Influence of Gestational Diabetes on the Programming of Metabolic Health Outcomes in Offspring

by

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ABSTRACT

Population health data suggests that the development of metabolic disease is influenced by early life events. Gestational diabetes (GDM) is a common complication of pregnancy, but its effects on the offspring are poorly understood. It is hypothesized that a diet high in fat and sucrose will cause excessive weight gain and obesity during pregnancy accompanied by hyperglycemia and hyperinsulinemia that are characteristic of GDM. It is also hypothesized that gestational exposure to GDM will cause obesity, hepatic steatosis and insulin resistance in the offspring when compared to the offspring from metabolically healthy, lean mothers. To test these hypotheses, female Sprague-Dawley rats were fed a high fat (45%) and sucrose (HFS) diet prior to mating in order to cause pre-pregnancy weight gain and glucose intolerance which is manifested as GDM during pregnancy. Lean control pregnant rats received low fat (LF; 10%) diets prior to and during pregnancy. The offspring were weaned at 3 weeks of age and randomly assigned to LF or HFS diets for 12 weeks and analyzed for insulin sensitivity and hepatic steatosis. Metabolomic analyses by mass spectroscopy as well as standard biochemical analyses were performed on liver samples from the offspring in order to assess the fetal programming effects of maternal GDM on hepatic metabolite levels in the offspring.

In support of the suggested hypotheses, the HFS diet caused excessive gestational weight gain, hyperinsulinemia and mild hyperglycemia in pregnant rats, which is characteristic of GDM. The young adult offspring of GDM dams gained more weight than offspring of lean dams. More specifically, offspring born to GDM dams and fed a postnatal low fat diet displayed similar weight gain patterns to offspring born from lean dams and fed a postnatal

high fat diet. This suggests that prenatal environment plays a crucial role in programming weight gain regardless of postnatal diet. This was accompanied by hepatic steatosis and *in vivo* insulin resistance compared to the offspring of lean dams. Metabolomic analysis showed a 10-fold increase in levels of the lipotoxic lipid, ceramide, in offspring from GDM dams, regardless of postnatal dietary condition. Increased expression of acetyl-CoA carboxylase-2 (ACC-2) and reduced expression of peroxisomal proliferator activated receptor-α and insulin receptor-β were also observed in the livers of the young adult offspring of GDM dams. These molecular alterations could be factors responsible for the gestational programming effects of GDM on hepatic steatosis and insulin resistance. GDM was shown to enhance the development of obesity, hepatic steatosis and insulin resistance in the offspring.

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"Knowledge speaks, but wisdom listens"

-Jimi Hendrix

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LIST OF ABBREVIATIONS

HFS	High Fat and Sucrose
LF	Low Fat
CoA	Coenzyme A
ACC-1	Acetyl-CoA-Carboxylase-1
ACC-2	Acetyl-CoA-Carboxylase-2
MetS	Metabolic Syndrome
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
GDM	Gestational Diabetes Mellitus
ATP	Adenosine Triphosphate
ETC	Electron Transport Chain
TCA	Tricarboxylic Acid
NADH	Nicotinamide Adenine Dinucleotide
FADH2	Flavin Adenine Dinucleotide
GLUT2	Glucose Transporter-2
GLUT4	Glucose Transporter-4
IR	Insulin Receptor
IRS1	Insulin Receptor Substrate-1
IRS2	Insulin Receptor Substrate-2
PI3K	Phosphotidylinositol-3-Kinase
PIP3	Phosphotidylinositol-3,4,5-Triphosphate

AKT-2	Serine-Threonine Protein Kinase-2
OGTT	Oral Glucose Tolerance Test
BMI	Body Mass Index
DNA	Deoxyribonucleic Acid
LEP	Leptin (Gene)
LEP-R	Leptin Receptor (Gene)
FTO	Fat Mass & Obesity Related (Gene)
MC4R	Melanocortin-4-Receptor (Gene)
TMEM18	Transmembrane Protein 18 (Gene)
ADIPOQ	Adiponectin (Gene)
CVD	Cardiovascular Disease
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
NAFLD	Non-Alcoholic Fatty Liver Disease
STZ	Streptozotocin
PGC1α	Peroxisome Proliferator-Activated Receptor Gamma Co-activator 1-Alpha
PCK1	Phosphoenol Pyruvate Carboxykinase-1
ROS	Reactive Oxygen Species
GI	Gastrointestinal
G6P	Glucose-6-Phosphate
G6PC	Glucose-6-Phosphatase
GCK	Glucokinase

TAG/TG	Triacylglycerol
DAG	Diacylglycerol
DGAT	Diglyceride Acyltransferase
CPT-1	Carnitine Palmitoyltransferase
FFA	Free Fatty Acid
PI	Phosphatidylinositol
TNFα	Tumor Necrosis Factor Alpha
IL-1β	Interleukin-1-Beta
HOMA-IR	Homeostatic Model Assessment-Insulin Resistance
SREBP-1c	Sterol Regulatory Element-Binding Protein-1-C
LC/MS	Liquid Crystallography Tandem Mass Spectroscopy
ASA	American Diabetes Association
JNC	Joint National Committee
NHANES	National Health and Nutrition Examination Survey
DECODE	Diabetes Epidemiology : Collaborative Analysis of Diagnostic Criteria in Europe
IDF	International Diabetes Federation
DIAGRAM	Diabetes Genetics Replication and Meta-analysis
HAPO	Hyperglycemia and Adverse Pregnancy Outcomes
ITT	Insulin Tolerance Test
GTT	Glucose Tolerance Test

CHAPTER 1 - INTRODUCTION

1.1 Nutrient Homeostasis

Glucose and fats are the major sources of energy that fuel the cells of the body. Following a meal, food is broken down into glucose and fats that are absorbed by the gastrointestinal tract and transported to the tissues via the bloodstream. The body's homeostatic mechanisms that regulate circulating glucose and fat levels is highly regulated. Dysregulation of this system is observed in metabolic disorders such as the metabolic syndrome and diabetes.

1.1.2 The Endocrine Pancreas

The pancreas is both an exocrine and endocrine organ that lies below the stomach and liver in the human body (Gittes, 2009). As an exocrine organ it functions to aid in digestion, secreting digestive enzymes from acinar cells into the bile duct leading to the duodenum (Weiss *et al.*, 2008). The endocrine portion of the pancreas is made up of many cellular clusters called the Islets of Langerhans. These islets contain four distinct cell types that secrete hormones into the circulation. The α-cells secrete glucagon, β-cells secrete insulin, Δ-cells secrete somatostatin, and γ-cells secrete pancreatic polypeptide (Nadal *et al.*, 1999; Gittes, 2009).

All islet cells play a role in the regulation of glucose homeostasis, but the α and β-cells are the most prominent cell types involved in maintaining energy homeostasis through the fine interplay of glucagon and insulin release (Quesada *et al.*, 2006). Glucagon is secreted between meals (during times of fasting) and stimulates glycogenolysis by the

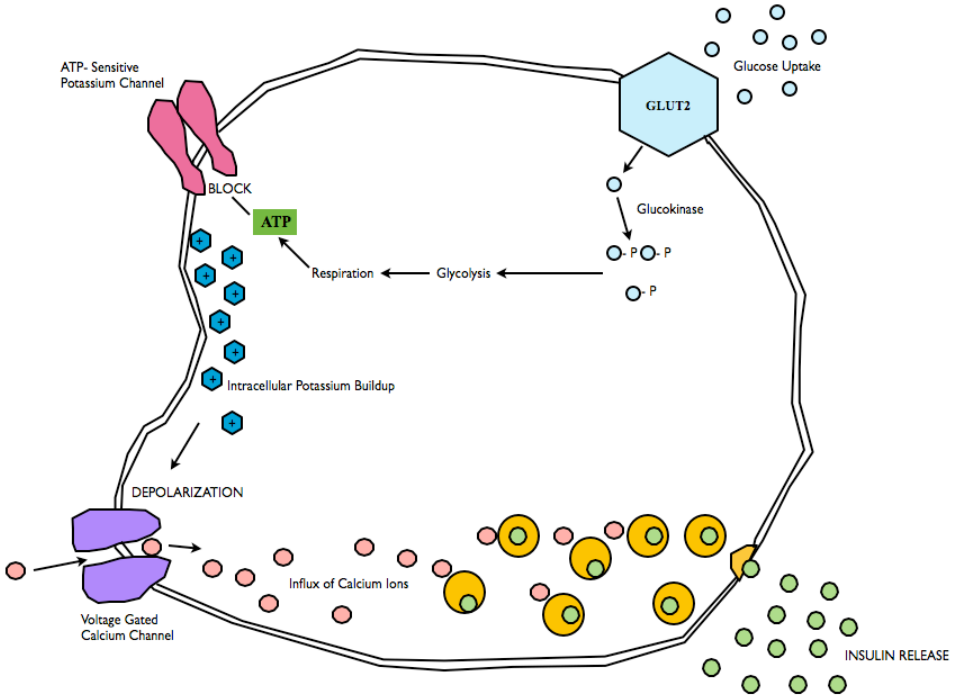
liver to produce glucose from stored glycogen for excretion into the circulation, thereby maintaining normal blood glucose levels. When glycogen stores are depleted, glucagon can also stimulate gluconeogenesis. Insulin acts in opposition to glucagon and is secreted by the β-cells following a meal and promotes the uptake of glucose from circulation into peripheral tissues (Vander Heiden et al., 2009; Bertram *et al*., 2006; Huttermann *et al.*, 2011).

1.1.3 Insulin & Insulin Signaling

Following a meal, β-cells sense the rise in circulating blood glucose through diffusing of glucose through glucose transporter-2 (GLUT2) and release insulin to coordinate the uptake of glucose into insulin-sensitive peripheral tissues, such as skeletal muscle, adipose tissue, reproductive tissue and the liver (Keane & Newsholme, 2014).

The pathways of glucose-stimulated insulin secretion have been well studied and occur as follows: Glucose enters the β-cell through the GLUT2 transporter where it is broken down via glycolysis. Glycolytic intermediates enter the TCA cycle and the electron transport chain (ETC) in the mitochondria where adenosine triphosphate (ATP) is produced. High levels of ATP within the β-cell causes a blockade in the ATP-sensitive potassium channel. The buildup of potassium within the β-cell causes a depolarization and opening of a voltage gated calcium channel. When calcium ions flood the β-cell insulin-containing vesicles are triggered to secrete insulin into circulation.(Kahn, 1994; Newsholme *et al.*, 2010; Ashcroft & Rorsman, 2012). (See Figure 1.1)

Figure 1.1 Pancreatic β-Cell Insulin Release

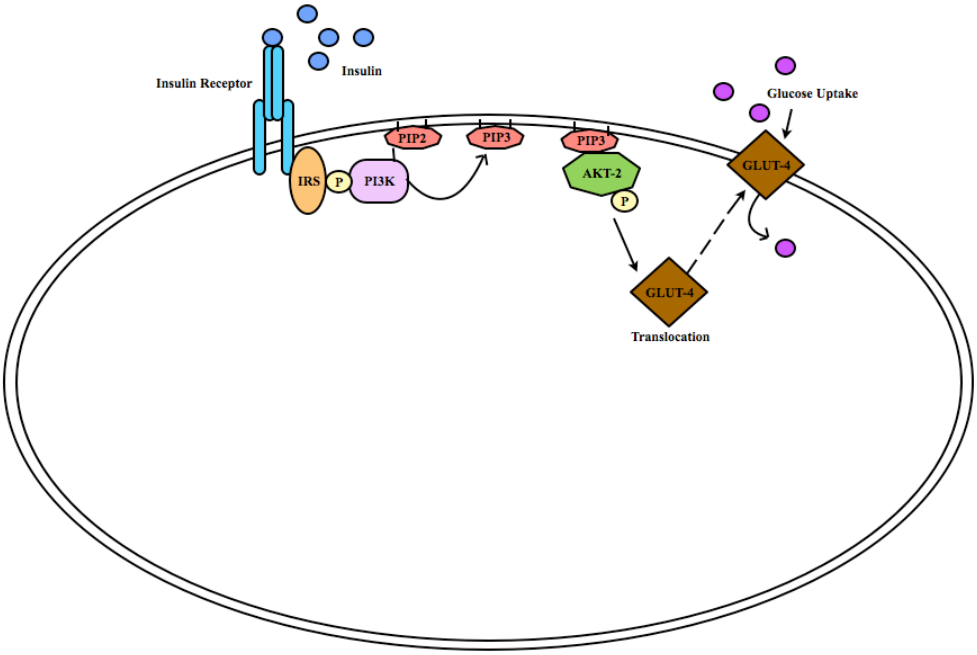


Glucose enters the pancreatic β-cell through the glucose transporter (GLUT2) and is broken down via glycolysis. Subsequently, ATP is produced through the electron transport chain (ETC). ATP acts to block the ATP- sensitive potassium channel. Intracellular buildup of potassium causes a depolarization event and a rapid influx of calcium ions into the cell. This calcium influx causes insulin to be released from storage granules into circulation.

Insulin signaling is highly complex and coordinated and involves many proteins and steps that results in the uptake of glucose into peripheral tissues from circulation. Insulin first acts by binding the insulin receptor (IR), an autophosphorylating tyrosine kinase receptor, which is made up of two α subunits, which remain extracellular, and two β subunits, which are transmembrane in nature (Cohen, 2006; Taniguchi *et al*., 2006; Prudente *et al.*, 2009). Insulin binding causes a phosphorylation event on a tyrosine residue of the β subunit and activates the receptor, which stimulates its tyrosine kinase activity and allows the phosphorylation of specific insulin receptor substrate (IRS) proteins (Keller & Lienhard, 1994; White, 2002; Cohen *et al*., 2006; Taniguchi *et al.*, 2006). The IRS proteins (IRS-1, 2, 3, 4) dock to the IR, become phosphorylated and mediate downstream insulin signaling through the activation of phosphotidylinositol-3-kinase (PI3K), subsequent production of phosphotidylinositol-3,4,5-triphosphate (PIP3) and the activation of the serine-threonine protein kinase, AKT-2 (Keller & Lienhard,1994; White, 2002; Chang *et al*., 2004). AKT-2 then acts to directly mediate the translocation of glucose transporter-4 (GLUT4) to the membrane where it can act to uptake glucose from circulation (Kahn, 1994; Chang *et al.*,2004) (See Figure 1.2).

Aside from the role of insulin in glucose uptake into peripheral tissues, it also possesses many other important roles in metabolic regulation. Insulin decreases the formation of glucose from pyruvate through gluconeogenesis in hepatic tissue. Insulin also promotes glycogen storage and fatty acid synthesis in hepatic tissue. (Blake & Trounce, 2014; Saltiel & Kahn, 2001)

Figure 1.2 Insulin Signaling Pathway



Insulin acts by binding the insulin receptor (IR), an autophosphorylating tyrosine kinase receptor, which subsequently recruits and phosphorylates insulin receptor substrate (IRS) proteins. Downstream insulin signaling occurs through phosphatidylinositol-3-kinase (PI3K) activation, production of phosphotidylinositol-3,4,5-triphosphate (PIP3), and activation of serine-threonine protein kinase (AKT-2), which acts to mediate glucose transporter (GLUT4) translocation to the membrane for glucose uptake.

The disruption of these processes is an accepted cause of chronic metabolic disease development, seen especially in type 2 diabetes (T2D) and gestational diabetes mellitus (GDM). (White, 2002) Insulin resistance can be explained, in part, by the development of visceral obesity, dyslipidemia, hepatic steatosis and lipotoxicity associated with the MetS. (Bikman *et al*., 2011)

1.1.4 Mitochondrial Function

Mitochondria are complex membrane-bound organelles found within eukaryotic cells, more commonly know as the "energy powerhouse of the cell". Mitochondria perform biochemical reactions to breakdown carbohydrates and fatty acids and utilize these substrates for ATP production (McBride *et al*., 2006; Schatz, 2007; Davis & Williams, 2012). Structurally, mitochondria contain two distinct membranes, therefore creating four separate and distinct portions, the outer membrane, inter-membrane space, inner membrane and the matrix (McBride *et al*., 2006; Schatz, 2007; Davis & Williams, 2012). The inner membrane is home to the ETC and ATP synthase, which are central in energy production.

Glucose is broken down via glycolysis, yielding pyruvate and ATP. Oxidation and reduction reactions act to degrade fatty acids as well as pyruvate from glycolysis producing intermediates which enter the tricarboxylic acid (TCA) cycle. Electron carrier coenzymes NADH and $FADH_2$ are generated by oxidation-reduction reactions as well as in the TCA cycle and are subsequently oxidized by the ETC. As a result, ATP is

generated through a process that is termed oxidative phosphorylation (Papa *et al*., 2012). As described in section 1.1.4, mitochondria in the pancreatic β-cell controls insulin secretion via ATP production.

The break down of fatty acids by the mitochondria occurs via a process known as β-oxidation. In the liver, β-oxidation of fatty acids can yield ketone bodies that may be released into the circulation allowing their utilization by extrahepatic tissues as an energy source during fasting periods (Rui, 2014). Carnitine palmitoyltransferase-1 (CPT-1) is required for fatty acid translocation into mitochondria and β-oxidation. Malonyl-CoA is produced by acetyl-CoA carboxylase-2 (ACC2) in mitochondria and acts to inhibit CPT-1 and subsequent β-oxidation (Abu-Elheiga *et al.*, 2000). Peroxisome proliferator-activated receptor-α (PPARα) is the chief regulator of β-oxidation within mitochondria. Increased expression of PPARα actually acts to boost fatty acid oxidation (Kersten *et al*., 1999). PPARgamma co-activator-1 (PGC1α) is also of interest as it has a role in modulating mitochondrial biogenesis.

Perturbations in any of these regulatory genes can interfere with oxidative metabolism and may play a causal role in the development of fatty liver, insulin resistance and subsequent T2D (ie-Metabolic Syndome (MetS)). Recent studies demonstrate that a reduction in mitochondrial respiration is associated with both insulin resistance and fatty liver development in humans (Peterside et al., 2003; Selak et al., 2003; Petersen *et al*., 2004). Furthermore, a reduction in mitochondrial biogenesis and decrease in total

mitochondrial copy number, respiratory enzymes, oxidative phosphorylation and fatty acid oxidation has been shown in various rodent models of obesity (Lenzen *et al*., 1996; Jonas *et al*., 1999; Szendroedi et al., 2012). The accumulation of toxic lipid intermediates, such as diacylglycerol, sphingolipids and ceramides is often observed when there is a defect in oxidative phosphorylation (See chapter 3.1). Dysfunctional oxidative phosphorylation is also associated with increased reactive oxygen species (ROS) production within many metabolic tissues (Sakai *et al.*, 2003; Sorriento *et al.*, 2014). This plethora of disturbances in the metabolic function of mitochondria is believed to predispose organisms to metabolic disease development.

1.1.5 Glucose Handling

The Fed-State:

Upon ingestion of a meal, complex carbohydrates are broken down in the gastrointestinal (GI) tract by digestive enzymes into glucose. Glucose enters circulation, causing the release of insulin from pancreatic β-cells, and reaches the liver through the portal vein circulatory system. Post-prandial elevations in blood glucose result in glucose uptake by the liver, where it undergoes conversion into glycogen via UDP-glucose-glycogen glucosyltransferase (glycogen synthase). Glycogen is a polysaccharide of glucose that is highly branched and serves as a long-term energy store that can be later used during fasting periods (Cohen et al., 1978; Radziuk & Pie, 2001).

