Maternal resveratrol supplementation in gestational diabetes prevents cardio-metabolic disease development and improves cardiac structure in the rat offspring

by

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Abstract

Gestational diabetes mellitus (GDM), a common complication of pregnancy, arises during the third trimester and is characterized by hyperglycemia. GDM increases cardio-metabolic disease risk in mothers and offspring. Current treatments have disadvantages. Resveratrol (RESV), a naturally produced polyphenol, has anti-oxidant and positive metabolic health effects. Thus, we hypothesized that RESV supplementation during the third trimester and lactation would improve maternal glucose tolerance and prevent cardio-metabolic disease in the offspring.

A diet-induced GDM model was utilized for this thesis. Different metabolic tests were performed. Echocardiography was used to assess cardiac structure and function. Immunoblotting, qPCR and cardiomyocyte isolations were performed to study mechanisms.

RESV supplementation prevented maternal glucose intolerance and cardio-metabolic disease development in the offspring by improving glucose homeostasis and inhibiting cardiac remodelling.

Supplementing maternal diets with RESV at the onset of GDM may become a newer intervention to protect mothers and their offspring from GDM-induced short and long-term consequences.

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Dedication

"Education is the most powerful weapon which you can use to change the world"

Nelson Mandela

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List of Abbreviations

Acc-2 – Acetyl-Coenzyme A Carboxylase 2

ACE – Angiotensin Converting Enzyme

AKT – Protein Kinase B

AMPK – 5’ Adenosine Monophosphate Protein Kinase

ANOVA – Analysis of Variance

ATGL – Adipose Triglyceride Lipase

ATP – Adenosine Triphosphate

BMI – Body Mass Index

CAD – Coronary Artery Disease

Cat – Catalase

CDA – Canadian Diabetes Association

CDC – Centre for Disease Control and Prevention

CHD – Coronary Heart Disease

CHRIM – Children’s Hospital Research Institute of Manitoba

CO – Cardiac Output

CVD – Cardiovascular Disease

CycA – Cyclophilin A

DAG – Diacylglycerol

DC – Diabetic Cardiomyopathy

DEXA – Dual X-ray Absorptiometry

DINT – Dietary Intervention

DNA – Deoxyribonucleic Acid

DOHaD – Developmental Origins of Health and Disease

eEF2 – Eukaryotic Elongation Factor 2

EF – Ejection Fraction

eIF2α – Eukaryotic translation Initiation Factor 2α

ELISA – Enzyme-Linked Immunosorbent Assay

FAO – Fatty Acid Oxidation

FBS – Fetal Bovine Serum

GDM – Gestational Diabetes Mellitus

GLUT-4 – Glucose Transporter Protein 4

GMC – Genetic Models Center

GPX1 – Glutathione Peroxidase 1

GSIS – Glucose Stimulated Insulin Secretion

GTT – Glucose Tolerance Test

gWAT – Gonadal White Adipose Tissue

GWG – Gestational Weight Gain

G-6-P – Glucose-6-Phosphate

HbA1c – Hemoglobin A1c Levels

H&E – Hematoxylin and Eosin

HFS – High Fat and Sucrose Diet

HG – High Glucose

HOMA-IR – Homeostatic Model Assessment for Insulin Resistance

HW:BW – Heart Weight to Body Weight

HW:TL – Heart Weight to Tibia Length

IFNα/β – Interferon α/β

IL-6 – Interleukin-6

IRβ – Insulin Receptor β

IRS-1 – Insulin Receptor Substrate-1

ITT – Insulin Tolerance Test

IVRT – Isovolumetric Relaxation Time

IVS – Interventricular Septal Thickness

LF – Low Fat Diet

LKB1 – Liver Kinase B1

LVAW – Left Ventricular Anterior Wall Thickness

LVID – Left Ventricular Interior Diameter

LVPW – Left Ventricular Posterior Wall Thickness

mRNA – Messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

NAD+ - Nicotinamide Adenine Dinucleotide +

NAFLD – Non-Alcoholic Fatty Liver Disease

NFκB – Nuclear Factor κB

NRf2 – Nuclear Factor-Like 2

OGTT – Oral Glucose Tolerance Test

PBS – Phosphate-Buffered Saline

Pcyt2 – CDP:Phosphoethanolamine Cytidylyltransferase 2

PE – Phosphatidylethanolamine

Pepck/Pck1 – Phosphoenolpyruvate Carboxykinase

PFA – Paraformaldehyde

PGC1α – Peroxisome Proliferator Activated Receptor-γ Co-Activator 1α

PI3K – Phosphatidylinositol 3 Kinase

PPARα – Peroxisome Proliferator-Activated Receptor α

pWAT – Perirenal White Adipose Tissue

p70S6K – Ribosomal Protein p70 S6 Kinase

qPCR – Quantitative Polymerase Chain Reaction

RESV – Resveratrol

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

SEM – Standard Error of the Mean

Serca2a – Sarco/endoplasmic Reticulum Calcium ATPase

SGLT2 – Sodium Glucose Co-Transporter 2

SIRT1 – Sirtuin 1

SOD – Superoxide Dismutase

Srebp-1c – Sterol Response Element Binding Protein 1C

TG – Triglycerides

TMRM – Tetramethylrhodamine, Methyl Ester, Perchlorate

TNF-α – Tumor Necrosis Factor α

T1D – Type 1 Diabetes

T2D – Type 2 Diabetes

T3 – Trimester 3

Chapter 1:

Introduction

1.1 The Obesity Epidemic:

Obesity is now widely recognized as a key risk factor for the development of many different types of chronic diseases. Obesity is generally defined as the excess accumulation of fat tissue which can have detrimental consequences for an individual's health (Corey and Kaplan 2014). This can be the result of an unbalanced food intake and calorie expenditure, leading to increased fat deposition and increased weight gain, mainly due to a societal switch to fatty food consumption and lack of exercise (Corey and Kaplan 2014; Lehnert et al. 2013; Swinburn et al. 2011). Clinicians currently use Body Mass Index (BMI) to assess whether their patients are obese or overweight. This index takes into consideration the person's weight and height and expresses the result in kg/m^2 (World Health Organization. 2017). The World Health Organization has set a BMI of 25 kg/m^2 or more as being overweight, while more than 30 kg/m^2 corresponds to obese individuals (World Health Organization. 2017). This method is also widely used in children, however, the values need to be standardized for both age and sex of the child to make proper comparisons (Adab, Pallan, and Whincup 2018). Other methods that doctors may use to assess fat distribution and content particularly in children include dual x-ray absorptiometry scans (DEXA), skinfold thickness, waist circumference, waist to height ratio, waist to hip ratio, and bioelectric impendence, although BMI is still believed to be the most accurate, as described by (Adab, Pallan, and Whincup 2018).

Obesity rates are increasing worldwide setting the stage for a global epidemic. Rates of obesity in the general population have tripled since 1975, and there are currently 2 billion overweight adults, of which 650 million are obese (World Health Organization. 2017). In addition, obesity in children has also been on the rise. In 2016, 41 million children were either overweight or obese (World Health Organization. 2017), and unfortunately these numbers are still expected to

continue rising if the issue is not addressed. This becomes particularly significant when these individuals reach adulthood and the chronic diseases associated with obesity start to emerge resulting in a reduced quality of life and an increased financial burden on the health care system (Cawley and Meyerhoefer 2012; Lehnert et al. 2013).

Being overweight or obese puts the individual at a greater risk for developing cardio-metabolic diseases, such as type 2 diabetes (T2D) and cardiovascular disease (CVD)(Ng et al. 2014). Cardio-metabolic disease/syndrome is a term that encompasses a broad spectrum of different risk factors associated with altered metabolism (Srivastava 2012). For example, glucose intolerance, insulin resistance, dyslipidemia and lipotoxicity, hypertension and increased adiposity are all risk factors which can affect the transition to T2D and/or CVD (Srivastava 2012). Another complication of obesity is non-alcoholic fatty liver disease (NAFLD), one of the most common diseases of the liver in the United States (Corey and Kaplan 2014; Ludwig et al. 1980; Vernon, Baranova, and Younossi 2011). Here, the increasing amount of triglycerides from the diet accumulate and get stored in the liver causing steatosis and triggering inflammation which can then damage the function of the liver and can lead to other liver diseases (Corey and Kaplan 2014; Vernon, Baranova, and Younossi 2011). Additionally, there is an association between hepatic steatosis and the development of insulin resistance and thus the progression to T2D (Kelley et al. 2003; Samuel and Shulman 2017; B. A. Wicklow et al. 2012).

1.1.1 Type 2 Diabetes Mellitus (T2D):

T2D is a chronic metabolic condition first reported in Egypt almost 3000 years ago (Ahmed 2002). T2D is characterized by glucose intolerance or hyperglycemia, insulin resistance, and pancreatic β-cell dysfunction (Olokoba, Obateru, and Olokoba 2012). One of the contributors for T2D development is obesity. Obesity may cause insulin resistance through the abnormal

accumulation of lipids in the liver, skeletal muscle and pancreas, which can then lead to whole body insulin resistance, hyperinsulinemia, glucose intolerance, and eventually β-cell failure (as reviewed in (van Herpen and Schrauwen-Hinderling 2008). The β-cell of the pancreas is the endogenous producer of insulin, which is required to take up glucose into tissues to allow for glucose utilization and energy production (Arshad, Karim, and Hasan 2014). Unlike T2D, type 1 diabetes (T1D) is characterized by a complete destruction of β-cells due to an autoimmune response, meaning that T1D patients have almost no insulin production (Salsali and Nathan 2006). T2D is the more common of the two, in fact, according to the Centre for Disease Control and Prevention (CDC) in the United States, almost 90-95% of diabetes cases belong to T2D group (Centers for Disease Control and Prevention 2011). In Canada, 29% of the population has diabetes or prediabetes and by 2025 that number will jump to 33% (Canadian Diabetes Association (CDA) 2015). Currently, there are more than 422 million people living with diabetes, and in 2015, 1.6 million people died from diabetes and its complications (World Health Organization 2017). Furthermore, diabetes is putting a great financial burden on our health care system as it is costing the Canadian government $14 billion per year (Canadian Diabetes Association (CDA) 2015). Consumption of diets with a high level of fat and a shift towards a more sedentary lifestyle, in addition to the environment and genetics, have influenced the development of T2D in more people than ever before (Hu et al. 2001; Olokoba, Obateru, and Olokoba 2012).

For a long time, T2D predominantly affected adults, but, more recently, the incidence of T2D in children has increased rapidly (Dana Dabelea et al. 2014; Todd, Srinivasan, and Pollin 2018). This could be partially explained by the rise in childhood obesity, genetic susceptibility and the environment, as reviewed by (Todd, Srinivasan, and Pollin 2018). Currently, Manitoba has the highest rate of T2D of Canadian youth with approximately 20 cases per 100,000 children per year

(Sellers, Wicklow, and Dean 2012). When T2D occurs in children, these children may develop complications of diabetes earlier in their lives (D. Dabelea et al. 2017; Dart et al. 2014; Jaiswal et al. 2018; Todd, Srinivasan, and Pollin 2018). For example, these children have a higher risk of developing renal complications than children with T1D (Dart et al. 2012). Additionally, having T2D increases the prevalence of cardiovascular risk factors and subsequent heart disease (Rodriguez et al. 2006). A diagnosis of T2D also puts individuals at a greater risk of hypertension, stroke, heart disease, retinopathy, neuropathy, and amputations (Centers for Disease Control and Prevention 2011). Current treatments for T2D involve lifestyle therapies such as healthy diets and regular exercise, the administration of insulin parenterally, or the oral anti-diabetic agents metformin, glyburide, and others to control glycemic levels (Lipscombe et al. 2018). They all have advantages and disadvantages and will be described in more detail in a subsequent section in this thesis.

1.1.2 Gestational Diabetes Mellitus:

As obesity levels increase, more women are entering their pregnancies either obese or overweight which puts them at an increased risk of developing pregnancy-related complications that can affect them and their children. Consequently, the cases of gestational diabetes mellitus (GDM) are increasing as obese women of reproductive age have a greater risk of developing the disease while pregnant (Simmons 2011). As GDM affects 1-14% of all pregnancies, it is considered to be a common complications of pregnancy (Ferrara 2007; Kim et al. 2010). GDM is a condition that usually manifests in the late second or early in the third trimester of pregnancy and is characterized by hyperglycemia, or high blood glucose levels, insulin resistance, glucose intolerance, and increased gestational weight gain. Normally, women with GDM will go back to having normal glucose levels after delivery, however they are at an increased risk of developing

T2D later on (American Diabetes Association 2015; Buchanan, Anny H. Xiang, and Page 2012; Jovanovic and Pettitt 2001; T J Pereira et al. 2015). This is in contrast to T2D where the patient will have hyperglycemia regardless of whether they are pregnant or not. However, once GDM is established, the condition is very similar to T2D since hyperglycemia, hyperinsulinemia and potentially β-cell dysfunction are all present (Buchanan, Anny H. Xiang, and Page 2012). Importantly, T2D and GDM share many of the same complications, such as cardiac disease and heart failure (American Diabetes Association 2015; Rodriguez et al. 2006).

Normal physiology dictates that during pregnancy, insulin sensitivity will decrease so that glucose will be spared for the fetus to ensure its proper development, but in healthy women, the pancreatic β-cells will produce more insulin to maintain proper blood glucose levels (Arshad, Karim, and Hasan 2014). However, in GDM, the increased production of insulin by the pancreas may not be sufficient to compensate for the insulin resistance of pregnancy, or there may be a dysregulation in the ability of the β-cell to secrete enough insulin when glucose levels are high (Arshad, Karim, and Hasan 2014; Banerjee 2018).

The diagnosis criteria for GDM has remained controversial in many parts of the world as different methods are currently being used in the United States, Canada and Europe (M. M. Agarwal 2015). In addition, the diagnostic criteria used and ethnicity of the patient can play a big role in determining the risk for GDM for each individual pregnancy (Kim et al. 2010). According to the Canadian Diabetes Association (CDA), two different approaches are used to determine whether a pregnant woman has GDM (Canadian Diabetes Association 2013). Firstly, 50 grams followed by 75 grams of glucose are consumed and then an oral glucose tolerance test (OGTT) is performed where blood glucose levels are measured at different time intervals. GDM will then be diagnosed if more than one result is not normal, for example, fasting blood glucose ≥ 5.3 mmol/L,

after 1 hour blood glucose ≥10.6 mmol/L, and after 2 hours ≥9.0 mmol/L. The second approach is to only consume 75 g of glucose followed by an OGTT. In this case, GDM will be diagnosed if fasting blood glucose ≥5.1 mmol/L, after 1 hour ≥10.0 mmol/L, and after 2 hours ≥8.5 mmol/L (Canadian Diabetes Association 2013). Even though these methods are the ones used in Canada, they have been shown to both under and overestimate the incidence of GDM (M. M. Agarwal 2015). The incidence of GDM increases as the rates of obesity also increase. In fact, estimates indicate that there are 300 million women who are obese worldwide (World Health Organization. 2017). In the United States alone, 60% of women of childbearing age are obese (Flegal et al. 2010). There are several risk factors for GDM development such as pre-conception obesity and obesity throughout gestation, being older than 35, ethnicity, increased gestational weight gain, previous pregnancy complicated with GDM, increased abdominal fat accumulation and a family history of diabetes (Pons et al. 2015). As GDM frequency continues to rise, the health care system will have to adapt to treat the potential complications associated with GDM pregnancies in both mothers and their children, meaning that the financial burden will also be problematic (Poston, Harthoorn, and Van Der Beek 2011).

1.2 The Developmental Origins of Health and Disease (DOHaD) Hypothesis:

Dr. Barker's Developmental Origins of Health and Disease (DOHaD) hypothesis revolutionized the way we study pregnancy environments and interpret the effects that it has on short and long-term health outcomes in the offspring. The DOHaD theory, as defined by Barker, states that any environmental exposure, such as over or under-nutrition and hyperglycemia, in utero will condition fetal development, thus, increasing the risk for developing chronic diseases in the offspring later on (D. J P Barker 2007). He studied how maternal undernutrition during

pregnancy resulted in obesity, cardio-metabolic disease and CVD in the offspring (D. Barker et al. 1993; D. J P Barker 2007; David J.P. Barker 1997). His main findings came from British hospital records from the 1920s which showed that maternal undernutrition caused low birthweights and they were associated with higher rates of ischemic heart disease, coronary artery disease (CAD) and death as both men and women aged (D. Barker et al. 1993; David J.P. Barker 1997).

