternal leptin levels (Lepercq et al., 2001). In line with other studies, we found increases in maternal serum leptin in mothers with GDM relative to control pregnancies (Figure 23B). Interestingly, GDM mothers also had significantly higher serum leptin levels than T2D mothers. It is worth noting that leptin shows a strong positive correlation with BMI, even in pregnancy (Soheilykhah et al., 2011). In our study, mothers with T2D had on average a lower BMI at delivery than mothers with GDM, potentially leading to reduced hyperleptinemia (Table 14). The correlation between maternal leptin and BMI was true in our study population as well, with significant increases in serum leptin levels observed with increasing BMI, across all groups (Table 15). Although a compelling correlation has been previously reported (A Kautzky-Willer et al., 2001; Z. Liu et al., 2003), it is difficult to determine whether maternal leptin is independently associated with development of GDM.

Maternal leptin levels in our study were strongly associated with cord blood leptin levels. Although some studies have reported that neonatal leptin is higher than maternal leptin (S. G. Hassink et al., 1997), others (Wolf et al., 2000) observed that cord blood leptin corresponded to maternal leptin levels, similar to this cohort. While it remains somewhat contested, only a small proportion of placental adiponectin is directed to the fetus, with the remainder destined for maternal circulation (Lepercq et al., 2001). Studies have shown that fetal adipose tissue is capable of producing leptin in increasing amounts throughout gestation (Lepercq et al., 2001). Cord blood leptin is a marker of fetal adiposity, as studies have shown it is positively correlated with birthweight and neonatal skinfold thickness (Geary et al., 1999; Teague et al., 2015; Tsai et al., 2004). It has been postulated that leptin in pregnancy may signal changing nutrient availability in the mother (Forhead et al., 2008) especially considering that cord blood leptin appears to be influenced by maternal glycemia (Côté et al., 2016) and fetal insulin (Forhead et al., 2008; Muhlhausler, Duffield, & McMillen, 2007).

It was previously reported that cord blood leptin was positively correlated with birthweight (S. G. Hassink et al., 1997; Ong et al., 1999). In our study, we observed that increased BMI lead to a trend of increasing cord blood leptin across all groups (control, GDM and T2D). Increased cord blood leptin showed positive association with increased GWG in mothers with GDM (Table 16), however this association was lost in mothers with T2D. As mentioned previously, mothers with GDM gained the least amount of weight in pregnancy. However, infants from

mothers with GDM tended to have higher cord blood leptin than those from T2D pregnancies, which may account for the observed difference. Overall, cord blood leptin increased with gestational age, and correlated positively with ponderal index, which is a measure of infant adiposity. This agrees with previous research that suggests that fetal adipose tissue produces leptin (Lepercq et al., 2001), and with progression of fetal adipose tissue development in the late gestation (Enzi, Zanardo, Caretta, Inelmen, & Rubaltelli, 1981). While not significant, infants from GDM and T2D pregnancies tended to have higher cord blood leptin relative to controls, similar to other cohort studies (Lepercq et al., 1998; Teague et al., 2015).

Most research suggests that higher cord blood leptin is linked to increased birth weight (Okereke, Huston-Presley, Amini, Kalhan, & Catalano, 2004; Teague et al., 2015). While the exact role of leptin in fetal growth is unclear, rodent studies have identified leptin receptors present in many fetal tissues including bone, cartilage, lung and hypothalamus (Hoggard et al., 1997; Matsuda et al., 1999). There is also data to suggest that larger infants may go through a period of postnatal "catch down" growth during which their rate of growth slows. Infants with higher cord blood leptin had slower growth between birth and four months (Sandra G. Hassink et al., 1997; Ong et al., 1999). This may be due to the fact that during this early life period, leptin sensitivity is intact and larger infants with higher leptin levels undergo "catch down" growth. In rodent studies, administration of leptin to neonates exposed to maternal undernutrition and low serum leptin rescued the metabolic phenotype and reduced hyperphagia (Vickers et al., 2005).

One study showed that both cord blood leptin and maternal glycemia were associated with methylation of genes associated with adipogenesis and activation of brown adipose tissue, potentially affecting the risk for adiposity, obesity and metabolic syndrome later in life (Côté et al., 2016). While the exact role is not entirely clear, higher levels of cord blood leptin are associated with larger infants, fetal insulin resistance (Walsh, Byrne, Mahony, Foley, & McAuliffe, 2014) and in general infants with higher cord blood leptin show slower growth in infancy (Ong et al., 1999) and lower adiposity and BMI z-scores at 3 years of age (Christos S. Mantzoros et al., 2009). Regardless of this, several large meta-analyses have concluded that high birthweight is associated with higher risk for overweight and obesity later in life (Kinge, 2017; Schellong, Schulz, Harder, & Plagemann, 2012; Zhao, Wang, Mu, & Sheng, 2012).

In cord blood, some studies have shown a positive correlation between leptin and insulin (Sandra G. Hassink et al., 1997; Ong et al., 1999). However, Wolf *et al* only observed a correlation between cord blood insulin and leptin in LGA infants (Wolf et al., 2000). In our study, we observed a positive correlation between cord blood leptin and cord blood insulin in control (Table 15) and GDM (Table 16) mothers, but this association was lost in pregnancies affected by T2D (Table 17). LGA is defined as birthweight >90th percentile for gestational age, and we found that infants of GDM and T2D mothers were in this category (Table 14), with birthweight-for-gestational age increasing progressively with duration of diabetes in pregnancy. While infants from GDM and T2D pregnancies were similar in birthweight, GDM infants were more frequently between the 10th and 50th percentiles. Depending on infant sex, infants in the GDM cohort were at the higher end of the birthweight curve and therefore had a higher proportion of LGA relative to T2D.

Leptin receptors have been found on fetal pancreatic islets in rodents (Islam, Sjöholm, & Emilsson, 2000). Thus, leptin may have a role in stimulating islet proliferation in the fetus. Additionally, leptin antagonism of islets in

the immediate neonatal period showed decreased β-cell area, suggesting that leptin may play a role in β-cell mass in the neonate (Attig, Larcher, Gertler, Abdennebi-Najar, & Djiane, 2011). In the adult system, leptin may stimulate insulin release (Ceddia, Koistinen, Zierath, & Sweeney, 2002). Moreover, results from cell culture (Leroy et al., 1996) and adult rodent studies (Saladin et al., 1995; Tan, Patel, Kaplan, Koenig, & Hooi, 1998) show that insulin levels may regulate leptin expression. In adult humans, insulin may positively regulate leptin expression over a longer period of time (Kolaczynski et al., 1996). Although placental leptin may only contribute to cord blood leptin in a negligible way, maternal insulin levels have been shown to upregulate placental leptin levels (Lepercq et al., 1998). In our mother-infant pairs we found that cord blood insulin correlated strongly with maternal HbA1c in control pregnancies, with hyperglycemia in the mother resulting in higher cord blood insulin in the offspring. The association was weaker in mothers with GDM and lost in T2D pregnancies (Tables 13-16). This correlation of cord blood insulin with maternal HbA1c has been previously reported (Westgate et al., 2006).

Cord blood insulin showed positive correlations with ponderal index and birthweight in infants from control (Table 15). and GDM groups (Table 16), however these associations were lost in the T2D group (Table 17). Even in non-diabetic pregnancies, macrosomic infants have been shown to display elevated cord blood insulin (Hoegsberg, Gruppuso, & Coustan, 1993). In the fetal system, insulin acts as a growth factor (Arshad, Karim, & Ara Hasan, 2014), and is positively correlated with birthweight and adiposity (Ong et al., 1999). Ponderal index takes both birthweight and length into account, much like a BMI z-score, and is a marker of infant proportionality. While similar to BMI z-score, it is used in infants and children as it is thought to more accurately diagnose obesity due to changing rates of growth in childhood (Zaniqueli et al., 2019). In this study it is possible that, unlike leptin and adiponectin, cord blood insulin may be more associated with overall growth than adiposity specifically, due to associations with birthweight, but not the ponderal index in infants from T2D pregnancies. It has been speculated that the roles of cord blood insulin and leptin are interlinked, and that in addition to its anabolic role, leptin mediates some of the effects of insulin on increased birthweight (Wolf et al., 2000). Interestingly, in control pregnancies cord blood leptin correlated more strongly with ponderal index and birth weight than gestational age, whereas in GDM and T2D pregnancies, the inverse was true. It is also important to note that only approximately half of the cord blood samples had insulin analysis completed and this reduced statistical power of the analysis. These results may indicate dysregulation of fetal growth in diabetic pregnancies, potentially mediated by changes in adipokine signalling.