The Fasted-State:

During short fasting periods (overnight and between meals), the liver breaks down glycogen and releases glucose into circulation with stimulation from glucagon where it enters other extra-hepatic tissues to be utilized for energy. This process is known as glycogenolysis. PEPCK (phosphoenolpyruvate carboxykinase) is the rate limiting enzyme in the gluconeogenic pathway (Beale *et al*., 2007). Another enzyme of interest in this pathway is glucose-6-phosphatase (G6PC), which acts to hydrolyze glucose-6-phosphate to free glucose molecules which can then exit the liver and enter circulation, thereby elevating blood glucose and maintaining homeostasis (Ghosh *et al*., 2002; Radziuk & Pie, 2001).

The process of *de novo* lipogenesis (See section 1.1.6) is also observed postprandially in the liver where glucose can be converted to fatty acids which can then serve to form triacylglycerol (TAG), another form of energy storage (Rui, 2014). During prolonged fasting periods, both glucose and TAG are released from the liver back into circulation where they may be utilized by extra-hepatic tissue for energy production. Glucose also enters the glycolytic pathway where it is initially phosphorylated by glucokinase (GCK) to form glucose-6-phosphate (G6P). The glycolytic pathway ends with the formation of pyruvate, which serves as a substrate for ATP production via the TCA cycle and oxidative phosphorylation within mitochondria (Wang *et al.*, 2013; Massa *et al.*, 2011; Mithieux, 1996). Pyruvate also serves as a substrate for *de novo* lipogenesis.

1.1.6 Lipid Handling

Dietary fats are ingested and absorbed in the GI tract by enterocytes in the small intestine. Within these enterocytes fatty acids are converted to TAG, which is secreted as a chylomicron particle into the lymphatic system of the gut (Morgantini *et al*., 2014; Randolph & Miller, 2014). Chylomicrons are lipoproteins containing TAGs as well as cholesterol and function to transport lipids from the GI tract to the rest of the body (Hussain, 2000).

During periods of fasting, the liver receives fatty acids that have been released by adipocytes into circulation and these fatty acids are generally oxidized by mitochondria for the production of ATP and ketone bodies. Ketone bodies can also be utilized as an energy source during periods of fasting or when carbohydrate abundance is low. The liver is also responsible for repackaging fatty acids into lipoproteins, which act to shuttle lipids to other tissues (Morgantini *et al*., 2014; Hussain, 2014; Randolph & Miller, 2014).

Ingesting large amounts of dietary carbohydrates, however, leads to a high concentration of circulating glucose and under these conditions the liver has the capability to not only utilize glucose directly as an energy substrate, but to also convert glucose to fatty acids through a process known as *de novo* lipogenesis. Over-production of fatty acids that exceed amounts needed for energy production or even phospholipid synthesis will cause an increase in TAG synthesis within the tissue. Hepatocytes utilize the glycerol-3-phosphate shuttle primarily for *de novo* TAG synthesis. This increase in *de novo*

lipogenesis and storage of TAG within the liver associated with the intake of high amounts of carbohydrates and fats which can disrupt the normal function in the insulin signaling cascade and lead to the development of liver steatosis, non-alcoholic fatty liver disease (NAFLD) and insulin resistance (Hellerstein, 1999; Acheson et al., 1988; McDevitt et al., 2001).

1.2 Obesity

Obesity can be defined as the accumulation of excess fat tissue at a level which becomes harmful to the body. When daily caloric intake exceeds caloric expenditure, individuals are left with a positive energy balance that leads to weight gain and consequently obesity (Kearney, 2010). Body Mass Index (BMI) is a measure commonly used in diagnosing obesity. BMI's above 30kg/m2 are deemed obese (Finucane *et al*., 2011; Misra & Khurana 2008).

Throughout the past several decades, the rates of obesity have climbed at a steady and significant pace and have reached epidemic levels in both the developed and developing world. In fact, between 1980 and 2008 the prevalence of obesity globally rose from 4.8 % to 9.8% in men and from 7.9% to 13.8% in women (Flegal *et al*., 2012). There is an estimated 500 million adults living with obesity according to 2008 statistics. North America accounts for the highest number of obese adults with over 30% of the population living with obesity (Finucane *et al*., 2011). Obesity has a large burden on the health care system as well, with approximately $147 billion in medical costs in the United States in 2008 alone (Finkelstein *et al*., 2009).

Aside from the adult obesity epidemic that has gripped the world, there has also been a frightening and significant increase in the prevalence of childhood onset obesity. From 1990 to 2010, the worldwide rates of childhood obesity have increased from 4.2% to 6.7% accounting for approximately 43 million children. This number is expected to

increase to roughly 60 million children by the year 2020 if current trends are not dealt with and reversed (de Onis *et al.*, 2010).

There are several factors that play a causal role in the development of obesity. Among these are environmental factors such as diet and lifestyle as well as genetics and epigenetics (Fuster, 2010; van Dijk *et al*., 2014). Regarding diet and lifestyle, there has been a globalization of a "western-style" diet due to reduced poverty across the world. "western-style" diets are defined as being calorie-dense and high in both unhealthy saturated fats and simple sugars (Halton *et al*., 2006; Odermatt, 2011; Pan *et al*., 2012). The widespread use of automated technologies has resulted in a shift from classically labor intensive work to sedentary employment and social environments that also contribute to the significant increase in obesity rates (Fuster, 2010). Over the past half century, levels of physical activity related to work and transportation significantly declined in the United States even though levels of activity associated with leisure have remained steady (Brownson *et al*., 2005).

Beyond the environmental factors described above, genetics and epigenetics also contribute to the increased prevalence, susceptibility and heritability of obesity. Genetic variations in the deoxyribonucleic acid (DNA) sequence have been studied extensively in populations with a high rate of obesity in an attempt to explain the genetic basis for obesity. Genome-wide association studies have revealed approximately 40 genetic loci that have been implicated in obesity and the regulation of BMI. Such genes include, LEP

(leptin), LEPR (leptin receptor), FTO (fat mass and obesity related), MC4R (melanocortin 4 receptor), TMEM18 (transmembrane protein 18) and ADIPOQ (adiponectin) (Graff *et al*., 2013; Mitchell *et al*., 2013; Pei *et al*., 2014). Polymorphisms in these genes, while linked with obesity and energy homeostasis, are very rare and not widely observed in the general population. Thus, it is unlikely, that these genetic mutations can explain the rapid increase in the obesity epidemic across the globe.

Epigenetic modifications to the genome have come to the forefront of the scientific community as the potential underlying cause to many chronic diseases. Epigenetics is an area within the field of genetics that studies the changes in gene activity that are heritable but not a result of modifications to the genetic sequence itself (DeWoskin & Million, 2013). Variations in DNA methylation patterns and/or histone modifications act to turn on or alternatively silence gene expression dependent on exposure to various environments (Schwenk *et al*., 2013; DeWoskin & Million, 2013). Recently, it became evident that epigenetic changes during crucial developmental periods (both in-utero and early postnatal) can also predispose children to a metabolic phenotype more susceptible to obesity and the development of chronic diseases such as T2D and cardiovascular disease (CVD) in adulthood (Schwenk *et al.*, 2013).

With an increase in obesity rates amongst both adults and children, there is a paralleled increase in chronic disease development related to metabolic disturbances. The accumulation of adipose tissue and development of obesity and its related pathologies,

such as hepatic steatosis, have been shown to induce insulin resistance that eventually leads to T2D and cardiovascular disease (CVD) complications (Danaei *et al.*, 2009; Whitlock *et al.*, 2009). In children, it has been shown that obesity increases the likelihood of chronic disease development much earlier in life (Singh *et al.*, 2008).

Abdominal or visceral obesity is seen as the core of the Metabolic Syndrome (MetS) and is causal in the development of the other related conditions (Canale *et al.*, 2013). Adipose tissue is not an inert entity but plays a crucial role in energy metabolism through the synthesis and secretion of bioactive hormones, such as leptin and adiponectin (Antuna-Puente *et al.*, 2008). In the case of increased adiposity and obesity, this regulatory process is interrupted which leads to further risk factor development and eventual chronic disease (Slanovic-Kuzmanovic *et al.*, 2013).

1.3 Metabolic Syndrome (MetS)

MetS is a collection of conditions, which, together, effect how the body stores and utilizes energy. The MetS was first described by Reaven in 1988 as the association of a cluster of different conditions that led to the development of T2D, CVD and eventually premature death. (Reaven, 1988; Qiao *et al.*, 2007) Obesity is a major driving force behind the MetS but other factors are also important, including glucose intolerance, insulin resistance, hypertension and dyslipidemia (Canale *et al*., 2013).

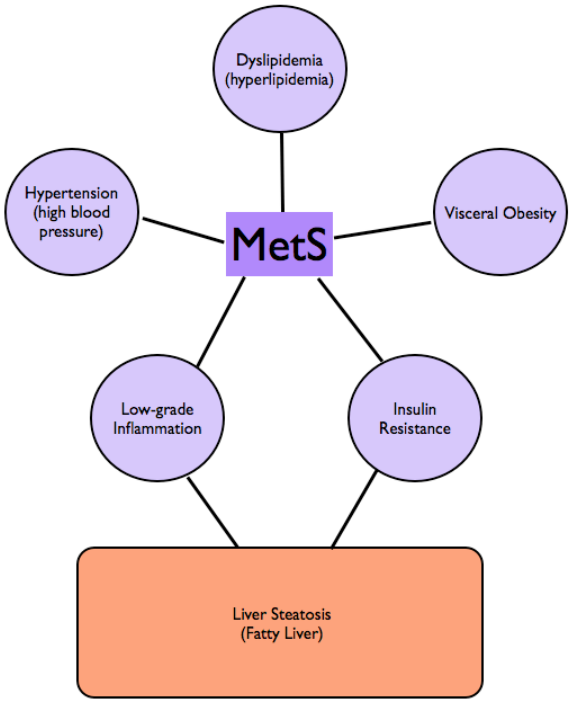
Glucose intolerance, as defined by the American Diabetes Association, is a state of decreased glucose uptake into peripheral tissues leading to elevated circulating glucose levels. After a two hour 75g oral glucose tolerance test (OGTT), individuals with plasma blood glucose levels between 7.8-11.0 mmol/L are deemed glucose intolerant (American Diabetes Association, 2005). Insulin resistance is a condition in which the actions of insulin cease to have a physiological effect on tissues and cells of the body. When insulin signaling fails in peripheral tissues, a state of hyperglycemia occurs (International Diabetes Federation: Diabetes Atlas 6th Edition). Hypertension, also known as high blood pressure, as defined by the the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC), is when subsequent readings of diastolic blood pressure are above 90mm Hg or when systolic blood pressure readings are consistently above 140mm Hg (JNC VI, 1997). Hypertension is highly associated with CVD development. Dyslipidemia is a condition where there is an abnormal amount of circulating lipids (Fredrickson & Lees, 1965). Due to changes in lifestyle and diets that

are more obesogenic in nature, dyslipidemia's are usually hyperlipidemic displaying an increase in circulating lipids (Chait & Brunzell, 1990). The National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) reported that individuals with MetS also had increased incidence of fatty liver and development of non-alcoholic fatty liver disease (NAFLD) (Adult Treatment Panel III, 2002). The presence of excess abdominal fat accumulation leads to increased levels of circulating free fatty acids, which redistribute within skeletal muscle tissue as well as the liver. Fatty liver plays a crucial role in the development of insulin resistance characteristic of MetS and subsequent T2D (Grundy *et al*., 2004) (See Figure 1.3).

More recently, other conditions have been included in the MetS, such as chronic low-grade inflammation (Grundy *et al*., 2004). Therefore, MetS is not a disease in itself, but a collection of risk factors that can predict future chronic disease development.

Due to the various definitions of MetS, exact epidemiological statistics are hard to achieve; however, data from the National Health and Nutrition Examination Survey (NHANES) in the United States reported a prevalence of 25.5% in adults over the age of 20 in 1999-2000 (Beltran-Sanchez *et al*., 2013; Samson & Garber, 2014). Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe (DECODE) released data from 9 separate population studies that indicated that 41% of men and 38%

Figure 1.3 Factors Contributing to the Metabolic Syndrome and Liver Steatosis



Visceral obesity, increased blood pressure (hypertension), hyperlipidemia, inflammation and insulin resistance are all contributing factors to the development of the metabolic syndrome (MetS). Visceral adiposity and hyperlipidemia can cause excess fat to accumulate within the liver (steatosis). Increased intrahepatic fat content plays a role in the development of insulin resistance and also illicits an inflammatory response.

of women between the ages of 47 and 71 met the inclusion criteria for MetS (Singh *et al*., 2013; Samson & Garber, 2014).

In younger populations where obesity is present, the prevalence of MetS is also staggeringly high. Data from NHANES from 1999 to 2002 showed an overall prevalence of 9%, but this number jumped significantly to 44% in overweight and obese children (Wright *et al.*, 2007; Samson & Garber, 2014). It is also important to note the close association between MetS in children and parental history of obesity (Whitaker *et al*., 1997; Parsons *et al*., 1999). Obese children are also at risk of developing fatty liver or hepatic steatosis. A recent study by Wicklow et al. displayed a strong correlation between the presence of fatty liver among obese adolescents and insulin resistance. In fact, obese adolescents presenting with NAFLD had a 55% reduction in insulin sensitivity with a two-fold increase in MetS diagnosis comparative to controls (Wicklow *et al*., 2012). It has also been recently shown by Dart et al. that youth diagnosed with T2D begin to have renal and neurological complications much earlier than youth diagnosed with Type 1 Diabetes Mellitus (T1D) (Dart *et al*., 2014). Since the obesity epidemic is rising at an alarming rate globally among adults and youth, it is only fair to expect a parallel increase in MetS and associated end stage chronic diseases such as CVD and T2D.

1.4 Diabetes

1.4.1 Type 2 Diabetes Mellitus (T2D)

T2D is a heterogeneous metabolic disorder characterized by the development of hyperglycemia that arises from the combination of insulin resistance in peripheral tissues and β-cell failure (Ashcroft & Rorsman, 2012). Type 1 Diabetes Mellitus (T1D) is different from T2D in that is an autoimmune disorder that results in complete deficiency of insulin (Seidell, 2000; Ashcroft & Rorsman, 2012; Holman, 2013). T2D is the most common form of diabetes mellitus, accounting for roughly 90% of all new diabetic cases worldwide (World Health Organisation, Geneva, 1999). Lifestyle, living environment, obesity and genetics are all major risk factors associated closely with the development of T2D (Ashcroft & Rorsman, 2012).

Statistics released from the International Diabetes Federation (IDF) in 2013 stated that worldwide, 382 million people suffer from T2D and that the disease accounts for roughly 4.6 million deaths annually. (International Diabetes Federation, 2011; Ashcroft & Rorsman, 2012) It has been projected, in a study from 2010, that by the year 2030, the prevalence of diabetes worldwide will reach 439 million adults (Shaw *et al*., 2010) ; however, the IDF estimates that this number could be closer to 592 million by 2035 (International Diabetes Federation: Diabetes Atlas 6th Edition). T2D also has an enormous burden on healthcare costs globally. Data suggests that an estimated $612 million USD are spent daily due to diabetes and its associated complications in the United States alone (Ashcroft & Rorsman, 2012).

There are many perturbations to normal metabolic health associated with T2D. Since insulin is the primary hormone involved in glucose, fat and even protein metabolism, defects in insulin secretion and/or the response of tissues to the actions of insulin can damage the metabolic health of individuals (Defronzo, 2010). Left unchecked, insulin resistance due to increased visceral adiposty can eventually lead to CVD and other T2D-associated macro-and microvascular pathologies such as retinopathy, nephropathy and central and peripheral neuropathies (Ayodele *et al*., 2004; Said, 2007).

Glycemic control is currently the major approach to treating T2D amongst adults and youth to reduce the risk of CVD and other macro-and microvascular complications (Greenfield *et al.*, 2009). Lifestyle modifications that increase physical activity and decrease consumption of foods high in saturated fats and simple sugars are used as a first-line treatment strategy for the prevention of T2D or the maintenance of glycemia in T2D patients. Therapeutic agents also exist to increase insulin release and peripheral sensitivity to insulin, as well as slow the absorption of glucose from the gut into circulation following a meal. Sulphonylureas and meglitinides increase insulin secretion from pancreatic β-cells to counterbalance insulin resistance (Elson & Meredith, 1998; Thule & Umpierrez, 2014). Biguanides, such as metformin, act as euglycemic agents to decrease postprandial blood glucose levels and increase insulin sensitivty (Elson & Meredith, 1998; Dunn & Peters, 1995). Glitazones act to increase insulin sensitivity through increasing activity of peroxisome proliferator-activated receptor gamma

(PPARγ), a transcription factor involved in regulating metabolism (Mudaliar & Henry, 2001).

Previously, T2D was viewed as a disease of aging but rates of youth-onset T2D have been rapidly increasing since the mid 1980s. Since prevalence of obesity among youth parallels the increase in worldwide obesity rates, children and adolescents, are increasingly at risk for the development of T2D (Hannon *et al*., 2005). During the early 1990s, T2D accounted for less than 3% of all newly diagnosed cases of diabetes among children in the United States. This number rose drastically to 45% by the year 2005 (Pinhas-Hamiel & Zeitler, 2005). Recent Canadian data suggests incidence of youth-onset T2D is seen between 1.54 and 20.55 cases per 100,000 youth under the age of 18 and is steadily increasing, with the highest rates seen in Manitoba (Dart *et al.*, 2014; Amed *et al*., 2010).

Aside from the increasing obesity epidemic in both adult and youth populations globally, there is also an underlying genetic property to T2D and its development. Genome wide association studies previously identified 56 susceptibility loci for the disease. These loci represent an estimated 10 % of total familial aggregations of T2D (Zeggini *et al.*, 2008; Voight *et al*., 2010; Morris *et al*., 2012). More recently, the Diabetes Genetics Replication and Meta-analysis (DIAGRAM) consortium uncovered 65 new genetic loci having direct implication in T2D development using large-scale genotyping methods (Hivert *et al*., 2014; Morris *et al*., 2012). A large majority of loci involved in T2D pathogenesis seem to

be involved in pancreatic β-cell function, insulin secretion and insulin processing. Loci associated with insulin sensitivity have also been found, though less frequently (Hivert *et al*., 2014; Dimas *et al.*, 2013).

1.4.2 Gestational Diabetes Mellitus (GDM)

GDM is defined as a state of glucose intolerance and hyperglycemia with first onset during pregnancy and is usually diagnosed during the third trimester (Buchanon *et al.*, 2012). Screening tests for GDM are generally administered at 24-28 weeks of gestation and involve an OGTT. A 75g oral glucose drink is administered in the fasting state and plasma glucose levels are evaluated before and 1 and 2 hours after administration of the drink. A diagnosis of GDM is made when one or more of the blood glucose readings fall at or above the threshold of 7.8mM (Canadian Diabetes Association Clinical Practice Guidelines). GDM is one of the most common complications of pregnancy, affecting 5-10% of women (Jovanovic & Pettitt, 2001; Ferrara, 2007). Worldwide, the prevalence of GDM has been steadily increasing over the last 20 years in part due to the obesity epidemic and increasing number of women delaying pregnancy until later in life (Ferrara, 2007; Reece *et al*., 2009). In 2002, it was suggested that between 1992-2002 the overall incidence of GDM rose slightly from 4% to 6% (Dabelea *et al*., 2005). Newer studies released by the same group in 2011 saw a significant increase in incidence of GDM to 10% between the years 1995-2009 (Xiang *et al*., 2011). In addition rates of GDM are even higher (upwards of 20%) in some Indigenous populations (Aljohani *et al*., 2008). Pregnancy is a natural state of insulin resistance that spares more glucose for the fetus

during crucial developmental periods (Buchanon *et al*., 2012). Because of this natural state of insulin resistance during pregnancy it is difficult to set threshold criteria for the diagnosis of GDM.