On the other hand, not only undernutrition during pregnancy plays a role in disease development. We recently published a review paper on how intake of diets rich in carbohydrates and fats during pregnancy can also predispose offspring to cardio-metabolic disease development via genetic programming through epigenetic mechanisms (S. Kereliuk, Brawerman, and Dolinsky 2017). Epigenetics is a term referring to changes occurring with gene expression without modifications in the deoxyribonucleic acid (DNA) sequence (P. Gluckman and Hanson 2008). It is believed that environmental conditions, such as intrauterine environment and nutrition state, can cause epigenetic modifications causing critical gene expression changes during fetal development as reviewed by (P. Gluckman and Hanson 2008). Histone methylation, DNA methylation and acetylation and non-coding ribonucleic acid (RNA) can all affect gene expression, but do not cause changes in DNA sequences (P. Gluckman and Hanson 2008). Therefore, these epigenetic modifications may occur early during development, thus predisposing the offspring to different health outcomes later on (P. Gluckman and Hanson 2008). The fact that diabetes during pregnancy can result in cardio-metabolic disease in the offspring was further established by Dr. Freinkel and his “fuel mediated teratogenesis” theory (Freinkel 1980). His theory states that the hyperglycemic environment the fetus is in can have an impact on the development of many different organs, such as the liver, pancreas, heart, etc., which are all responsible for maintaining proper metabolic health in the offspring (Freinkel 1980). Further studies have shown a clear association between

hyperglycemia during pregnancy and cardio-metabolic disease development in the offspring, as summarized in (Kaaja and Rönnemaa 2008).

1.2.1 Predictive Adaptive Response and the Mismatch Hypothesis:

Another important concept is the predictive adaptive response and the mismatch hypothesis. Here, the developing fetus is believed to undergo several changes in gene expression to be able to properly adapt to the intrauterine environment it is in. These changes in gene expression are meant to prepare the offspring for life after birth with the assumption that they will be exposed to a similar environmental condition (Bateson et al. 2004; P. D. Gluckman 2012; P. Gluckman and Hanson 2008). However, when the postnatal diet or conditions differ from those during development, then this mismatch can increase the susceptibility of the offspring to develop cardio-metabolic disease, such as obesity, T2D and CVD (Bateson et al. 2004; P. D. Gluckman 2012; P. Gluckman and Hanson 2008). This hypothesis was clearly observed in studies that looked at the Dutch famine and the Leningrad Siege cohorts. During the Second World War, the Netherlands suffered a famine where food availability was scarce, however, once the war ended conditions went back to normal (G.-P. Ravelli, Stein, and Susser 1976). Children who were conceived prior to the famine but were born during the famine years were born restricted, had a reduction in glucose utilization and tolerance, were more obese and had larger waist circumferences. They also had hyperlipidemia and an increased risk for developing cardio-metabolic disease such as T2D and CAD, and increased death rates than children who were born once the famine ended and nutrition status had gone back to normal, suggesting the importance of the mismatch hypothesis (Abeelen van et al. 2012; Painter et al. 2006; A. C. Ravelli et al. 1999; A. C. J. Ravelli et al. 1998; G.-P. Ravelli, Stein, and Susser 1976). On the other hand, studies looking at the Leningrad Siege cohort did not see any associations between intrauterine

environments and cardio-metabolic disease risk in the offspring (S. A. Stanner and Yudkin 2001; S. a Stanner et al. 1997). In this population, people suffered from starvation both during the war and after the war ended, therefore the predictive adaptive response may have taken place in utero to prepare the children to live life with fewer nutrient availability, and thus protect them from disease development (S. A. Stanner and Yudkin 2001; S. a Stanner et al. 1997; Yudkin and Stanner 1998).

1.3 GDM Effects on Mothers and Offspring:

The DOHaD hypothesis, the predictive adaptive response and the mismatch hypothesis could all be playing a role in GDM patients and their offspring. Even though GDM only occurs during pregnancy and usually reverts back to normal conditions after delivery, its effects can have both short and long-lasting complications for both mother and offspring health (Simmons 2011). Some of the maternal complications include an increase in caesarean section delivery, eclampsia-preeclampsia or high blood pressure during pregnancy, lower rates of breast feeding initiation, increased obesity, and the development of T2D and CVD (Poston, Harthoorn, and Van Der Beek 2011). GDM can also adversely affect the offspring as it increases the risk for large for gestational age offspring, or macrosomia, primarily due to increased insulin signalling which acts as a growth factor and to increase storage of fat as a response to hyperglycemia exposure in the placenta (Arshad, Karim, and Hasan 2014). Having a large for gestational age child can lead to shoulder dystocia which is a serious complication that may occur during delivery (Hod et al. 1991; Kaaja and Rönnemaa 2008). Additionally, there is an increased risk of birth defects or malformations in the digestive, genital, limb, nervous and cardiac systems of children born to overweight or obese mothers which could potentially turn into a more serious chronic disease or to infant death (Persson et al. 2017). Furthermore, hypoglycemia, due to increased production of fetal insulin in response

to the hyperglycemic environment, and hyperbilirubinemia have also been described as critical risks associated with GDM (Hod et al. 1991; Kaaja and Rönnemaa 2008). Unfortunately, as these offspring get older, they can develop obesity, T2D, and CVD earlier than children born to healthy women (Capobianco et al. 2016).

Therefore, the exposure of the fetus to the high levels of blood sugar during gestation may play a significant role in generating an adverse fetal environment which could result in disease development later on.

1.3.1 Diabetic Cardiomyopathy – one of the most common complications affecting GDM-exposed offspring

Due to an aging population, the obesity epidemic and the increasing number of people with diabetes, heart failure is becoming the leading cause of death and morbidity worldwide (Jia, Hill, and Sowers 2018). Individuals living with diabetes experience a higher risk of heart failure and CVD, so as the incidence of diabetes continues to rise, so will the incidence of heart failure and CVD (Jia, Hill, and Sowers 2018; Shindler et al. 1996). Diabetic cardiomyopathy (DC) is a condition where there is myocardial dysfunction without the presence of coronary artery disease (CAD), valvular disease (i.e. disease affecting cardiac valves which are responsible for blood flow within the heart), hypertension (i.e. high blood pressure), or dyslipidemia (i.e. high levels of lipids in the circulation) as reviewed by (Jia, Hill, and Sowers 2018). The term was first coined by Rubler in 1972 after studying four adults who had both diabetes and heart failure at the time of death, but did not have any other risk factors (Rubler et al. 1972). It is estimated that 60% of patients with T2D have DC (Mohammadshahi, Haidari, and Soufi 2014). DC can go undetected for many years, as symptoms only appear once the heart cannot function properly any longer (Falcão-Pires and Leite-Moreira 2012). During that time, the heart undergoes several structural and functional

changes. For example, there is increased fibrosis and stiffness development, increased ventricular hypertrophy (i.e. increased thickening of the heart muscle along the ventricle), and diastolic dysfunction (i.e. abnormal cardiac relaxation which prevents proper filling with blood) that occur before the development of systolic dysfunction (i.e. abnormal cardiac excitation which causes less blood to be pumped through the body) and full blown heart failure (Falcão-Pires and Leite-Moreira 2012; Jia, Hill, and Sowers 2018; Mohammadshahi, Haidari, and Soufi 2014).

Many factors are believed to have a role in the etiology of DC including left ventricular hypertrophy, metabolic dysfunction such as glucose intolerance and insulin resistance, oxidative stress, mitochondrial dysfunction and apoptosis (Falcão-Pires and Leite-Moreira 2012). However, the main factor behind the development of DC is believed to be hyperglycemia (Falcão-Pires and Leite-Moreira 2012). In fact, patients with T1D had a 30% increased risk for heart failure for every 1% increase in glycated hemoglobin A1c levels (HbA1c), which is a measure of how much glucose is in the bloodstream in a 3 month period (Lind et al. 2011). While T2D patients had an 8% risk of heart failure for every 1% increase in HbA1c (Stratton et al. 2000). These findings were independent of obesity, smoking, high blood pressure, dyslipidemia and coronary heart disease (Lind et al. 2011; Stratton et al. 2000). Also, high blood glucose levels can increase the concentrations of growth factors and free fatty acids leading to abnormal substrate utilization and abnormal lipid and calcium homeostasis, which can all lead to oxidative stress and apoptosis (Falcão-Pires and Leite-Moreira 2012). Additionally, hyperglycemia can cause the mitochondria, the energy source of cells, to have a dysfunctional electron transport chain resulting in a reduction in adenosine triphosphate (ATP) generation and increasing levels of reactive oxygen species (ROS) production which can then damage DNA and proteins increasing apoptotic rates of

cardiomyocytes, or heart muscle cells, and in skeletal muscle cells (Duncan 2011; Falcão-Pires and Leite-Moreira 2012; Petersen et al. 2004).

Studies have found that diabetes-induced hyperglycemia can damage mitochondria causing a reduction in oxidative phosphorylation and in fatty acid oxidation, which are required for proper ATP production (Duncan 2011; Petersen et al. 2004). These mitochondrial changes can also reduce gene expression of the peroxisome-proliferator-activated receptor (PPAR) γ-coactivator-1α (PGC-1α) which is involved in mitochondrial biogenesis (Duncan 2011). As the heart prefers fatty acid oxidation (FAO) over glucose oxidation for energy generation, since FAO produces more energy, a reduction in the process can have detrimental effects on the heart tissue, as fewer ATP molecules would be produced leading to hypertrophy and cell death (Duncan 2011; Mazumder et al. 2004). Interestingly, T2D mice had enhanced FAO mainly due to an increase in fatty acid transporters which increased uptake and further oxidation to produce energy (Carley et al. 2007). The increased fatty acid uptake can also result in saturated mitochondrial enzymes, thus the remaining fatty acid will not be oxidized, but rather will get secreted back into circulation, and will be subsequently converted to triglycerides to be stored in the liver or heart tissue leading to increased insulin resistance (Aguirre et al. 2014; Carley et al. 2007; Sparks et al. 2005).

The main consequence of having faulty mitochondria is increased oxidative stress which may have a significant role in the pathogenesis of DC, namely in cardiac hypertrophy development (Mohammadshahi, Haidari, and Soufi 2014). This increase in ROS can damage cardiomyocytes causing hypertrophy, fibrosis, stiffness and eventually apoptosis (Mohammadshahi, Haidari, and Soufi 2014). Another hypothesis is that inflammation may also play a role in DC as it is known that hyperglycemic environments can be associated with chronic low-grade inflammation (Jia, Hill, and Sowers 2018). This inflammation can cause changes in gene expression leading to

abnormal responses (Jia, Hill, and Sowers 2018). Most of these studies have looked at T2D cases, however less research has been done on T1D patients and DC development. But, there is some evidence suggesting that T1D patients could also suffer from DC, the difference is that insulin therapy may be masking the phenotype making it harder to study, characterize, and diagnose (as reviewed in (Holscher, Bode, and Bugger 2016).

1.3.2 Cardiac Hypertrophy:

One of the main predictors and features of DC and heart failure is cardiac hypertrophy. Cardiac hypertrophy, or the thickening of the heart muscle, can be described as being either physiological or pathological (Chung and Leinwand 2014). In physiological conditions, both continued exercise training done by athletes for example, and pregnancy will naturally cause the heart to increase in size and thickness to compensate for the increased blood flow required during intense bouts of exercise or during pregnancy, however this will result in normal or enhanced heart function, thus referred to physiological hypertrophy (Chung and Leinwand 2014; Hytten and Paintin 1963). During pregnancy, the left ventricle of the heart hypertrophies to compensate for the increased blood volume required to move nutrients from the mother to the fetus (Hytten and Paintin 1963; Li et al. 2012). This is a complicated process highlighted by changes in gene expression, signalling pathways and sex hormones, particularly estrogen, as reviewed by (Li et al. 2012). In pathological conditions, such as T2D or hypertension, there is increased pressure and wall stress for the heart to pump against. In order to compensate, the heart muscle enlarges or remodels to deal with the increased pressure overload (Chung and Leinwand 2014).

These natural compensations by the heart can result in either concentric hypertrophy, where the ventricular wall thickness is affected but the chamber size is not, or eccentric hypertrophy where both walls and chamber size are dilated or enlarged (Chung and Leinwand 2014; Chung,

Yeung, and Leinwand 2012). Eventually, these compensatory mechanisms will become maladaptive and will not be sufficient any longer, so the heart will not be able to generate enough pumping force to deal with the increased pressure, resulting in a decline in cardiac function and subsequent heart failure (Chung and Leinwand 2014).

Physiological and pathological hypertrophy can activate different signalling pathways and will respond to different stimuli which can be used to be able to differentiate between the two (Chung and Leinwand 2014; Mcmullen et al. 2003). Generally, physiological hypertrophy will activate beneficial pathways, such as increased angiogenesis, so that more blood vessels are available to carry more oxygen and nutrients (Chung and Leinwand 2014). However, pathological hypertrophy will not result in increased angiogenesis and will instead activate the angiotensin receptor and β-adrenergic signalling making a bad situation worse since these pathways are associated with increasing blood volume, blood pressure and cardiac contractility (Chung and Leinwand 2014). Therefore, cardiac hypertrophy development, whether it is physiological or pathological, under the right conditions, can turn into diabetic cardiomyopathy, which could eventually progress into heart failure.

1.4 Type 2 Diabetes and GDM Treatments:

Currently, the clinical treatments used in patients that have T2D include lifestyle therapies and pharmacological interventions. According to the 2018 clinical practice guidelines from Diabetes Canada, the first line treatment consists of lifestyle changes, namely moderate exercise and the consumption of a proper and balanced diet, to maintain healthy blood glucose levels (Lipscombe et al. 2018). If that fails, the next step is to prescribe oral glucose-lowering drugs such as metformin and glyburide. Metformin therapy is safe, has a low cost, and it may provide beneficial heart effects as well (Lipscombe et al. 2018). But, if these agents were to fail,

then insulin injections may be prescribed, as well as some newer second-line glucose lowering agents, such as the sodium glucose co-transporter 2 inhibitors (SGLT2, i.e. Empagliflozin) which were shown to lower blood glucose levels, by directly inhibiting the reabsorption of glucose from the kidney back into circulation, resulting in increased excretion of glucose through the urine, and were also shown to be beneficial for the prevention of CVD and mortality (Lipscombe et al. 2018; Zinman et al. 2015). However, SGLT2 inhibitors have not been tested on GDM patients yet. Therefore, as GDM shares many of the same features as T2D, clinical treatment approaches for GDM have been similar to T2D. We recently published a review article detailing the different GDM treatments available today and how they impact both mother and offspring short and long-term health outcomes in both animal and human studies (Brawerman and Dolinsky 2018). For this thesis, a few of the examples provided in the paper will be discussed here.

1.4.1 Lifestyle Therapy Effects on Maternal and Offspring Health: - Clinical Data

It is widely accepted that consuming a healthy diet and exercising regularly will improve the health status of the individual whether they are pregnant women or not. Many studies have been performed to assess the effectiveness of diet and exercise on health outcomes of mothers and their offspring. Of note, the DALI Lifestyle Study followed 436 obese pregnant women from 9 different European countries for two years who were between 20 and 35 weeks of gestation and were randomly assigned to four different lifestyle interventions (Simmons et al. 2017). The interventions were healthy eating, exercise, a combination of the two and usual care. This study found that both eating healthy and exercising during pregnancy reduced gestational weight gain when compared to controls and to mothers who only ate healthy or exercised (Simmons et al. 2017). However, this study failed to show any improvements in fasting or fed glucose levels, insulin levels, or the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), which

is a measure of how insulin resistant a person is, suggesting that lifestyle therapy alone is not enough to prevent GDM (Simmons et al. 2017). Briefly, the HOMA-IR is a mathematical equation used to assess insulin sensitivity and pancreatic β-cell function which is responsible for insulin secretion (Wallace, Levy, and Matthews 2004). This equation multiplies fasting insulin blood levels by fasting blood glucose levels and divides it by a constant (i.e. 22.5) (Wallace, Levy, and Matthews 2004). A high HOMA-IR value is associated with increased insulin resistance (Wallace, Levy, and Matthews 2004). In contrast, the RADIEL trial from Finland followed 293 obese pregnant women and had a similar intervention strategy to that of the previous study (Koivusalo et al. 2016). Combined lifestyle therapy improved gestational weight gain, but more importantly it prevented GDM development (Koivusalo et al. 2016). In fact, the incidence of GDM in women who were treated with lifestyle therapy was 13.9% compared to 21.6% of the controls, suggesting that lifestyle therapy alone was sufficient to stop GDM development in this cohort (Koivusalo et al. 2016). The Brazilian PAMELA study looked at the effects of exercise and strength training in 213 pregnant women for a 16 week period during their second and third trimesters while 426 pregnant women did not receive extra training sessions (Ginar Da Silva et al. 2017). They found that exercise did not reduce gestational weight gain nor prevent GDM development; however adverse effects were not observed in the newborns (Ginar Da Silva et al. 2017). A study from a Spanish population of 510 pregnant women that were randomly assigned to three sessions of supervised moderate exercise per week or sedentary control groups from an earlier point during pregnancy in the first trimester found that both maternal and offspring health outcomes were improved (Barakat et al. 2013). Even though exercise did not prevent GDM development, mothers had a reduction in gestational weight gain, as well as a reduced risk of having a Caesarean delivery and large for gestational age offspring (Barakat et al. 2013).