It is important to consider the effect of obesity as well as hyperglycemia, as we observed maternal BMI was related to circulating adipokine levels. As maternal BMI increased, levels of circulating maternal adiponectin decreased and maternal leptin increased (Figure 23G-H). This is in line with what is known about adipokine secretion and obesity. Adiponectin exhibits an inverse relationship with adiposity (Arita et al., 1999) and leptin is positively correlated with adipose tissue mass (Fried, Ricci, Russell, & Laferrère, 2000). While we did not observe significant changes to cord blood adiponectin when separated by BMI class, we did note a moderate positive correlation between cord blood adiponectin and maternal BMI in only the GDM group (Table 16). Based on this, it is possible that maternal glycemia masked some of the effects of obesity in Figure 23G.

It has been established that maternal adiponectin levels are lower in women with GDM (Worda et al., 2004) and individuals with T2D across populations (Cruz et al., 2004; Snehalatha et al., 2003) (Kizer et al., 2012; Shanshan Li, Hyun Joon Shin, Eric L. Ding, & Rob M. van Dam, 2009). However, in our study we found significantly lower levels of maternal adiponectin in mothers with GDM, as well as in comparison to mothers with T2D. While this was initially surprising, as mothers with T2D have experienced hyperglycemia and insulin resistance for a longer period of time, treatments could have impacted adiponectin levels. Individuals with T2D were diagnosed prior to enrollment and received standard nutritional and physical activity advice and, if glycemic targets were not met, insulin and/or medication to manage their diabetes. Mothers diagnosed with GDM were treated with dietary counselling and, if blood glucose was not effectively controlled, received insulin when necessary. Both *in vivo* and *in vitro* results using adipose tissue explants from diabetic and non-diabetic patients showed that the drug metformin was capable of increasing adiponectin gene expression (Zulian et al., 2011). In addition, in male subjects with T2D insulin therapy significantly increased circulating adiponectin (Katsiki et al., 2011). In our cohort, most mothers with T2D were treated with insulin and a minority were treated with metformin. Considering the above noted effects on weight loss, the impact of insulin and metformin on adiponectin levels, pharmacological interventions may have had an impact on the maternal serum adiponectin levels in our study.

While a large body of research supports the increased metabolic risk of excessive GWG in pregnancy, some studies suggest that for obese women, pre-pregnancy BMI may be more indicative of gestational complications than GWG (Nohr et al., 2008). Given the inverse relationship between adiponectin secretion and adiposity, it is also important to note that in our study the mothers in the control group gained the most weight during pregnancy (18.25 kg on average) and women with GDM gained the least (Table 14), correlating with pre-pregnancy weight. Overall, maternal adiponectin showed a strong positive correlation with GWG. However, this correlation was negative in mothers with GDM (Table 16). While the GDM group showed the expected trend of decreased serum adiponectin with increasing GWG, the fact that the control group had the highest rate of GWG and higher levels of adiponectin could account for this correlation among mothers without diabetes. In T2D mothers, circulating adiponectin could be increased by pharmacological interventions mentioned above and contribute to a positive correlation with even modest GWG. Interestingly, other studies have reported that women with lower BMI tend to gain more weight than overweight or obese women in pregnancy (Nohr et al., 2008; Rasmussen, 2009; Teague et al., 2015), although this could be attributed in part to counselling or interventions.

As discussed in Chapter 1, maternal adiponectin does not cross the placenta and appears to exert its effects on the intrauterine environment by signalling through placental receptors (T. Jansson et al., 2012). Additionally, fetal adiponectin is postulated to have the opposite effect of circulating adiponectin in adults. Upregulation of fetal adiponectin serves to promote fetal growth and may potentiate insulin resistance (Luo et al., 2013b; R. C. Vasavada et al., 2000). The project VIVA cohort found an association between increased cord blood adiponectin and increased childhood adiposity at 3 years of age (Christos S. Mantzoros et al., 2009). In rodent studies, fetal adiponectin promotes adiposity (L. Qiao et al., 2012). Thus, the strong positive correlation we found between maternal and cord blood adiponectin, and increased birthweight in the GDM group is consistent with the function of adiponectin during development. A similar positive correlation has been previously reported (Luo et al., 2013a). Luo *et al* reported that

maternal adiponectin was negatively correlated with cord blood leptin (Luo et al., 2013a), an association that we observed only in mothers without diabetes in pregnancy (Table 15). Interestingly, mothers with GDM showed a positive correlation between maternal adiponectin and cord blood leptin, and this may be due to the much lower circulating adiponectin in mothers with GDM relative to both control and T2D mothers (Figure 23A). There are currently few studies reporting the effect of GDM on cord blood adiponectin. However, our results agree with those reported by the ACHOIS study and by Cortelazzi *et al*, wherein infants born to mothers with GDM had lower cord blood adiponectin (Cortelazzi et al., 2007; Pirc et al., 2007).

Luo *et al* did not report any association between maternal diabetes or BMI and cord blood adiponectin levels (Luo et al., 2013a). Another study reported a negative correlation between high molecular weight (HMW) cord blood adiponectin and maternal pre-pregnancy BMI in mothers with diabetes in pregnancy (Teague et al., 2015). We did not note any significant effect of increasing BMI on cord blood adiponectin (Figure 23F). , This may illustrate that fetal adiponectin functions solely in the fetal compartment and is more closely associated with fetal growth than maternal BMI (Laudes et al., 2009). LGA is defined as birthweight >90th percentile for gestational age, and in our study, we found that infants of GDM and T2D mothers fell into this category more frequently. In contrast to the action of adiponectin, leptin may increase placental amino acid transport, positively regulating fetal growth (N. Jansson, Greenwood, Johansson, Powell, & Jansson, 2003). In line with this, we observed an overall positive correlation between maternal leptin and birthweight. Increasing birthweight may be associated with risk of obesity and T2D later in life (reviewed in (T. J. Parsons, Power, Logan, & Summerbelt, 1999)) and preterm birth (<37 weeks) is associated with increased risk of respiratory morbidities, hypertension and metabolic syndrome later in life (Khashu, Narayanan, Bhargava, & Osiovich, 2009). We observed a trend towards increased birthweight and earlier deliveries on average in our GDM and T2D groups, relative to control, indicating that even with standard interventions, GDM and T2D may still pose a risk for LGA.

Both adiponectin and leptin show sexual dimorphism in cord blood, and associations with outcomes including childhood BMI and adiposity have been shown to be sex dependent (Ashley-Martin et al., 2020). Cord blood leptin levels have been reported to be significantly lower in males (Matsuda et al., 1997). In one study associations between cord blood adiponectin and childhood BMI and adiposity were correlated only in boys (Ashley-Martin et al., 2020). In adults, circulating adiponectin is higher in females than males (Boyne et al., 2010; U. Pajvani et al., 2003) and there appears to be a similar sex difference at the fetal stage (S. Basu et al., 2009). In our data set we did not have information regarding neonatal sex, which could mask differences in cord blood adipokines, introducing additional variability. Additionally, infant sex affects the curve on which birthweight is judged. In our T2D group, the average birthweight could be LGA or average depending on whether the infants were male or female.

Along with the lack of information on neonatal sex, another limitation in this study includes the size of the cohort. In particular the relative dearth of control pairs, as the control group includes only 14 mother-infant pairs for which a number of clinical characteristics were missing. A number of mothers were located and delivered outside the Winnipeg Health Region, and the clinical statistics that were collected varied widely. As a result, some correlations were relatively weak or missing. While we had access to information regarding parity, we were unable to determine

whether any mothers in this study had a previous GDM diagnosis which could potentially help explain associations observed between diabetes status, adipokines and parity. Additionally, we measured total adiponectin. Since adiponectin exists in multiple isoforms in circulation, measuring total adiponectin may capture only a portion of the effect of hyperglycemia on adipokines. However, some studies that measured cord blood HMW adiponectin noted similar associations with total adiponectin (Ballesteros et al., 2011; S. M. Lee et al., 2016).

Risk for caesarean delivery has been reported as an outcome in other studies, and this information was missing from our cohort. There is also evidence that labour and vaginal delivery may impact maternal levels of adiponectin (Fazeli Daryasari et al., 2019). We set out to clarify key differences between GDM and T2D in pregnancy. However, it is important to note that therapeutic interventions for both GDM and T2D may impact our analysis, specifically circulating adipokines, as mothers with T2D were frequently given insulin or metformin and both have been shown to affect circulating adiponectin levels.