Women who have a history of GDM are at higher risk of preeclampsia, caesarean delivery and its potential morbidities (Yogev *et al*., 2004). It is projected that up to 50% of women with GDM will develop T2D within 20 years after pregnancy (Bellamy *et al.*, 2009 ; England *et al.*, 2009). It is probable that GDM and T2D share many of the same risk factors as T2D (Mao & Gao, 2012), though knowledge of the specific genes and environmental interactions that drive the development of GDM remain to be established.

Insulin resistance acquired as a natural property of pregnancy may not account for the pathogenesis of the condition as there can be maintenance of normoglycemic conditions in cases of mild to moderate insulin resistance. This suggests that β-cells are able to compensate and release enough insulin to maintain normoglycemia throughout pregnancy in most instances. Elevated blood glucose levels throughout gestation, however, have been associated with negative long-term effects on the metabolic health of offspring (Meztger *et al*., 2008; Buchanon *et al*., 2012). There has been strong evidence to suggest that T2D during pregnancy predisposes offspring to the development of obesity, MetS and eventually T2D (Franks *et al.*, 2006). Until recently the effects of a more mild state of hyperglycemia seen in GDM on metabolic health of offspring was relatively unknown. The Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) Study Cooperative Research Group looked at 25,505 pregnant women at 15 centers in nine different

countries to assess the effects of mild hyperglycemia on fetal outcome. This study showed a strong association between GDM conditions and an increase in offspring birth weight as well as increased cord-blood serum C-peptide (Meztger *et al.*, 2008). Studies have also shown that intrahepatic fat content is significantly increased in neonatal offspring born to obese mothers presenting with GDM, thus predisposing them to the development of NAFLD (Brumbaugh *et al*., 2013; Brumbaugh & Friedman, 2014). Other studies have reported that offspring from mothers consuming diets high in saturated fats during gestation are significantly more obese, glucose intolerant and insulin resistant compared to offspring from healthy mothers (Dudley *et al*., 2011; Franks *et al*., 2006). There is a strong association between maternal nutritional and metabolic health status and the direct metabolic outcomes of offspring.

The mechanisms by which deleterious changes in metabolic regulation occurs is an area of great interest. Fetal exposure to a hyperglycemic gestational milieu could permanently re-program gene expression by an epigenetic mechanism. Initially, the altered gene expression profile may be an adaptive response to prepare the fetus to be born into a "stressful" environment, though it could become maladaptive postnatally and contribute to obesity, MetS and T2D. Mechanistically, epigenetic modifications such as DNA methylation and histone modification have been shown to be responsible for perturbing gene expression during crucial developmental periods in offspring exposed to various prenatal stressors such as alcohol, folate deficiency, teratogenic chemicals, amongst others (Darney *et al.*, 2011; Allan *et al*., 2014; Gueant *et al.*, 2013; Eleazu *et al*., 2013).

1.4.3 Rodent Models of Gestational Diabetes Mellitus

The study of GDM in a preclinical, animal-based system is necessary to tease out the many possible mechanisms that predispose offspring to the development of chronic metabolic diseases later in life. To date, there have been several different animal models utilized to study the question of how maternal obesity or GDM influences fetal programming. The majority of these studies have used streptozotocin (STZ) during pregnancy to induce a diabetic state (Jawerbaum *et al.*, 2010). STZ is a naturally occurring compound that is toxic to pancreatic β-cells. Toxicity is achieved through uptake by the β- cell glucose transporter-2 (GLUT2), where it acts as an alkylating agent to cause DNA damage (Eleazu *et al*., 2013). Destruction of β-cells results in overall insulin deficiency and severely elevated blood glucose concentrations that reach 20mM and above. STZ has also been associated with low birth weight pups in contrast to the macrosomic offspring seen in GDM pregnancies. Birth defects are also common with STZ as it has teratogenic effects on organogenesis (Eleazu *et al*., 2013). Neither insulin deficiency, severe hyperglycemia or low birth weight offspring are hallmarks of GDM. Therefore, results from these studies should be considered objectively. Some studies have used multiple low-dose STZ; however this approach has led to a wide variation in plasma glucose levels being reported (Caluwaerts *et al*., 2003). Therefore, the development of a more physiologically-relevant model of GDM would greatly aid in our understanding of this disease and its impact on the metabolic health of offspring.

Since the worldwide obesity epidemic is a major player in the development of insulin resistance, MetS and subsequent T2D, the establishment of appropriate rodent models of maternal obesity that utilise "western-style", high-fat and high-sugar diets appear to more accurately model the effects of maternal obesity on fetal outcomes (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Dudley *et al*., 2011; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012; Taylor *et al*., 2005). This approach of diet-induced obesity during pregnancy has been shown to mimic the condition of maternal obesity more so than the human GDM condition. Pregnant rats and mice utilized in these studies were not hyperglycemic during pregnancy, nor did the researchers assess glucose and insulin tolerance; however, high-fat and high-sugar diets have been shown to cause hyperglycemia, hyperinsulinemia as well as glucose intolerance in other rodent models (Sasidharan *et al*., 2013; Omar *et al.*, 2012; Islam & Wilson, 2012). These rodent models also yielded macrosomic or large for birth weight offspring, also commonly observed in the human GDM condition (Cisse *et al.*, 2013; Vambergue & Fajardy, 2011; Kiss *et al.*, 2009).

While it might seem like an attractive approach to use genetic mouse models of diabetes and obesity to study GDM, these models have limitations. For example the *ob/ob* mouse lacks leptin that is functional. Leptin is an adipokine, a hormone released by adipocytes that plays a role in regulating food intake and appetite (Brennan, 2006). *Ob/ob* mice are obese, hyperinsulinemic, hyperglycemic and are unable to supress food intake (Garthwaite, 1980); however, female *ob/ob* mice are infertile and cannot be used to study programming effects of maternal obesity and hyperglycemia on fetal metabolic health

(Ingalls, 1950). *db/db* mice, a knockout model of the leptin receptor, display the same characteristics as the *ob/ob* mouse and are not suitable for reproductive studies. (Jones, 1957)

Yamashita *et. al,* developed a genetic model of GDM utilising heterozygous female *Lepr db/+* mice. These particular mice develop diabetes during pregnancy and are glucose intolerant and hyperglycemic. These mice also produce macrosomic offspring with elevated circulating insulin levels comparative to offspring from control mice (Yamashita, 2003); however, few women with diabetes during pregnancy have mutations in the Lepr gene and a more "global" model of GDM is required to understand this disease.

Huang *et. al,* used a prolactin receptor-deficient mouse model to display the importance of the prolactin receptor during pregnancy. Signal transduction through the prolactin receptor is required for the increase in β-cell number during pregnancy to compensate for the natural insulin resistance associated with pregnancy (Huang, 2009). It was shown that heterozygous $Prlr^{+/-}$ mice were hyperglycemic during pregnancy due to decreased β-Cell number and overall lower circulating insulin levels compared to control. The offspring from these prolactin receptor-deficient mice also displayed lower β-cell number and increased plasma glucose levels (Huang, 2013).

More recently, Isganaitis *et. al,* used an insulin receptor substrate-1 (IRS1) haploinsufficient mouse model to assess fetal programming through gestational insulin resistance. These mice display hyperinsulinemia and overall insulin resistance while maintaining normal blood glucose levels throughout pregnancy. The male offspring of IRS1 haploinsufficient (*IRS1-het*) mice were both glucose intolerant and hyperinsulinemic by one month of age compared to wild-type control male offspring. The male offspring from *IRS1-het* also developed fasting hyperglycemia and increased liver fat accumulation compared to wild-type control male offspring despite no changes in adiposity between the groups (Isganaitis, 2014).

1.5 Developmental Origins of Disease

1.5.1 The Barker Hypothesis

Historically, chronic disease development was explained solely through postnatal environmental and lifestyle factors, such as diet, and the interaction of these factors with individual inherited genetics. Initially, developmental programming was recognized as an adaptive and advantageous process necessary for the overall health and survival of the species in response to a particular environment. Fetal tissue development occurs in various critical stages when there is an increase in cellular proliferation, differentiation and growth, that fuels tissue growth. It is also known that different tissues develop during different times (Widdowson *et al.*, 1972; Barker, 1990). It has been known for half a century that birth weight is highly correlated to intrauterine environment. A study from 1955 displayed that among half-siblings, those who were related through a common mother displayed similar birthweights, with a correlation coefficient of 0.58, compared to half-siblings with a common father, with a correlation coefficient of 0.1 (Morton, 1955). Looking at rates of infant mortality in both England and Whales post 1911, there was an increase in neonatal deaths where both low birth weight and poor maternal health status was noted (Baird, 1949; Baird, 1980; Barker & Osmond, 1987). It was also shown through follow-up studies of patients born more than 60 years previously that those who weighed more at birth had lower death rates from heart disease and stroke (Barker, 1986). Moreover, Barker observed, through a study of 449 men and women over the age of 50, that low birth and placental weight showed positive correlation with increased blood pressure and risk of hypertension (Barker, 1990). Undernutrition and low birth weight

was later shown to also be associated with increased rates of both MetS and T2D later in life (Barker, 1993). Thus, the *in utero* environment was strongly associated with fetal undernutrition during gestation and this strongly correlated with poor fetal outcome and the increased rates of cardiovascular disease, MetS (Syndrome-X) and T2D as adults, demonstrating the role and importance of the in-utero environment on the programming of the metabolic health of the offspring. As a result of these observations, associating poor maternal health with low birth weight and subsequent adult CVD rates, David Barker formulated the novel hypothesis that maternal health and nutritional status during gestation have a crucial role in fetal programming and susceptibility of the offspring to the onset of chronic diseases in adulthood (Barker, 1990).

1.5.2 Undernutrition During Pregnancy

The "thrifty phenotype" hypothesis proposed by Hales and Barker in 1992 suggested that undernutrition during gestation could cause an adaptive response by the fetus which causes a decrease in both structural and functional units of important metabolic tissues, including nephrons, cardiomyoctes and pancreatic β-cells. Underdevelopment of β-cells in the pancreas was postulated to be the cause of insulin resistance seen in low birth weight individuals. 468 men made up the study on which this hypothesis was formed. The percentage of these men suffering from impaired glucose tolerance and T2D was high in males born at low birth weight and the percentage declined in males born at higher birth weights (Hales & Barker, 1992).

The fetal insulin hypothesis suggested genetic mutations disrupting insulin function could be the cause of low birth weight and T2D later in life (Frayling & Hattersley, 2001 & Ozanne *et al.*, 2002). However, these mutations are extremely rare and could not be the main underlying component of the epidemic prevalence of the disease. However, it is important to note that such genetic mutations do result in youth onset T2D. It has been shown that mutations in the gene encoding glucokinase, involved in initiating the glycolytic pathway by phosphorylating glucose to glucose-6-phosphate, resulted in both low birth weight and youth onset T2D (Hattersley *et al.*, 1998).

The fetal salvage hypothesis suggested peripheral tissue insulin resistance within the fetus was the main cause of disease susceptibility later in life. Since nutrients are sparse in the undernourished, developing fetus, insulin resistance allows glucose to be spared and utilized by essential organs, such as the brain, which is crucial for survival. This comes at the expense of other non-essential tissues such as skeletal muscle (Hofman, 1997 & Brenseke *et al.*, 2013).

The mismatch hypothesis suggests that programming in response to adverse *in utero* environments, such as undernutrition, followed by subsequent exposure to a completely different environment postnatally, such as overnutrition (Bateson, 2004). In fact, lower birth weight individuals that went on to grow up in a more socioeconomically affluent society postnatally had much higher incidence of T2D and CVD alike (Gluckman & Hanson, 2013 & Kanaka-Gantenbein, 2010). Increased incidence of T2D in developing

countries, such as India, may be explained by this phenomenon, as there have been significant improvements in mechanization and technology as well as food cost and availability that promotes a more sedentary and obesogenic lifestyle (Bavdekar *et al*., 1999). However, it does little to explain the remarkable rise in the incidence of obesity and T2D in North America where most of society has been affluent for decades.

Over the past two decades, epidemiological evidence shows a consistent correlation between *in utero* programming to undernutrition, low birth weight and the development of many chronic diseases in adulthood, especially T2D and CVD. However, there is clear debate over the precise mechanisms that are driving fetal programming.

1.5.3 Maternal Overnutrition, Obesity and Diabetes During Pregnancy

Since most of the first clinical evidence to suggest fetal programming due to adverse in-utero conditions was aimed at maternal undernutrition, it has been of great interest over the past decade to investigate the opposite end of the spectrum, maternal overnutrition, and its programming effects on fetal predisposition to chronic disease in adulthood. Of particular interest, what clinical epidemiology data has been acquired to suggest maternal overnutrition, obesity and diabetes during pregnancy has a direct correlation with fetal predisposition to the same metabolic dysfunctions.

In the United States, it has been reported that up to 60% of women are overweight at the beginning of the gestational period and roughly 30% of these women are obese by

standard definition, with BMI's greater than 30kg/m2 (Hinkle *et al.*, 2012). It has been suggested that maternal obesity allows the transfer of excess nutrients, such as glucose and lipids, to the fetus during gestation. Several epidemiological studies have been carried out and demonstrate the effect of overnutrition and diabetes on fetal development. Studies in Pima Indian populations in Arizona were among the first to show a correlation between the presence of diabetes during pregnancy and increased rates of youth onset T2D in children born to diabetic mothers compared to non-diabetic mothers (Franks, 2006). A study by Franks et al. showed a positive association between third-trimester glucose tolerance and blood glucose levels with birth weight. Moreover, decreased tolerance to glucose during the third-trimester resulting in elevated blood glucose levels was associated with higher incidence of T2D amongst Pima youth (Franks, 2006). A follow up study by Clausen et al. reported that obesity development was increased two-fold in offspring from GDM or T1D mothers comparative to offspring born to healthy mothers (Clausen *et al*., 2009). Metzger *et. al*, carried out The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study which provided data suggesting maternal hyperglycemia at levels below GDM diagnosis levels lead to macrosomic, or, large for birth-weight offspring (Metzger, 2008). Other studies have correlated high maternal triglyceride and fatty acid levels during gestation with an increase in fetal adiposity after birth (Harmon *et al*., 2011). Obesity and hyperglycemia associated with diabetes during pregnancy programs increased hepatic fat stores in neonates (Modi, 2011). This has been hypothesized to "prime" the fetal liver for postnatal fat accumulation and storage leading

to steatohepatitis, especially in response to a childhood diet high in saturated fats and simple sugars (Brumbaugh & Friedman, 2013).

More recently, epigenetic studies have been performed on peripheral blood leukocytes of non-diabetic Pima Indians who were either born to a diabetic or non-diabetic mother to assess the role of DNA methylation with the increased rates in youth onset T2D seen in this population. It has been previously established that β-cell development occurs largely during the *in utero* and early postnatal time periods and roughly 50% of the adult β-cell population has been accrued by one years of age (Rahier *et al.*, 1981). More recently the differential expression in promoter regions of genes involved in pancreas formation, insulin signaling and overall β-cell function have been reported in offspring born to diabetic mothers that could be causal for youth onset T2D (Del Rosario, 2014).

Epigenetic modifications, more specifically, methylation to the peroxisome proliferator-activated receptor γ coactivator 1α gene (PGC1α), has been shown to be important in the development of insulin resistance in the liver since PGC1α is an important modulator of energy homeostasis in this tissue type (Sookoian *et al*., 2010). Rat offspring born to mothers consuming high saturated fat diets had significantly lower mitochondria number within liver tissue, however, only male offspring showed signs of insulin resistance and decreased expression of PGC1α (Burgueno *et al*., 2013). Furthermore, a recent study showed that rat offspring of mothers fed a diet high in saturated fat during pregnancy, had increased expression of PCK1 in the fetal livers. PCK1 displayed variations in histone modifications leading to increased expression of PCK1 (Strakovsky, 2011). This could

program increased glucose production and release from fetal liver tissue, thus causing post-prandial hyperglycemia, which could lead to the development of insulin resistance, MetS and T2D.

There is a clear link between overnutrition, obesity and hyperglycemia observed in diabetic pregnancies that have severe implications for metabolic health outcomes in offspring. Epidemiological studies have associated T2D and GDM during pregnancy to adverse fetal outcomes and an overall predisposition to obesity, MetS and T2D amongst youth but the full mechanisms have yet to be elucidated. Epigenetic studies have begun to associate *in-utero* environment with changes in gene expression that effect metabolic homeostasis, but these studies have only scratched the surface and few studies have examined the effect of GDM on the offspring.

1.6 Thesis Objectives

There has been a staggering increase in the rates of obesity, MetS and subsequent chronic disease development worldwide. Aside from genetic and lifestyle factors that predispose youth to the development of metabolic disease, such as T2D, it has been established that adverse *in utero* environments also have a substantial role. It has been known for half a century that during gestation, nutrient insufficiency programs the metabolic state of the offspring and predisposes the offspring for the early onset of conventionally adult diseases such as CVD and T2D. It has also been established that exposure to hyperglycemia *in utero* also programs energy metabolism in the offspring. There are several important objectives for this thesis. Firstly, the development of a clinically-relevant rodent model of the human GDM condition is necessary. Utilizing a high fat and sucrose diet prior to and throughout pregnancy I aimed to achieve gestational characteristics associated with human GDM, including, hyperglycemia, hyperinsulinemia and macrosomic fetus development. Secondly, I aimed to understand how exposure to different postnatal diets, either low fat or high fat and sucrose, affects metabolic health in offspring from GDM mothers compared to offspring born from metabolically healthy mothers.

1.7 General Hypotheses

It was hypothesized that feeding female sprague-dawley rats a high fat and sucrose (HFS) diet prior to, and throughout gestation would cause hyperglycemia, hyperinsulinemia and insulin resistance that is characteristic of clinically defined GDM.

It was also hypothesized that the rat offspring born from GDM dams will be more susceptible to the development of several characteristic features of the MetS, compared to offspring from lean control dams.

CHAPTER 2 - MANUSCRIPT INTENDED FOR PUBLICATION

Maternal gestational diabetes mellitus increases the susceptibility of young rat offspring to hepatic steatosis and insulin resistance.

Running title: Gestational diabetes and hepatic steatosis in the offspring

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2.1 Abstract

Background: Population health data suggests that the development of metabolic disease is influenced by early life events. Gestational diabetes (GDM) is a common complication of pregnancy, but its effects on the offspring are poorly understood. Progress has been hampered by the lack of an appropriate animal model system. Aims: We hypothesize that diet-induced GDM causes obesity, hepatic steatosis and insulin resistance in the offspring. Methods: Female Sprague-Dawley rats were fed a high fat (45%) and sucrose (HFS) diet to cause GDM. Lean control pregnant rats received low fat (LF; 10%) diets. The offspring were weaned at 3 weeks of age and randomly assigned to LF or HFS postnatal diets for 12 weeks then analyzed for insulin sensitivity and hepatic steatosis. Results: GDM rats exhibited excessive gestational weight gain, hyperinsulinemia and mild hyperglycemia. The young adult offspring of GDM dams gained more weight than the offspring of lean dams, which was accompanied by hepatic steatosis and in-vivo insulin resistance compared to the offspring of lean dams. The increased expression of acetyl-CoA carboxylase-2 and reduced expression of peroxisomal proliferator activated receptor-α and insulin receptor-β in the livers of the young adult offspring of GDM dams could be driving factors responsible for the development of obesity, hepatic steatosis and insulin resistance. Conclusions: GDM enhances the development of obesity, hepatic steatosis and insulin resistance in the offspring.