Thus, it seems that lifestyle therapies have contradictory effects on the prevention of GDM development. However, a healthy maternal weight at conception reduces the risk of GDM development (Nelson, Matthews, and Poston 2010). Additionally, exercising during pregnancy may be associated with potential risks such as uterine contractility stimulation and a reduction of glucose supply to the fetus which can lead to fetal hypoglycemia (Nelson, Matthews, and Poston 2010). Thus, duration and intensity of exercise should also be assessed to ensure adherence and prevent potential consequences in either mother or offspring health (Nelson, Matthews, and Poston 2010). Clinical trials have also found that exercise during pregnancy can lower the availability of glucose for the fetus to develop (Nelson, Matthews, and Poston 2010). This is because the mother utilizes glucose to produce energy while exercising, so the fetus will not be exposed to a high glucose concentration, potentially decreasing fetal growth. This could be beneficial for lowering the risk of macrosomia, but it also increases the risk of fetal hypoglycemia, which could be fatal (Nelson, Matthews, and Poston 2010). Therefore, duration and intensity of maternal exercise is crucial to ensure the offspring develops properly.

1.4.2 Lifestyle Therapies during Pregnancy in Animal Models:

Animal models provide a controlled way of studying how different lifestyle therapies can affect both maternal and offspring health. Furthermore, as the lifespan of some animals used in scientific studies is shorter to that of humans, we can identity any long-term effects on the offspring. For example, one study randomly assigned either a chow diet or a high energy obesogenic diet to female rats before becoming pregnant (Zambrano et al. 2010). Before breeding, some of the rats consuming the high energy diet were given the control diet instead to determine whether dietary changes before breeding would have any benefits and in this study they were defined as the dietary intervention group (DINT). The DINT dams exhibited a lower body weight

than the dams that consumed the obesogenic diet (Zambrano et al. 2010). At weaning, the male offspring from DINT dams had a reduction in leptin, insulin, serum triglycerides levels and fat mass, while at postnatal day 120, glucose levels, HOMA-IR values and fat cell size were all reduced (Zambrano et al. 2010). In another study, female rats consumed either a low or a high fat diet up until the second trimester of pregnancy; however, at the beginning of the third trimester until birth, some rats had their diets restricted (Giraudo et al. 2010). They found that the 11 week-old offspring of the high fat fed dams that had their diet restricted had reduced body weights when compared to the offspring of dams that consumed the high fat diet throughout (Giraudo et al. 2010). Thus, restricting food intake specifically in the third trimester of pregnancy prevented obesity in the offspring. Other studies have also shown that obesity development differs between the males and female offspring of dams fed a high fat and sugar diet during pregnancy. In one specific study, female offspring were resistant to the intrauterine exposure to a high fat and sugar diet as they did not gain as much weight as the male offspring did, suggesting females are capable of dealing better with the additional nutrient supply in utero, primarily due to differences in sex specific genes or hormones (Gallou-Kabani et al. 2007).

In terms of exercise during pregnancy, many different studies have examined how moderate exercise affects maternal and offspring health. In one study, female mice were randomly divided into an exercise group having access to a running wheel, or sedentary group prior to pregnancy up until weaning (Carter et al. 2012). Importantly, the offspring did not exercise and their results showed that exercise during pregnancy had positive outcomes on both insulin and glucose tolerance in both male and females, while males, and not females, had a higher percentage of lean body mass and reduced fat mass (Carter et al. 2012). In a follow up study looking only at the female offspring, they found that the female offspring from the dams that exercised during

pregnancy were more glucose tolerant, had lower insulin levels, reduced hepatic gluconeogenesis and higher glucose uptake in skeletal muscle, while the heart had reduced glucose uptake when compared to the female offspring from the sedentary dams (Carter et al. 2013). Similar findings were reported in more recent studies from different research groups that used rodent models that were subjected to different exercise protocols at different time points in pregnancy (Fernandez-Twinn et al. 2017; Stanford et al. 2015, 2017; Vega et al. 2015). Generally, these studies also found that offspring of dams that had exercised during pregnancy had reductions in circulating glucose, insulin, leptin and triglyceride levels, as well as reduced percent body fat, adiposity, and improved hepatic gluconeogenesis in both male and female offspring when compared to the offspring from sedentary dams (Fernandez-Twinn et al. 2017; Stanford et al. 2015, 2017; Vega et al. 2015).

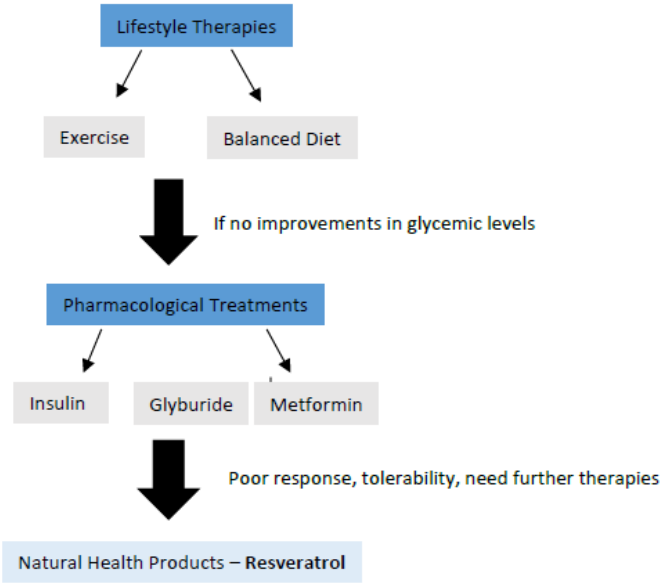
Additionally, another study found that exercise throughout pregnancy improved cardiovascular parameters in the adult male offspring (Beeson et al. 2018). Using a diet-induced model, obese female mice had access to a treadmill for five days per week from one week before pregnancy until E17. They did not find any changes in body weights at 8 weeks of age, however, maternal exercise prevented cardiac hypertrophy development and cardiac dysfunction, but did not prevent high blood pressure in the male offspring (Beeson et al. 2018). Interestingly, exercise prior to conception and throughout pregnancy may also affect epigenetic markers in the offspring suggesting a mechanism for fetal programming (Laker et al. 2014). In the skeletal muscle of the offspring of mothers that exercised, the promoter of the *PGC1α* gene was hypomethylated, while the PGC1α messenger RNA (mRNA) levels were increased, suggesting more mitochondrial biogenesis (Laker et al. 2014). These offspring also had improved glucose and insulin tolerance when compared to the offspring of mothers that did not exercise (Laker et al. 2014). Therefore,

lifestyle therapy prior and during pregnancy, namely diet restriction and exercise, have positive health outcomes in the offspring, and seem to be more conclusive than human studies.

1.5 Therapeutic Approaches:

Lifestyle therapies are a good starting point for the treatment of GDM in pregnant women, and evidence from animal models suggests that these lifestyle therapies are also beneficial to the long-term health of the offspring. On the other hand, if these lifestyle interventions fail to maintain proper maternal blood glucose levels, then different therapeutics may be prescribed (Fig. 1).

Fig. 1: Current Interventions for GDM



First line therapies include lifestyle therapies like exercise and diet. Second line therapies include pharmacological treatments such as insulin, glyburide and metformin, and finally newer therapies can include natural health products like Resveratrol.

1.5.1 Insulin:

Injection of insulin or its analogues, such as NPH, Lispro, Aspart, and Detemir, have all been studied extensively and were found to be generally safe and effective when given during pregnancy to lower blood glucose levels (Bergel et al. 2016). Insulin, first purified by Dr. Banting in Toronto, is naturally secreted from β-cells of the pancreas after glucose enters the cell and subsequently generates ATP (Wilcox 2005). This ATP will close the potassium-ATP-dependent channels, depolarizing the cell and opening voltage gated calcium channels. The entry of calcium allows insulin vesicles to get secreted, so that insulin can bind to insulin receptors in adjacent cells triggering a signalling cascade resulting in glucose transporter protein 4 (GLUT-4) translocation and entry of glucose into cells (Wilcox 2005). However, this increase in insulin concentration can result in hypoglycemia, so using the correct dosage is critical (Bergel et al. 2016; Simmons 2015). On the other hand, insulin use has been shown to decrease the risk of pregnancy-related complications like pre-eclampsia, Caesarean section and shoulder dystocia (Simmons 2015). It is important to emphasize that insulin is a growth factor, thus studies have shown associations between insulin administration, gestational weight gain and macrosomia, or large for gestational age offspring (Scholl and Chen 2002). Insulin itself does not cross the placenta as it is a large molecule, so it is safe to use during pregnancy (Bergel et al. 2016; Simmons 2015). However, insulin can interact with placental lipid carriers thereby signalling for the accumulation of fat in the fetus (Ruiz-Palacios et al. 2017). In a study where GDM mothers were treated with insulin, there was an association with higher expression of endothelial lipase and lower expression of lipoprotein lipase in the placenta with increased fetal abdominal circumference (Ruiz-Palacios et al. 2017). This association implies that there was a higher accumulation of fat in the fetus, thus explaining the macrosomic phenotype observed with insulin therapy (Ruiz-Palacios et al. 2017).

Therefore, insulin therapy efficiently lowers blood glucose levels in GDM patients, but the risks associated with its use are serious and include hypoglycemia and macrosomia.

1.5.2 Metformin:

Metformin is an oral anti-diabetic drug that made its reappearance in the pharmaceutical market in 1995 when studies suggested that metformin could prevent insulin resistance and could lower blood glucose levels without causing weight gain (Bailey 2017). Today, metformin is the gold standard for the treatment of T2D after the 1998 UKPDS study found that it lowered blood glucose levels, did not cause hypoglycemia or weight gain, and it reduced CVD and death rates in patients who took the drug long-term (Turner 1998). Metformin works by upregulating a key energy homeostatic sensor in the cells, the 5' adenosine monophosphate protein kinase (AMPK), thereby reducing endogenous gluconeogenesis in the liver and increasing peripheral insulin sensitivity so more glucose can be taken up (Feig and Moses 2011; Rena, Pearson, and Hardie 2017). The use of metformin during pregnancy was very limited back in the 1970s as it was unknown what effect it might have on the developing fetus (Lindsay and Loeken 2017). Later studies, particularly the Metformin in Gestational Diabetes (MiG) Trial, showed that metformin administration during pregnancy did not cause any significant neonatal complications and there were fewer hypoglycemic events when compared to mothers who used insulin instead (J.A. Rowan et al. 2008). These findings provided evidence that metformin may be a good alternative to insulin therapy. Additionally, metformin treatment of GDM mothers reduced their risk of developing postpartum T2D by 40% (Aroda et al. 2015). Interestingly, metformin crosses the placenta, but studies have not reported serious adverse effects on the fetus. One study reported that two year old children of mothers given metformin therapy for GDM had increased mid upper arm circumference, larger bicep and subscapular skinfolds compared to the children of insulin-treated

mothers (Janet A. Rowan et al. 2011). Additionally, 18 month-old children of mothers given metformin were heavier and taller than the children of mothers who were treated with insulin (Ijas et al. 2015). Thus, metformin may be a good alternative to insulin therapy having fewer side effects than insulin for the mothers, however, it may result in larger children.

1.5.3 Glyburide:

Glyburide or Glibenclamide, belongs to the family of sulfonylureas that target the pancreatic β-cell to increase insulin secretion by closing the sulfonylurea receptor 1 subunit of the ATP-sensitive potassium ion channel, thereby depolarizing the membrane, opening voltage gated calcium ion channels and allowing insulin vesicles to be secreted (Malek and Davis 2016) therefore lowering blood glucose levels. A systematic review and meta-analysis of randomized controlled trials found that glyburide treatment during GDM increased the risk of pregnancy related complications such as macrosomia and large for gestational age offspring, while maternal and neonatal hypoglycemia were also a concern (Amin et al. 2015). However, no long-term studies have been done to assess the incidence of post-partum T2D development in mothers. Additionally, after some contrasting results, glyburide was shown to cross the placenta (Hebert et al. 2009; Schwartz et al. 2015). In some studies, low dose glyburide did not increase the risk of preterm birth, malformations or macrosomia (Glover et al. 2016), but large doses increased birth weights in the offspring, while maternal hypoglycemia, a common complication of glyburide, was not observed (Díaz et al. 2017; Glover et al. 2016). One breakthrough study examined the effects of glyburide treatment versus insulin for the treatment of GDM. Glyburide was as efficient as insulin in lowering blood glucose levels without causing macrosomia associated with insulin (Langer et al. 2000). Eventually, more studies were designed to compare both treatments and the results were very controversial as some studies found insulin therapy to be better than glyburide or vice versa

in terms of weight gain, hypoglycemic events, neonatal complications, macrosomia and caesarean sections (Camelo Castillo et al. 2015; Koren et al. 2016; Mirzamoradi et al. 2015). Therefore, even though glyburide is effective for GDM treatment, maternal and fetal hypoglycemia as well as macrosomia remain a concern.

In order to better understand what the best choice is for GDM therapy, different studies have been designed that compared all three therapies during GDM. Recent systematic reviews and randomized controlled trials have reached the same conclusion that glyburide was more strongly associated with macrosomia, neonatal hypoglycemia, increased gestational weight gain, and decreased fasting glucose levels, pre-eclampsia, increased rates of preterm birth and other complications when compared against insulin and metformin treatments (Balsells et al. 2015; George et al. 2015). Furthermore, patients taking glyburide tended to fail reaching optimal glycemic levels and experienced more hypoglycemic events (Nachum et al. 2017). Thus, based on these comparisons, metformin should be the drug of choice over both insulin and glyburide for the treatment of GDM since it is associated with the lowest risk of maternal and fetal hypoglycemia, macrosomia, preeclampsia, and other complications.

1.6 Natural Health Products:

Due to the contradicting results arising from studies comparing insulin, metformin and glyburide therapies, there is a need for new treatments, particularly ones that do not cause macrosomia or fetal hypoglycemia. Furthermore, prescribing medications during pregnancy is a significant concern as there is a shortage of evidence about their direct effects on the developing fetus as well as their long-term effects on the health outcomes in the offspring. Additionally, lifestyle therapies may be difficult to comply with and maintain throughout pregnancy. Thus, the

use of natural health products and vitamins are gaining popularity for disease prevention and treatment during pregnancy.