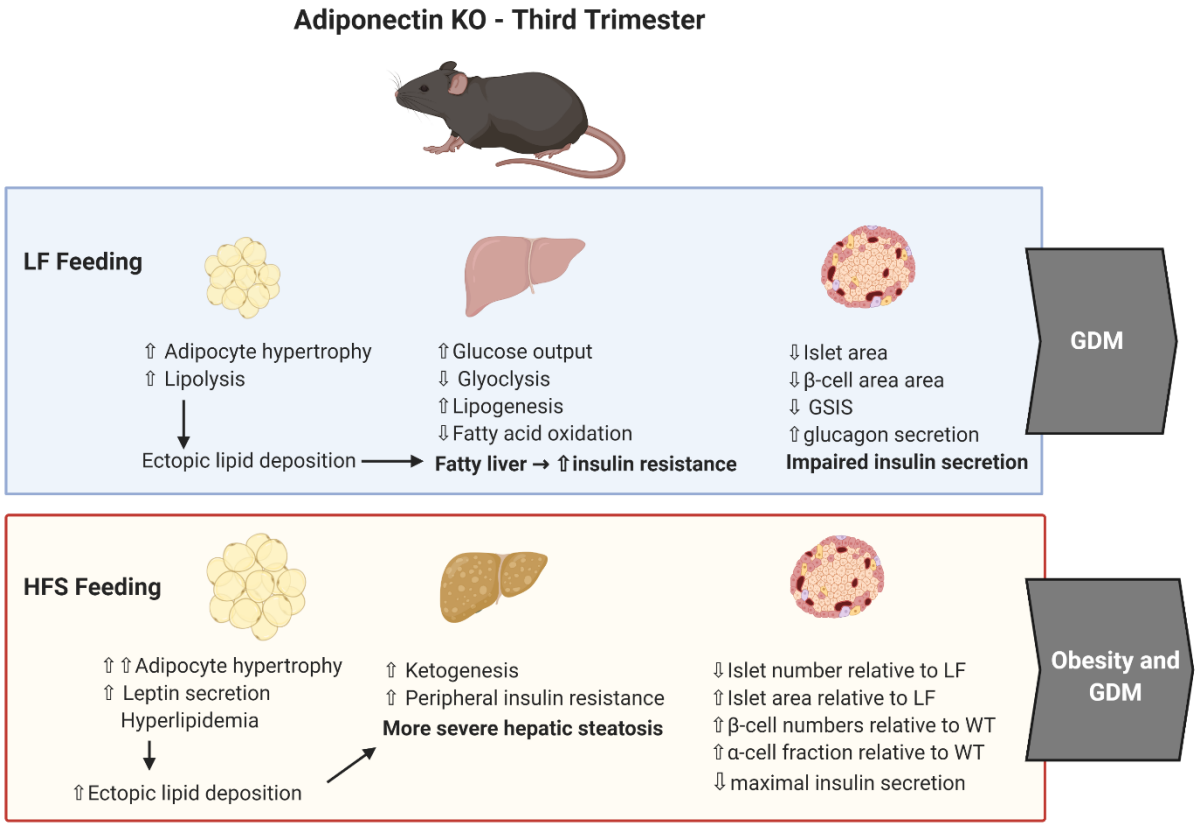
Taken together these results suggest alterations of adiponectin and leptin in mothers with GDM and T2D was associated with birthweight, and importantly that GDM exhibits a distinct adipokine profile compared to T2D. On average, mothers with GDM were heavier prior to pregnancy, had a higher BMI at delivery, but showed the lowest GWG relative to control or T2D. The heaviest babies were from GDM pregnancies and this was associated with the lowest maternal serum adiponectin and the highest leptin. Cord blood adipokine levels, despite being secreted independently from maternal adipokines, were also impacted by maternal diabetes status and reflected other risk factors such as gestational age at delivery and birth weight. Maternal obesity was also a significant determinant of adipokine levels. The increased risks associated with exposure to hyperglycemia in pregnancy make it necessary to better understand the mechanisms that underlie the distinct etiology of GDM and pre-pregnancy T2D.

Chapter 8

8.1 Discussion, Conclusions and Future Directions:

The overall hypothesis of this thesis was that adiponectin deficiency in pregnancy leads to the development of GDM and increases fetal growth. We hypothesized that a lack of adiponectin in pregnancy would disrupt hepatic lipid metabolism, leading to the development of hepatic steatosis and worsening insulin resistance, contributing to GDM development. We also hypothesized that the added metabolic stress of HFS diet consumption would further impair the ability of adiponectin KO mice to adapt to pregnancy. Since adiponectin impacts insulin secretion and β-cell survival, we also hypothesized that lack of adiponectin in pregnancy impaired β-cell adaptations to pregnancy. We further aimed to determine whether increasing adiponectin in the third trimester could improve the metabolic phenotype of GDM. Using an adiponectin KO mouse model, we determined that lack of adiponectin was sufficient to induce a GDM-like phenotype in the third trimester, characterized by hyperglycemia and impaired glucose tolerance brought on by dysregulation of gluconeogenesis, hepatic steatosis, β-cell dysfunction and mitochondrial dysfunction (Figure 24). Adiponectin KO mice were more susceptible to the deleterious effects of HFS diet consumption in pregnancy, leading to more severe hepatic steatosis and impaired glucose homeostasis. Moreover, we showed that increasing adiponectin levels in pregnancy rescued the metabolic dysfunction that characterized GDM. Additionally, we were able to show that low levels of circulating adiponectin were associated specifically with GDM in a cohort of pregnant First Nations women. We also found that exposure to GDM, T2D and obesity during pregnancy is associated with alterations in cord blood levels of adiponectin and leptin, potentially influencing both fetal and postnatal growth.

Figure 24: Adiponectin KO mice develop GDM in the third trimester, which is exacerbated by the addition of HFS diet consumption.



In the third trimester of pregnancy, female adiponectin KO mice were hyperglycemic and developed adipocyte hypertrophy, increased hepatic lipogenesis and disrupted mitochondrial metabolism even when consuming a LF diet. LF-fed adiponectin KO mice also developed hepatic steatosis, impaired hepatic insulin sensitivity and dysregulated islet hormone secretion in the third trimester relative to WT controls. Consumption of a HFS diet in pregnancy lead to increased visceral adiposity, hyperleptinemia and hyperlipidemia, hepatic steatosis and more severe impairments to islet function in the third trimester in adiponectin KO mice.

Prior to pregnancy, female adiponectin KO mice were metabolically similar to WT mice. Consumption of a HFS diet caused increased weight gain in both adiponectin KO and WT mice. However, weight gain of adiponectin KO mice was similar to WT mice in agreement with previous studies using adiponectin KO mice (R. Guo, Zhang, Turdi, & Ren, 2013; Kubota et al., 2002; Maeda et al., 2002). Moreover, non-pregnant adiponectin KO mice were normoglycemic and glucose tolerant. Other studies have shown that adiponectin KO mice only show metabolic dysfunction after the addition of an obesogenic diet (Asano et al., 2009; R. Guo et al., 2013; Y. Liu et al., 2013; Maeda et al., 2002), suggesting that a lack of adiponectin sensitizes mice to the effects of a secondary metabolic "challenge".

Pregnancy requires the balance between providing glucose for the growing fetus and maintaining normoglycemia in the mother. Reversible adaptive changes in energy metabolism and hormone secretion are a necessary process during pregnancy. One physiologic mechanism necessary to make more glucose available for the fetus is for the maternal tissues to become more insulin resistant (Patrick M. Catalano et al., 1999; Sivan, Homko, Chen, Reece, & Boden, 1999). Pre-existing obesity and insulin resistance or excessive gestational weight gain followed by the development of more profound insulin resistance occurs during pregnancy and can lead to GDM (Fagulha et al., 1997; Plows et al., 2018; Sonagra, Biradar, K, & Murthy D S, 2014). A second physiologic mechanism in pregnancy is to increase maternal insulin secretion to counteract the insulin resistance of maternal tissues (Fagulha et al., 1997). The increased insulin secretory capacity is provided by increasing the number of β-cells as well as the amount of insulin which individual β-cells secrete (J. A. Parsons et al., 1992; Sorenson & Brelje, 1997). Insufficient β-cell compensation during pregnancy can also cause GDM (Plank, Frist, LeGrone, Magnuson, & Labosky, 2011; H. Zhang et al., 2010). We showed that during pregnancy, adiponectin KO mice had impairments in glucose homeostasis compared to non-pregnant adiponectin KO females. A previous study using pregnant adiponectin KO mice did not compare glucose homeostasis and insulin sensitivity before and during gestation (Liping Qiao et al., 2017).