2.2 Introduction

Obesity and its associated co-morbidities are reaching epidemic proportions. (Seidell, 2000) In addition, the proportion of young adults with obesity has increased dramatically in recent decades, (Ogden *et al*., 2006) with several obesity-related pathologies, including hepatic steatosis reaching record levels (Brumbaugh *et al.*, 2013; Dabelea *et al*., 2000). Obesity has a complex pathophysiology characterized by inherited factors and environmental variables, though genetics can not completely account for the increased prevalence. While the consumption of calorie-dense foods and a sedentary lifestyle are driving forces behind the obesity epidemic, population health data and experimental animal model systems established that alterations in the prenatal environment during crucial periods of development have long-lasting effects that influence disease susceptibility in the offspring. As a result, a theory was formulated that perturbations to the maternal environment during fetal development permanently program biological systems and increase susceptibility of offspring to chronic diseases (Barker, 1995); however, studies using animal models of gestational diabetes mellitus (GDM) with controlled prenatal and postnatal conditions are lacking.

GDM is a common complication of pregnancy, affecting 5-10% of women, a figure that is increasing in parallel with the obesity epidemic. (Reece *et al*., 2009) Currently, little information about mechanisms that predispose women to the development of GDM is available, though a significant proportion of women who develop GDM are overweight, obese and insulin resistant (Catalano et al., 1999; Catalano et al., 1991). Clinical studies confirm the short-and long-term health implications of GDM for both mother and child. (Gillman *et al.*,

2003; Moore, 2010; Reece *et al.,* 2009) Mothers diagnosed with diabetes during pregnancy are more likely to have infants that are overweight at birth (Franks *et al.*, 2006; Gillman *et al.,* 2003) and children that are at higher risk for obesity, hepatic steatosis and type 2 diabetes (T2D). (Brumbaugh *et al.*, 2013; Dabelea *et al*., 2000)

Our objective is to determine how maternal GDM influences the metabolic health of rodent offspring and characterize the mechanisms involved. The majority of previous studies administered streptozotocin (STZ) to pregnant rodents in order to induce GDM. This approach causes severe hyperglycemia and low birth weight offspring. (Jawerbaum & White, 2010) We show that a rat model of high fat and sucrose diet (HFS)-induced GDM mimics several clinical features of GDM during human pregnancy. We also show that GDM increases the susceptibility of rat offspring to obesity, hepatic steatosis and insulin resistance.

2.3 Methods

2.3.1 Diet-Induced GDM Model

All procedures were approved by the Animal Welfare Committee of the University of Manitoba. Rats were given *ad libitum* access to the respective diets and water and housed two per cage. Female Sprague-Dawley rats were obtained at 4 weeks of age from the University of Manitoba colony and randomly allocated to a low-fat (LF) diet (10% fat, Research Diets D12450B) or a high-fat and sucrose (HFS) diet (45% fat, Research Diets D12451) for 6 weeks to induce glucose intolerance. Females were then mated with lean males within the animal facility and diets continued throughout pregnancy and the suckling period. At the time of birth, litters were reduced to eight pups to avoid competition for food and subsequently weaned at 3 weeks of age. After weaning, offspring were randomly allocated to LF or HFS diets for 12 weeks. Four experimental groups were created: Offspring from LF-fed (termed Lean) dam that were fed a post-weaning LF diet, offspring from the Lean dam receiving a post-weaning HFS diet, offspring from a HFS-fed (termed GDM) dam fed a post-weaning LF diet, and offspring from a GDM dam fed a post-weaning HFS diet. Rats were anesthetized by overdose of sodium pentobarbital and blood was collected by cardiac puncture. For immunoblot analysis of the insulin signaling cascade, a separate set of rats were fasted 2-hours prior to intraperitoneal injection with insulin (1 mU/kg) and euthanized as above 15 minutes after injection. Tissues were dissected, rinsed in PBS, weighed and immediately freeze clamped in liquid nitrogen and stored at -80°C for future analyses.

2.3.2 Analysis of mRNA and Protein Expression

RNA was isolated from tissue using a Qiashredder column and further purified using the RNeasy kit (Qiagen, Valencia CA). The QuantiTect SYBR Green PCR kit (Qiagen) was used to monitor amplification of cDNA on an ABI-7500 real-time PCR detection machine (Applied Biosystems, Foster City, CA). Eukaryotic initiation factor 2a was used as the reference gene to normalize cDNA. SDS-PAGE and immunoblotting was performed using antibodies from Cell Signaling Technologies (Danvers, MA), as described previously. (Dolinsky *et al*., 2011)

2.3.3 Determination of Circulating Factors

Circulating concentrations of insulin, leptin and adiponectin were determined using colorimetric ELISA assays (Millipore, St. Charles, MO) according to the manufacturer's instructions. Free fatty acids were determined by colorimetric assay (Wako Chemicals, Richmond, VA).

2.3.4 Histology and Morphometry

Oil red O and hematoxylin/eosin staining was performed by the University of Manitoba Core Platform for Histology according to standard procedures. The internal diameters of 10 consecutive adipocytes from 10 randomly selected fields on each slide was measured under light microscopy using a digital micrometer under 40x magnification and averaged.Hepatic steatosis in HE stained liver sections was evaluated and scored by a blinded and experienced pathologist: 0= no visible steatosis, 1= 5-19%, 2=20-30%, and 3= above 30%

2.3.5 Metabolic Measurements

At twelve weeks post-weaning, an insulin tolerance test (ITT) was performed as described previously, (Dolinsky *et al*., 2011) and blood glucose from the tail was measured using an ACCU-CHEK® advantage glucose meter (Roche Diagnostics). Glucose tolerance tests (GTT) were also performed at twelve weeks post weaning as described previously, (Dolinsky *et al.*, 2011) and blood glucose from the tail was measured using an ACCU-CHEK® advantage glucose meter (Roche Diagnostics). Liver was homogenized and TG and glycogen content determined by non-radioactive, colorimetric assays (Biovision: Milpitas, CA) according to the manufacturer's instructions.

2.3.6 Statistical Analysis

Data are presented as mean (+/-) SEM. Comparisons between two groups were evaluated as unpaired t-test. Differences in measurements performed among four groups were analyzed using two-way ANOVA and a Bonferroni post hoc test with both diet and GDM as sources of variation. The level of significance was defined as $p < 0.05$. For the offspring studies, the litter is the unit of analysis.

2.4 Results

2.4.1 Maternal characteristics and glucose homeostasis

After 6 weeks, the female rats that consumed the HFS diet (termed the GDM group) weighed 1.2-fold more (Fig. 2.1A, $p<0.05$) than the LF diet group (termed the Lean group) and were glucose intolerant (data not shown). Subsequently, female rats were mated with LF-diet fed male rats. During pregnancy, the GDM dams continued to consume the HFS diet and increased their body weight by 24% while the lean LF-fed group increased their body weight by 13% (Fig. 2.1A). Thus, the HFS diet induced a remarkable 1.9-fold greater gestational weight gain in GDM rats compared to lean rats (Fig. 2.1B, $p<0.01$). During pregnancy, differences in energy intake were not observed between the GDM groups (Fig. 2.1C, $p=0.17$). Blood glucose levels were not different between lean and GDM groups prior to mating (Fig. 2.1D, $p=0.33$), although HFS-diet experienced significant hyperglycemia (Fig. 2.1D, $p<0.001$) and impaired glucose tolerance (Fig. 2.1E) in the GDM rats compared to the lean rats at mid-gestation. In the GDM rats, steady-state mid-gestational insulin levels were increased 2.3-fold compared with lean rats (Fig. 2.1F, $p<0.001$). In addition, GDM reduced mid-gestational levels of the insulin sensitizing adipokine, adiponectin by ~40% (Fig. 2.1G, $p<0.01$). Furthermore, the HOMA-IR index was increased 2.9-fold during pregnancy, compared to the lean rats (Figure 2.1H, $p<0.001$), suggesting that the consumption of the HFS diet contributed to whole body insulin resistance during pregnancy. Therefore the HFS diet-induced GDM rat model utilized in this study shares many of the features characteristic of the clinical presentation of GDM (Catalano et al., 1999).

2.4.2 Birth characteristics of the offspring

While maternal GDM did not influence the number of pups (Fig. 2.2A, $p=0.9$), nor the gender distribution in the litter (Figs. 2.2B, $p=0.63$), GDM did cause a 1.2-fold increase in the birth weight, compared with pups from lean mothers (Fig. 2.2C, $p<0.001$). The body length of the newborn pups was also significantly increased (Fig. 2.2D, $p<0.001$), indicating that the HFS-induced model of GDM caused fetal macrosomia, which is also commonly observed in clinical cases of GDM (Gillman et al., 2003).

To investigate the influence of fetal GDM on the postnatal response to LF and HFS diets, at the time of weaning (3 weeks of age), pups from lean and GDM dams were randomly assigned to either a LF or a HFS diet. As expected, 4 weeks post-weaning, the consumption of the HFS diet increased the body weight by both the lean and GDM male offspring more than the LF diet (Fig. 2.3A $p<0.001$); however, after 4 weeks male LF fed GDM offspring weighed significantly more than the lean offspring (Fig. 2.3A, $p<0.001$). Furthermore, the male GDM offspring that consumed the HFS diet weighed significantly more than the lean offspring after 5 weeks (Fig. 2.3A, $p<0.001$). Interestingly, the weights of the lean offspring consuming the HFS diet were not significantly different from the GDM offspring that consumed the LF diet (Fig. 2.3A). While the offspring that consumed HFS diets gained significantly more weight over the course of the experiment than those offspring consuming the LF diet (Figure 2.3B, $p<0.001$), male GDM offspring consuming either the LF diet or the HFS diet gained significantly more weight than the lean offspring in both dietary groups (Fig. 2.3B, $p<0.01$). Interestingly, these effects were less pronounced in the female offspring at this age (Table 2.1). While energy intake was

increased in the lean offspring that consumed the HFS diet compared with the LF-fed controls (Fig. 2.3C, $p<0.05$), the energy intake of GDM offspring consuming the LF diet was not different compared with the LF fed offspring from lean dams. Interestingly the energy intake of the GDM offspring consuming the HFS diet was similar to the LF-fed GDM offspring while energy intake was significantly reduced when compared with the lean offspring consuming the HFS diet (Fig. 2.3C, $p<0.01$). Therefore, maternal GDM appeared to program body weight in the offspring, independent of postnatal energy consumption.

As predicted, the consumption of the HFS diet by both lean and GDM offspring had elevated gonadal and perirenal fat pad mass (Fig. 2.3D, $p<0.01$). Offspring from GDM dams had similar gonadal fat pad mass as the lean offspring, though the perirenal fat pad mass was increased in the GDM offspring compared to the lean offspring (Fig. 2.3D, $p<0.05$). Visually, the adipocytes from the perirenal fat pad of GDM offspring were larger than the lean offspring (Fig. 2.3E). Consistent with this observation, the HFS diet and GDM was also associated with a significant increase in the adipocyte diameter (Fig. 2.3F, $p<0.05$). Corresponding to the increased fat mass in the HFS-fed offspring, plasma levels of leptin were elevated in both lean and GDM offspring compared to their respective LF-fed controls (Fig. 2.3G, $p<0.001$), although there were no differences in the plasma leptin concentrations between the offspring of lean and GDM dams. The postnatal HFS-diet reduced plasma levels of adiponectin in the lean pups (Fig. 2.3H, $p<0.05$) and interestingly the consumption of HFS diets by the pups from GDM dams did not affect the adiponectin levels. These findings suggest that GDM predisposes offspring to have

larger adipocytes, especially in response to postnatal HFS diets, although this did not significantly impact the levels of circulating adipokines.

2.4.3 Effects of Maternal GDM on glucose homeostasis in the offspring

Although 11 weeks of HFS diet did not result in fasting hyperglycemia in any of the experimental groups (Fig. 2.5A), offspring of lean dams receiving a HFS diet had higher fasted plasma insulin concentrations than offspring of lean dams receiving the LF diet . Offspring of GDM dams had much higher circulating insulin levels compared to the corresponding lean control offspring (Fig. 2.5B, $p<0.01$). The elevated HOMA-IR index of the GDM offspring (Figure 2.5C, $p<0.01$) suggested that GDM predisposes young rat offspring to develop whole body insulin resistance irrespective of the diet consumed. To investigate this further, the rat offspring of lean and GDM dams that were fed either LF or HFS diets were subjected to GTTs and ITTs. Interestingly, the glucose tolerance of the GDM offspring was similar to the lean offspring on both the LF and HFS diets (Fig. 2.5D-F). In contrast, the GDM offspring exhibited an impaired response to insulin (Fig. 2.5G, H) compared to the lean offspring, in both the LF and HFS diet groups (Fig. 2.5I, $p<0.05$). These findings suggest that maternal GDM contributed to a pronounced insulin resistance in the offspring. To further investigate the molecular mechanism underlying insulin resistance in the GDM offspring, we measured the expression of the insulin receptor and downstream kinases in the liver following acute administration of insulin to offspring immediately prior to sacrifice. Interestingly the expression of insulin receptor-β was markedly reduced in the GDM offspring (Fig. 2.8A, $p<0.05$). Moreover, the

phosphorylation of phosphatidylinositol-3 kinase and Akt at activating sites was also reduced in the GDM offspring (Fig. 2.8B, 2.8C, $p<0.05$). These findings suggest that maternal GDM contributed to a pronounced hepatic insulin resistance in the offspring.

2.4.4 Effects of Maternal GDM on liver morphology and metabolism in the offspring

Since the liver is an insulin-sensitive organ that has a significant role in the control of blood glucose, we examined how hepatic glycogen content and gluconeogenic gene expression was affected by maternal GDM. Notably, offspring of lean and GDM dams had similar hepatic glycogen levels that were not influenced by the postnatal diet that was consumed (Fig. 2.7A). Similarly, the mRNA expression of the rate limiting enzymes of gluconeogenesis, glucose-6 phosphatase (G6pd) and phosphoenolpyruvate carboxykinase (Pck1) were not significantly different across all the groups of rat offspring (Fig. 2.7B, C).

Next, we examined the liver for signs of pathology using hematoxylin and eosin (HE) stained sections. We detected histological evidence of steatosis in all of the sections from rats that had consumed the HFS diet (Fig. 2.4A, B). Steatosis appeared to be predominantly observed in zone 1 (Fig. 2.4A). However, a larger proportion of the offspring from GDM dams had histologically detectable evidence of macrovesicular steatosis in the liver (Fig. 2.8C). In addition, a larger proportion of the offspring from GDM dams had detectable microvesicular steatosis (Fig. 2.8D).

To examine the biochemical changes in the offspring that were associated with the observed liver steatosis, we measured hepatic TG levels and observed that the offspring

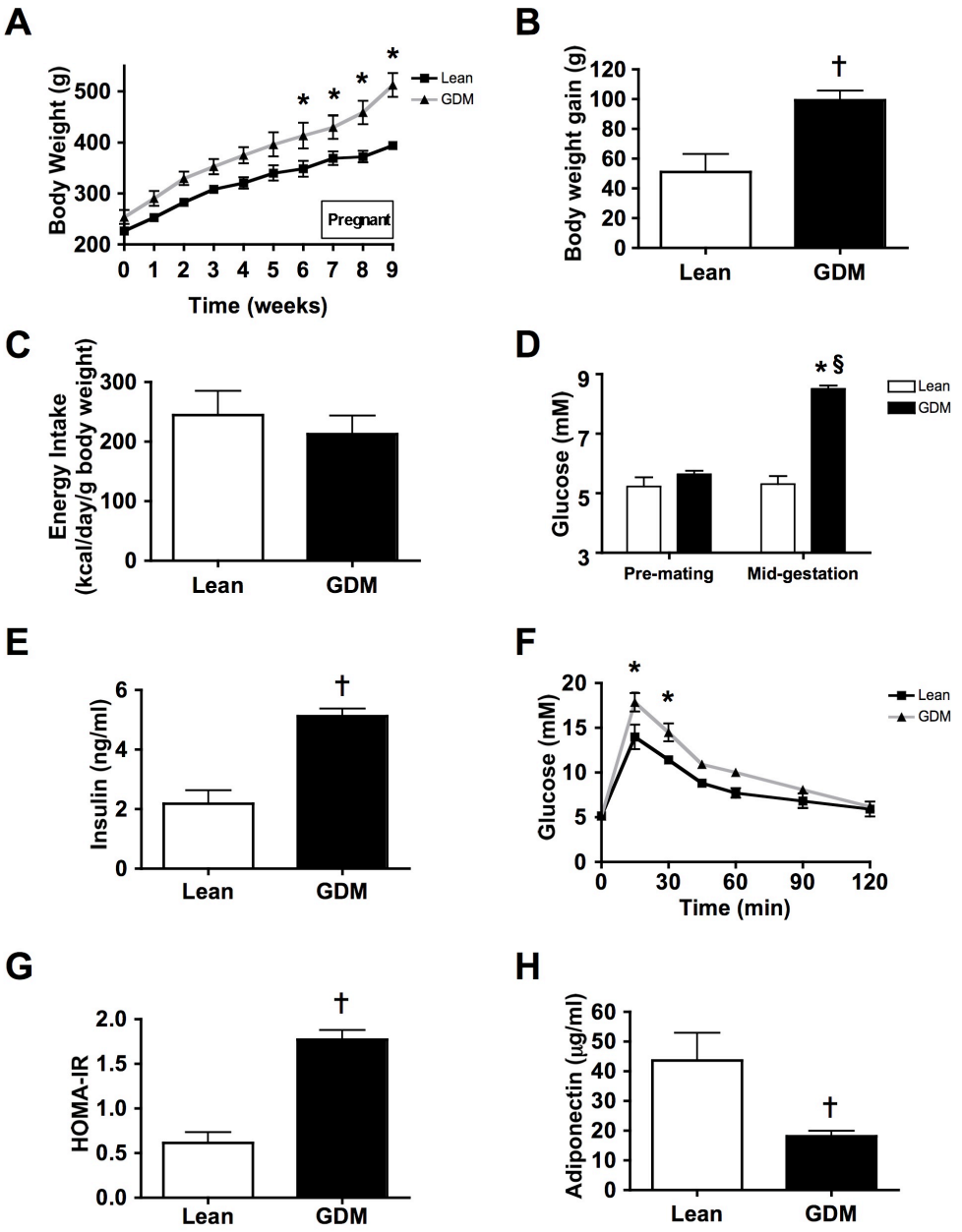
from GDM dams had higher TG levels compared to the offspring from lean dams (Fig. 2.6A, $p<0.05$). These findings corresponded with the histological observations. Fasted serum TG levels were not altered in the rat offspring (Fig. 2.6B), although the fasted serum free fatty acid (FFA) levels were elevated in the offspring from GDM dams, compared to the offspring of lean dams (Fig. 2.6C, $p<0.05$). To investigate the mechanisms underlying these observations, we measured the mRNA expression of enzymes and transcription factors involved in hepatic lipid metabolism. While acetyl-CoA carboxylase (ACC)-1 (Acca) was similar in all groups of rats (Fig. 2.6D), ACC-2 (Accb) expression was markedly increased in the livers of HFS-fed offspring of GDM dams (Fig. 2.6E, $p<0.05$). Sterol response element binding protein (SREBP)-1c (srebp1c) mRNA expression was significantly reduced in the livers of LF-fed offspring of GDM dams, while srebp1c expression was significantly increased in HFS-fed offspring from GDM dams (Fig. 2.6F, $p<0.05$). On the other hand, SREBP-2 gene expression was not significantly altered (Fig. 2.6G). Interestingly, we observed that peroxisomal proliferator activated protein-α (Ppara) expression was significantly reduced in the livers of both the LF and HFS-fed offspring of GDM dams (Fig. 2.6H, $p<0.05$), and a trend for reduced PPAR-γ coactivator-1α (Pgc1a) was observed (Fig. 2.6I). Collectively these findings suggest that GDM in the maternal environment programs the postnatal response of the liver to a HFS diet, resulting in hepatic steatosis. This may involve altered responses in key genes that favour the synthesis and storage of lipids in the liver at the expense of their oxidation.