1.6.1 Resveratrol:

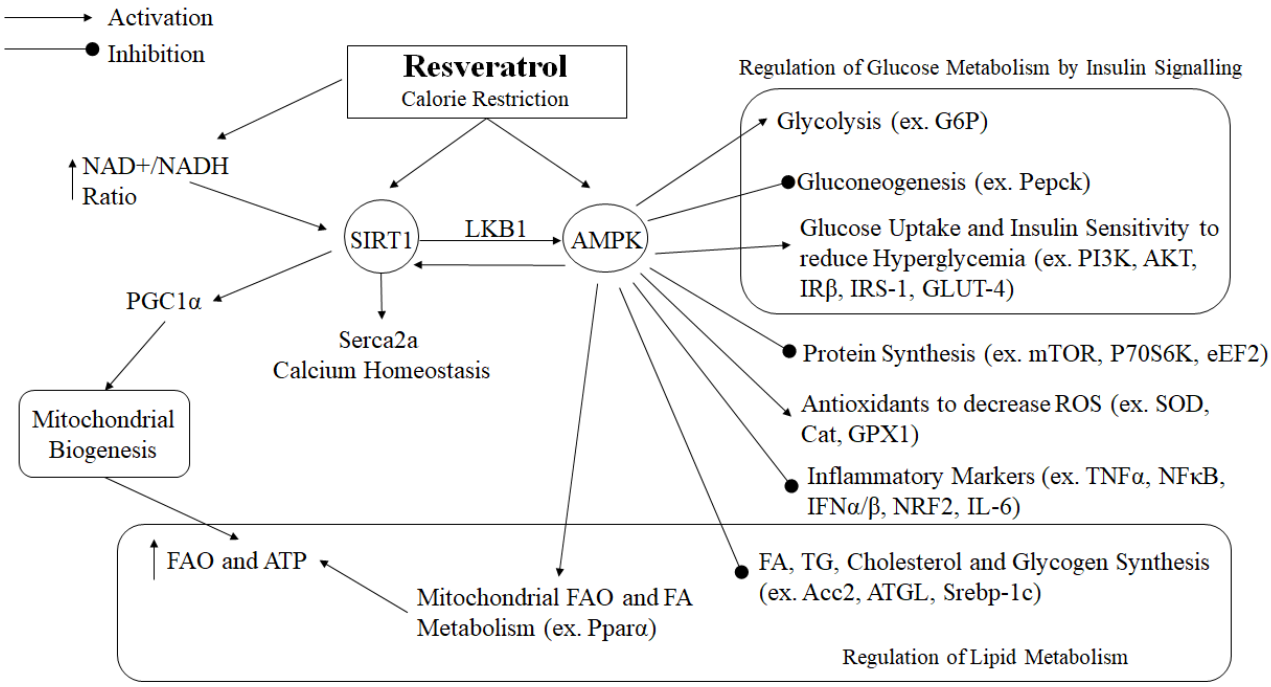
Resveratrol (RESV) is a compound naturally produced by plants in response to environmental threats, such as bacteria and parasites, and is one of the most widely studied polyphenolic compounds (Zordoky, Robertson, and Dyck 2015). RESV is produced by several different vegetables and fruits, for example grapes, berries, peanuts, and Japanese knotweed (Vernon W. Dolinsky and Dyck 2011). RESV was first identified by M. Takaoka in 1939 after its successful isolation from a plant's root (Catalgol et al. 2012). However, RESV research became popular after the "French paradox" was described. In 1992, researchers noticed that coronary heart disease (CHD) incidence in the French population was low even though the French are known for consuming diets high in saturated fats (Renaud and de Lorgeril 1992). They argued that the moderate drinking of red wine, which is highly consumed in France, may be protecting the population from CHD (Renaud and de Lorgeril 1992). Even though other compounds and antioxidants are present in red wine, the high RESV concentration led researchers to believe that RESV could be the protective compound (Catalgol et al. 2012). Further studies have shown RESV to have pleiotropic activity on multiple signalling pathways and RESV supplementation in animal models has been reported to improve health outcomes in animals suffering from different chronic disorders, such as cancer, diabetes, Alzheimer's disease and CVD, suggesting this compound could be used as a broad-spectrum treatment for these chronic conditions (Diaz-Gerevini et al. 2016). Furthermore, RESV was shown to activate the same signalling pathways as exercise and calorie restriction implying that this compound could be an add-on to the lifestyle therapies prescribed for GDM (V.W. Dolinsky et al. 2012; Timmers et al. 2011) (Fig. 2)

RESV can be found in both trans and cis isomers, however the trans form is the most biologically active and the most stable (Catalgol et al. 2012). Even though RESV has low bioavailability, many studies have reported that RESV has anti-inflammatory, anti-apoptotic, anti-oxidant characteristics and can also efficiently lower blood glucose levels (Mohammadshahi, Haidari, and Soufi 2014; Roberts et al. 2014; Turan, Tuncay, and Vassort 2012), suggesting that RESV can be a good agent to be used against hyperglycemia, diabetes and GDM. Both, diabetes and GDM, are characterized by increased oxidative stress and low grade inflammation which can cause further damage and disease development (Coutinho et al. 2018). RESV studies have reported increased activation of anti-oxidants, like glutathione peroxidase, catalase, and superoxide dismutase (SOD), and decreased expression of inflammatory molecules such as Nuclear Factor κB (NFκB), Nuclear Factor like 2 (NRf2), Tumor Necrosis Factor α (TNFα), Interferon α and β (IFNα/β), and Interleukin 6 (IL-6) (Coutinho et al. 2018; Mohammadshahi, Haidari, and Soufi 2014). Therefore, RESV could also be used to target the increased ROS and inflammation associated with diabetes and GDM. Additionally, RESV was shown to prevent cardiac hypertrophy, a key risk factor for diabetic cardiomyopathy, in animal studies (Vernon W Dolinsky et al. 2013).

RESV has many cellular targets; however the main signalling pathways involved are AMPK, an important regulator of energy homeostasis as it controls glucose metabolism and fatty acid oxidation among other things, and the nicotinamide adenine dinucleotide (NAD) + dependent histone deacetylase, Sirtuin 1 (SIRT1) , which its activation is also energy state dependent and is associated with changes in gene expression (C. K. Singh et al. 2013; Turan, Tuncay, and Vassort 2012) (Fig. 2). RESV supplementation is also believed to be safe as few side effects have been

reported in human studies, namely constipation or diarrhea, nausea and indigestion only in patients that consumed more than 1g/day (B. Wicklow et al. 2015).

Fig. 2: Proposed molecular pathways affected by RESV supplementation with a focus on glucose and lipid metabolism



Several molecules from these pathways were targeted to investigate the effect of GDM on their expression and whether RESV supplementation rescued their expression in liver and cardiac tissue of the 15 week old young adult male rat offspring. Arrow indicates activation, while black dot indicates inhibition. NAD+: Nicotinamide Adenine Dinucleotide; PGC1α: Peroxisome Proliferator Activated Receptor-γ Co-Activator 1α; SIRT1: Sirtuin 1; LKB1: Liver Kinase B1; AMPK: 5' Adenosine Monophosphate Protein Kinase; Serca2a: Sarco/Endoplasmic Reticulum Calcium ATPase; G6P: Glucose-6-Phosphate; Pepck: Phosphoenolpyruvate Carboxykinase; PI3K: Phosphatidylinositol 3 Kinase; AKT: Protein Kinase B; IRβ: Insulin Receptor β; IRS-1: Insulin Receptor Substrate-1; GLUT-4: Glucose Transporter Protein 4; mTOR: Mammalian Target of Rapamycin; P70S6K: Ribosomal Protein p70 S6 Kinase; eEF2: Eukaryotic Elongation Factor 2; SOD: Superoxide Dismutase; Cat: Catalase; GPX1: Glutathione Peroxidase 1; TNFα: Tumor Necrosis Factor α; NFκB: Nuclear Factor κB; NRf2: Nuclear Factor-Like 2; IL-6: Interleukin-6; IFNα/β: Interferon α/β; FA: Fatty Acids; TG: Triglycerides; Acc2: Acetyl-CoA Carboxylase 2; ATGL: Adipose Triglyceride Lipase; Srebp-1c: Sterol Response Element Binding Protein 1c; FAO: Fatty Acid Oxidation; Pparα: Peroxisome Proliferator-Activated Receptor α; ATP: Adenosine Triphosphate.
Adapted from the following: (Abbasi Oshaghi et al. 2017; Coutinho et al. 2018; V. Dolinsky and Dyck 2014; Mohammadshahi, Haidari, and Soufi 2014; Price et al. 2012; C. K. Singh et al. 2013; Turan, Tuncay, and Vassort 2012)

1.6.2 Maternal Resveratrol Supplementation Studies:

Currently, there is a lack of clinical studies looking at the effects of RESV supplementation during pregnancy. Two studies found that RESV supplementation in overweight pregnant women reduced blood pressure and also lowered lipid and blood glucose levels, thereby reducing the chance of developing GDM (Ding et al. 2017; Malvasi et al. 2017). Recently, more animal research studies have been performed looking at the effects of RESV supplementation either before or throughout gestation and lactation on the health of the dams and offspring. In terms of maternal health, RESV supplementation prior and during pregnancy in Japanese macaques that consumed a high fat diet prevented gestational weight gain, improved uterine artery blood flow and glucose homeostasis when compared to the controls that consumed a high fat diet without RESV (Roberts et al. 2014). In other studies using rodents, such as the db/+ leptin receptor deficient mouse model consuming a chow (i.e. regular) diet, RESV supplementation improved glucose levels, insulin sensitivity, reduced triglycerides and circulating cholesterol and increased anti-oxidant expression (C. K. Singh et al. 2011, 2013; Yao et al. 2015).

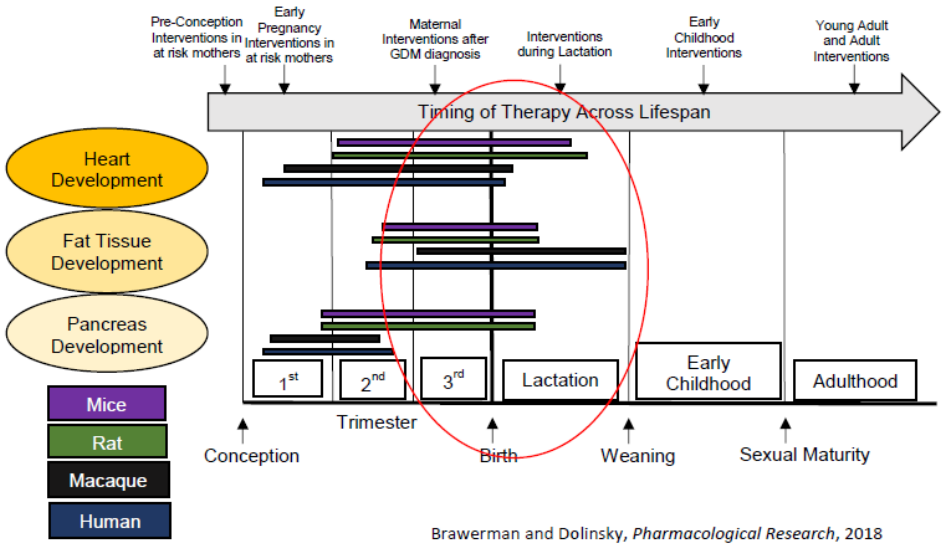
1.6.3 Offspring Studies:

The safety of RESV supplementation during pregnancy on fetal and offspring health in animal models has been assessed. Generally, RESV was found to be safe, well tolerated, and non-teratogenic (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; Williams et al. 2009). Different dosages and timing of supplementation have been tested, but generally, with positive results. RESV supplementation (4g/kg of diet) throughout gestation prevented small for gestational age offspring exposed to severe hypoxia (Bourque et al. 2012). Other studies using different animal models, dosages, and timing of intervention found that RESV supplementation increased fetal

survivability, decreased obesity, increased hepatic AMPK activity, reduced hepatic gluconeogenesis and improved insulin sensitivity in both male and female offspring (Yao et al. 2015; Zou et al. 2017). Maternal RESV supplementation during the lactation period reduced hepatic triglycerides, and hepatic steatosis, inhibited fatty acid synthesis and reduced obesity without affecting food intake by upregulating AMPK and SIRT1 in the male offspring (Tanaka et al. 2017). Interestingly, RESV had no effect on offspring from dams that consumed a low fat diet, but when compared to offspring of dams that had a high fat and sucrose diet supplemented with RESV, they had reduced body weight and adipose tissue content, suggesting that RESV may not have a significant role in healthy populations (Ros et al. 2018). Only one study reported a serious adverse effect of RESV on the offspring when RESV supplementation was started pre-conception and continued throughout pregnancy. Japanese macaques exposed to the RESV diet had a 42% enlargement of their fetal pancreatic mass, although islet and β-cell mass were not changed (Roberts et al. 2014). Also, insulin gene expression was not affected, and even though α-cell mass was reduced, there was an increase in glucagon expression (Roberts et al. 2014). The authors suggested there was an increase in α-cell proliferation as ki-67,which is a marker for proliferation, was up-regulated in these animals (Roberts et al. 2014).

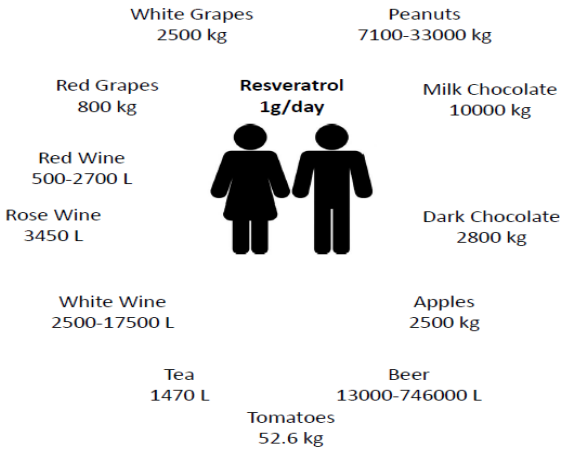
Other problems with RESV include the timing of therapy (whether it occurs prior, during pregnancy or during lactation) (Fig. 3), and dosage (Fig. 4), as bioavailability is low and high doses or amounts may be required to reach proper therapeutic levels. Additionally, more research is needed to elucidate the long-term health outcomes and any potential adverse events in the offspring as these are currently unknown.

Fig. 3: Timing of therapy across lifespan and the effect on selected tissue development in different animal models



This graphic shows the importance of timing of therapy and how that timing can interact with fetal development. As GDM is diagnosed at the beginning of the third trimester, most treatments are given at that point, which can have some effects on heart, pancreatic, and fat tissue development. Red circle highlights the timing of RESV supplementation in this study. Brawerman, Gabriel M., and Vernon W. Dolinsky. 2018. "Therapies for Gestational Diabetes and Their Implications for Maternal and Offspring Health: Evidence from Human and Animal Studies." *Pharmacological Research* 130: 52–73.

Fig. 4: Amount of different foods people have to consume to achieve 1g/day of Resveratrol



Adapted from (Weiskirchen and Weiskirchen 2016) "Resveratrol: How much wine do you have to drink to stay healthy?" *Adv. Nutr*. 7:706-718. In order to consume 1g/RESV/day, large amounts of different foods are required, thus new formulations are necessary.

1.7 GDM Animal Model:

Due to the ethical concerns of prescribing therapeutics to pregnant women for research, many different animal models have been designed to study the effects of GDM on both mothers and their offspring. These animal models provide us with a safer and more ethical way of testing out different treatments and interventions as well as different timings and dosages which we can later translate into clinical trials. GDM in humans is characterized by high blood glucose levels, glucose intolerance, insulin resistance and increased gestational weight gain in mothers and macrosomia in children with the onset near the end of the second trimester or beginning of the third (Buchanan, Anny H. Xiang, and Page 2012; Jovanovic and Pettitt 2001; Troy J. Pereira et al. 2015). Most current animal models may manifest some of these phenotypes, but not all. Different chemicals have been used to induce glucose and insulin intolerance, such as Streptozotocin, which is used to destroy β-cells of the pancreas, however in GDM β-cell destruction does not occur, thus this would not be a proper model to use (Pasek and Gannon 2013). Other models may use genetic manipulations, such as the db/db mouse which has a mutated leptin receptor, thus affecting appetite, and closely resembles the characteristics of GDM (Pasek and Gannon 2013). However, the homozygous mutant female is not fertile, and only the heterozygous can be used. But, clinically, most women do not tend to have this mutation (T J Pereira et al. 2015). Consequently, many models have been using diets rich in fat to induce obesity, which is a key risk factor for the development of GDM, and more closely resembles the clinical phenotype of GDM (Pasek and Gannon 2013).

1.7.1 GDM Animal Model previously characterized in the Lab:

Previously in the Dolinsky Lab, an animal model was designed to assess how accurate it would be at resembling a GDM pregnancy. Female Sprague-Dawley rats were randomly fed either a low fat diet or a high fat and sucrose diet for 6 weeks prior to mating to induce either a pre-diabetic and/or obesity state (Troy J. Pereira et al. 2015). They were then mated with lean males and followed throughout pregnancy. During the third trimester, after the onset of GDM, a glucose tolerance test (GTT) was performed to ensure that a glucose intolerance phenotype was observed. Based on this model, the dams that consumed the high fat and sucrose diet had increased gestational weight gain, impaired glucose tolerance, hyperinsulinemia, and moderate hyperglycemia mid-gestation (Troy J. Pereira et al. 2015). All of these observations are very similar to the clinical progression of GDM, suggesting that this model may resemble some of the features of the disease as well. The offspring also exhibited similar responses that are observed in children of mothers with GDM, such as increased obesity, macrosomia, insulin resistance, glucose intolerance, and other early markers of cardio-metabolic disease (Troy J. Pereira et al. 2015). Therefore, for my thesis work, we selected this model, but with some modifications to allow for the pregnant dams to consume the RESV diet instead (Refer to Materials and Methods and Fig. 4).