The presence of hyperglycemia during pregnancy in adiponectin KO mice is likely derived from a combination of factors. Firstly, hyperglycemia in pregnancy can result in part from impairments to peripheral glucose uptake. Insulin resistance in the third trimester has been shown to reduce glucose uptake by skeletal muscle and liver in numerous animal models including rats (Saad et al., 1997), rabbits (Gilbert, Pere, Baudelin, & Battaglia, 1991) and ewes (Bell & Bauman, 1997). Results from qPCR in liver tissue in the third trimester showed that expression of hepatic glucokinase (*Gck*), the rate limiting enzyme of glycolysis, was not significantly altered in WT mice with the administration of insulin. Although many models have noted impairments to glucose uptake and metabolism by the liver, as mentioned above, other studies have failed to observe these impairments. A canine model of pregnancy in which glucokinase levels and activity were measured noted reductions in levels of GK with high fat feeding in pregnancy, however, this did not impact enzyme activity (Coate et al., 2013). It has been shown that GK may be required to suppress hepatic glucose output in response to hyperglycemia (Barzilai, Hawkins, Angelov, Hu, & Rossetti, 1996; Torres et al., 2009). As we have shown previously, WT mice were not hyperglycemic and did not have excessive hepatic glucose output in the third trimester. This indicates that glucose uptake and earlier steps of glycolysis are functional in adiponectin KO mice in the third trimester, but point to potential defects in complete glycolysis, and control of glucose metabolism.

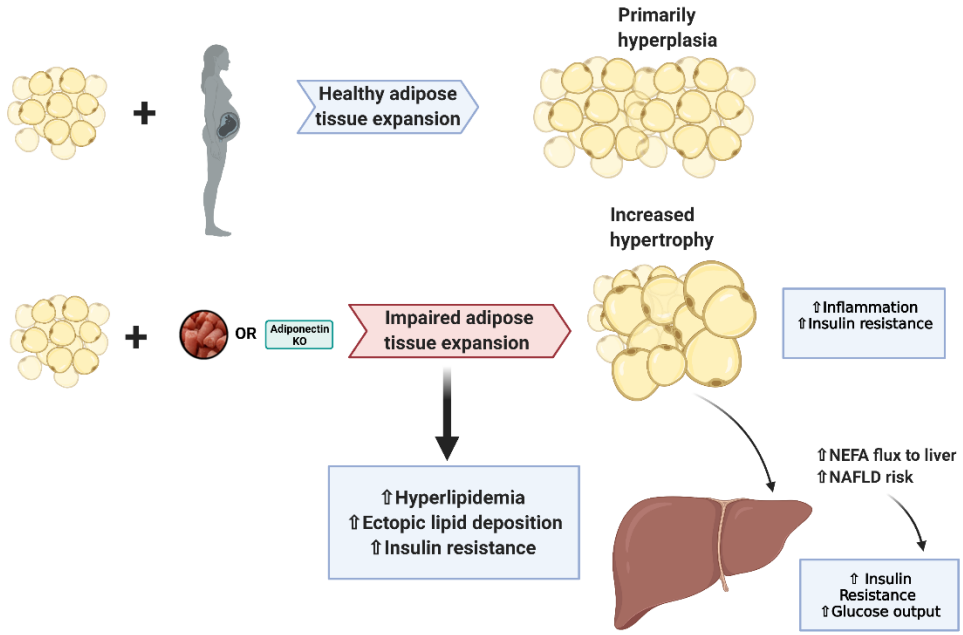
Pyruvate kinase is downstream of GK in the glycolysis pathway and converts phosphoenolpyruvate to pyruvate for TCA cycle metabolism and complete oxidation to ATP. When we measured the expression of the hepatic isoform of pyruvate kinase (*Pklr*) we found no differences in adiponectin KO mice compared with WT even after insulin administration. Insulin should increase activity of hepatic pyruvate kinase, however diabetes is known to suppress the effects of insulin on hepatic PK in rats (Parks & Drake, 1982). With this in mind, it is possible that tissue collected within 10 minutes of insulin injection did not allow enough time to show changes to PK gene expression, and that insulin resistance associated with pregnancy and adiponectin KO may extend the amount of time required for insulin to show its effects, as demonstrated by Parks *et al* (Parks & Drake, 1982).

Secondly, dysregulated insulin-mediated suppression of hepatic gluconeogenic genes contributes to elevated hepatic glucose production and glucose intolerance. Insulin resistance can prevent adequate suppression of hepatic gluconeogenesis, and lead to increased blood glucose. While Catalano *et al* did not observe hepatic insulin resistance in lean pregnant women (Catalano et al., 1992), increased hepatic gluconeogenesis has been implicated in the development of GDM as a by-product of insulin resistance (Butte, 2000; P. M. Catalano, L. Huston, S. B. Amini, & S. C. Kalhan, 1999; Di Cianni, Miccoli, Volpe, Lencioni, & Del Prato, 2003). Additionally, adiponectin suppresses hepatic glucose output (Zhou et al., 2005). We found increased expression of hepatic gluconeogenic genes in adiponectin KO mice in the third trimester relative to WT mice, which agrees with previous research on the role of adiponectin in the regulation of gluconeogenesis (Liping Qiao et al., 2017; Zhou et al., 2005). Notably, while *Pck1* and *Pck2* were unchanged in WT mice, adiponectin KO mice had a marked increase in expression of *Pck1* and *2*, and a trend towards increased *G6pase* even after insulin administration. This suggests that lack of adiponectin combined with insulin resistance leads to impaired suppression of gluconeogenic genes in pregnancy. In line with these findings, pyruvate tolerance tests *in vivo* showed that adiponectin KO mice had increased glucose production from pyruvate in the third trimester. This observation confirmed that increased hepatic glucose output in adiponectin KO mice may have been partly responsible for the hyperglycemia observed in the third trimester. Overall, these findings agree with our hypothesis that adiponectin KO mice develop hyperglycemia and impaired glucose tolerance in the third trimester in the absence of obesity, relative to WT controls.

GDM is strongly associated with overweight and obesity (C. Kim, 2010). We found that adiponectin KO mice were not obese during pregnancy, did not exhibit excessive gestational weight gain, and that hyperglycemia during pregnancy was independent of obesity. Individual fat depots are not the same and the mechanism of adipose tissue expansion, particularly in pregnancy, may have implications for metabolic health (Rojas-Rodriguez et al., 2015). Adipocyte hyperplasia is associated with better glucose control and hypertrophy, as we observed in adiponectin KO mice, and has been linked to increased inflammation and insulin resistance (Hoffstedt et al., 2010) (Figure 25). Adiponectin is known to promote heathy expansion of adipose tissue (Tao et al., 2014) and the expansion of adipose tissue prevents ectopic deposition of lipids in peripheral tissues which can lead to worsening insulin resistance (Gray & Vidal-Puig, 2007). A major finding was the development of hepatic steatosis, a common complication of obesity, in pregnant adiponectin KO mice even with LF diet consumption. It is therefor possible that in pregnant adiponectin KO mice impaired adipose tissue expansion contributed to increased hepatic fat deposition, and hepatic steatosis and

the accumulation of lipotoxic lipids contribute to hepatic insulin resistance and dysregulated hepatic glucose output (Samuel et al., 2004; Trauner, Arrese, & Wagner, 2010) (Figure 25).

Figure 25: Impaired adipose tissue expansion can increase ectopic lipid deposition



Impaired adipose tissue expansion (hypertrophy as opposed to hyperplasia) due to high fat-diet feeding or adiponectin deficiency can lead to increased adipose tissue inflammation and insulin resistance. This contributes to hyperlipidemia and ectopic lipid deposition in other tissues such as the liver. Increased hepatic lipid deposition leads to NAFLD, which is associated with hepatic insulin resistance and increased glucose output all of which can lead to GDM in pregnancy.

Less is known about the connection between fatty liver and GDM. Hepatic metabolism is central to energy homeostasis as the liver is major insulin sensitive organ and the primary source of *de novo* glucose production. In the livers from pregnant adiponectin KO mice we found evidence of histologically detectable hepatic steatosis. We also showed that adiponectin KO mice had increased levels of triglyceride synthesis and increased lipogenic gene expression. In addition, adiponectin KO mice have impairments to mitochondrial respiratory capacity in pregnancy and these defects were particularly pronounced when utilizing fatty acids for energy. These results point to defects in hepatic lipid metabolism with worsening insulin resistance in GDM development. We noted increased uptake of lipid precursors by hepatocytes from non-pregnant adiponectin KO mice and this corresponded with dramatic increases in cholesterol synthesis and secretion, as well as triglyceride and diglyceride synthesis from oleate. These results suggest that adiponectin deficiency may "prime" the hepatocytes of female mice to develop fatty liver in pregnancy (Sunny et al., 2011; Trauner et al., 2010).