Table 2.1 Metabolic Characteristics of Female Rat Offspring

	Lean		GDM	
	Low fat diet	**High fat and sucrose diet**	**Low fat diet**	**High fat and sucrose diet**
Body weight gain (g)	307.8 ± 17.2	270.8 ± 15.7	373.2 ± 26.9	352.3 ± 23.7
Tibia length (mm)	41.8 ± 1.0	42.2 ± 0.4	40.6 ± 0.7	42.3 ± 0.9
Energy intake (kcal/ day/ kg body weight)	174 ± 13	242 ± 20	166 ± 11	153 ± 9
Gonadal fat mass (g)	12.73 ± 1.5	18.70 ± 1.6 *	11.14 ± 0.9	20.5 ± 4.1 *
Perirenal fat mass (g)	13.48 ± 2.3	25.02 ± 3.3 *	11.76 ± 0.8	19.03 ± 3.9 *
Fasting glucose (mM)	5.3 ± 0.1	5.7 ± 0.3	5.1 ± 0.3	5.3 ± 0.3
Fasting insulin (ng/mL)	2.9 ± 0.3	3.2 ± 1.1	3.8 ± 0.8	5.5 ± 1.2
HOMA-IR	1.4 ± 0.2	1.7 ± 0.4	1.3 ± 0.3	1.4 ± 0.6

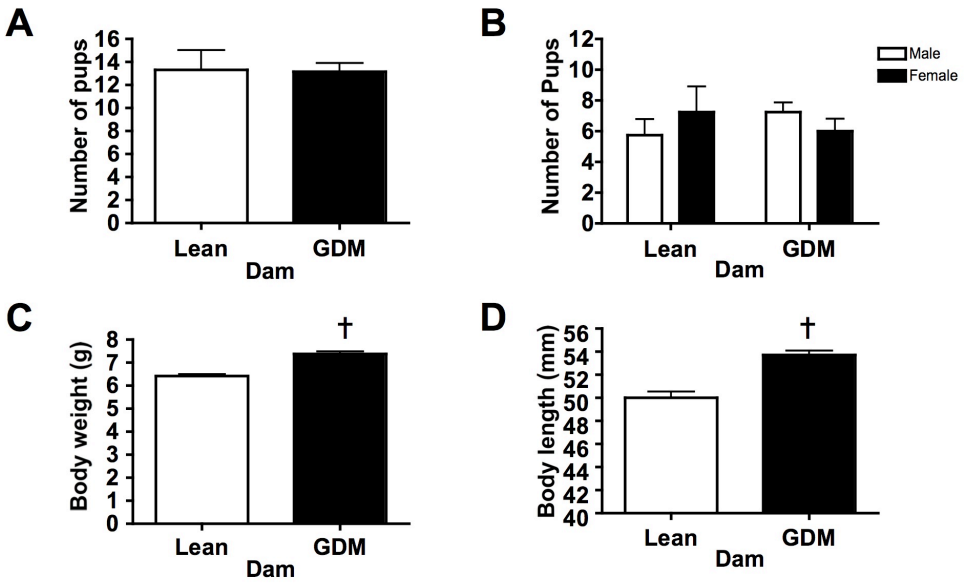
**p-values represent significant differences (<0.05) between maternal environments: Lean vs. GDM by student's t-test (n=6)*

Figure 2.1 Effect of a maternal high-fat and sucrose diet (HFS) on maternal body composition and metabolic parameters during pregnancy



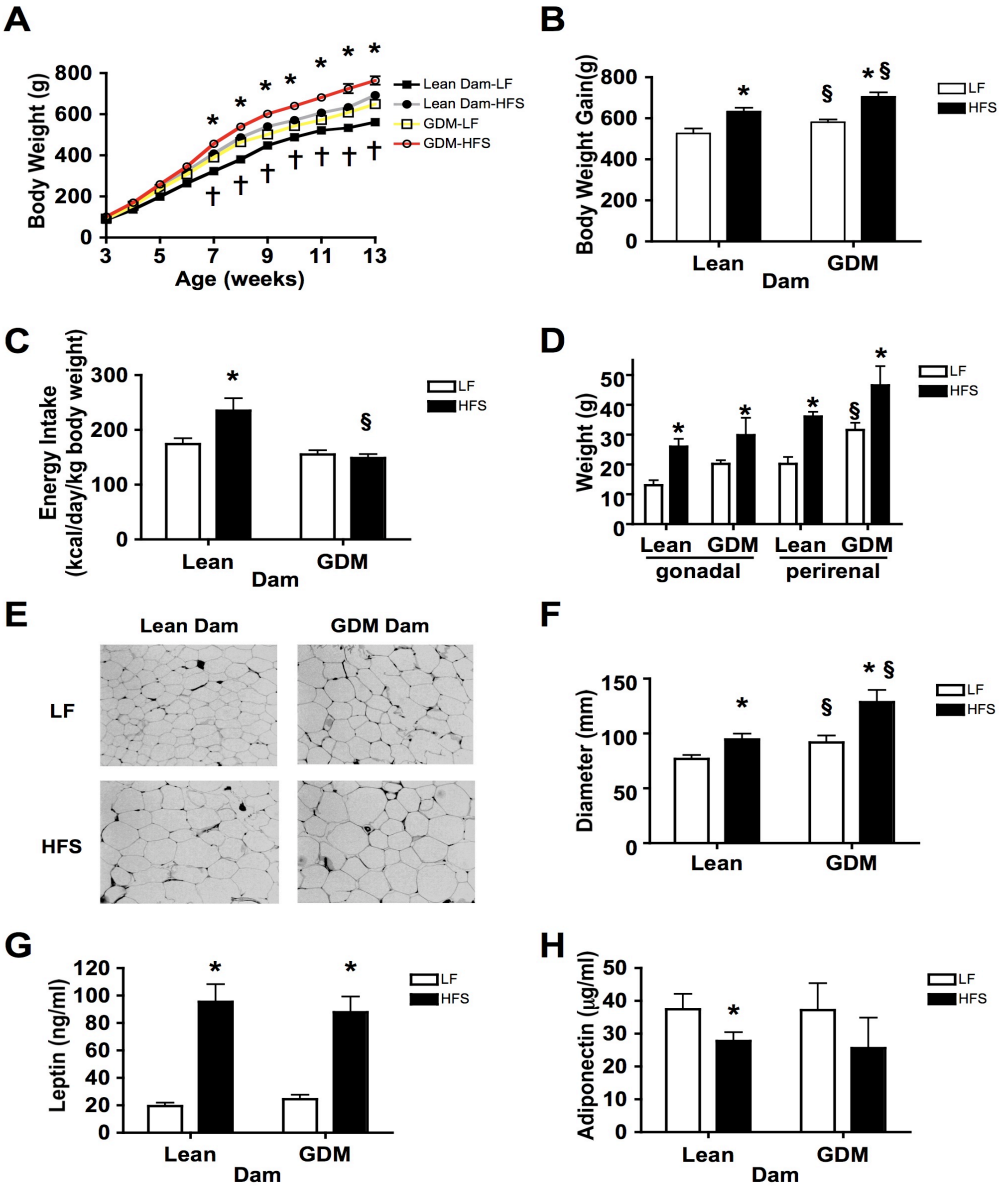
*(A) Change in body weight over time, (B) Gestational weight gain and (C) Energy intake adjusted by body weight. (D) Pre-mating & Mid-gestational blood glucose levels, (E) Glucose tolerance test, (F) Plasma insulin, (G) HOMA Index, and (H) Plasma adiponectin levels. *p-values represent significant differences (<0.05) between LF: Low fat diet (10% fat 3.85 kcal/g), HFS: (45% fat, 4.73 kcal/g) groups by student's t-test (n=6 by group).*

Figure 2.2 Effect of maternal GDM on newborn rat pup offspring body composition and metabolic parameters



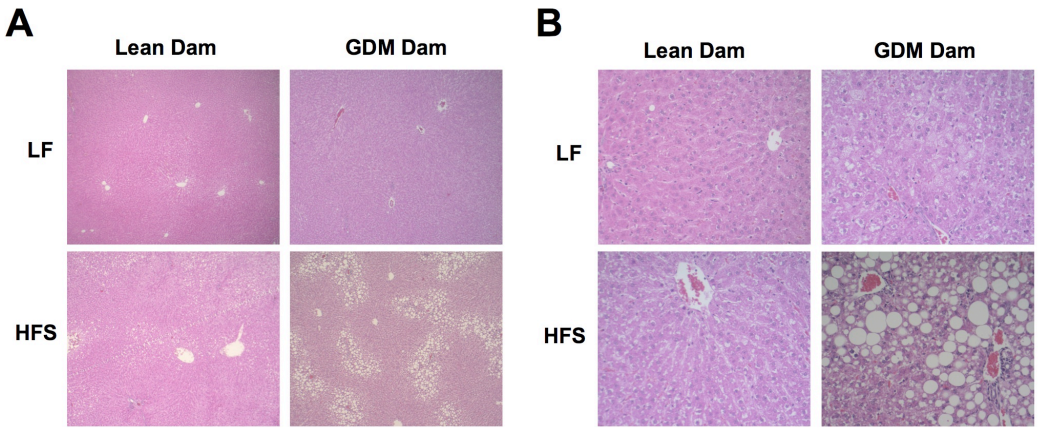
*(A) Litter size, (B) Gender distribution, (C) Pup birth weight, (D) Body length at birth, *p-values represent significant differences (<0.05) between maternal environments: Lean: Low fat diet (10% fat 3.85 kcal/g), GDM: High fat and sucrose diet (45% fat, 4.73 kcal/g) by student's t-test (n=6 by group).*

Figure 2.3 Effect of maternal GDM and postnatal diet on body composition



*(A) Change in body weight over time, (B) Calculated body weight gain (C) absolute energy intake adjusted by body weight, (D) Individual fat pad weights adjusted to tibia length. (E) Representative histological images of perirenal fat tissue (magnification 40X), (F) Average intra-abdominal adipocyte diameter. (G) Plasma leptin and (H) adiponectin concentrations. LF: Low fat diet (10% fat 3.85 kcal/g), HFS: High fat and sucrose (45% fat, 4.73 kcal/g) diet. p-values next to each label represent the significance in the effect for each source of variation (postnatal diet or GDM) as calculated by ANOVA. *p<0.05 in Bonferroni post-hoc test comparing GDM and control offspring receiving the same diet (n=6 by group)*

Figure 2.4 Effect of maternal GDM and postnatal diet on hepatic metabolism in the offspring



C

Macrovesicular steatosis scores

	0	1	2	3
Lean-LF	87.5%	12.5%	0%	0%
GDM-LF	0%	100%	0%	0%
Lean-HFS	14.3%	85.7%	0%	0%
GDM-HFS	20%	40%	20%	20%

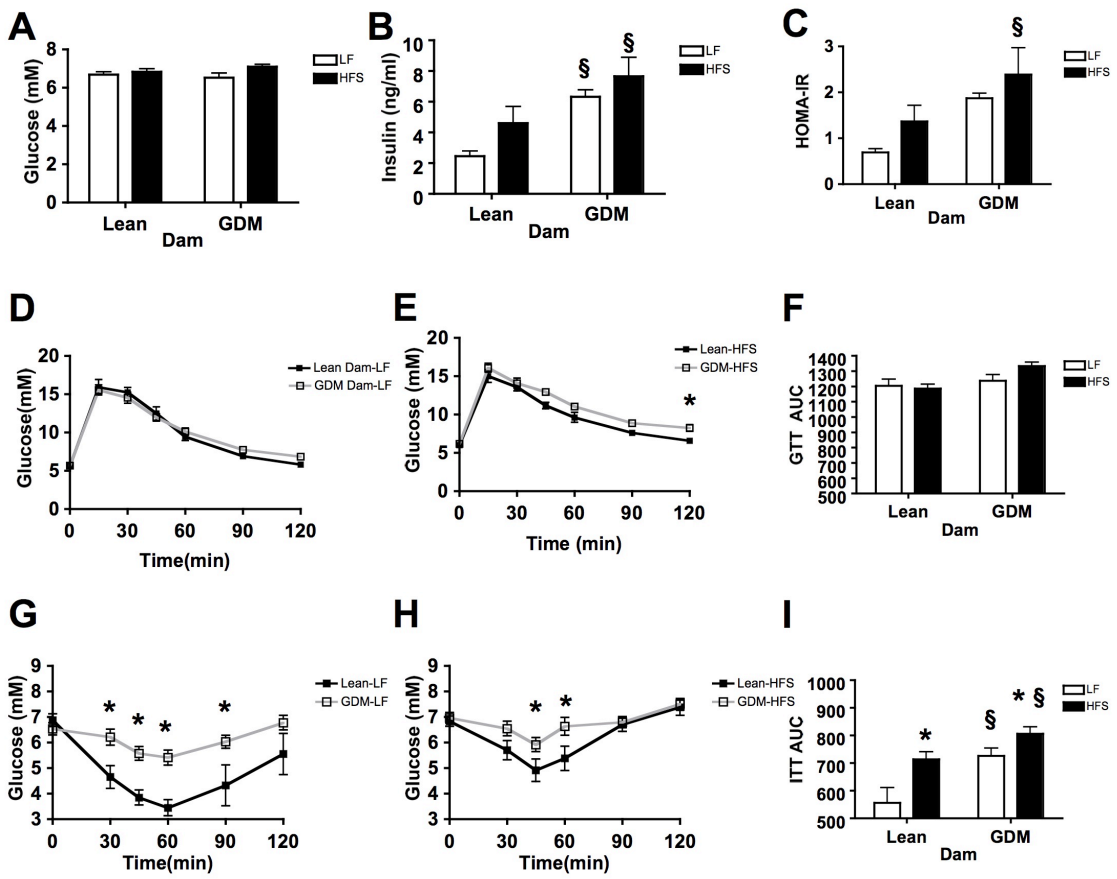
D

Microvesicular steatosis scores

	0	1	2	3
Lean-LF	62.5%	25%	12.5%	0%
GDM-LF	0%	100%	0%	0%
Lean-HFS	0%	83%	17%	0%
GDM-HFS	0%	40%	20%	40%

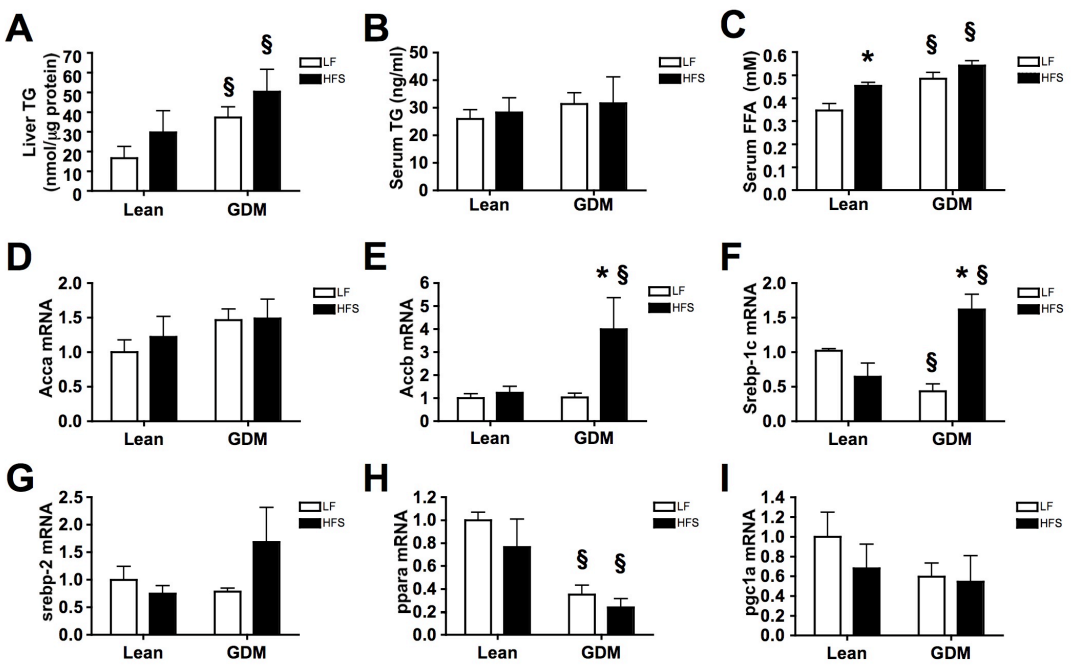
(A) 2x and (B) 40x Representative HE-stained liver sections (C) Macrovesicular steatosis scores. (D) Microvesicular steatosis scores. (n=6)

Figure 2.5 Effect of maternal GDM and postnatal diet on glucose homeostasis in the offspring



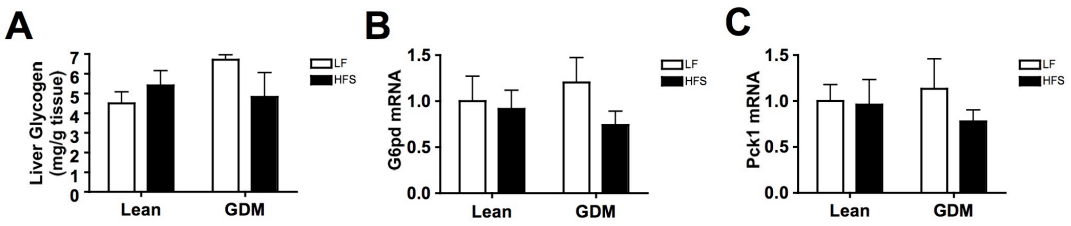
*(A) Fasting blood glucose and (B) Fasting plasma insulin concentrations, (C) HOMA index, (D and E) glucose tolerance test (GTT), (F) area under the curve (AUC) of the GTT, (G and H) insulin tolerance test (ITT) and (I) AUC of the ITT. 12-week old offspring fed a low fat (LF; 10% fat 3.85 kcal/g) or a high fat and sucrose (HFS; 45% fat, 4.73 kcal/g) diet from weaning. p-values next to each label represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA. P-values represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA. *p<0.05 in Bonferroni post-hoc test comparing postnatal LF vs. HFS diet groups and §p<0.05 in Bonferroni post-hoc test comparing GDM and control offspring receiving the same diet (n=4-6 by group).*

Figure 2.6 Effect of maternal GDM and postnatal diet on hepatic lipid metabolism in the offspring



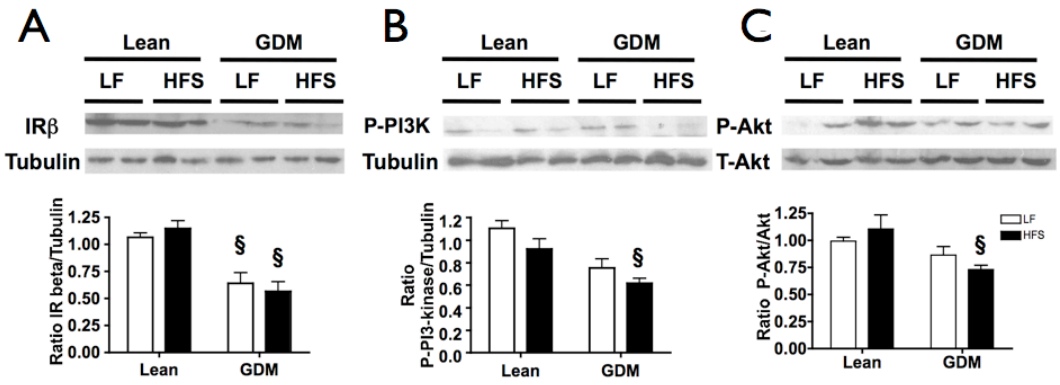
*(A) Liver triacylglycerol (TG) levels, (B) Serum TG and (C) free fatty acid (FFA) levels. (D) Acetyl-CoA carboxylase-1 (Acca) and (E) Acc-2 (Accb) mRNA expression in liver. (F) Sterol response element binding protein (Srebp)-1c and (G) Srebp-2 mRNA expression in liver. (H) Peroxisomal proliferator activated protein receptor-α (Ppara) and (I) PPAR-Coactivator-1α (Pgc1a) mRNA expression in liver. Gene expression was evaluated by quantitative real-time PCR and normalized to the eukaryotic inititation factor 2a housekeeping gene. P-values represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA. *p<0.05 in Bonferroni post-hoc test comparing postnatal LF vs. HFS diet groups and §p<0.05 in Bonferroni post-hoc test comparing GDM and control offspring receiving the same diet (n=4-6 by group).*

Figure 2.7 Effect of maternal GDM and postnatal diet on hepatic glucose metabolism in the offspring



(A) Liver glycogen levels, (B) Glucose-6-phosphate dehydrogenase (G6pd) mRNA expression and (C) Phosphoenolpyruvate carboxykinase mRNA expression (Pck1). Gene expression was evaluated by quantitative real-time PCR and normalized to the eukaryotic inititation factor 2a housekeeping gene. (n=4-6 by group).