1.8 Thesis Objectives

Obesity levels are rising worldwide, and with this increase, more women are entering their pregnancies either obese or overweight, thus putting them at a greater risk of developing GDM. Previous research has shown that a GDM pregnancy predisposes mothers to have increased obesity and an increased risk for developing cardio-metabolic disease later on, such as T2D and CVD. Similarly, GDM has detrimental effects on the offspring as they are also at a greater risk for cardio-metabolic disease development. Current treatments include lifestyle therapies, such as diet and exercise, and therapeutics such as insulin, glyburide and metformin. These treatments can be efficient at lowering blood glucose levels; however they are all associated with serious adverse events such as maternal and fetal hypoglycemia, macrosomia and potential delivery complications. Even though these treatments may work in a subset of the population, failure rates are also rising, therefore there is the need for new therapies to be researched and brought into the market. Natural health products such as RESV, which has been shown to lower blood glucose levels and improve cardio-metabolic parameters, may be a good therapy option for these patients. However, appropriate dosages, timing of therapy, and the long term effect of RESV supplementation on the offspring are currently unknown. I used a diet-induced GDM animal model previously established in the Dolinsky lab, where rats were fed the high fat diet supplemented with RESV during the third trimester of pregnancy and lactation only. My first aim was to show what effects RESV would have on maternal metabolism, such as glucose tolerance and gestational weight gain compared to dams not exposed to RESV. My second aim was to study the effects of RESV supplementation on the metabolism in the young adult offspring (e.g. at 15 weeks of age) compared to the offspring from Lean and GDM dams. And finally, since our lab has observed that GDM exposure induced

cardiac hypertrophy in the male offspring, my third aim was to assess what effect RESV supplementation had on cardiac structure and function in the 15 week-old offspring.

1.9 General Hypotheses:

I hypothesized that maternal RESV supplementation during the third trimester of pregnancy and lactation would prevent glucose intolerance and improve metabolic parameters in the dams.

I also hypothesized that maternal RESV supplementation would prevent metabolic health disorders such as glucose intolerance, obesity and hepatic steatosis in the 15 week-old young adult rat offspring.

In addition, I hypothesized that maternal RESV supplementation would prevent cardiac hypertrophy and improve cardiac function in the 15 week old young adult male rat offspring.

Materials and Methods:

Diet-Induced GDM Model and Resveratrol Treatment

The University of Manitoba's Central Animal Care committees approved all animal procedures. 3 week-old female Sprague-Dawley rats were received from the Genetic Models Center (GMC) core facility (University of Manitoba) and were housed 2 per cage. They were randomly fed either a low-fat (LF) diet (10% kcal fat, Research Diets, D12450B) or a high fat and sucrose (HFS) diet (45% kcal fat, Research Diets, D12451) for 6 weeks to induce obesity prior to pregnancy. All rats were then mated with chow diet (i.e. regular rodent food) fed males within the animal facility at the Children's Hospital Research Institute of Manitoba (CHRIM) (one male per two female rats) and diets were continued throughout pregnancy and lactation. Pregnancy was confirmed 10 days after male breeders were separated from females via echocardiography scans. Pregnant females were then housed alone until birth. Since GDM is typically diagnosed later in pregnancy, a subgroup of female rats that consumed a HFS diet were switched to a HFS diet supplemented with Resveratrol (45% kcal fat + 4g/kg RESV, Research Diets, D10020402) at the beginning of the third trimester (i.e. day 14 of gestation) until birth and throughout lactation. This dosage, which is equivalent to ~147.6 mg/kg/day based on the amount of food consumed per rat per day, was previously determined to improve insulin sensitivity, glucose response, exercise capacity, be safe and non-teratogenic during pregnancy (Bourque et al. 2012; V. W. Dolinsky et al. 2011; V.W. Dolinsky et al. 2012; Vernon W Dolinsky et al. 2013). Therefore, we created three different experimental mothers; Lean (fed LF diet), GDM (fed HFS diet) and GDM+RESV (fed HFS diet + RESV). Pre-breeding, trimester 1, 2, and 3 random and fasted blood glucose levels were measured from the tail vein using the ACCU-CHEK® Aviva glucose meters and glucose

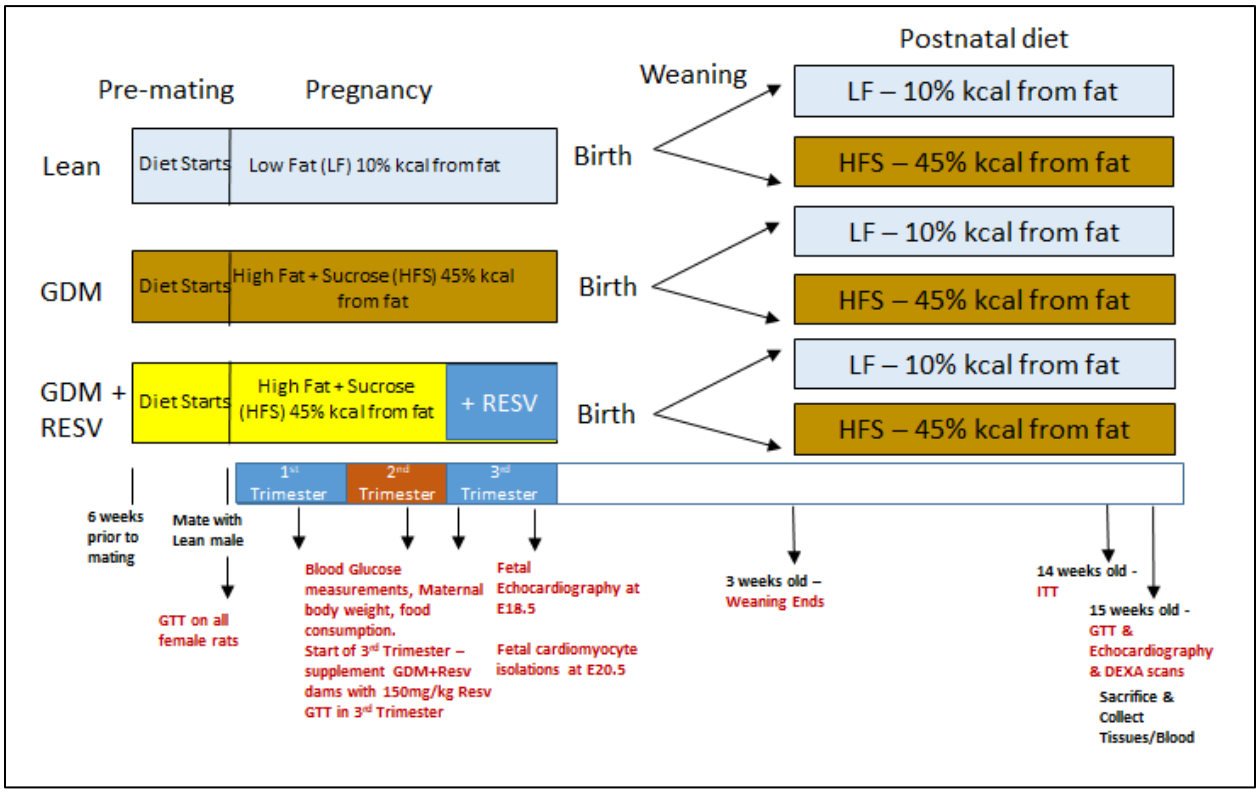
strips (Roche Diagnostics). Serum was collected at all three trimesters for later analysis. Maternal food consumption and body weights were measured weekly. Fetal echocardiography was done at e18.5 to assess fetal heart structure and function.

Assessment of Postnatal Diet Effect on Offspring:

Within 24 hours of giving birth, total litter size, sex, pup weight and length were all assessed, and litters were reduced to eight pups to avoid competition for food. Fetal hearts, livers and kidneys were collected and placed in liquid nitrogen and subsequently stored away in -80°C freezer for future studies. After weaning, at 3 weeks of age, offspring were randomly assigned to consume either a LF or a HFS diet for 12 weeks to examine the interaction between prenatal exposure and the postnatal diet and were housed 2 per cage when possible. Thus, six experimental groups were created; Offspring from lean dams consuming either LF or HFS postnatal diet (Lean-LF or Lean-HFS), offspring from GDM dams consuming either a LF or a HFS postnatal diet (GDM-LF or GDM-HFS), and finally offspring from a GDM+Resv dam consuming either a LF or a HFS postnatal diet (GDM+Resv-LF or GDM+Resv-HFS). Offspring food consumption and body weights were measured weekly. At 14 weeks of age, an insulin tolerance test (ITT) was performed on the offspring, while at 15 weeks of age a glucose tolerance test (GTT) was done. Similarly, at 15 weeks of age, dual x-ray absorptiometry scans (DEXA) and echocardiography were performed. Finally, at 15 weeks of age, rats were fasted for 4 hours prior to sacrifice. Some rats were injected with saline, while others were injected with Insulin (1 mU/kg) 15 minutes prior to sacrifice to allow for insulin-activated pathway studies. Rats were then euthanized by an overdose injection of sodium pentobarbital, while whole blood was collected into Vacutainer® tubes coated with 10.8 mg EDTA (BD, Franklin Lakes, NJ, USA) via cardiac puncture. These tubes were then centrifuged at 4000 rpm for 20 minutes at 4°C and the serum was aliquoted into

1.5mL Eppendorf tubes and stored at -80°C for later analyses. Hearts, kidneys, livers, lungs, fat pads, soleus and anterior tibialis muscle were dissected, weighed and placed in liquid nitrogen and later stored at -80°C for future experiments or in 10% formalin or 4% paraformaldehyde (PFA) to fix tissues for histological analyses. Another set of rats were sacrificed at e20.5-21 for cardiac myocyte isolations (Fig. 5).

Fig. 5: Experimental Animal Model



Three different experimental dams were generated by feeding them either a low fat (Lean control), high fat and sucrose (GDM), or high fat and sucrose + Resveratrol (GDM+RESV). The latter was given at the start of the third trimester of pregnancy until the end of lactation. Throughout pregnancy, dams had their blood glucose levels measured, and a T3 glucose tolerance test was performed at ~e18. Fetal echocardiography was performed at ~e18.5, while fetal cardiomyocytes were isolated at ~e20.5. After a 3 week lactation period, offspring were randomly assigned to either a low fat or a high fat and sucrose diet until 15 weeks of age. At 14 weeks of age, insulin tolerance tests were performed. At 15 weeks of age, glucose tolerance tests, DEXA scans and echocardiography were done. Offspring were then sacrificed and their tissues and serum collected for later analyses. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, GTT: Glucose Tolerance Test, ITT: Insulin Tolerance Test, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, DEXA: Dual X-ray Absorptiometry Scans.

Insulin and Glucose Tolerance Tests (ITTs and GTTs):

Prior to mating and during the third trimester of pregnancy (i.e. ~e18), female rats were given a glucose tolerance test to determine glucose response and whether resveratrol offered any improvements as previously described (V. W. Dolinsky et al. 2011). Briefly, female rats were fasted overnight for the pre-breeding GTT while pregnant rats were fasted for 4 hours for the trimester 3 GTT. Fasted blood glucose from the tail vein was measured with the aid of ACCU-CHEK® Aviva glucose meters and glucose strips (Roche Diagnostics) and serum was then collected using a microvette® (Sarstedt, Germany). A glucose solution (2g/kg) was intraperitoneally injected into the rats. Blood glucose levels were measured at times 0, 15, 30, 45, 60, 90, and 120 minutes. In the offspring, at 14 and 15 weeks of age, insulin and glucose tolerance tests were performed as previously described (V. W. Dolinsky et al. 2011). Offspring were fasted for 4 hours for the ITT and for 24 hours for the GTT procedure. The GTT was performed as described above. For the ITT, an insulin solution (Sigma, St. Louis, MO, USA) (2g/kg) was intraperitoneally injected into the rats. Blood glucose levels were measured at times 0, 30, 45, 60, 90, and 120 minutes. HOMA-IR index was calculated by multiplying fasting blood insulin concentration and fasting blood glucose levels divided by 22.5, as previously described (Wallace, Levy, and Matthews 2004).

Assessing Circulating and Tissue Factors:

Circulating concentrations of insulin (ALPCO®, Salem, NH, USA), free fatty acids (Cayman Chemical, Ann Arbor ,MI, USA), and triglycerides (Wako Diagnostics, Mountain View, CA, USA) from serum were assessed using colorimetric and fluorometric ELISA (Enzyme-Linked Immunosorbent Assay) assay kits by following the manufacturer's instructions.. Liver and cardiac

tissue were homogenized to measure triglyceride content via colorimetric assays (Biovision, Milpitas, CA, USA) by following the manufacturer's instructions.

Dual-Energy X-ray Absorptiometry Scans:

To further study fat distribution, content, and mass, plus bone mineral content and density, DEXA (Dual Energy X-Ray Absorptiometry – Hologic Discovery ®) scans were performed on 15 week-old rat offspring at CHRIM (Children's Hospital Research Institute of Manitoba) facilities. A technician blinded to the offspring experimental groups took all the images once rats were anesthetized using 5% Isoflurane gas for induction and 2% Isoflurane gas for maintenance. Rats were placed back into their cages before they regained consciousness.

Echocardiography Scans:

To study the effects of the prenatal and postnatal diets on cardiomyopathy development in the offspring exposed to GDM and subsequently with Resveratrol, in vivo echocardiography using the Vevo 2100 high frequency ultrasound system (Central Animal Care Small Animal Imaging Core Facility at the U of M) was used. A technician blinded to the experimental groups took all echocardiography images including data from all rats in utero (e18.5) and in young adulthood stages (15 weeks of age). I performed a blinded analysis utilizing the Vevo 2100 cardiac measurement software package to assess cardiac structure and function. Left ventricular wall thickness, intraventricular septal thickness and left ventricular chamber size and volumes were used to assess morphological changes, while cardiac output and left ventricular ejection fraction were measured to assess systolic function of the heart. Isovolumetric relaxation time and mitral valve E/A ratio were used to assess diastolic function.

Liver Histology/pWAT Histology:

Hematoxylin and Eosin (H&E) staining of formalin fixed liver and adipose tissue was performed by the University of Manitoba Histology Core Platform according to their standard procedures. Individuals blinded to the offspring diets took liver images using the Zeiss Lab.A1 AX10 microscope from the CHRIM microscopy core platform. Images were taken at both 20x and 40x magnification, and 10 pictures of different sections per slide were taken to assess hepatocyte size and fat droplet accumulation. Perirenal white adipose tissue (pWAT) was stained using the same procedure as mentioned above, and images were taken by a blinded individual using the same microscope as mentioned above. Images were taken at 20x magnification, and 6-10 pictures of different sections per slide were taken to assess adipocyte number and size. Image analyses was performed using ImageJ software (NIH, USA).

Western Blot:

For western blotting, frozen cardiac and liver tissue from both saline and insulin injected animals were homogenized using ice-cold sucrose buffer containing 20mM Tris-HCl at pH 7.4, 50mM NaCl, 50mM NaF, 5mM sodium pyrophosphate, 0.25M sucrose, and supplemented with protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO, USA), 1M dithiothreitol (DTT), and 20mM sodium orthovanadate. Briefly, 20-30mg of tissue was homogenized with 300uL of lysis buffer using a homogenizer and the lysate was then centrifuged at 4000 rpm for 10 minutes at 4°C to remove nuclei and cellular debris. The Bradford protein assay was then used to calculate protein concentrations (Bio-Rad). 20-25 ug of protein was separated using 8-10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots were then visualized using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL,

USA) and a developer. All primary antibodies were purchased from Cell Signalling Technologies (Danvers, MA, USA), and used at 1:1000, unless otherwise noted (Table 1). Secondary horseradish peroxidase-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and were used at concentrations of 1:5000-1:20000. Protein expression was normalized to either total protein expression or to Tubulin expression and then to Lean-LF controls. Band intensity was analyzed using ImageJ software (NIH, USA).