In one study by Koliaki *et al*, obesity and insulin resistance in patients, both with and without hepatic steatosis, was associated with reduced serum adiponectin (Koliaki et al., 2015). In pregnancy, the liver plays a critical role in regulating the metabolic switch between glucose and fatty acid oxidation that is required to mediate the needs of maternal and fetal nutrition in the third trimester (Boden, 1996). This switch is facilitated by the preceding phase of anabolic adaptations and the progressive insulin resistance of pregnancy. In primary hepatocytes isolated from non-pregnant WT and adiponectin KO mice fed a LF diet we observed increased respiration when cells were using glucose. However, in cells isolated from mice fed a HFS-diet fed adiponectin KO mice showed decreased mitochondrial respiration relative to WT. This finding adds further evidence that adiponectin KO mice do not adapt to additional metabolic stress. In a study of obese human patients with and without hepatic steatosis, obesity and insulin resistance were associated with increased hepatic mitochondrial respiration (Koliaki et al., 2015). However, in patients with advanced to hepatic steatosis, hepatic mitochondrial respiration was not elevated (Koliaki et al., 2015). An increase in mitochondrial oxidative capacity was also observed in a mouse model of diet induced obesity (Buchner et al., 2011). We noted more severe impairments to hepatic mitochondrial respiration when primary hepatocytes from adiponectin KO mice utilized fatty acids, compared to WT mice in the third trimester. At this point in pregnancy, fatty acid oxidation should be increased as a mechanism to provide energy for maternal nutrition (Boden, 1996; Knopp et al., 1981).

Hepatocytes from adiponectin KO mice in the third trimester showed reduced ATP production, increased ketogenesis and increased synthesis of triglycerides relative to WT mice when utilizing fatty acids. This may suggest the presence of incomplete β-oxidation in pregnant adiponectin KO mice. It has been determined that non-pregnant adiponectin KO mice developed NAFLD, and this is accompanied by dysregulated mitochondrial morphology and impaired activity of mitochondrial respiratory chain function (Zhou et al., 2005). The authors of this study also observed increases in TNF-α, a pro-inflammatory cytokine, that is increased as a result of increased ROS and lipid peroxidation (Zhou et al., 2005). Due to its many insulin sensitive actions, hepatic insulin resistance has a number of metabolic consequences, and hepatic steatosis in pregnancy could be a factor that induces increased hepatic glucose output and resulting hyperglycemia.

The transcription factor FoxO1 regulates a number of metabolic processes including gluconeogenesis and lipogenesis in the liver (W. Zhang et al., 2006). Hepatic gluconeogenic gene expression is increased by FoxO1 (Y. Wang et al., 2014). We showed marked increases in hepatic *Foxo1* expression in pregnant adiponectin KO mice. Additionally, phosphorylation of FoxO1 (by AKT, downstream of insulin) should inhibit activity of FoxO1. However, FoxO1 phosphorylation appeared to be reduced in adiponectin KO mice suggesting a potential mechanism for insulin-resistance induced increase of hepatic gluconeogenesis in these animals. FoxO1 activation was shown to suppress lipogenesis (through *Srebp1c* (Deng et al., 2012)) and increase VLDL secretion, factors that mitigate hepatic steatosis in rodent models where FoxO1 is constitutively expressed in the liver (W. Zhang et al., 2006). In adiponectin KO mice, we observed an increase in hepatic lipogenic genes, without significant changes in *Srebp1c* expression with or without insulin administration. It is important to note that the *FoxO* family of transcription factors respond rapidly to lower insulin concentrations in comparison to *Srebp1c* (Rebecca A. Haeusler et al., 2014; Shimomura et al., 2000),

therefor changes to *Srebp1c* expression may not have been observable within 10-15 minutes of insulin administration, representing a limitation to this particular analysis.

In a healthy pregnancy, insulin resistance is not accompanied by hyperglycemia due to increased insulin secretion by the endocrine pancreas. We did not observe any significant increase in serum insulin in pregnant adiponectin KO mice relative to WT mice. Typically, hyperinsulinemia accompanies insulin resistance (S. H. Kim & Reaven, 2008). Maeda *et al* noted that non-pregnant adiponectin KO mice prior to pregnancy only became hyperinsulinemic with consumption of a HFS diet (Maeda et al., 2002). However, neither adiponectin KO nor WT controls showed increase in serum insulin in the third trimester with HFS diet consumption. Qiao *et al* noted a progressive increase in serum insulin throughout gestation in wildtype C57B6 mice (Liping Qiao et al., 2017). However, they also reported lower serum insulin in adiponectin KO mice relative to WT in the third trimester (Liping Qiao et al., 2017). Since insulin secretion tends to increase in order to overcome insulin resistance in the third trimester, it is possible that compensatory mechanisms by the β-cell to increase insulin secretion capacity are exhausted by late gestation when animals are exposed to the stress of a HFS diet in pregnancy. It is important to note that insulin secretion can be regulated in a circadian and diurnal fashion (Kalsbeek & Strubbe, 1998). Although mice were fasted for 1 hour prior to sacrifice we did not sacrifice animals at the same time of day and this may impact the serum insulin levels observed in our study.

At the level of the endocrine pancreas, we found that adiponectin KO mice had reduced glucose stimulated insulin secretion in the third trimester as well as increased secretion of glucagon from α-cells under high-glucose conditions. Moreover, adiponectin KO mice tended to have reduced total islet area and β-cell positive area in the third trimester relative to WT mice. This may indicate that lack of adiponectin in pregnancy hinders the adaptive responses in the pancreatic islet to the metabolic demands of late pregnancy. Collectively these factors contribute to the development of hyperglycemia in pregnancy, as increased serum glucagon has been reported in women with GDM (Feng et al., 2020). Impaired suppression of glucagon secretion earlier in gestation may be associated with poor glucose control late in gestation in women with GDM (Horie et al., 2019). Due to its role in stimulating hepatic gluconeogenesis, (A. Young, 2005) increased serum glucagon could directly impact hyperglycemia in pregnancy, particularly in combination with increased insulin resistance and impaired compensatory adaptations by the β-cell, which is implicated in the development of GDM (C. Homko, E. Sivan, X. Chen, E. Reece, & G. Boden, 2001).

Other studies have shown that a lack of adiponectin in pregnant mice decreased β-cell positive area and total islet area in pregnant adiponectin KO mice relative to WT controls (Liping Qiao et al., 2017). In our study, we observed a decrease in total islet area and β-cell area in adiponectin KO mice relative to WT at the end of the third trimester. However, our sample size was low. We did not observe marked changes to β-cell numbers in adiponectin KO compared to WT mice fed a LF diet in the third trimester. This observation may be due to the fact that in rodent models, β-cell proliferation reaches its maximum in mid-gestation and has been shown to return to control levels by e18-19, the point when pancreases were collected in our study (Ernst et al., 2011; J. A. Parsons et al., 1992; Sebastian Rieck & Kaestner, 2010). The work by Qiao *et al* did not compare β-cell and islet mass in adiponectin KO and WT in non-pregnant and pregnant mice (Liping Qiao et al., 2017). This could reveal the extent of compensatory failure in

the absence of adiponectin. We did note that islet number, islet area and β-cell area appeared to increase in the third trimester of WT mice fed a LF diet relative to non-pregnant mice. These changes were less pronounced in HFS fed WT mice and in adiponectin KO mice, regardless of diet, though these observations are based on a limited sample size.

Additionally, while we did not perform experiments to determine the mechanism behind any increase in islet or β-cell mass, research suggests that the mechanisms by which β-cell mass is increased differs between rodents and humans (reviewed in (Genevay, Pontes, & Meda, 2010)), potentially limiting the translatability of such experiments. Human islets isolated from pregnant women show less dramatic increases in β-cell mass relative to rodents and there is more evidence of increased proliferation and neogenesis rather than hypertrophy in pregnant women compared to rodent models (A. E. Butler et al., 2010; Van Assche et al., 1978). However, lineage studies have identified β-cell neogenesis from progenitor cells as a mechanism of increasing β-cell mass in pregnant rodents potentially narrowing the mechanistic gap between human and rodent islets (Abouna et al., 2010). Additionally, the scarcity of human islets during pregnancy leads to heterogeneity in study populations. For example, research by Butler *et al* included women with inflammatory conditions, and at different stages of gestation which may confound observations (A. E. Butler et al., 2010).

We also observed that adiponectin KO mice had impaired insulin secretion in the third trimester with reduced glucose stimulated and maximal insulin secretion relative to WT controls. The addition of a HFS diet further impaired insulin secretory capacity. This extends previous findings that adiponectin potentiates glucose stimulated insulin secretion (Bratanova-Tochkova et al., 2002; Okamoto et al., 2008; Straub & Sharp, 2004; Wijesekara et al., 2010). Adiponectin receptors have been found on the β-cell. Hence, the primary action of adiponectin in islets is most likely on the β-cell. Nonetheless, we also observed impaired glucose mediated inhibition of glucagon secretion from islets isolated from adiponectin KO mice in the third trimester. While serum glucagon was not measured, glucagon signalling to the liver can increase glucose output, which we observed in adiponectin KO mice in the third trimester.