Figure 2.8 Effect of maternal GDM and postnatal diet on glucose homeostasis in the offspring



*(A) Levels of insulin receptor(IR)-β protein normalized to tubulin. (B) Levels of phosphorylated tyrosine-458 phosphatidylinositol 3-kinase (P-PI3K) normalized to tubulin. (C) Levels of phosphorylated serine-473 Akt (P-Akt) were normalized to total Akt. p-values next to each label represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA. P-values represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA. *p<0.05 in Bonferroni post-hoc test comparing postnatal LF vs. HFS diet groups and §p<0.05 in Bonferroni post-hoc test comparing GDM and control offspring receiving the same diet (n=6 by group).*

2.5 Discussion

Women with pre-existing obesity are at elevated risk for the development of GDM and giving birth to large-for-gestational-age infants (Taylor, 2007). The majority of rodent studies use STZ administered during pregnancy in order to induce insulin deficiency and a diabetic pregnancy (Jawerbaum & White, 2010); however, GDM is mostly characterized by the inability of the islet cells to adapt to the enhanced insulin resistance at mid-gestation rather than insulin deficiency. The consumption of a high (60%, kcal) fat diet (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Dudley *et al*., 2011; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012; Taylor *et al*., 2005) or a "cafeteria"-style diet (Holemans *et al.*, 2004; Kjaergaard *et al*., 2014) by pregnant rats has been used as a model of maternal obesity. Unlike the preceding studies, we initiated the HFS diet prior to mating in order to induce weight gain and glucose intolerance before gestation. The HFS diet was continued throughout pregnancy and we observed the development of hyperglycemia that returned to normoglycemic levels following birth of the litter. Using this approach, GDM was likely a consequence of the additive effects of pre-existing glucose intolerance and pregnancy that resulted in elevated gestational weight gain, mid-gestational hyperinsulinemia and insulin resistance, as well as larger newborn pups, which are all characteristic of human GDM. (Catalano *et al*., 1991; Catalano *et al*., 1999)

While maternal high-fat diet feeding was used to investigate the effect of maternal obesity on offspring (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Dudley *et al*., 2011; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012; Taylor *et al*., 2005;

Holemans *et al.*, 2004; Kjaergaard *et al*., 2014), the present study is the first to investigate the influence of GDM on the postnatal response to LF and HFS diets in young rat offspring. These preceding studies of maternal obesity reported different effects on birth weights, with some studies suggesting that maternal obesity increased birth weight, (Kjaergaard *et al*., 2014; Ogden *et al*., 2006) reduced birth weight (Dudley *et al*., 2011; Howie *et al.*, 2009), or had no effect (Buckley *et al.*, 2005; Caluwaerts *et al.*, 2007; Holemans *et al.*, 2004; Sun *et al.*, 2012; Taylor *et al.*, 2005). These observations could be due to differences in the amounts of fat and sucrose in the respective diets or the timing of the diets that resulted in the variability in maternal fasting plasma glucose and insulin levels in the maternal obesity models reported in the preceding studies. On the other hand, the utilization of STZ during pregnancy consistently reduced fetal weight, regardless of the dosage and also increased fetal mortality and birth defects (Jawerbaum & White, 2010). We report that in our model of HFS diet-induced GDM, we consistently observed an increased weight and length of newborn rat pups, likely as a response to maternal hyperinsulinemia and the increased availability of calories from saturated fats and simple sugars through the maternal consumption of the HFS diet. While we observed that the male offspring were more sensitive to the development of obesity than the female offspring, we terminated our experiments when the rats were young adults and longer-term future studies will investigate whether female GDM offspring also develop a more severe metabolic syndrome later in life than the female offspring of lean dams. Since human GDM is associated with poor metabolic health outcomes for the offspring (Gillman *et al*., 2003; Moore, 2010; Reece *et al*., 2009) our data present a very attractive

model for future investigations of the biological and molecular effects of GDM on the offspring.

There is a clear link between the accumulation of lipids in the tissues and the development of insulin resistance (McGarry, 2002; Sinha *et al*., 2002). The fetal liver and adipogenesis are key targets of altered in utero conditions (Symonds *et al.*, 2009). The data presented herein identified putative fetal programming of key genes involved that stimulate hepatic lipid synthesis (*Accb, Srebp-1c*) and reduced the expression of a major regulator of lipid oxidation (*Ppara*) in the offspring of GDM dams that appeared to mediate the development of hepatic steatosis in the offspring of GDM dams. Previous reports showed that maternal diet-induced obesity during pregnancy increased the percent body fat content (Buckley *et al*., 2005; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012) as well as liver TG content (Buckley *et al*., 2005) in adult chow-fed offspring. Our study is the first to investigate the influence of prenatal GDM and the postnatal response to LF and HFS diets on hepatic steatosis in the offspring, as well as identify genes that contributed to GDM-induced hepatic TG accumulation. The liver fat content of adolescents is a significant determinant of insulin sensitivity independent of whole body and visceral fat mass (Wicklow *et al.*, 2012). In agreement with this observation, we showed that the young adult offspring of GDM dams had impaired insulin sensitivity, which was further impaired by the postnatal consumption of a HFS diet. In addition, reduced expression of insulin receptor-β in the offspring of GDM dams appeared to have significant effects on the phosphorylation/activity of downstream

signalling kinases in the insulin signal transduction pathway (ie- phosphatidylinositol 3-kinase and Akt). Therefore, our data show that the postnatal consumption of the HFS diet by the offspring of GDM dams increased the severity (i.e.- accelerates the onset) of obesity, hyperinsulinemia, insulin resistance and fatty liver. These observations demonstrate that the interaction between GDM and the postnatal diet contribute to the early onset of dyslipidemia and insulin resistance in the rat offspring.

Collectively, our findings highlight the influence of pre-pregnancy weight on the development of GDM as well as the influence of GDM on the development of obesity, hepatic steatosis and insulin resistance in young adult offspring. In developed countries, 15-20% of reproductive age women are obese and >40% of women gained excessive weight during their pregnancy. (Seidell, 2000) Correspondingly, the prevalence of GDM has increased. (Gaillard *et al*., 2013; Holemans *et al.*, 2004) This trend has put increasing numbers of the children at risk for obesity and could contribute to increases in the pediatric incidence of T2D (Franks *et al*., 2006; Shields, 2006; Young *et al*., 2002). Our findings emphasize the importance of treating GDM as a preventative measure against poor metabolic health outcomes in children.

Conflict of Interest

None.

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CHAPTER 3 -METABOLOMIC & PHOSPHOLIPID ANALYSIS

3.1 INTRODUCTION

3.1.1 Hepatic Steatosis, Lipotoxicity and Insulin Resistance

The consumption of "western-style" diets high in saturated fats and simple sugars plays a major role in the development of obesity, the MetS and T2D (Canale *et al*., 2013; Halton *et al*., 2006; Odermatt, 2011). It has also been established that excess visceral adiposity and the subsequent harboring of lipids in non-adipose tissues, such as liver, leads to the development of tissue steatosis indicative of non-alcoholic fatty liver disease (NAFLD).

NAFLD is defined by a total fat-to-tissue weight ratio between 5% and 10%. (Neuschwander-Tetri & Caldwell, 2003). This condition can progress to steatohepatitis, hepatocellular carcinoma and eventual liver failure and death. (Farrell & Larter, 2006) NAFLD does not effect the entire obese population, although it has been suggested that between 20-30% of the American population suffers from NAFLD. (Machado *et al*., 2006 ; Clark *et al*., 2003)

The accumulation of TAGs within the liver plays a small role in the development of insulin resistance, which may progress to T2D, especially in children (Grundy *et al*., 2004; Wicklow *et al*., 2012; Samson & Garber, 2014; Lee *et al*., 2014). TAGs are formed mainly through a conjugation of three fatty acid chains to a glycerol backbone via the Kennedy pathway. While TAGs are a marker of tissue steatosis, on their own, they are largely inert and do not interfere with insulin sensitivity. On the other hand, evidence suggests that the accumulation of lipotoxic lipids such as diacylglycerols (DAGs) and

sphingolipids interfere with the insulin signal transduction cascade and cause insulin resistance.

3.1.2 Diacylglycerol (DAG)

The *de novo* synthesis of DAG is initiated by a double acylation event between glycerol-3-phosphate and acyl-coenzyme A (acyl-CoA) to form phosphatidic acid. Phosphatidic acid is subsequently de-phosphorylated to make DAG. DAGs also play a crucial role in TAG synthesis through diacylglycerol acyltransferase (DGAT). DAGs can also be formed when TAGs are broken down to generate glycerol and fatty acids for energy production by TAG lipase. (Choi & Diehl, 2008; Nagle *et al.*, 2009)

Recently, there has been evidence to suggest that DAGs play a crucial role in insulin resistance in both skeletal muscle and liver tissue by activating protein kinase C (PKC) (Samuel *et al*., 2004; Weiss *et al*., 2003).

Samuel & Shulman performed a study in which rats were fed a short-term high fat diet. This short-term feeding was seen to elevate the DAG content of the liver and was also seen to increase PKC-ε activation. Furthermore, there was a significant decrease in insulin receptor substrate-2 (IRS-2) phosphorylation and activation indicative of dysfunction in the insulin signaling pathway (Samuel & Shulman, 2012). A follow-up study was performed utilizing rats that were treated with a PKC-ε antisense oligonucleotide to knock down its activity *in vivo*. Results showed a significant decrease

in insulin resistance within liver tissue even when levels of DAGs and TAGs were not significantly changed (Samuel & Shulman, 2012). Neschen *et al*. used a knockout mouse model lacking the gene for mitochondrial glycerol-3-phosphate acyltransferase, thus blocking DAG synthesis within the liver. These mice were shown to maintain insulin sensitivity. (Neschen *et al*., 2005) Savage et al. similarly used antisense oligonucleotides to knockdown acetyl-CoA carboxylase 1 and 2 (ACC-1/ACC-2) in rats, thus increasing fatty acid oxidation. This increase in fatty acid oxidation led to a decrease in liver DAG content, decrease in PKC-ε activity and overall maintenance of insulin sensitivity (Savage *et al*., 2006). Thus, there is a clear association that can be made between increased DAG accumulation within the liver and the development of insulin resistance.

3.1.3 Sphingolipids

Sphingolipids also have an important role in hepatic steatosis development. Sphingolipids are components of eukaryotic membranes that act to form a stable outer leaflet of plasma membranes and have also been shown to play a role in regulating various signal transduction pathways, including the insulin signaling pathway (Ohanian & Ohanian, 2001 ; Bartke & Hannun, 2009). Ceramide, glycosphingolipid and sphingosine are sphingolipids that have been implicated in T2D development (Langeveld & Aerts, 2009 ; Summers, 2006).

High levels of sphingolipids are consistently observed in animal models of obesity. Yetukuri *et al.* showed that the degree of liver steatosis seen in *ob/ob* mice was correlated to the amount of ceramide (Yetukuri, 2007). Mechanistically, it has been proposed that

increased liver lipid content along with increased inflammatory cytokine production drives sphingolipid formation (Day & James., 1998; Day & Saskena, 2002). Obese rodent models showed a significant elevation in both sphingolipids and tumor necrosis factor - alpha (TNF-α) compared to non-obese controls (Turinsky *et al.*, 1990 ; Xu *et al*., 2003 ; Li *et al*., 2005). A rat model studying the correlation between saturated fats and sphingolipid production showed that after a 6 hour lard oil infusion there was a 60% increase in liver ceramide levels compared to control rats (Holland *et al.*, 2007). Human studies have also shown that inflammatory levels of TNF-α were significantly higher in patients with NAFLD compared to controls.

Aside from the direct link made between sphingolipid accumulation and NAFLD, there is also data suggesting these lipids, especially ceramide, can have a direct role in T2D development primarily by causing β-cell apoptosis in response to inflammation and high fat diets. (Lang *et al*., 2011; Chandra *et al*., 2001)

Inflammatory cytokines, such as TNF-α and interleukin-1 beta (IL-1β) are known to have cytotoxic effects on the β-cells of the pancreas and ceramide synthesis is activated by these inflammatory cytokines (Kim & Lee, 2009; Lang *et al*., 2011). Studies performed by Ishizuka *et al*. and Sjoholm have demonstrated that exposing MIN6, insulin producing, cells to TNF-α and interleukin-1-beta (IL-1β) *in vitro* resulted in increased ceramide levels. Furthermore, endogenous generation or also exogenous delivery of

ceramide had similar cytotoxic effects on β-cells as the aforementioned cytokines through endogenous generation or also exogenous delivery (Ishizuka *et al*., 1999; Sjoholm 1995).

β-cells are also sensitive to free fatty acids (FFA) during periods of extended exposure and ceramide has also been implicated in this mechanism of induced apoptosis via FFA in these cells. In fact, a precursor of ceramide, palmitate, has been shown to have apoptotic effects on pancreatic islets in various rat models of diabetes. Interestingly, these results are also similar in healthy rats and in humans (Shimabukuro *et al*., 1998; Maedler *et al.*, 2001; Lupi *et al.*, 2002).

Sphingolipids have become of particular interest in the story of NAFLD and T2D pathogenesis and future experiments are required to more rigorously to tease out exactly how they act in vivo.

3.1.4 Phosphatidylethanolamine

Phosphatidylethanolamine (PE) is a phospholipid residing on the inner leaflet of the plasma membrane in eukaryotic cells that have a role in membrane fusion and cell division (Emoto *et al.*, 1996). De-novo synthesis of these phospholipids occurs via the PE-Kennedy pathway ending with the conjugation of cytidine diphosphate (CDP) - ethanolamine to DAG and formation of PE. PE can also be synthesized when phosphatidylserine is decarboxylated (Fullerton *et al*., 2009). It has been shown, by Fullerton *et al*. that cytidine triphosphate (CTP):phosphoethanolamine

cytidylyltransferase (Pcyt2) is the enzyme responsible for the synthesis of CDP-ethanolamine and when this enzyme is made deficient in a mouse model (Pcyt2(+/-)) it hinders PE biosynthesis leading to an increase in DAG and subsequent increase in TAG formation. As a result, fatty liver, hyperlipidemia, obesity and insulin resistance characteristic of MetS develops in these mice suggesting an important role in proper regulation of PE biosynthesis via Pcyt2 has an important role in liver metabolism (Fullerton *et al*., 2009; Pavlovic & Bakovic, 2013).

3.1.5 Phosphatidylinositol

Phosphatidylinositols (PIs) are important cytosolic membrane phospholipids responsible for controlling and regulating many signal transduction pathways, cytoskeletal development, and membrane trafficking among other functions. Phosphatidylinositol is synthesized utilizing cytidine diphosphate DAG and *myo*-inositol (Gardocki *et al*., 2005). The phosphorylation of these PIs at the 3,4 or 5 position of the inositol rings results in phosphoinositide formation. There are seven possible phospoinositide species that mediate signal transduction by binding cytosolic portions of membrane proteins or cytosolic proteins themselves (Cantley, 2002; Berridge & Irvine, 1989).

Phosphatidylinositol-3-kinases (PI3Ks) are of particular importance when discussing insulin signaling and T2D pathogenesis. There are three classes of PI3Ks that play a regulatory role in growth and metabolism, however, class IA PI3K functions to regulate insulin signaling (Engelman *et al*., 2006). It is known that dysfunction and overall depletion in IA PI3K downstream of the insulin receptor plays a role T2D development

(Taniguchi *et al*., 2006). The PI3K-Protein Kinase B (AKT) signaling pathway is crucial in regulating downstream response to activation of the insulin receptor (IR) and insulin receptor substrate (IRS) molecules. It has been previously shown that the use of PI3K inhibitors can act to block GLUT4 translocation to the membrane for glucose uptake (Zhou *et al*., 2004). It is also known that in the liver, this signaling pathway acts to inhibit glucose production and release into the circulation by inhibiting the transcription of gluconeogenic genes (Thong *et al*., 2005).

In addition, dysregulation of myoinositol synthesis, which is an important precursor of the signaling molecules mentioned above, could also contribute to insulin resistance. For example, Nissen *et al*. reported a higher plasma level of myo-inositol in piglets from intrauterine growth restricted (IUGR) pregnancies comparative to high weight offspring (Nissen *et al*. 2011). Dessi *et al*. performed metabolomic analysis on the urine of 26 human IUGR neonates and compared the metabolite profile to 30 control neonates from normal births. Myo-inositol was also observed to be significantly higher in the urine of IUGR neonates comparative to control neonates (Dessi *et al*., 2011). There is current evidence that IUGR predisposes offspring to development of metabolic disorders later in life and dysregulation in myo-inositol could effect the insulin signaling cascade causing insulin resistance and eventual T2D. Thus, adverse fetal programming of reduced PI biosynthesis and phosphoinositide formation through PI3K can have negative effects on insulin signaling and is known to play a role in T2D pathology.

3.1.6 General Hypothesis

Studies that investigate the fetal programming effects of GDM on the entire lipid metabolome of the livers of offspring from a diabetic pregnancy have not been performed. Given the important role of the liver in regulating lipid metabolism as well as controlling blood glucose levels, our primary aim was to perform the first metabolomics analysis of lipid levels in the livers of the young adult offspring of GDM dams via liquid chromatography-tandem mass spectroscopy. We hypothesize that gestational exposure to diabetes will increase hepatic levels of lipotoxic lipids and reduce levels of lipids necessary for the insulin signaling cascade in the offspring.

3.2 METHODS

3.2.1 Extraction of Water Soluble Compounds

Adapted from Koutsidis et al. (2008)

Analysis of total liver tissue lipids was performed using a lipid soluble extraction as described in detail below, performed in duplicate and stored at -80C for further analysis. Frozen rat liver tissue from 15 week old male offspring was crushed into powder form and homogenized in deionized water. Norvaline (1.5mg/ml deionized water) was added to the homogenate as an internal standard and shaken by hand vigorously for 5 minutes. Centrifugation for 30 minutes at 3000 g at 4°c followed. The supernatant was decanted and filtered through Whatman 54 filter paper into new tubes. Remaining residues were reconstituted with deionized water and underwent centrifugation for 15 minutes at 3000 g at 4°c. Extracts were combined and filtered a second time through Whatman 54 filter paper into new tubes. 1mL of filtered extract was transferred to a 3000 Da molecular weight cut-off ultrafiltration tube and centrifuged for 4 hours at 3000 g at 4°C. The filtrate was collected and vacuum-dried prior to metabolomic analysis.