Table 1. Primary antibodies used for immunoblotting analyses

Antibody	**Company**	**Catalog Number**	**Working Dilution**
p-AKT (Ser473) and AKT	Cell Signalling Technology ®	9271/9272	1:1000
p-PI3K (Tyr458/Tyr199)	Cell Signalling Technology ®	4228	1:500
α-Tubulin	Cell Signalling Technology ®	2144	1:1000
SOD2	Abcam	13534	1:400
Serca2a	Cell Signalling Technology ®	4388	1:1000
ATGL	Cell Signalling Technology ®	2138	1:1000
p-mTOR (Ser2448) and mTOR	Cell Signalling Technology ®	2971/2972	1:1000
p-P70S6K (Thr389) and P70S6K	Cell Signalling Technology ®	9205/9202	1:1000
Gpx-1	Abcam	22604	1:1000
Catalase (D5N7V)`	Cell Signalling Technology ®	14097	1:1000
p-eEF2 (Thr56)	Cell Signalling Technology ®	2331	1:5000
eEF2	Cell Signalling Technology ®	2332	1:1000
p-IRβ (Tyr1150/1151) (19H7)	Cell Signalling Technology ®	3024	1:2500
IRβ (4B8)	Cell Signalling Technology ®	3025	1:1000
p-IRS-1 (Ser1101) and IRS-1 (D23G12)	Cell Signalling Technology ®	2385/3407	1:1000

Antibodies and dilutions used for immunoblotting of liver and heart tissue of 15 week old male rat offspring. AKT: Protein Kinase B, PI3K: Phosphatidylinositol 3 Kinase, SOD2: Superoxide Dismutase 2, Serca2a: Sarco/endoplasmic Reticulum Calcium ATPase, ATGL: Adipose Triglyceride Lipase, mTOR: Mammalian Target of Rapamycin, P70S6K: Ribosomal Protein P70 S6 Kinase, Gpx-1: Glutathione Peroxidase 1, eEF2: Eukaryotic Elongation Factor 2, IRβ: Insulin Receptor β, IRS-1: Insulin Receptor Substrate 1

RNA Isolation and qPCR:

For RNA isolation, 20-30 mg from saline injected liver tissue were homogenized using 600 uL of lysis buffer provided in the kit, and was further homogenized using a homogenizer column as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). The PureLink™ RNA Mini Kit (Ambion Life Technologies, Carlsbad, CA, USA) was used to isolate and purify RNA using the on-column PureLink® DNase treatment protocol without modifications. RNA integrity was assessed by running samples on 1% agarose gel and visualized using the FluorChem® HD2 (Alpha Innotech, Germany). RNA concentration was measured with the NanoDrop 2000 (Thermo Fisher Scientific, Rockford, IL, USA). 1ug of RNA was converted to cDNA using the ProtoScript® II First Strand cDNA Synthesis kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. The QuantiTect® SYBR® Green PCR Kit (Qiagen) was used to monitor the amplification of cDNA on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The CFX software was then used to visualize the expression of genes which were plated in duplicate and analyzed using the 2-ΔΔCT method as described in (Livak and Schmittgen 2001). Briefly, the target gene Ct was subtracted from the reference genes Ct creating the first ΔCT. This value was then subtracted from the Ct corresponding to the Lean-LF control gene expression generating the ΔΔCT. Finally, the following equation was used to calculate the relative fold-change in expression (2^-ΔΔCT). Additionally, data were normalized to the geometric mean (geomean) of eukaryotic translation initiation factor 2a (*eIF2a*) and cyclophilin A (*cycA*) expression which were used as the reference genes as they were constant across all groups. Thus,

gene expression of target genes was expressed relative to the geomean of the housekeeping genes. In this method, the geometric average of multiple housekeeping genes was taken resulting in a more accurate representation than if taking the regular average of those genes alone (Vandesompele et al. 2002). Therefore, instead of normalizing the data to a single gene, the data was normalized to two different housekeeping genes, which provides a more accurate way of normalizing qPCR data (Vandesompele et al. 2002). Data was then normalized to the Lean-LF expression of each target gene. All primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). Primer sequences were validated and are provided in Table 2.

Table 2. Primer sequences used for quantitative real-time PCR

Gene	Forward Primer 5′ – 3′	Reverse Primer 5′ – 3′
Acc2	AGAACTCGATGACCCTTCA	TCAGATTTTCCAAGACGC
Srebp-1c	CCTCTCTGGAAGCCTT	ACTGGCTCCTCTTTGAT
Pparα	GATGACCTGGAAAGTCCCTTATC	AGCTCCCTAAGTACTGGTAGTC
Pcyt2	CCATCAAGTGGGTGGATGAA	CATTGCCATGAACGCAGAAG
Sod2	GCGACCTACGTGAACAATCT	CTGAAGAGCAACCTGAGTTGTA
Pgc1α	GACACGAGGAAAGGAAGACTAAA	GTCTTGGAGCTCCTGTGATATG
CycA	TCCAAAGACAGCAGAAAACTTTCG	TCTTCTTGCTGGTCTTGCCATTCC
EIF2a	CCTGAAGTGTGATCCTGTGTTT	CCAAATCCAGCCAGCACTAATA
Pepck-1	ATGCTGATCCTGGGCATAAC	CATCACCCACACATTCAACTTTC
G-6-P	GTGGTTGGAGACTGGTTCAA	CACGGAGCTGTTGCTGTAATA

Validated primer sequences used for qPCR in liver tissue of 15 week old male rat offspring. *Acc2*: Acetyl CoA-Carboxylase 2, *Srebp-1c*: Sterol Response Element Binding Protein 1c, *Pparα*: Peroxisome Proliferator Activated Receptor α, *Pcyt2*: CTP:phosphoethanolamine cytidylyltrasferase 2, *Sod2*: Superoxide Dismutase 2, *PGC1α*: Peroxisome Proliferator Activated Receptor γ Coactivator 1α, *CycA*: Cyclophilin A, *EIF2a*: Eukaryotic Translation Initiation Factor 2a, *Pepck-1*: Phosphoenolpyruvate Carboxykinase-1, *G-6-P*: Glucose-6-Phosphate

Cardiomyocyte Isolations:

To study the function of cardiac myocytes upon different diet exposures, fetal (e20.5-21) ventricular cardiomyocytes from offspring of Lean, GDM and GDM+Resv dams were isolated as described by (Kovacic et al. 2003) with some modifications. Briefly, fetal rat hearts at e20.5-21 were isolated and stored in ice-cold 1 X Phosphate Buffered Saline (PBS) solution. Hearts were rinsed 3 times with 1x PBS and the atria and any other debris on the hearts were removed. The ventricles were then minced with curved iris scissors and were transferred to a non-vented T25 tissue culture flask containing 17mL of cold 1x PBS, 1mL of filter sterilized 0.5% DNase/2% Collagenase and 0.5 mL 2% Trypsin. This flask was agitated on a rotary shaker at 80 rpm for 20 minutes at 37°C to start the first dissociation. The contents were then mixed with 20 mL of pre-warmed DF20 media (plus 20% fetal bovine serum (FBS), 1% penicillin/streptomycin and 50ug/mL gentamicin) and centrifuged at 800 rpm at room temperature for 2 minutes. The supernatant was discarded, and 1X PBS, DNase/Collagenase and Trypsin were added to the pellet and placed in the rotary shaker for another 20 minutes at 37°C to start the second dissociation. The contents were transferred to a 50mL falcon tube containing 20mL of pre-warmed DF20 media and spun at 800 rpm at room temperature for 1 minute. The supernatant was collected into another 50mL falcon tube and spun at 1700 rpm at room temperature for 7 minutes, the supernatant was again removed, and 20 mL of pre-warmed DF20 media was added to the pellet. 1X PBS, DNase/Collagenase and Trypsin were added to the pellet from the second dissociation to start the third dissociation by placing the flask on the rotary shaker for another 20 minutes at 37°C. After the final incubation, all the supernatants were mixed and spun at 1700 rpm at 4°C for 7 minutes. The supernatant was removed, while the pellet was resuspended with 12mL of pre-warmed plating media (DMEM/F12 (1:1), Horse Serum, FBS, 1% Penicillin/Streptomycin and 50 ug/mL

gentamicin). This was filtered through a 100 uM nylon cell strainer into a vented T75 tissue culture flask and placed into a 37°C incubator for 1 hour. After 1 hour, the supernatant was transferred into another T75 flask and incubated for an hour. After incubation, the supernatant was collected into a 50mL falcon tube and spun at 1000 rpm for 2 minutes at room temperature. The resulting pellet was then diluted with pre-warmed plating media. After cell counting, the cells were plated on primaria dishes (Falcon) at a density of 500,000 cells/well. The next day, cells were washed with 1x PBS as needed.

MitoSOX Experiment:

To assess reactive oxygen species production, namely superoxide, by the mitochondria in the fetal cardiomyocytes, the molecular dye MitoSOX™ Red (Invitrogen™, #M36008) was applied to the cells. Briefly, cardiomyocytes were washed twice with warm HBSS media, and 0.5mL of MitoSOX solution (plus Hoescht 1:1000) was added to each well. The plate was covered with aluminum foil and incubated at 37°C for 20 minutes. The wells were washed with warm HBSS, then again but for 5 minutes on a rocker at room temperature, and one final wash back in the biosafety cabinet. Cells were then fixed with 4% para-formaldehyde (PFA) for 15 minutes in a cold room. Wells were washed 3 times with cold 1x PBS. 0.5mL of cold 1X PBS was added to the cells, the sides of the plate were parafilmed and the plate was wrapped in foil until imaging using the Olympus Epifluorescence and Calcium Imaging Microscope (CHRIM) using the Cy3 (510/580 nm) and Dapi (350/461 nm) filters with 40X objective.

MitoTracker Experiment:

Similarly, MitoTracker™ Deep Red FM (Invitrogen™, #M22426) dye was used to assess mitochondrial number within each well. Cells were washed twice with warm 1x PBS. MitoTracker (plus Hoescht 1:1000) solution was added to the wells, the plate was wrapped in foil and incubated at 37°C for 30 minutes. After incubation, the wells were washed twice with warm 1x PBS, and then fixed for 15 minutes in the cold room with 4% PFA. Cells were then washed twice with 1x PBS. 0.5mL of cold 1x PBS was added to the cells, the sides of the plate were parafilmed and wrapped in tin foil until imaging using the Olympus Epifluorescence and Calcium Imaging Microscope with the Cy5 (644/665 nm) and Dapi (350/461 nm) filters with 40X objective.

TMRM Experiment:

Finally, mitochondrial membrane potential was observed by applying TMRM (Tetramethylrhodamine, Methyl Ester, Perchlorate) (Biotium, #70017, Fremont, CA, USA) dye. Cells were washed once with warm 1x PBS, and then TMRM solution (plus Hoescht 1:1000) was added to each well. The plate was covered with aluminum foil and incubated at 37°C for 30 minutes. Wells were then washed once with 1x PBS, and the cells were immediately imaged using the Olympus Epifluorescence and Calcium Imaging Microscope with Cy3 (548/573 nm) and Dapi (350/461 nm) filters with 40X objective. Hoescht 33342 (Thermo Scientific™, #62249) dye was used in all three experiments at a concentration of 1:1000 to stain the nucleus blue.

Maternal and Offspring Islet Isolations:

This was performed by members in Dr. Christine Doucette's Laboratory. After sacrificing the animal, the pancreas was perfused through the bile duct with cold Collagenase V solution (Sigma, St. Louis, MO, USA). The pancreas was then removed and placed into a 50mL conical tube which contained 5mL of cold Collagenase V solution. The tube was placed in a water bath at 37°C for 15 minutes with manual shaking at 2-3 minute intervals to allow for proper pancreatic digestion. 30-35 mL RPMI (11.1 mM glucose, Invitrogen) + 10% FBS + 1% P/S + 1% L-glutamine, Invitrogen) was then added to the solution, and the tube was put on ice. In order to create a homogenous solution, the tube was inverted and centrifuged at 290 x g for 30 seconds. The supernatant was removed, and 20 mL of cold 1X PBS was used to wash the pellet by centrifugation at 290 x g for 30 seconds. The pellet was re-suspended and the homogenous solution was filtered through a 100 mm cell strainer (VWR, Pennsylvania, USA) which was previously wetted with 1-2 mL of 1X PBS, into a 50 mL tube. The strainer was washed with 3 mL 1X PBS while tapping to increase liquid flow. Islets trapped on the strainer were washed with 10 mL 1 X PBS. A sterile 100 mm tissue culture plate was used to capture the islets from the strainer after rinsing with 15 mL of cold media. A dissection microscope (Olympus SZ61; NCL 150 Illuminator) was used to manually pick individual healthy looking islets. 200-300 islets were picked and centrifuged at 290 x g for 30 seconds. The resulting pellet was re-suspended in 300 mL RLT buffer (Qiagen, Valencia CA, USA) and was placed in the -80°C freezer until needed.

Glucose-Stimulated Insulin Secretion (GSIS) Assays:

This was performed by members in Dr. Christine Doucette's Laboratory. 8-20 islets per group were washed with cold KRB and were then incubated at 37°C in 2.8 mM glucose for 1 hour. The tubes were put on ice and the KRB removed. 2.8 or 16.7 mM of cold glucose KRB was added to the islets which were then incubated at 37°C for 30 minutes. A mouse insulin ELISA kit (ALPCO, Salem, NH, USA) was used to measure insulin concentration in the media. Total insulin content in the islets were measured as above and the insulin concentration was normalized to DNA content of the islets.

Statistical Analysis:

Data are presented as the mean +/- standard error of the mean (SEM). GraphPad Software (La Jolla, CA, USA) was used for all statistical analyses. One and Two-way Analysis of Variance (ANOVA) were used to assess differences among maternal and postnatal diets with a Bonferroni post hoc test to look at differences between specific groups. Any comparisons between two groups were done using an unpaired t-test. Repeated measures tests were performed when necessary. $p<0.05$ was defined as the level of significance.

Chapter 2:

Effects of Resveratrol Supplementation during Pregnancy on Maternal Metabolism.

2.1 Introduction:

Worldwide obesity has more than tripled since 1975 and is reaching epidemic levels (World Health Organization. 2017). Since more individuals are living a sedentary lifestyle, our diets are becoming fattier and less healthy, and there are increased changes in genetics and in the environment, then obesity levels will continue to rise, particularly among the younger population (Corey and Kaplan 2014; Lehnert et al. 2013; Swinburn et al. 2011; World Health Organization. 2017). Obesity can be problematic as many conditions and diseases are known to be partially caused by the increased fat accumulation, such as cardio-metabolic diseases, namely T2D and CVD (Ng et al. 2014; Srivastava 2012). As obesity levels rise, more women of child-bearing age will also be starting their pregnancies either obese or overweight (Simmons 2011). The Developmental Origins of Health and Disease (DOHaD) theory had been suggested to explain how the maternal intrauterine environment during development could have serious repercussions on offspring health outcomes in both the short and long-term as molecular programming may be taking place, thus increasing their risk for developing chronic diseases later on (D. Barker et al. 1993; D. J P Barker 2007; David J.P. Barker 1997).

Gestational diabetes mellitus (GDM) is considered to be one of the most common complications of pregnancies (Ferrara 2007; Kim et al. 2010). GDM has been found to affect 1-14% of pregnancies, and these numbers are expected to rise as obesity levels keep increasing in the population (Ferrara 2007; Flegal et al. 2010; Kim et al. 2010). GDM is characterized by increased gestational weight gain, mild hyperglycemia and insulin resistance with the onset at the beginning of the third trimester of pregnancy (American Diabetes Association 2015). Importantly, GDM is a transient condition, where blood glucose levels tend to go back to normal after parturition, however, it can have long-lasting effects on both mothers and their offspring

(American Diabetes Association 2015; Buchanan, Anny H. Xiang, and Page 2012; Jovanovic and Pettitt 2001). GDM during pregnancy has been found to predispose mothers to eclampsia-preeclampsia, greater risk of caesarean sections, reduction in breast feeding initiation, increased obesity, and eventually, glucose intolerance and insulin resistance, T2D and CVD development (Poston, Harthoorn, and Van Der Beek 2011). A GDM pregnancy can also have serious problems in the health of the offspring, such as macrosomia, birth defects or malformations, hypoglycemia, and cardio-metabolic disease development (Arshad, Karim, and Hasan 2014; Hod et al. 1991; Kaaja and Rönnemaa 2008; Persson et al. 2017). These offspring effects will be discussed in the next chapter.

Current treatments include lifestyle therapies, such as diet and exercise, to maintain proper glycemic levels during pregnancy and the oral-antidiabetic drugs metformin and glyburide, while insulin injections may also be required in serious cases where women do not respond to previous treatments (Lipscombe et al. 2018). These interventions generally work in the population, however they may increase the risk for macrosomia and both fetal and maternal hypoglycemia which could cause harm to the mother and her infant, as recently reviewed in (Brawerman and Dolinsky 2018). Additionally, we do not fully understand the long-term effects that interventions administered during pregnancy could have on the developing baby. Therefore, newer treatments for GDM should be explored to circumvent problems associated with existing interventional options (Fig. 1).