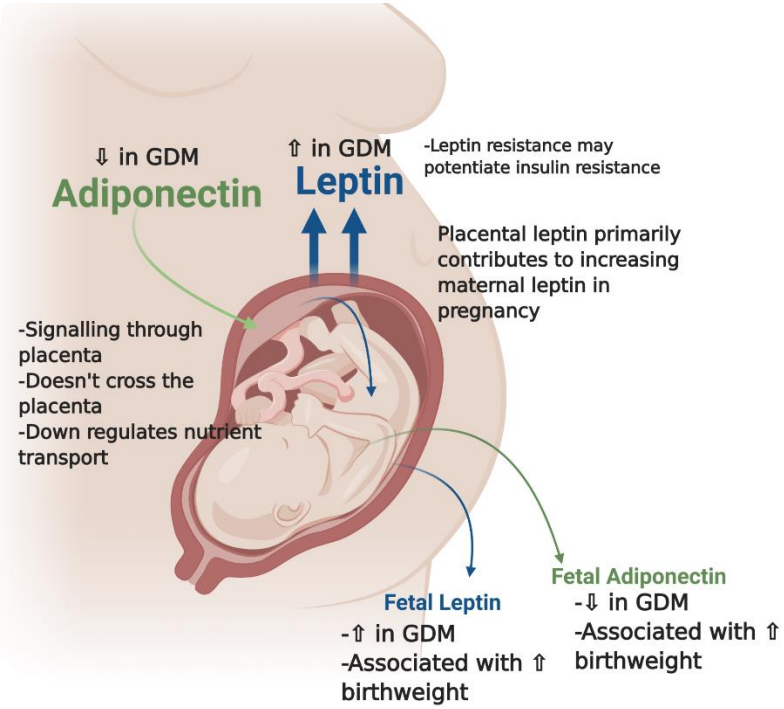
We also observed higher fractions of α-cell positive area in pregnant adiponectin KO mice relative to WT mice. There is evidence to suggest that hyperglucagonemia and increased pancreatic glucagon are seen in obesity and T2D (Dunning & Gerich, 2007; Starke et al., 1984; Stern et al., 2019). Placental lactogen can increase α-cell proliferation and α-cell mass in late gestation (Quesada-Candela et al., 2020). However, we showed that HFS diet consumption reduced α-cell content in WT mice. Interestingly the α-cell content was unaffected by diet in adiponectin KO mice. There is little literature that addresses the mechanism of action of adiponectin in the α-cell. Insulin secretion from the β-cell has been shown to exert paracrine effects on the α-cell and influence glucagon secretion (Roger H Unger & Orci, 2010). Since GSIS was impaired in the absence of adiponectin in pregnancy, it is possible that reduced insulin also alleviates the paracrine effect of insulin that suppresses glucagon secretion.

To determine whether increasing adiponectin could rescue diabetes during pregnancy, we used adenoviral mediated adiponectin expression in the third trimester as a proof of concept. Overall, we noted that adiponectin supplementation improved hepatic steatosis, hyperglycemia, glucose tolerance and suppressed gluconeogenesis in pregnant mice relative to GFP controls. These findings agree with previous studies that show adiponectin

supplementation improves hyperlipidemia, hyperglycemia and glucose tolerance in pregnant adiponectin KO mice fed a chow diet (Liping Qiao et al., 2017). In our study it was especially interesting to note these phenotypic improvements given the fact that serum adiponectin did not increase to the same level as that of WT mice. This may be due to the fact that tissue collection occurred ~6 days following adenovirus injection. Other studies noted significant decreases in plasma levels of adiponectin transcript and protein between 2 hours and 7 days post plasmid-delivery (Y. Ma & Liu, 2013). One potential explanation is the fact that we administered adenovirus by tail vein injection. Hepatic sequestering of viral gene therapy (Shayakhmetov et al., 2004; Turner et al., 2011) could lead to paracrine signalling of adiponectin through hepatic AdipoR2, keeping signalling from supplemented adiponectin localized. This could lead to improvements in hepatic metabolism that seem disproportionate to the increases in circulating adiponectin that result from supplementation.

Diabetes during pregnancy affects fetal growth and development (Geurtsen et al., 2019; Lawlor, Lichtenstein, & Långström, 2011). As discussed in previous chapters, fetal adiponectin behaves as a growth factor potentiating fetal growth, and LF-fed WT mice had significantly smaller pups when dams were supplemented with adiponectin in pregnancy (Table 11). Since maternal adiponectin does not cross the placenta it impacts fetal growth through placental signalling (Figure 26). We examined the interaction between maternal and cord blood adipokine levels, and their relationship to maternal diabetes status and infant bodyweight in First Nations mothers with GDM, pre-gestational T2D and controls without diabetes in pregnancy from the NextGen cohort study (Jabar et al., 2019). While we found that women with GDM had lower adiponectin and higher leptin levels in serum relative to women without diabetes in pregnancy, women with GDM also had an adipokine profile in the third trimester that was distinct from T2D. In the non-diabetes control group, we observed an inverse relationship between maternal adiponectin and maternal age at delivery, pre-pregnancy weight, maternal HbA1c, serum leptin, birthweight and gestational age. However, in mothers with diabetes in pregnancy (in particular GDM), some of these associations were disrupted. For instance, mothers with GDM were the only group to show a positive correlation between maternal adiponectin and birthweight, despite an overall negative correlation observed in the cohort and in control, and T2D mothers.

Figure 26: Disruption of maternal and fetal adipokines by GDM can lead to alterations in maternal metabolism during pregnancy, and dysregulated fetal growth



Maternal adiponectin does not cross the placenta but signalling through placental receptors can impact fetal growth and nutrition. Placental leptin can contribute to maternal hyperleptinemia which is present in GDM and can potentiate insulin resistance already present in GDM. Studies have reported that cord blood adiponectin is reduced in infants born to mothers with GDM, and in contrast to the actions of maternal adiponectin, fetal adiponectin may have a role in promoting fetal adiposity and insulin resistance. Fetal leptin is primarily derived from fetal adipose tissue rather than maternal or placental sources and is increased in infants exposed to GDM; higher cord blood leptin is associated with increased birthweight.

It is important to note the limitations inherent in this analysis, particularly that for some variables, missing data could alter some associations, including data from samples collected early in the study or at remote locations. Nonetheless, the strongest finding from the Next Gen cohort was that mothers with GDM had the lowest levels of serum adiponectin relative to mothers with T2D as well as controls. Additionally, the effect of dietary and pharmacological interventions for managing diabetes on the levels of adipokines should be considered. Women with T2D are likely managing their diabetes with insulin or in some cases with metformin, both of which may increase serum adiponectin levels (Katsiki et al., 2011; Zulian et al., 2011). Alternatively, dietary and lifestyle counseling to reduce GWG may impact serum adiponectin and alter associations. Nevertheless, we noted altered serum and cord blood levels of adiponectin and leptin in mothers and neonates with GDM, which when taken with results from animal models, may suggest a role for adipokine signalling in the development of GDM.

In the NextGen cohort, newborns exposed to diabetes in pregnancy (whether GDM or T2D) had increased birthweight and were more likely to be born LGA. These observations agree with previous findings on diabetes in pregnancy and risk of LGA and fetal macrosomia (Baeten et al., 2001; Buchanan et al., 2012). Fetal adiponectin and

leptin generally show a positive association with fetal growth (Figure 26), an association that was generally confirmed in our study. However, both adipokines have been known to show significant sexual dimorphism (Ashley-Martin et al., 2020; Matsuda et al., 1997). We did not have access to neonatal sex in our mother-infant dyads. Therefore, infant sex is a potential source of variation that could impact the strength of our associations.

In our adiponectin KO mice, we did not observe any significant differences in litter sizes relative to WT mice. HFS diet consumption led to smaller pups in WT mice and adiponectin supplementation reduced pup size in WT mice fed a LF diet but had no effect on pup size in adiponectin KO mice. Qiao *et al* reported that fetuses from adiponectin KO dams fed a HFD were smaller than their WT counterparts at e17.5 (L. Qiao et al., 2012). These results are in line with results reported in a review by Christians *et al* wherein HFD consumption had no effect on litter size in mice (Christians, Lennie, Wild, & Garcha, 2019). Additionally, it has been reported that mice consuming a HFD either have smaller pups (Edlow et al., 2016) or no changes to pup size at all (reviewed in (Christians et al., 2019)), an observation that is also impacted by neonatal sex (Edlow et al., 2016). Maternal obesity has not been linked strongly to inter-uterine growth restriction when the confounding effect of preeclampsia is corrected for (Cedergren, 2004), so it is possible that fetal growth due to maternal nutrition may not be adequately modeled in mice, given these discrepancies. It was also reported that HFD feeding affected fetal growth in mice in a time-dependent manner, in some cases decreasing fetal growth up to gestational-day 15 but not impacting neonatal size at term (Sferruzzi-Perri et al., 2013), which is when most of our measurements were recorded. In our study, it is possible that without allowing mice to give birth, variability in gestational age at the time of weighing (due to changes in the method of detection of pregnancy) lead to variability in pup weight. Previous studies have reported that a lack of adiponectin in pregnancy can impact neonatal fat deposition. However, weighing pups does not take into account body composition, and differences in lean mass and fat deposition between WT and adiponectin KO pups were observed as early as e17.5 (L. Qiao et al., 2012). It is possible that without a change in birthweights between WT and adiponectin KO mice, the metabolic phenotype may still be disrupted in our model, however we did not examine body composition of the pups (L. Qiao et al., 2012).