3.2.2 Extraction of Lipid Soluble Compounds

As described by Folch, Lees & Sloane (1957)

Frozen rat liver tissue from 15 week old male offspring was crushed into powder form and homogenized in 0.025% anyhydrous calcium chloride solution. Homogenate was transferred to 15mL glass tubes and nonadecanoic acid (C19:0 ; 100μg/ml) was added as an internal standard. This solution was vortexed. A chloroform and methanol solution

(C:M, 2:1, v:v) was added to each tube and centrifuged for 12 minutes at 2000rpm at 4°C. After centrifugation, samples were left at 4°c for 2 hours to achieve phase separation. The lower phase (chloroform and lipids) was transferred to a new, pre-weighed, 15mL glass tube and dried down under N2 gas with low heat (45-50°c). Total lipid weight was calculated by subtracting the original weight of the glass tube from the final weight of the tubes with dried lipid. To make sure no tissue was incorporated into the weight of total lipids, two subsequent rinsing steps were performed with C:M (2:1) and dried down under N2 gas with low heat (45-50°C). A 1:1, C:M solution was added to each tube before liquid chromatography tandem mass spectroscopy (LC/MS) analysis.

3.2.3 LC-QTOF-MS Analysis

Performed by Dr. Michel Aliani, St. Boniface Research Center

Analysis of total liver tissue lipids was performed using a lipid soluble extraction as described previously (Folch et al., 1957), in duplicate and stored at -80C until further analysis. Prior to analysis, liver lipid extracts were reconstituted with 100 µL of 80% acetonitrile prepared in deionized water. Metabolomics analysis was performed on an 1290 Infinity Agilent HPLC system coupled to a 6538 UHD Accurate Q-TOF LC/MS from Agilent Technologies (CA, USA) equipped with a dual electrospray ionization (ESI) source. A 3 x 50 mm, 2.7µ Agilent Poroshell column (Agilent Technologies) was used to separate metabolites while the column temperature was maintained was at 60°C. The mobile phases A and B were water and acetonitrile, with 0.1% formic acid. A sample size of 2 µL was injected by maintaining the HPLC flow rate at 0.7 mL/min with a gradient program of: 0, 0.5, 16, 17 and 22 min with 30, 30 100, 100 and 30% of solvent B,

respectively. A post-run time of 2 min was buffered before injecting the next sample. The auto-sampler was maintained at a temperature of 15°C. The mass detection was operated using dual electrospray with reference ions of m/z 121.050873 and 922.009798 for positive mode; and m/z 119.03632 and 980.016375 for negative mode. The main parameters for MS were as followed: gas temperature, 300°C; drying N2 gas flow rate, 11L/min; Nebulizer pressure, 50 psig; fragmentor voltage, 175V; skimmer voltage 50V and OCTRF Vpp voltage, 750V. Targeted MS/MS mode was used to identify the potential biomarkers. As part of the MassHunter Software, the collision energy was applied by setting an appropriate equation having a slope value of 5 and offset value of 2.5. A full range mass scan from 50-3000 m/z with an extended dynamic range of 2 GHz standardized at 3200 was applied. Data acquisition rate was maintained at the rate of 3spectra/s at a time frame of 333.3 ms/spectra with a transient/spectrum ratio of 1932.

3.2.4 Phospholipid Extraction

Prior to running a phosphorus assay, lipids were extracted from liver tissue samples (2mg protein, 1ml PBS, 5ml C:M, 2:1). This mixture was vortexed and subsequently centrifuged for 20 minutes at 2000rpm at 4°C. After centrifugation, the bottom phase was extracted and dried down under N2 gas with low heat (45-50°c). After drying, samples were reconstituted in chloroform and loaded on a thin layer chromatography (TLC) plate. The TLC plate runs for 1.5 hours in the following solvent system to separate various phospholipid species (70/30/12/4/1) (chloroform, methanol, acetic acid, formic

acid, water). Lipids on the TLC plate were then stained with iodine for visualization and scraped.

3.2.5 Phosphorous Assay

As described by Rouser et al. (1966)

Performed by Dr. Laura Cole, Manitoba Institute of Child Health

Standards were placed in individual tubes marked 0, 5, 10, 25, 50, 100, 200ul and subsequently dried in a heat block at 180°C for 5 minutes or until dry. The standard range is 10-200 nmoles. 70% perchloric acid was added to the dried phospholipid samples and standards and again heated at 180°C for 2 hours. After the samples and standards have been allowed to cool, water was added to each tube. Ammonium molybdate was then added and vortexed immediately. Next, ascorbic acid was added and vortexed immediately. The samples and standards were then incubated in a hot (95°C) water bath for 15 minutes. Samples and standards were allowed to cool and then centrifuged at 2000rpm for 5 minutes at room temperature. Samples and standards were placed into a 96 well plate and absorbance is read at 820 nm.

3.3 RESULTS

Effects of Maternal GDM on Liver Lipid Soluble Metabolites

Offspring liver extractions were analyzed via liquid chromatography-tandem mass spectroscopy (LC-QTOF-MS) for variations in metabolite profile between offspring born to either GDM or lean dams, therefore creating two groups for analysis. To further investigate how in utero exposure to GDM influenced hepatic metabolism we performed unbiased metabolic profiling of over 9000 metabolite entities with specific masses using LC-QTOF-MS analysis of hepatic lipid extracts. Among these entities, 286 had a significant ($p<0.05$) 2-fold change in abundance in the offspring of GDM dams compared to the offspring of lean dams (Figure 3.1). Among these 110 showed a 10-fold change between the two distinct groups (Figure 3.2) The significant amount of change noted between the two groups was further displayed with the generation of a heat map. (Figure 3.3) and indicated a significant and widespread change in the liver lipid metabolite profile with offspring from GDM dams comparative to the offspring from lean dams, regardless of postnatal diet.

Effects of Maternal GDM on Liver Sphingolipid Content

Offspring liver tissue was analyzed via liquid crystalography tandem mass spectroscopy (LC-QTOF-MS) for variations in sphingolipid content. Offspring born to GDM dams, regardless of postnatal diet showed a significant increase in ceramide content. (Table 3.1) Most notably, ceramide (d18:2/16:0) showed an 82.0 fold increase compared to offspring coming from lean control dams. Another ceramide species (d18:0/16:0) showed a 34.0 fold increase compared to offspring coming from lean control dams irrespective of

postnatal diet. Looking at direct dietary group comparisons between LF-fed offspring born to lean dams and HFS-fed offspring born to GDM dams, there was a 160.9 fold increase in ceramide (d18:2/16:0) in HFS fed offspring born to GDM dams. (Table 3.2)

Effects of Maternal GDM on Liver Diacylglycerol Content

Next, we assessed offspring liver tissue, as mentioned above via LC-QTOF-MS for variations in DAG content. There was a significant increase in several DAG species within livers of offspring born to GDM dams compared to those coming from lean control dams. Most notably, DAG (18:1/18:2/0:0) showed a 57.0-fold increase and DAG (18:1/18:3/0:0) showed a 33.8 fold increase in offspring from GDM dams irrespective of postnatal diet compared to those from lean control dams (Table 3.1). Looking at direct dietary group comparisons between LF-fed offspring born to lean dams and HFS-fed offspring born to GDM dams there was an overall 35.7 fold increase in total DAG metabolite content in HFS-fed offspring liver born to GDM Dams. Most notably, DAG species (18:1/18:3/0:0) and (18:0/18:1/0:0) showed a 90.0 and 103.1.0-fold increase, respectively in HFS-fed offspring born to GDM dams compared to LF-fed offspring from lean control dams (Table 3.2).

Effects of Maternal GDM on Liver Phosphatidylethanolamine Content

Next, we assessed offspring liver tissue, as mentioned above via LC-QTOF-MS for variations in PE content. There was a significant decrease in several PE species within livers of offspring born to GDM dams compared to those coming from lean control dams.

Most notably, PE species PE (18:1/18:3) showed a 40.6-fold decrease and PE (20:4/20:4) showed a 14.4-fold decrease in offspring from GDM dams irrespective of postnatal diet compared to those from lean control dams (Table 3.1). Looking at direct dietary group comparisons between LF-fed offspring born to lean dams and HFS-fed offspring born to GDM dams there was an overall decrease in PE species. Most notably, PE (18:1/18:3) showed a 94.8- fold decrease in HFS-fed offspring born to GDM dams comparative to LF-fed offspring born to lean control dams.

Effects of Maternal GDM on Liver Phosphatidlyserine Content

We then assessed offspring liver tissue, as mentioned above via LC-QTOF-MS for variations in PS content. There was a general decrease in PS species within livers of offspring born to GDM dams compared to those coming from lean control dams. PS (18:0/20:2) and PS (18:0/20:3) showed a 3.6 and 2.8 fold decrease in offspring from GDM dams irrespective of postnatal diet compared to those from lean control dams (Table 3.1). Looking at direct dietary group comparisons between LF-fed offspring born to lean dams and HFS-fed offspring born to GDM dams there was an overall decrease in PS species. PS (18:0/20:2) and PS (18:0/20:3) showed a 5.6 and 4.6-fold decrease, respectively in offspring fed HFS diets born to GDM dams compared to LF-fed offspring born to lean control dams. (Table 3.2)

Effects of Maternal GDM on Liver Phosphatidylinositol Content

We then assessed offspring liver tissue, as mentioned above via LC-QTOF-MS for variations in PI content. There was a significant decrease in PI species within livers of offspring born to GDM dams compared to those coming from lean control dams. PI (18:1/20:3) and PI (22:2/16:1) showed a 10.0 and 51.7-fold decrease, respectively in offspring from GDM dams irrespective of postnatal diet compared to those from lean control dams (Table 3.1). Looking at direct dietary group comparisons between LF-fed offspring born to lean dams and HFS-fed offspring born to GDM dams there was an overall decrease in PI species. PI (18:1/20:3) and PI (22:2/16:1) showed a 24.7 and 58.3-fold decrease, respecitvely in offspring fed HFS diets born to GDM dams compared to LF-fed offspring born to lean control dams (Table 3.2).

Effects of Maternal GDM on Liver Phosphorus Assay Results

In order to validate the effects of the LC-QTOF-MS results on overall total phospholipid levels in the liver tissue, standard biochemical analysis by phosphorus assay was performed. Lipids were extracted from offspring liver samples from four distinct dietary groups (offspring from either GDM or lean dams and subsequently fed a postnatal HFS or LF diet) and individual phospholipid species were separated by thin-layer chromatography. The content of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (CL) were determined by a phosphorus assay (Table 3.3).

When looking at PS between Lean/LF and GDM/HFS there was a significant decrease noted with a p-value 0.04454 (t-Test). A significant reduction in PE levels between Lean/ LF and GDM/HFS groups were observed with a p-value of 0.00807 (t-Test). Finally, when looking at CL levels between Lean/LF and GDM/HFS groups there was, again, a significant decrease noted with a p-value of 0.0376 (t-Test). Differences in the levels of PC and PI were not observed between the groups. One-way analysis of variants (ANOVA) was also performed across all groups. One-way ANOVA analysis indicated significance for PE levels across all groups, p-value of 0.0195 (Table 3.3).

Table 3.1 Representative sphingolipid, diacylglycerol, phospholipid metabolic entities altered (< 2-fold) by GDM in the livers of 15 week-old rat offspring as determined by LC-QTOF-MS analysis

Lipid	Formula	$[M + H]^+$	Fold Change (GDM vs Lean)
Ceramide			
Ceramide (d18:2/16:0)	C34H65NO3	536.5052	+82
Ceramide (d18:1/22:0)	C40H79NO3	622.6161	+3.8
Ceramide (d18:1/18:0)	C36H71NO3	566.5549	+15.7
Ceramide (d18:1/24:0)	C42H83NO3	640.6467	+6.7
Ceramide (d18:1/24:1)	C42H81NO3	648.6323	+13.7
Ceramide (d18:0/16:0)	C34H69NO3	540.5392	+34
Diacylglycerol (DG)			
DG (18:1/18:2/0:0)	C39H70O5	619.5312	+57
DG (18:1/18:3/0:0)	C39H68O5	617.5157	+33.8
DG (16:0/22:1/0:0)	C41H78O5	651.5922	+29
DG (16:0/16:1/0:0)	C35H66O5	567.4983	+12.3
Phosphatidylethanolamine (PE)			
PE (14:0/22:0)	C41H82NO8P	748.5869	-40.6
PE (18:3/20:3)	C43H74NO8P	764.5251	-13.5
PE (18:3/20:2)	C43H76NO8P	766.5400	-10.2
PE (20:3/20:4)	C45H76NO8P	790.5385	-3.5
PE (18:2/18:3)	C41H72NO8P	738.5095	-15
PE (20:4/20:4)	C45H74NO8P	788.5243	-14.4
Phosphatidylserine (PS)			
PS (18:0/20:2)	C44H82NO10P	816.5749	-3.6
PS (P-20:0/18:3)	C44H80NO9P	798.5659	-2.8
Phosphatidylinositol (PI)			
PI (18:1/20:3)	C47H83O13P	887.5643	-10
PI (22:2/16:1)	C47H85O13P	889.5781	-51.7

Diacyglycerol (DG), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI) (n=6/group) LC-QTOF-MS analyses, a moderated t-Test

(P<0.05) and volcano plots (> 2-fold changes and P<0.05) were performed using MPP software (version 12.6)

Table 3.2 Representative sphingolipid, diacylglycerol, phospholipid metabolic entities altered (< 2-fold) by GDM in the livers of 15 week-old rat offspring as determined by LC-QTOF-MS analysis

Entity	Lean-LF vs. Lean-HFS	Lean-LF vs. GDM-LF	Lean-LF vs. GDM-HFS
	Fold-change	Fold-change	Fold-change
Ceramide			
Ceramide (d18:2/16:0)	**+1.4**	**+1.7**	**+160.9**
Ceramide (d18:0/16:0)	**+2.8**	**55.6**	**73.5**
Diacylglycerol (DG)			
DG (18:1/18:2/0:0)	**+2.8**	**+35.7**	**+35.7**
DG (18:1/18:3/0:0)	**+2.7**	**+90**	**+90**
DG (16:0/22:1/0:0)	**+4.1**	**+57.7**	**+57**
DG (18:0/18:1/0:0)	**+2.9**	**+9.2**	**+103.9**
DG (16:0/16:1/0:0)	**+3.2**	**+1.5**	**+50.2**
Phosphatidylethanolamine (PE)			
PE (18:1/18:3)	**+2.0**	**-2.6**	**-94.8**
PE (18:3/20:3)	**-4.9**	**-6.5**	**-25.4**
PE (18:3/20:2)	**+1.7**	**-9.6**	**-12.5**
PE (20:3/20:4)	**+2.6**	**-1.1**	**-8.5**
PE (18:2/18:3)	**-3.6**	**-6.2**	**-27.3**
PE (20:4/20:4)	**-4.3**	**-6.3**	**-26.8**
Phosphatidylserine (PS)			
PS (18:0/20:2)	**-1.6**	**-1.6**	**-5.6**
PS (18:0/20:3)	**+3.0**	**-4.0**	**-4.6**
Phosphatidylinositol (PI)			
PI (18:1/20:3)	**-1.8**	**-3.3**	**-24.7**
PI (22:2/16:1)	**+1.1**	**-45.1**	**-58.3**

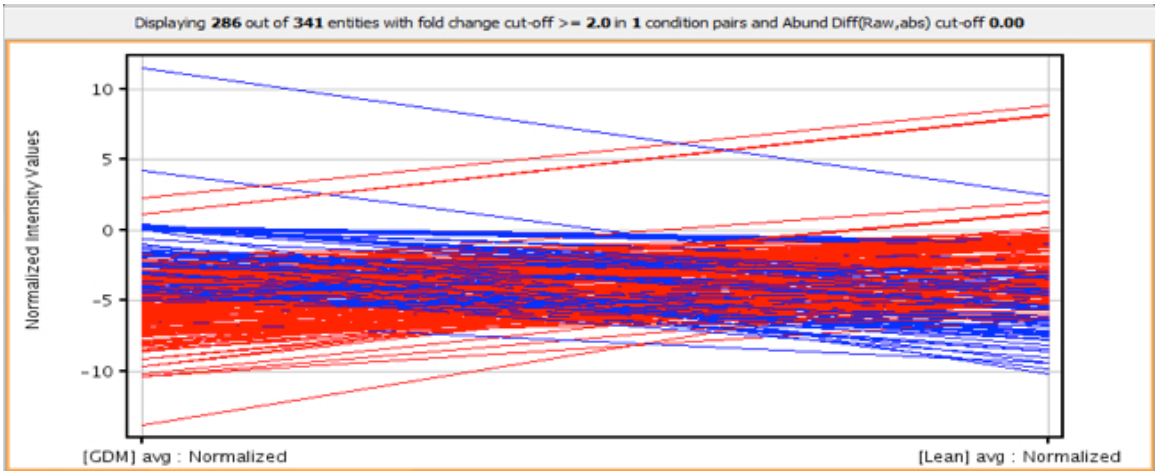
Diacyglycerol (DG), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI) (n=6/group) LC-QTOF-MS analyses, a moderated t-Test (P<0.05) and volcano plots (> 2-fold changes and P<0.05) were performed using MPP software (version 12.6)

Table 3.3 Litter Liver Phospholipid Assay Results Across Each Dietary Group

	PC	PS	PI	PE	CL
	Average nmol/mg	Average nmol/mg	Average nmol/mg	Average nmol/mg	Average nmol/mg
Lean/ LF	175.368	7.847 +/- 2.916	25.169 +/- 5.401	63.733 +/- 15.574	7.337 +/- 1.749
Lean/ HFS	155.224	6.643 +/- 2.165	19.034 +/- 5.420	50.221 +/- 13.017	5.759 +/- 1.525
GDM/ LF	149.072	6.379 +/- 1.848	21.573 +/- 3.333	48.753 +/- 11.232	5.942 +/- 2.090
GDM/ HFS	152.639	5.515 +/- 1.437	21.233 +/- 4.383	46.876 +/- 6.724	5.771 +/- 1.190
	One-Way ANOVA (all groups) p=0.297 t-Test Lean/LF vs. GDM/HFS p=0.07277	One-Way ANOVA (all groups) p=0.1524 t-Test Lean/LF vs. GDM/HFS p=0.04454*	One-Way ANOVA (all groups) p=0.0653 t-Test Lean/LF vs. GDM/HFS p=0.1089	One-Way ANOVA (all groups) p=0.0195 * t-Test Lean/LF vs. GDM/HFS p=0.00807*	One-Way ANOVA (all groups) p=0.1344 t-Test Lean/LF vs. GDM/HFS p=0.0376*