Maternal exercise has been shown to have some benefits when done by obese pregnant women or women who already have GDM as they did not experience as many hypoglycemic events as women on glyburide or metformin did, and even had lower blood glucose levels when compared to women that did not exercise (Barakat et al. 2013; Koivusalo et al. 2016). However,

exercise intensity and duration is still problematic which explains why exercising during pregnancy is also associated with poor compliance (Nelson, Matthews, and Poston 2010). Resveratrol (RESV) is a natural health product that has been found to mimic several of the beneficial effects that we observe with exercise training on metabolic tissues. This is because RESV activates similar molecular pathways as exercise, so it may become a potential add-on therapy (V. Dolinsky and Dyck 2014; Vernon W. Dolinsky and Dyck 2011; Timmers et al. 2011). Importantly, RESV was reported to efficiently decrease blood glucose levels (Mohammadshahi, Haidari, and Soufi 2014; Yao et al. 2015). Furthermore, in animal studies, RESV supplementation during pregnancy further decreased maternal hyperglycemia, improved insulin sensitivity, reduced triglycerides and cholesterol levels and increased the expression of anti-oxidants, potentially providing a better intrauterine environment for fetal development (C. K. Singh et al. 2011, 2013; Yao et al. 2015). Finally, RESV was also found to be safe, well tolerated and non-teratogenic in both human and animal studies (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; B. Wicklow et al. 2015; Williams et al. 2009).

To date, no studies have been performed to assess the effects of RESV supplementation for both mother and offspring short and long-term health outcomes in a diet-induced GDM model (only in a genetic db/+ model, (Yao et al. 2015)). Additionally, this is the only study that administered the intervention at the onset of GDM (i.e. third trimester), as most previous studies supplemented diets with RESV either before gestation, throughout pregnancy and lactation, or only during lactation which may not be clinically significant (Bourque et al. 2012; Roberts et al. 2014; C. K. Singh et al. 2011, 2013; Tanaka et al. 2017; Yao et al. 2015; Zou et al. 2017).

Our objective was to determine whether 4g/kg (~146 mg/kg/day) of RESV supplementation in the third trimester of pregnancy, at the onset of GDM, and lactation would

prevent maternal glucose intolerance and improve metabolic parameters in pregnant rats. We hypothesized that RESV supplementation would reduce blood glucose levels, thus improving maternal intrauterine environment, and preventing GDM-related complications on the mothers. We showed that RESV supplementation did prevent glucose intolerance, lowered blood glucose levels in the third trimester and improved insulin secretion. We also showed no significant changes occurred in litter sizes or sex distribution and no serious adverse events were observed with RESV supplementation.

2.2 Materials and Methods – Refer to page 37.

2.3 Results:

2.3.1 Maternal RESV supplementation during pregnancy prevented glucose intolerance, restored blood glucose levels, and improved insulin secretion, without affecting gestational weight gain in the rat dams:

After 6 weeks on their respective diets, the HFS fed females had a tendency of weighing more, a 1.17 fold increase, than the lean group (Fig. 6A), however their pre-breeding GTT was not different (Area under the curve for Lean 1089 ± 29.46 vs. GDM 1189.6 ± 30.14). This is consistent with the clinical phenotype of GDM where there is no glucose intolerance prior to pregnancy. Females were then mated with chow fed lean males. During pregnancy, the HFS fed dams continued gaining weight at a higher pace than the LF fed dams, in turn, their gestational weight gain (GWG) was significantly higher, exhibiting a 2.36 fold increase, than the Lean dams (Fig. 6B). At the beginning of the third trimester of pregnancy, which is when GDM is diagnosed clinically, a subgroup of females consuming the HFS diet were supplemented with the RESV diet (termed GDM+RESV). Consumption of RESV diet did not affect the GWG associated with a HFS diet because their GWG had a 2.48 fold significant increase when compared to the Lean dams

(Fig. 6B). But, maternal food consumption from the third trimester of pregnancy was significantly lower in the GDM+RESV dams when compared to the Lean dams (Fig. 6C). After calculating the energy intake per kg of body weight, there were no differences among the maternal diets (Fig. 6D and Table 3).

Closer to the end of pregnancy (i.e. ~18) a trimester 3 GTT was performed on the pregnant rats to assess glucose intolerance and GDM development. Even though fasting blood glucose levels were unchanged, 15 minutes after glucose injection, the GDM dams had a 1.5 times significant increase in blood glucose concentration when compared to both GDM and GDM+RESV dams (Fig. 6E and 6F). Additionally, it took 2 hours for the GDM dams to reach basal levels, while it only took 90 minutes for both Lean and GDM+RESV dams to reach their basal levels (Fig. 6E). Thus, the GDM dams were more glucose intolerant than both Lean and GDM+RESV dams (Fig. 6E and 6F), suggesting there was an inability of GDM dams to take up glucose as efficiently as either Lean or GDM+RESV dams. However, with maternal RESV supplementation glucose response was normal (Fig. 6E and 6F), implying that these animals can better respond to the increased sugar levels. Additionally, random blood glucose levels were measured before mating and at each trimester of pregnancy, and a ~1.6 fold significant increase in blood glucose in the second trimester for both GDM and GDM+RESV dams was observed when compared to the Lean dams. Interestingly, during the third trimester (i.e. after the change to RESV diet), blood glucose levels significantly decreased in the GDM+RESV dams to the same levels observed in the Lean, while the GDM dams still exhibited blood glucose levels that were 1.33 times higher than both Lean and GDM+RESV dams (Fig. 6G). These findings suggested that GDM+RESV dams were capable of dealing with the extra sugars better than the GDM dams.

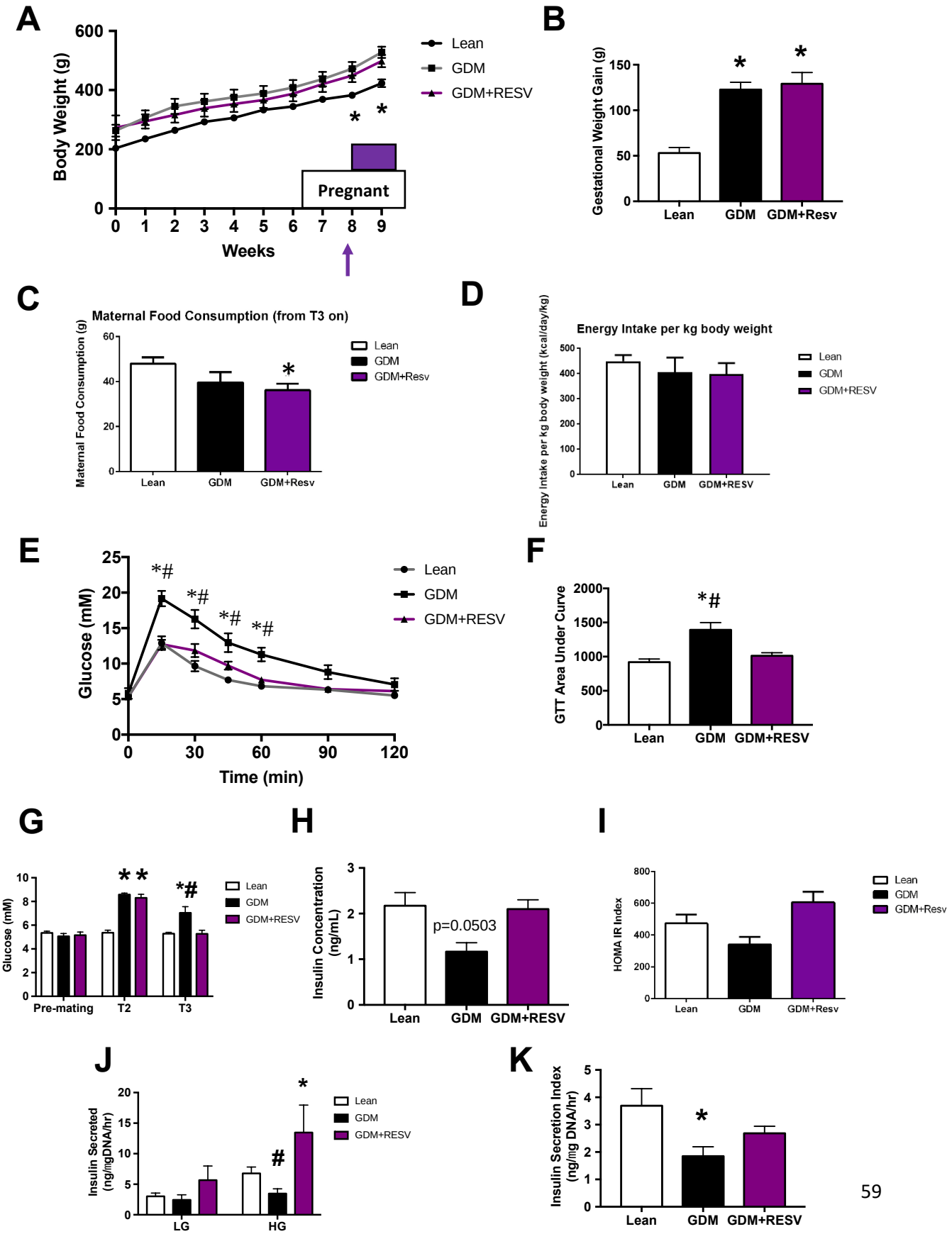
As blood glucose levels are closely tied with how much insulin is generated by the β-cells of the pancreas, the insulin concentration in the maternal serum during the third trimester of pregnancy was quantified using an ultra-sensitive ELISA kit (ALPCO®). The GDM dams had almost half the concentration of insulin observed in the Lean and GDM+RESV dams (Fig. 6H). This result could explain the reduction in blood glucose levels observed in those animals. The HOMA-IR index, a measure of insulin resistance, showed a similar trend, indicating an increase of almost double in the GDM+RESV dams compared to the GDM dams (Fig. 6I). Even though the HOMA-IR was increased in the GDM+RESV dams, they did not show signs of insulin resistance or impaired glucose metabolism. Additionally, as part of a collaboration with Dr. Christine Doucette's laboratory at CHRIM, glucose stimulated insulin secretion (GSIS) assays were done on the islets that were isolated from pancreas extracted from the dams. In high glucose (HG) media, insulin secretion was impaired in the islets from GDM dams, as they secreted almost half of what the Lean dams secreted, whereas GDM+RESV dams were able to secrete 3.87 times more insulin than the GDM dams (Fig 6J and 6K). However, with low glucose concentrations in the media, insulin secretion by isolated islets was similar among all three groups of dams (Fig. 6J). These findings imply that RESV rescued insulin secretion after exposure to glucose in the third trimester in GDM+RESV dams.

Table 3. Average daily food consumption by pregnant mothers (from T3 to end of lactation)

Average Daily Food Consumption by mothers (from T3 to end of lactation)			
	Lean	GDM	GDM+Resv
Food Consumption (g)	47.99 +/- 8.84	39.63 +/- 7.99	36.19 +/- 8.27 *
Amount of RESV consumed (mg/kg/day)	-	-	147.6 +/- 18.93
Energy Intake (kcal/day)	184.8 +/- 34	187.4 +/- 37.8	171.2 +/- 39.1
Energy Intake (kcal/day/kg body weight)	449.5 +/- 73.4	405.7 +/- 100.7	398.3 +/- 98.3

Daily amount of food consumed and energy intake by pregnant mothers from T3 to the end of lactation. N=3-10. *p values represent significant differences (<0.05) between GDM+RESV and Lean after One Way ANOVA with Bonferroni post-hoc tests. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, T3: Trimester 3

Fig. 6: Maternal RESV supplementation during pregnancy prevents glucose intolerance, restores blood glucose levels, and improves insulin secretion, without affecting gestational weight gain.



(A) Change in body weights over time – arrow indicates timing of RESV supplementation, (B) Gestational Weight Gain, (C) Food Consumption, (D) Energy Intake adjusted to body weight, (E) Trimester 3 GTT, (F) GTT Area Under the Curve, (G) Blood Glucose Levels pre-pregnancy and throughout pregnancy, (H) Insulin Concentrations at Trimester 3, (I) HOMA-IR Index, (J) Insulin Secretion, (K) Insulin Secretion Index. Maternal body weights and gestational weight gain N= 6-9, Maternal food consumption and energy intake N=3-10, T3 GTT N=6-8, T3 insulin concentration N=4-8, Blood glucose levels N= 2-8, Insulin assays N= 5-18. p-values represent significance (<0.05) after One Way, Two Way ANOVA, Repeated Measures ANOVA or multiple T-tests where applicable with Bonferroni post-hoc tests. *$p<0.05$ vs. Lean. #$p<0.05$ vs. GDM+RESV. GTT: Glucose Tolerance Test, HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

2.4 Discussion/Conclusion:

The objective was to determine the effects of resveratrol supplementation during the third trimester of pregnancy and lactation on female Sprague-Dawley rats exposed to GDM. Previous clinical studies have found that RESV supplementation decreased the risk of developing GDM by improving both lipid and blood glucose levels, with the addition of also lowering blood pressure in overweight pregnant women (Ding et al. 2017; Malvasi et al. 2017). Thus, rats consumed a high fat and sucrose diet for 6 weeks before mating to induce obesity, an important risk factor for the development of GDM. After 6 weeks on the diet, a pre-breeding GTT was performed to assess whether these rats were glucose intolerant prior to being mated, and as expected, there were no differences. Since these animals were not pregnant, the no change in pre-breeding GTT suggests that consuming a high fat diet is not by itself a trigger for glucose intolerance, but rather a second hit or stressor (i.e. pregnancy) is required for the onset. This was more clearly observed after performing a trimester 3 GTT. Since GDM usually occurs at the beginning of the third trimester of pregnancy, a GTT was done near the end of pregnancy (i.e. ~e18) to determine if at this time point the rats were glucose intolerant. Indeed, the GDM dams exhibited an abnormal response, implying that they were not able to clear up the increased glucose load as efficiently as the Lean dams did. More importantly, the GDM+RESV dams exhibited a very similar response to that of the Lean, so their glucose tolerance was normal. These improvements in glucose homeostasis after RESV supplementation were also observed in other animal studies (Roberts et al. 2014; C. K. Singh et al. 2011, 2013; Yao et al. 2015).

Surprisingly, gestational weight gain was not improved by RESV supplementation. In fact, the GDM+RESV dams had a similar GWG when compared to the GDM dams, and a significantly higher GWG when compared to the Lean. This is the opposite of what I was expecting to observe

as other research has shown a decrease in GWG (Roberts et al. 2014). However, Roberts et al. used a Japanese macaque animal model, their RESV dosage was 0.37% mixed in with the diets, and these animals consumed RESV for 3 months before they were mated and throughout gestation (Roberts et al. 2014). These differences in dosage, timing and animal model may explain the different results observed. Interestingly, GDM+RESV dams consumed less food than the other two groups of dams from the beginning of the third trimester until lactation, however their energy intake was the same. This suggests that one week on the RESV diet was not enough to counteract the effects of almost 9 weeks on the HFS diet on weight gain.

More importantly, and as other studies have also shown (C. K. Singh et al. 2011, 2013; Yao et al. 2015), RESV supplementation reduced blood glucose levels in the third trimester, suggesting that these animals had a better response to deal with the increase in glucose load. This further agrees with the T3 GTT results, where more glucose may had been taken up by the cells from the circulation, reducing the blood glucose levels measured. At the same time, the insulin concentration in these dams was measured using serum collected at time 0 of the T3 GTT. GDM+RESV dams had more insulin in the circulation than GDM dams did, which could be a reason as to why blood glucose levels decreased in these animals. Since the GDM dams had the least amount of insulin in their serum, it was hypothesized that a defective insulin secretion by the β-cells of the pancreas in GDM dams caused a reduction in insulin in the circulation which could have induced hyperglycemia during pregnancy. This hypothesis appeared to be correct as both human and animal studies have found associations between β-cell failure and GDM development, as reviewed in (Banerjee 2018). To further elucidate maternal β-cell function, I collaborated with Dr. Christine Doucette's laboratory (at CHRIM) to measure the amount of insulin secretion by the β-cell upon exposure to low levels of glucose (i.e. control levels) or high glucose levels (i.e. after

consumption of a meal). They found that when exposed to the high glucose levels, GDM+RESV dams were able to secrete more insulin than both GDM and Lean dams, while GDM dams had the least amount of insulin secretion from all three groups. This data suggests that the β-cell may be dysfunctional in GDM dams, while RESV supplementation may be maintaining proper β-cell function, and thus proper insulin and glucose response. Other studies have measured circulating insulin levels and also found them to be increased after RESV supplementation, however they did not assess maternal β-cell function (C. K. Singh et al. 2011, 2013; Yao et al. 2015). Thus, this study is unique in that we showed a plausible explanation as to how RESV supplementation prevented maternal glucose intolerance by increasing insulin secretion from β-cells of the pancreas.