Rodent models allow for many lines of experimental investigation, including developmental programming, where direct translation to human disease is not always possible. However, key differences in fetal development may impact comparisons, particularly considering the differences in length of pregnancy and how it may relate to organogenesis (Azad 2017). Additionally, more than 97% of human pregnancies are singleton pregnancies whereas rodent pregnancies contain litters of multiple fetuses which impacts fetal size and nutrition (Dahl Andersen et al., 2018). We should consider these differences when comparing the impact of adiponectin on birthweight in our knockout model and in patients from the Next Gen cohort, as we did not note any significant effect of adiponectin KO on birthweight in our mouse model. These differences suggest that findings from offspring in rodent studies may not translate directly to human studies but still may provide a mechanistic foundation.

Previous work from our lab focused on the intrauterine exposure to GDM and the effect of developmental programming on the offspring. HFS diet feeding in rats showed increased gestational weight gain, impaired glucose tolerance and hyperglycemia in pregnancy (Pereira et al., 2015). Rats fed a HFS diet in pregnancy showed increased serum insulin mid-gestation and reduced serum adiponectin (Pereira et al., 2015). Further, offspring exposed to GDM

in pregnancy were at increased risk for development of hepatic steatosis, even when consuming a LF diet postnatally (Pereira et al., 2015). Additional work in this model showed that exposure to GDM in pregnancy appeared to impact the adaptation of the islets to pregnancy including reduced GSIS and decreased islet content when offspring consumed a postnatal HFS diet (Prasoon Agarwal et al., 2019). In the context of our study, future work could examine the impact of aging in male and female offspring from adiponectin KO and WT mothers fed a LF or HFS diet to adulthood with the subsequent examination of how adiponectin deficiency during pregnancy can impact long-term offspring metabolic health outcomes.

Based on our results and previous studies (Liping Qiao et al., 2017), adiponectin supplementation has therapeutic potential for GDM. Maternal adiponectin supplementation may also be protective against the effects of exposure to GDM on offspring weight gain (Aye et al., 2015; Rosario et al., 2012). To date there are no studies evaluating the effect of adiponectin supplementation in humans although the effect of existing drugs and natural health products on adiponectin levels is an active area of research (Y. Liu, Vu, & Sweeney, 2019). In pregnancies affected by GDM, diet and lifestyle interventions are often prescribed to attempt to control hyperglycemia without the use of medications (reviewed in (Brawerman & Dolinsky, 2018)). Weight loss and exercise (Becic, Studenik, & Hoffmann, 2018; Kriketos et al., 2004; Saunders et al., 2012) also increase adiponectin levels. Thus, increased adiponectin could be a mechanism underpinning exercise and weight loss-induced improvements of metabolic health in women with GDM.

If hyperglycemia is not controlled adequately with lifestyle modification, both insulin and metformin have been used in pregnancy. Although both are safe and effective in pregnancy, increasing levels of insulin has been associated with increased gestational weight gain and incidence of hypoglycemia (Barnes et al., 2016; Bergel, Hadar, Toledano, & Hod, 2016; Scholl & Chen, 2002). Insulin therapy in pregnancy has also been associated with increased risk of macrosomia and increased fetal fat deposition (Barnes et al., 2016) which may also impact fetal adipokine secretion. Studies have shown conflicting results regarding the effect of metformin on adiponectin levels. Some studies show decreases in adiponectin levels with metformin treatment (Cannon Megan et al., 2014; Huypens, Quartier, Pipeleers, & Van de Casteele, 2005) whereas others show increases in secretion from subcutaneous adipose tissue, but not visceral adipose tissue after metformin treatment (Zulian et al., 2011). However, increased adiponectin *in vivo* after metformin could be due to the effect of metformin on weight loss (Rowan, Hague, Gao, Battin, & Moore, 2008).

8.2 Future Directions

In Chapter 3 we established that adiponectin KO mice develop impaired glucose tolerance and hyperglycemia in the third trimester that is not present in non-pregnant mice. While we investigated some of the underlying mechanisms, such as determining that increased gluconeogenesis may contribute to hyperglycemia, there is also some evidence to suggest hepatic insulin resistance is more severe in pregnant adiponectin KO mice. Experiments to assess downstream markers of insulin signalling in the liver, such as insulin receptor beta and IRS would provide insight into the effect of adiponectin deficiency on hepatic insulin signalling in pregnancy. Additionally, while we did not note any changes to hepatic AKT phosphorylation (a downstream effector of insulin) in adiponectin KO mice, studies suggest that adiponectin may exert downstream effects in an AMPK mediated manner (T. Yamauchi et al., 2002).

Adiponectin is also thought to suppress lipogenesis by inhibiting *Srebp1c* via AMPK (Awazawa et al., 2009). Discordant results were shown by one study in which non-pregnant chow-fed adiponectin KO mice had marked decreases in hepatic lipogenic gene expression, and reduced hepatic fat accumulation (Q. Liu et al., 2012). These results conflict with mechanistic studies that implicate adiponectin signalling in the suppression of hepatic lipogenesis (Awazawa et al., 2009; Terry P Combs et al., 2004; Pettinelli & Videla, 2011; Rogers, Ajmo, & You, 2008; Ruderman et al., 2003; A. Xu et al., 2003). Qiao *et al* reported increased expression of genes involved in hepatic triglyceride synthesis in pregnant adiponectin KO mice (Liping Qiao et al., 2017). Other studies have reported conflicting observations, with Qiao *et al* showing no changes to hepatic AMPK phosphorylation in pregnant adiponectin KO mice on a chow diet, and Guo *et al* showing impaired phosphorylation only with the consumption of a HFS diet in non-pregnant adiponectin KO mice (R. Guo et al., 2013; Liping Qiao et al., 2017). Thus, determining whether AMPK phosphorylation is impacted downstream of adiponectin in the livers of our pregnant adiponectin KO mice could clarify the mechanism by which a lack of adiponectin leads to increased lipid deposition in the liver in the third trimester.

We did not observe any significant effect of adiponectin deficiency on litter size or pup size. In the future, we aim to investigate the effect of adiponectin deficiency and supplementation on the offspring. As discussed in Chapter 7, dysregulated adipokines in pregnancy can impact growth and metabolic health later in life (Lekva et al., 2017; Paulsen et al., 2019). Therefor, we aim to determine the impact of exposure to adiponectin deficiency in pregnancy on the offspring, and the combined effect of HFS-diet feeding on growth, glucose tolerance and insulin resistance through to adulthood. This will also allow us the ability to better isolate any sex-specific differences.

A major finding of this study was the development of hepatic steatosis in pregnant adiponectin KO mice, even in the absence of obesity. We determined that this may be due in part to increases in *de novo* lipogenesis, however the effect of adiponectin deficiency on hepatic lipid uptake, synthesis and secretion may require further investigation. Since oleate uptake was reduced in the third trimester in adiponectin KO mice, it may be illuminating to quantify fatty-acid transport proteins and fatty acid binding proteins that are necessary for long-chain fatty acid uptake, such as CD36 and members of the FABP family. Synthesis and secretion of cholesterol esters in the livers of adiponectin KO mice was significantly reduced relative to non-pregnant mice. Additionally, while we measured serum cholesterol, we did not differentiate between HDL, LDL or VDLD species, which could provide a more complete picture. VLDLs in particular are important for secretion of hepatic triglycerides. Apolipoprotein B100 (ApoB100) is a component of lipoproteins and a rate-determining step in VLDL formation (Charlton, Sreekumar, Rasmussen, Lindor, & Nair, 2002), and as such decreased synthesis of ApoB100 may increase NAFLD risk and can be affected by lipid-mediated ER-stress (Ota, Gayet, & Ginsberg, 2008). Measuring hepatic ApoB100 may be informative for interpreting hepatic lipid secretion results, particularly as pertains to cholesterol. There were significant differences between hepatic synthesis and secretion of lipids in adiponectin KO mice in the third trimester relative to non-pregnant mice, but adiponectin KO mice were not hyperlipidemic relative to WT mice in the third trimester, despite the fact that increased serum lipids are common in the third trimester (Mazurkiewicz et al., 1994). In order to determine whether serum lipids are increased in pregnancy to a greater degree in adiponectin KO mice, it would be beneficial to measure serum lipids (triglycerides and FFA) in non-pregnant mice for reference.