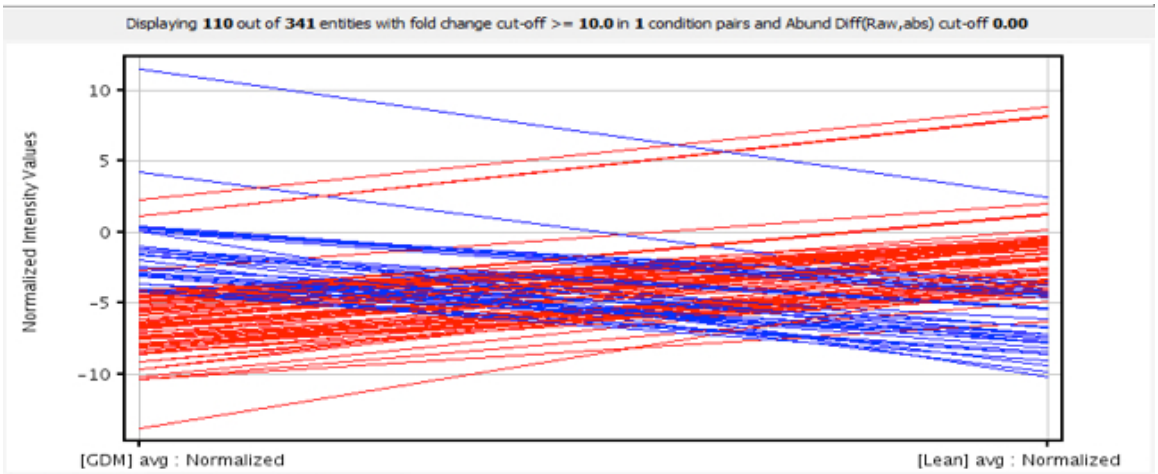
*P-values represent the significance in the effect for each source of variation (diet or GDM) as calculated by ANOVA.and T-Test *p<0.05; Diacyglycerol (DG), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI), LF (Low Fat Diet-Postnatal), HFS (High Fat Diet-Postnatal), Lean (LF fed Dam), GDM (HFS fed Dam) (n=8-10/group)*

Figure 3.1 Litter Liver Metabolites: Fold Change >2.0 Between GDM and Lean Dam Conditions (LC/MS)



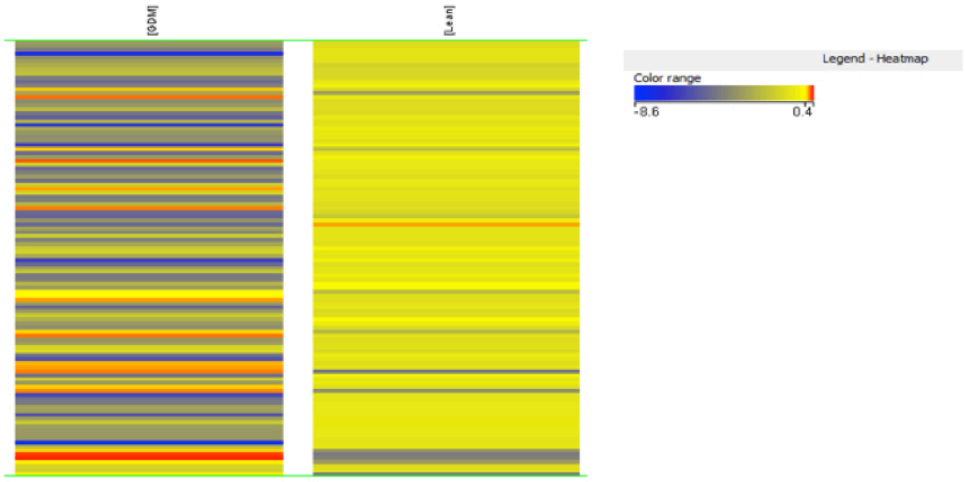
"Entities" = metabolites
Blue indicates a fold decrease in normalized intensity values of metabolites between offspring born to GDM dams and those born to lean dams, Red indicates a fold increase in normalized intensity values of metabolites between offspring born to GDM dams and those born to lean dams. LC-QTOF-MS analyses, a moderated t-Test (P<0.05) and volcano plots (> 2-fold changes and P<0.05) were performed using MPP software (version 12.6)

Figure 3.2 Litter Liver Metabolites: Fold Change >10.0 Between GDM and Lean Dam Conditions (LC/MS)



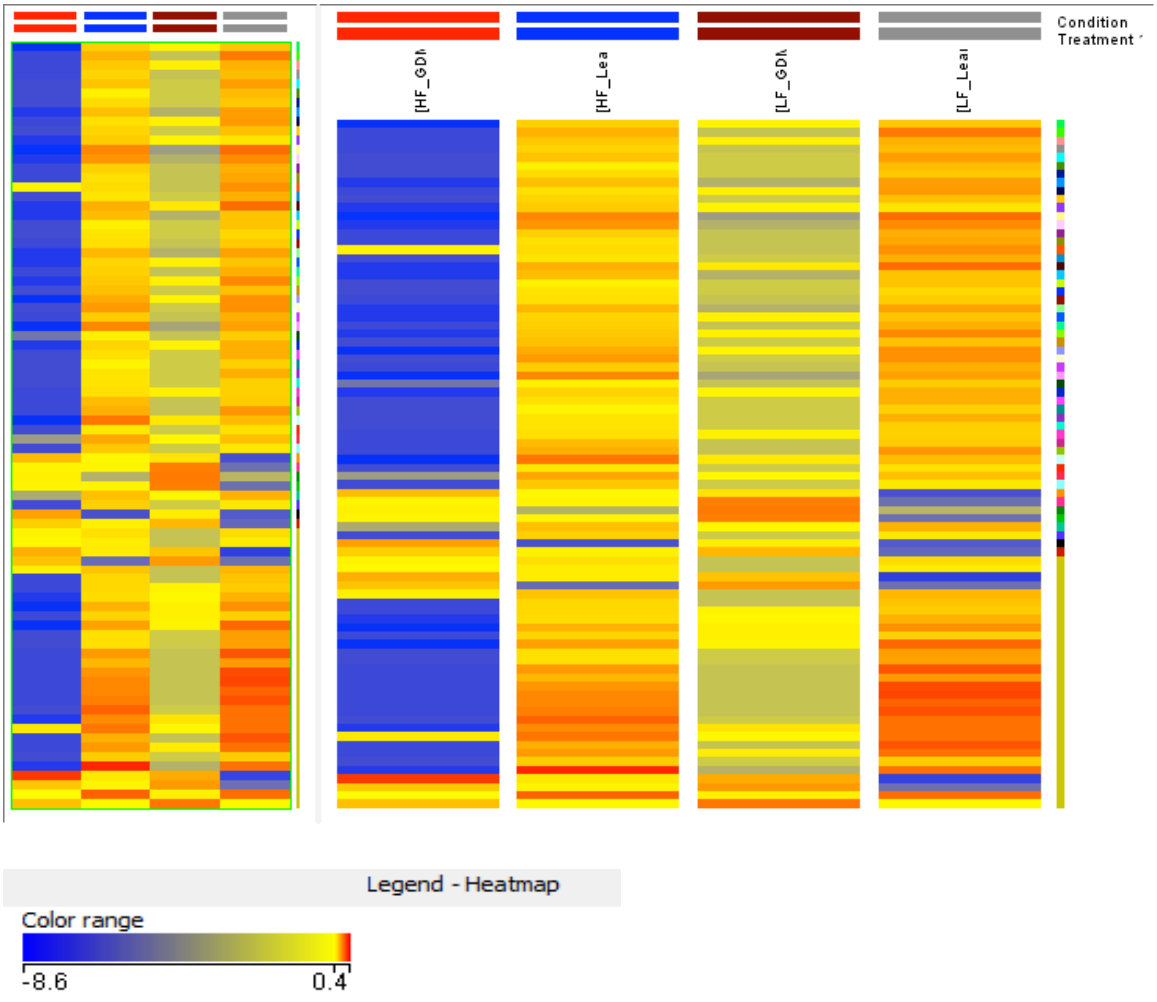
"Entities" = metabolites
Blue indicates a fold decrease in normalized intensity values of metabolites between offspring born to GDM dams and those born to lean dams, Red indicates a fold increase in normalized intensity values of metabolites between offspring born to GDM dams and those born to lean dams. LC-QTOF-MS analyses, a moderated t-Test ($P<0.05$) and volcano plots (> 2-fold changes and $P<0.05$) were performed using MPP software (version 12.6)

Figure 3.3 Heat Map: Changes in Litter Liver Metabolites Between GDM and Lean Dam Conditions (LC/MS)



Lean (LF fed Dam), GDM (HFS fed Dam),"Yellow" = No fold change in metabolite, "Red" = Fold increase in metabolite,"Blue" = Fold decrease in metabolite

Figure 3.4 Heat Map: Changes in Litter Liver Metabolites Between GDM and Lean Dam Conditions in Offspring Fed Postnatal HFS or LF Diets (LC/MS)



LF (Low Fat Diet-Postnatal), HFS (High Fat Diet-Postnatal), Lean (LF fed Dam), GDM (HFS fed Dam) "Yellow" = No fold change in metabolite, "Red" = Fold increase in metabolite, "Blue" = Fold decrease in metabolite

3.4 CONCLUSIONS

Metabolomic analysis of liver lipid soluble metabolites from rat offspring uncovered an array of changes amongst many lipotoxic lipid species, such as sphingolipids (ceramides) and DAGs that were significantly increased in the offspring of GDM rats. This increase in lipotoxic lipid species could potentiate metabolic disease development in offspring born to GDM dams.

Furthermore, it was noted that several PE and PI species were significantly decreased in offspring born to GDM dams. Though biochemical analysis of total PI phosphorus did not show a significant difference between any dietary group, further research is required in order to determine the importance of these individual PI species for controlling insulin sensitivity. While an overall decrease in PI species could have implications in disrupting insulin signaling and thus predisposing offspring to insulin resistance and eventual T2D development, a decrease in a few species of PI that act to maximize optimal insulin signaling could potentially have the same effect.

Interestingly, it was observed that PE levels from metabolomic analysis and phosphorus assay were significantly decreased in offspring born to GDM dams compared to offspring born to lean dams. Phosphorus assay also showed significantly decreased CL levels in

HFS offspring born to GDM dams compared to LF offspring born to lean dams. Previous research shows that a reduction in PE levels caused by genetic deficiency in a gene required for PE biosynthesis (ie- Pcyt2+/- mice), caused a compensatory increase in the accumulation of DAG and TAG in the liver (Fullerton *et al.*, 2009). Ultimately this resulted in insulin resistance and hepatic steatosis (Fullerton *et al*., 2009), similar to the rat offspring from GDM dams I describe in my thesis project. In addition, reduced CL levels could impair mitochondrial respiration (Mejia *et al*., 2014). These findings will be investigated in more detail in future experiments.

In conclusion, consistent with our hypothesis, gestational exposure to diabetes increased hepatic levels of lipotoxic lipids (DAG and ceramide) and reduced levels of lipids (ie- PE and PI) that maintain insulin sensitivity, in the offspring.

GENERAL DISCUSSION

It has been established that women presenting with obesity prior to and throughout pregnancy have elevated risk for developing GDM. Pre-existing obesity is also associated with giving birth to large-for-gestational-age infants (Taylor & Poston, 2007. The vast majority of rodent studies to date have utilized STZ to create a GDM model. STZ is a chemical toxic to pancreatic β-cells that is administered during gestation to induce overall insulin deficiency and a diabetic pregnancy (Jawerbaum *et al.*, 2010). GDM, however, is characterized by the inability of islet cells to adapt to insulin resistance that is commonly observed at mid-gestation.

The consumption of a high-fat (60%, kcal) diet (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Dudley *et al*., 2011; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012; Taylor *et al*., 2005) or a "cafeteria"-style diet (Holemans *et al.*, 2004; Kjaergaard *et al.*, 2014) by rats during gestation has frequently been used as a model to study maternal obesity. These studies, however, initiated diet consumption after pregnancy. We initiated the HFS diet 6 weeks prior to mating to induce appropriate weight gain and glucose intolerance before gestation. This diet was also fed to our rats throughout pregnancy. We observed the development of hyperglycemia by mid-gestation that returned to normoglycemic levels following birth of the litter. The utilisation of the HFS diet prior to mating caused glucose intolerance that when paired with pregnancy, a natural state of insulin resistance, caused elevated gestational weight gain, mid-gestational

hyperinsulinemia and insulin resistance, all characteristic of the human GDM condition. Litters from HFS-fed GDM dams also had larger newborn pups, which is also characteristic of human GDM (Catalano *et al.*, 1999; Catalano *et al*., 1991).

While maternal high-fat diet feeding was used to investigate the effect of maternal obesity on offspring (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Dudley *et al*., 2011; Howie *et al*., 2009; Samuelsson *et al*., 2008; Sun *et al*., 2012; Taylor *et al*., 2005), the present study is the first to investigate the influence of GDM on the postnatal response to LF and HFS diets in young rat offspring. Several of these studies described above have reported different effects of maternal obesity on birth weight. Studies have suggested the presence of maternal obesity is associated with increased birth weight, (Kjaergaard *et al.*, 2014; Samuelsson *et al*., 2008) reduced birth weight, (Dudley *et al*., 2011; Howie *et al*., 2009) or had no apparent effect (Buckley *et al*., 2005; Caluwaerts *et al.*, 2007; Holemans *et al*., 2004; Sun *et al*., 2012; Taylor *et al*., 2005). These variable observations could be due to differences in the respective diets because varying levels of fat and sucrose could play a role. The timing of the diets could also explain these variations in birth weight, maternal fasting plasma glucose and insulin levels. On the opposite end of the spectrum, studies mentioned above that have utilised STZ during pregnancy consistently reported an overall reduction in birth weight irrespective of dosage. STZ administration during pregnancy has also been closely associated with increased fetal mortality and birth defects (Jawerbaum *et al.*, 2010). Our model of HFS diet-induced GDM was consistent in observing both increased birth weight and birth length of newborn sprague-dawley rat

pups. This overall size increase, or macrosomia can likely be explained as a response to maternal hyperinsulinemia and the increased availability of calories from saturated fats and simple sugars through the maternal consumption of the HFS diet. While we observed that the male offspring were more sensitive to the development of obesity than the female offspring, we terminated our experiments when the rats were young adults and longer-term future studies will investigate whether female GDM offspring also develop a more severe metabolic syndrome later in life than the female offspring of lean dams. Since human GDM is associated with poor metabolic health outcomes for the offspring, (Gillman *et al*., 2003; Moore, 2010; Reece *et al.*, 2009) the data presented herein suggests our model to be of clinical relevance and may be utilised for future investigations of both biological and molecular effects of GDM on the offspring.

There has been a clear association made that links the accumulation of lipids in tissues, particularly liver and skeletal muscle, and the development of insulin resistance (McGarry, 2002; Sinha *et al.*, 2002). The fetal liver and adipogenesis are key targets of altered in utero conditions (Symonds *et al*., 2009). The data presented in this work have identified putative fetal programming of a number of key genes that are involved in stimulating hepatic lipid synthesis (*Accb, Srebp-1c*). It was also shown that the expression of a major regulator of lipid oxidation (*Ppara*) was also reduced in the offspring of GDM dams. These changes in gene expression appeared to be instrumental in the development of hepatic steatosis in these offspring. There have been several reports that associated maternal diet-induced obesity during pregnancy with an increase in body

fat content (Buckley *et al.*, 2005; Howie *et al.*, 2009; Samuelsson *et al*., 2008; Sun *et al.*, 2012) as well as liver TG content (Buckley *et al*., 2005) in adult chow fed offspring. Our study is the first to investigate the influence of prenatal GDM and the postnatal response to LF and HFS diets on hepatic steatosis in the offspring, as well as identify genes that contributed to GDM-induced hepatic TG accumulation.

With a particular interest in the mechanisms driving the development of hepatic steatosis, NAFLD and subsequent insulin resistance and T2D development, it is noted that sphingolipids such as ceramide, glycosphingolipid and sphingosine are implicated in T2D development (Langeveld & Aerts, 2009 ; Summers, 2006). High levels of sphingolipids are consistently observed in animal models of obesity. Yetukuri *et al.* showed that the degree of liver steatosis seen in *ob/ob* mice was correlated to the amount of ceramide (Yetukuri, 2007). Mechanistically, it has been proposed that increased liver lipid content along with increased inflammatory cytokine production drives sphingolipid formation (Day & James., 1998; Day & Saskena, 2002). There is also data suggesting these lipids, especially ceramide, can have a direct role in T2D development primarily by causing β-cell apoptosis in response to inflammation and high fat diets (Lang *et al*., 2011; Chandra *et al*., 2001). Our data from LC/MS analysis has shown, are consistent with the above findings, and demonstrate an increase in several ceramide species within the liver tissue of offspring born from GDM dams regardless of postnatal diet comparative to lean control offspring. A significant accumulation of ceramide within the liver could explain the insulin resistance seen in offspring from GDM dams.

Furthermore, there has been recent evidence published to suggest that DAG buildup also plays a crucial role in the development of insulin resistance in both skeletal muscle and liver tissue by activating protein kinase C (PKC) (Samuel *et al*., 2004; Weiss *et al*., 2003). Overnutrition and consumption of "western-style" diets acts to increase fatty acid delivery and uptake into the liver at a rate that exceeds the capacity of this tissue to utilize them for TAG synthesis or for energy production via oxidation in the mitochondria (Weiss *et al*., 2003; Nagle *et al*., 2009). Our data presented herein has also shown, through LC/MS analysis, a significant increase in liver DAG content from offspring of GDM dams regardless of postnatal diet comparative to lean control offspring. This could potentially be another explanation for the insulin resistance seen with these offspring. It is more likely that both DAG and ceramide accumulation together function to increase hepatic steatosis, inflammation and insulin resistance. Further experiments are required to evaluate whether the accumulation of DAG and ceramide alters the PKC and insulin signal transduction cascades in the livers of the GDM offspring.

PE, an important inner membrane leaflet phospholipid plays an important role in cell division. Recent published data suggested that the knockout or deletion of one allele of the rate-limiting enzyme involved in *de novo* PE synthesis, Pcyt2, caused a significant decrease in PE synthesis and a marked increase in DAG and TAG content, fatty liver, and insulin resistance characteristic of MetS (Fullerton *et al.*, 2009; Pavlovic & Bakovic, 2013). LC/MS analysis of liver tissue from our HFS fed offspring born to GDM dams

showed a significant decrease in PE content while displaying an increase in lipotoxic lipid species (sphingolipids, DAG) as mentioned above comparative to LF fed offspring born to lean dams. These offspring have also displayed significant fatty liver development and insulin resistance comparative to their control counterparts. These results could be due to dysregulation in Pcyt2 leading to a decrease in PE biosynthesis and the subsequent buildup of lipotoxic lipid species causing insulin resistance and eventually leading to T2D; however, further mechanistic analysis is needed.

The fat content of adolescent livers has been shown to be a significant contributing factor to overall insulin sensitivity that is independent of both whole body and visceral fat mass (Wicklow *et al*., 2012). We have shown that, in agreement with the above findings, the young adult offspring coming from GDM dams had impaired insulin sensitivity compared to control offspring. This state of insulin resistance was further impaired by the addition of postnatal consumption of a HFS diet. Reduced expression of insulin receptor-β in the offspring of GDM dams was also noted and appeared to have significant effects on the phosphorylation and overall activity of phosphatidylinositol 3-kinase and Akt, downstream signalling kinases in the insulin signal transduction pathway. The data presented herein shows that the consumption of the HFS diet postnatally by the offspring of GDM dams accelerated the rate at which obesity, hyperinsulinemia, insulin resistance and fatty liver appeared. These observations demonstrate that the interaction between GDM and the postnatal diet contribute to the early onset of dyslipidemia and insulin resistance in the rat offspring.

Our findings have shown the degree of influence that pre-pregnancy weight and obesity has on the development of GDM. Furthermore, it has been shown that GDM is strongly associated with the development of obesity, hepatic steatosis and insulin resistance in young adult rat offspring. In developed countries, 15-20% of reproductive age women are obese and >40% of women gained excessive weight during their pregnancy (Seidell, 2000). Correspondingly, the prevalence of GDM has increased (Gaillard *et al.*, 2013; May, 2007). This trend has put increasing numbers of the children at risk for obesity and could contribute to increases in the pediatric incidence of T2D (Franks *et al*., 2006; Shields, 2006; Young *et al.*, 2002). Our findings emphasize the importance of treating GDM as a preventative measure against poor metabolic health outcomes in children.

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