When we measured mitochondrial function of primary hepatocytes by OCR, we found that pregnant adiponectin KO mice had reduced maximal oxidative capacity when using glucose and overall oxidative capacity was impaired during fatty acid oxidation. Based on levels of citrate synthase, mitochondrial content appears to be unchanged however more direct observations of mitochondrial content and structure may reveal diet or genotype mediated differences. Adiponectin is postulated to play a role in mitochondrial function leading to dysregulation of OXPHOS proteins (M. E. Pepin et al., 2019) and in cardiac tissue lacking adiponectin receptors, mitochondrial ROS production was increased (Koentges et al., 2015). Increased hepatic ROS can lead to worsening insulin resistance, and potentially using dye-based tools such as MitoSOX in primary hepatocytes could determine the effect of adiponectin deficiency in pregnancy on the build up of ROS. It would be important to quantify proteins in the OXPHOS family, given the changes observed to oxidative capacity, to pinpoint potential defects in the hepatic electron transport chain in adiponectin KO mice.

We aimed to investigate whether supplementation with adiponectin could improve the GDM phenotype in adiponectin KO mice in the third trimester. These experiments showed that even relatively small increases to circulating adiponectin resulted in improved glucose tolerance, decreased hepatic glucose output and improvements to hepatic steatosis. One limitation of these analyses was the low sample numbers, limiting the capacity for statistical analysis. We have endeavored to increase the number of samples per group, repeating our histological analyses in order determine with more certainty the impact of adiponectin supplementation on hepatic steatosis development in the third trimester. It would also be beneficial to determine the effect of adiponectin supplementation on mitochondrial respiration in primary hepatocytes, specifically whether restoring adiponectin signalling could improve fatty acid oxidation in the third trimester in adiponectin KO mice. With respect to the modest increases in serum adiponectin following supplementation, we considered the possibility of hepatic sequestering of adiponectin leading to paracrine adiponectin signalling and improved hepatic metabolic function in pregnancy. It would be informative to confirm whether adiponectin delivered by tail-vein injection localizes in the liver. Liver tissue samples from adiponectin supplemented mice have been fixed in 4% PFA and embedded in OCT which would allow the visualization of adiponectin using immunofluorescence.

More experiments would be beneficial to further solidify the impact of adiponectin supplementation on pregnant adiponectin KO mice. For instance, western blots could be performed on targets that exhibit differential regulation in adiponectin KO mice without supplementation. These include ACC phosphorylation, FASN and gluconeogenic targets to determine if gene expression changes translate to changes in enzyme levels. In addition, it would also be informative to measure GSIS and islet area on pancreas from dams administered the adiponectin adenovirus to determine whether increasing adiponectin restores β-cell area in pregnant adiponectin KO mice (Qiao et al., 2017a). In particular, we could determine whether adiponectin supplementation could ameliorate the additive effects of HFS diet consumption on islet function in the third trimester of adiponectin KO mice, given the protective role of adiponectin in the β-cell.

In chapter 6 we showed that adiponectin KO mice have impaired GSIS and increased glucagon secretion in the third trimester relative to WT mice. Morphological analyses point to impairments in structural adaptation to

pregnancy in adiponectin KO mice fed a HFS diet, but additional samples are required for adequate statistical comparison. Since the pancreatic islet undergoes significant morphological changes during gestation, we aim to compare third trimester pregnant to non-pregnant mice and evaluate the effect of adiponectin deficiency, and the additive effect of HFS diet consumption. To this end, more non-pregnant samples are required for sufficient statistical power and comparison. Part of the adaptive mechanism to pregnancy involves decreased apoptosis and increased proliferation of β-cells; quantifying markers of cell death and proliferation can provide a more complete picture of the adaptive capacity of adiponectin KO mice in pregnancy, so we aim to measure Ki-67 (a marker of proliferation (W. Yang et al., 2020)) and caspase (a marker of cell death (Tomita, 2010)) in the same pancreatic sections used to quantify insulin and glucagon. Adiponectin may exert its effects on the β-cell by preventing apoptosis particularly when it is triggered by lipotoxicity. In this study we did not determine whether adiponectin KO mice had increased apoptosis in islets and whether there were changes in β-cell neogenesis or hyperplasia with exposure to HFS diet feeding during pregnancy. There are a handful of studies examining the effect of adiponectin on islet morphology. However, very few studies specifically look at the impact of adiponectin on the adaptation to pregnancy and the combinatorial effects of HFS diet feeding and adiponectin deficiency on primary islets has rarely been examined. In mouse models and in INS-1 cells, adiponectin may promote survival in isolated pancreatic β-cells under lipotoxic conditions increasing proliferation and preventing apoptosis (Rakatzi et al., 2004; Ye et al., 2014; Ye et al., 2015).

While we observed impairments to KCL-stimulated insulin release and glucose-stimulated insulin secretion in adiponectin KO mice, gene expression analysis would help pinpoint the location of the actual defects, for example whether there are impairments to glucose uptake or ATP synthesis in the β-cell. Adiponectin also increases ERK activation in the β-cell promoting proliferation and preventing apoptosis, a pathway that has been implicated as an adaptive mechanism in response to pregnancy (Gupta et al., 2007; Rieck et al., 2009; Wijesekara et al., 2010; Ye et al., 2015). To this end, we have been collecting mRNA from pancreatic islets isolated at the end of the third trimester with the ultimate goal of performing RNAseq. There is some research to suggest that adiponectin may improve beta-cell function in pregnancy (Liping Qiao et al., 2017), but little evidence showing the effect of adiponectin on the α-cell. Determining whether adiponectin receptors are present in the α-cell would be a novel finding. Further, we could elucidate the effect of adiponectin administration on the secretory capacity of pancreatic islets from adiponectin KO mice (with and without HFS-diet induced obesity) relative to WT controls *in vitro*, to clarify the role of adiponectin in the adaptation of islet to pregnancy.

Analysis of maternal serum and cord blood form pair-matched mothers and babies in a First Nations cohort of diabetes in pregnancy. In this cohort we found that mothers with GDM had a distinct adipokine profile relative to both T2D and control pregnancies. One limitation of this study was the relatively low number of control pregnancies without any diabetes in pregnancy. Increasing the number of control samples could increase the interpretive capacity for correlations observed in this cohort. Additionally, the majority of mothers in this study were obese or overweight and increased recruitment of mothers with a BMI below 26kg/m^2 could help to control for the effect of obesity on adipokine levels in pregnancy and cord blood. While we knew that mothers with diabetes in pregnancy (GDM or T2D) received interventions ranging from diet and lifestyle management to pharmacological therapies. Accessing and

including this data, thereby being able to account for therapeutic interventions and their potential confounding factors would improve analysis. One complication of pregnancies affected by diabetes is LGA infants; our interpretation of this outcome is limited by the absence of neonatal sex, which determines the curve on which birthweight is measured. In fact, BMI Z-score is the more common measure for infant size and this measure may better account for variability in our cohort and provide greater insight to the correlation between maternal diabetes status, serum adipokines and infant growth.

In summary, the research outlined in this thesis supports the overarching hypothesis that adiponectin deficiency contributes to GDM (Figure 24). Taken together, these results suggest an important role for adiponectin signalling in the development of fatty liver, and the subsequent predisposition towards GDM in the third trimester. The defects in hepatic metabolism and glucose intolerance develop in pregnant adiponectin KO mice in the absence of obesity. Our study shows that supplementation with adiponectin improves the fatty liver phenotype and suppresses hepatic glucose output correcting hyperglycemia in the third trimester. Although supplementation with adiponectin has been previously shown to improve metabolic adaptation to pregnancy in chow fed mice (Liping Qiao et al., 2017), ours is the first study to show the additive effect of HFS diet feeding on adiponectin KO mice in pregnancy as well as alterations in lipid synthesis and the impairment in mitochondrial respiratory capacity in primary hepatocytes. While Qiao *et al* reported some impairments to compensatory β-cell mass in adiponectin KO mice, ours is the first study to measure glucose stimulated insulin secretion from islets of pregnant adiponectin KO mice as well as report alterations in α-cell structure and function (Liping Qiao et al., 2017). Results from the Next Generation Cohort make it clear that with respect to adipokines, GDM is a condition distinct from T2D in pregnancy with distinct implications for maternal metabolism and neonatal growth. Since intrauterine exposure to diabetes induces maladaptive developmental programming, it is imperative to understand mechanisms that contribute to the development of GDM in order to provide better therapeutic and preventative options.

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