As RESV supplementation seemed to improve glucose homeostasis in the dams, it is possible that the fetal environment may be healthier and better for proper fetal development than the environment found in the GDM dams. Furthermore, no serious adverse events were observed, suggesting that RESV supplementation during pregnancy is likely to be well tolerated and safe. The only potential side effect associated with RESV supplementation is gastrointestinal problems, such as constipation. After dissection of the mothers, we did notice that some constipation may have occurred in the intestines of these animals. However, the lack of serious side effects was also observed in other clinical and animal studies (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; B. Wicklow et al. 2015; Williams et al. 2009). The long-term effects of RESV on the offspring (up to 15 weeks of age) in this model will be described in the next chapter of this thesis.

Taken together, these maternal data seems to show that RESV supplementation during the third trimester of pregnancy, at the onset of GDM, and during lactation prevents glucose intolerance, lowers blood glucose levels and improves β-cell function thereby increasing insulin

secretion. Additionally, no serious side effects were observed. Since RESV supplementation had these positive outcomes in the dams, it is possible that the fetal environment may have also improved providing protection from GDM-induced complications in the offspring.

Chapter 3:

Effects of Resveratrol Supplementation during Pregnancy on the Metabolism of the 15-Week-Old Young-Adult Rat Offspring

3.1 Introduction:

This work is based on a manuscript in preparation for scientific review.

As obesity levels rise worldwide, particularly among women of child-bearing age, so are the cases of gestational diabetes mellitus (GDM) (Simmons 2011; World Health Organization. 2017). GDM is considered to be one of the most prevalent complications of pregnancy, as anywhere from 1 to 14 percent of all pregnancies can be affected (Ferrara 2007; Kim et al. 2010). GDM is characterized by increased gestational weight gain with the onset of hyperglycemia, glucose intolerance and insulin resistance during pregnancy that is typically diagnosed in the second or third trimester (American Diabetes Association 2015; Buchanan, Anny H. Xiang, and Page 2012; Jovanovic and Pettitt 2001; Troy J. Pereira et al. 2015). GDM is very similar to type 2 diabetes (T2D) because the development of both conditions are characterized by obesity, insulin resistance and the metabolic syndrome (Poston, Harthoorn, and Van Der Beek 2011; Rodriguez et al. 2006).

A GDM pregnancy is associated with birth complications in newborns such as shoulder dystocia, macrosomia, neonatal hypoglycemia, and other malformations (Arshad, Karim, and Hasan 2014; Hod et al. 1991; Kaaja and Rönnemaa 2008; Persson et al. 2017). Consistent with the Developmental Origins of Health and Disease (DOHaD) theory (D. Barker et al. 1993; D. J P Barker 2007; David J.P. Barker 1997), human studies have shown an association between exposure to GDM and later cardio-metabolic disease development in the offspring (B. Wicklow et al. 2018) and as recently reviewed in (Prasoon Agarwal et al. 2018). These observations have been confirmed in animal model studies of maternal obesity and GDM, as reviewed in (S. Kereliuk, Brawerman, and Dolinsky 2017). For example, we recently showed that a diet-induced GDM pregnancy in rats led to increased obesity, insulin resistance and hepatic steatosis development in

the 15 week old rat offspring even after the consumption of a low fat diet postnatally (Troy J. Pereira et al. 2015). Furthermore, consuming a high fat and sucrose diet postnatally exacerbated the metabolic effects in the offspring (Troy J. Pereira et al. 2015). Therefore, it is crucial to treat GDM in order to prevent adverse health outcomes in the offspring.

While insulin, metformin and glyburide are used in pregnant women with GDM and are generally considered safe, they are associated with some potential adverse effects such as maternal and fetal hypoglycemia and fetal macrosomia, as reviewed in (Brawerman and Dolinsky 2018). Maternal exercise interventions during pregnancy have been shown to have some positive effects on maternal glucose homeostasis, as well as the prevention of fetal programming effects in the offspring (Beeson et al. 2018; Brawerman and Dolinsky 2018). However, initiation and adherence to a program of exercise may be difficult for many sedentary and overweight pregnant women.

Resveratrol (RESV) is a natural polyphenolic compound that is produced by plants which has been found to mimic many of the positive cardio-metabolic effects of exercise (V. Dolinsky and Dyck 2014). RESV was found to be safe, well tolerated and non-teratogenic in animal studies of pregnancy (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; Williams et al. 2009). In addition, RESV reduced body weights, prevented hepatic steatosis and cardiac hypertrophy and improved glucose homeostasis and insulin sensitivity in the offspring in models of severe hypoxia, obesity and GDM (Bourque et al. 2012; Tanaka et al. 2017; Yao et al. 2015; Zou et al. 2017). However, no studies have been performed to date on a diet-induced GDM model to determine what effects RESV supplementation during pregnancy would have on the short and long-term health outcomes in the offspring. Additionally, this is the first study that supplemented maternal diets with RESV specifically in the third trimester of pregnancy, which is when GDM is typically diagnosed, while other studies supplemented with RESV during different time points throughout gestation when

GDM may not necessarily be present (Bourque et al. 2012; Roberts et al. 2014; C. K. Singh et al. 2011, 2013; Tanaka et al. 2017; Yao et al. 2015; Zou et al. 2017).

Thus, the objective of this study was to examine the effects of supplementing maternal diets with 4g/kg of RESV, a dosage equivalent to 147.6 mg/kg/day, during the third trimester of pregnancy and lactation on the metabolic health outcome in the 15 week old rat offspring. Also, we wanted to determine the outcome of consuming either a LF or a HFS postnatal diet on metabolic syndrome development in the offspring and whether previous maternal RESV consumption would protect the offspring from disease development (Fig. 5). Based on the work described in the preceding chapter (i.e. Chapter 2), maternal RESV supplementation prevented glucose intolerance, improved β-cell function of the pancreas, increased insulin secretion and lowered blood glucose levels in the dams, potentially providing a better intrauterine environment for proper offspring development. Therefore, we hypothesized that RESV supplementation would prevent metabolic health disorders such as obesity, glucose intolerance and hepatic steatosis in the 15 week-old rat offspring. In this chapter, we showed that RESV supplementation prevented fetal macrosomia, obesity, and hepatic steatosis, while it also improved glucose homeostasis and insulin sensitivity in the rat offspring. In addition, the expression of several metabolic genes in the liver of the offspring were modified by maternal RESV supplementation. We also observed differences between male and female offspring response to RESV supplementation, particularly in terms of obesity and fat accumulation.

3.2 Materials and Methods: Refer to page 37.

3.3 Fetal Offspring Results:

3.3.1 Maternal RESV supplementation was safe during pregnancy and prevented macrosomia in males but not in females:

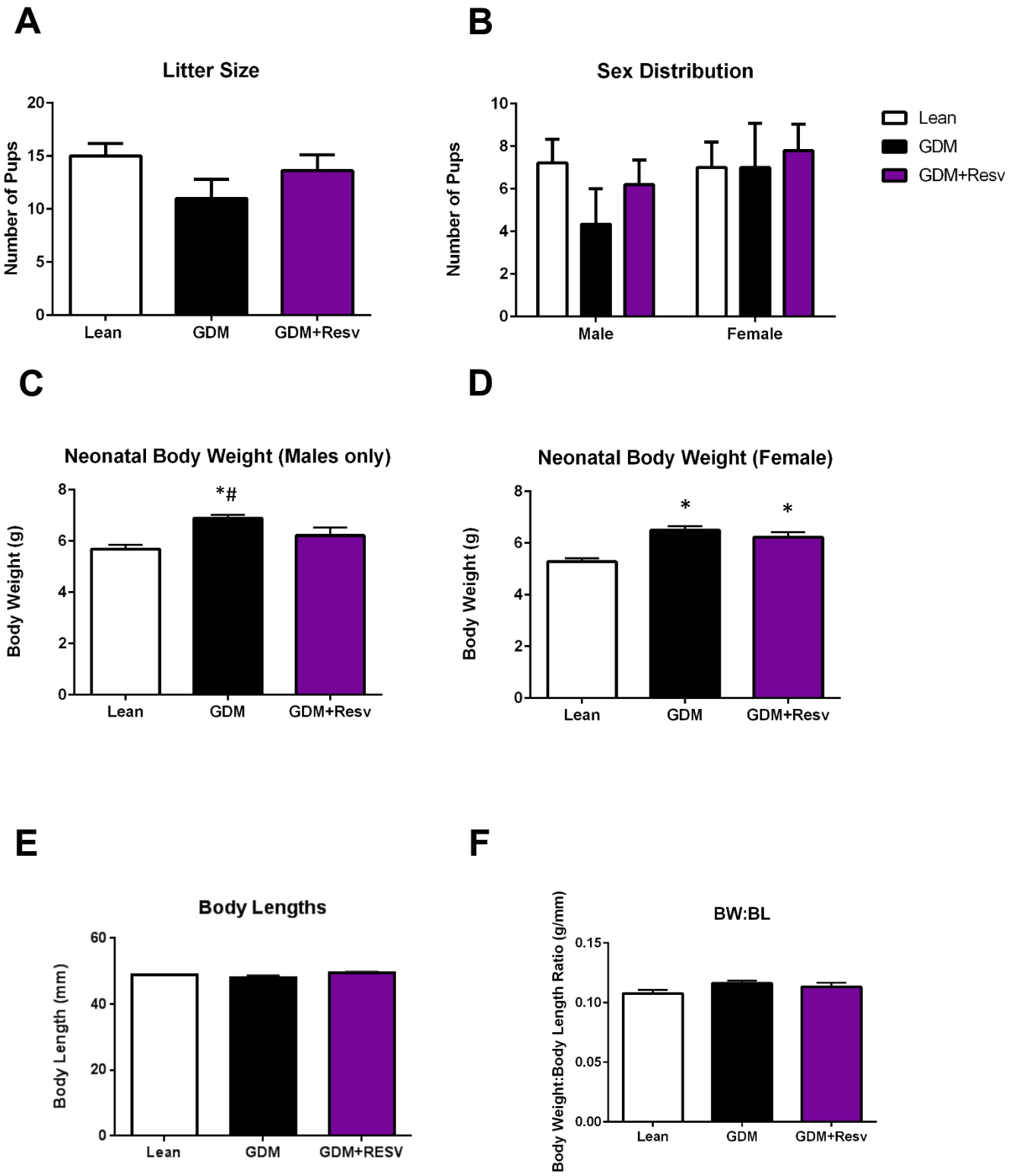
Within 24 hours of offspring birth, pups were sexed, weighed, and their lengths were measured. No changes in litter sizes or sex distribution were observed (Figs. 7A and 7B, and Table 4), implying that RESV supplementation did not have adverse effects during development. Pups were then reduced to eight to avoid competition for food, and were left alone with their respective mothers for the three week lactation period where these pups consumed the same diet their mothers did (i.e. LF, HFS, or HFS+RESV diets). Surplus neonatal rats were then sacrificed and their hearts, livers and kidneys were collected for future analyses. Male offspring from GDM dams exhibited a significant 1.21 fold increase in body weight at birth when compared to the Lean offspring, and had a 1.11 significant fold increase when compared to GDM+RESV offspring (Fig. 7C). Thus, GDM neonates were large for gestational age, or macrosomic, a common clinical consequence observed in GDM pregnancies (Arshad, Karim, and Hasan 2014; Scholl and Chen 2002). On the other hand, female offspring from both GDM and GDM+RESV dams exhibited a significant 1.23 and 1.18 fold increase respectively when compared to the Lean offspring. However, there were no changes when GDM female offspring was compared to GDM+RESV offspring (Fig. 7D). Thus, these changes may imply that different sex specific characteristics may be affecting the response to RESV providing benefits in males and no effects in females. Additionally, body lengths did not differ among neonates (Fig. 7E). So, after normalizing body weights to body lengths, no changes were observed, suggesting that the body weights were not influenced by the size of the animal (Fig. 7F).

Table 4. Litter Sizes and Sex Distribution

Litter Sizes				Sex Distribution:			
				Males		Females	
	Average	St. Dev.		Average	St. Dev.	Average	St. Dev.
Lean	15	3.898718		7.222222	3.34581	7	3.605551
GDM	11	4.427189		4.333333	2.886751	7	3.605551
GDM+Resv	13.625	4.206712		6.2	2.588436	7.8	2.774887

Upon 24 hours of offspring birth, litter sizes were calculated and offspring sexed. No significant differences were observed. Litter Size N=6-11; Sex Distribution N=3-9. One-Way ANOVA with Bonferroni post-hoc tests was used. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol

Fig. 7: Maternal RESV supplementation did not cause adverse developmental effects and prevented macrosomia in males but not in females.



(A) Litter Size, (B) Sex Distribution, (C) Male Neonatal Body Weights upon birth, (D) Female Neonatal Body Weights upon birth, (E) Neonatal offspring body lengths (F) Body Weight to Body Length Ratio. Litter Size & Sex Distribution by Litter N=3-11, Body Weights N=28-53. p-values represent significance (<0.05) after One Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean, #p<0.05 vs. GDM+RESV. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

3.4 15-Week-Old Young-Adult Offspring Results:

3.4.1 Maternal RESV supplementation prevented the increase in obesity in the young adult male rat offspring, but not in the female offspring:

After the three week lactation period, offspring were randomly weaned onto either a LF or a HFS diet for the remainder of the study, until 15 weeks of age, when offspring were sacrificed and their serum and tissues collected (i.e. important to remember these offspring did not consume RESV diet postnatally after the lactation period). Body weights and food consumption were recorded every week. As expected, male offspring that consumed the HFS diet postnatally gained more weight than the male offspring that consumed the LF diet when comparing offspring from the same maternal groups, as shown in Fig. 8A. However, Lean-LF offspring gained the least amount of weight among all offspring groups, while GDM-HFS and Lean-HFS gained the most (Fig. 8A), showing the impact a HFS diet has on the offspring even when exposed to a LF diet prenatally, consistent with the mismatch hypothesis. Of note, GDM+RESV-LF and GDM+RESV-HFS offspring were in the middle of the body weight growth curve, indicating a significant reduction in their body weights when compared to both GDM-HFS and Lean-HFS (Fig. 8A). Body weights measured at 15 weeks of age prior to sacrifice showed a significant 1.17 fold increase in GDM-HFS offspring when compared to Lean-LF, and significant 1.14 and 1.21 fold increases when compared to GDM-LF and GDM+RESV-HFS, respectively (Fig. 8B). Female offspring exhibited a completely different response in terms of body weights. In fact, the GDM+RESV-HFS female offspring were the most obese when compared to all the other groups (Fig. 8C). When body

weights were measured at 15 weeks of age prior to sacrifice, the same result was seen, as the GDM+RESV-HFS female offspring were 1.32 times heavier than the Lean-LF offspring, while all other groups had similar body weights regardless of maternal or postnatal diets (Fig. 8D), once again highlighting the sex specific responses that GDM in utero and RESV exposure may have on metabolism in the offspring. Furthermore, upon examination of food consumption and energy intake in both male and female offspring, no changes were observed (Figs. 8E, 8F, 8G, and 8H, Table 5). Thus, differences in metabolic rates and substrate utilization could have led to alterations in fat storage, which could partially explain the reduction in body weights observed in the GDM+RESV male offspring even when energy intake among all offspring groups was the same.

3.4.2 A high fat and sucrose postnatal diet resulted in increased fat accumulation in the male rat offspring regardless of maternal diet, while RESV supplementation increased fat mass in females:

To further evaluate fat content and distribution in the young adult male rat offspring, dual energy x-ray absorptiometry (DEXA) scans were performed at the CHRIM facilities by a blinded and experienced technician. Upon visual examination of the body shape of the offspring, there appeared to be more abdominal fat in the offspring fed a HFS diet postnatally when compared to offspring fed a LF diet, regardless of maternal diet (Fig. 9A). DEXA results further showed a significant 1.5 fold increase in percent body fat in the GDM-HFS offspring when compared to the Lean-LF offspring, and a significant 1.23 and 1.38 fold increases when compared against Lean-HFS and GDM-LF offspring, respectively (Fig. 9B). Interestingly, the GDM+RESV-HFS offspring had 1.3 times more percent body fat than both Lean-LF and GDM+RESV-LF offspring (Fig. 9B), confirming that the postnatal HFS diet was a significant determinant for body fat percentage. The increase in abdominal fat mass in the HFS fed offspring was further examined by weighing fat pads from 15 week-old offspring at sacrifice. Perirenal white adipose tissue (pWAT),

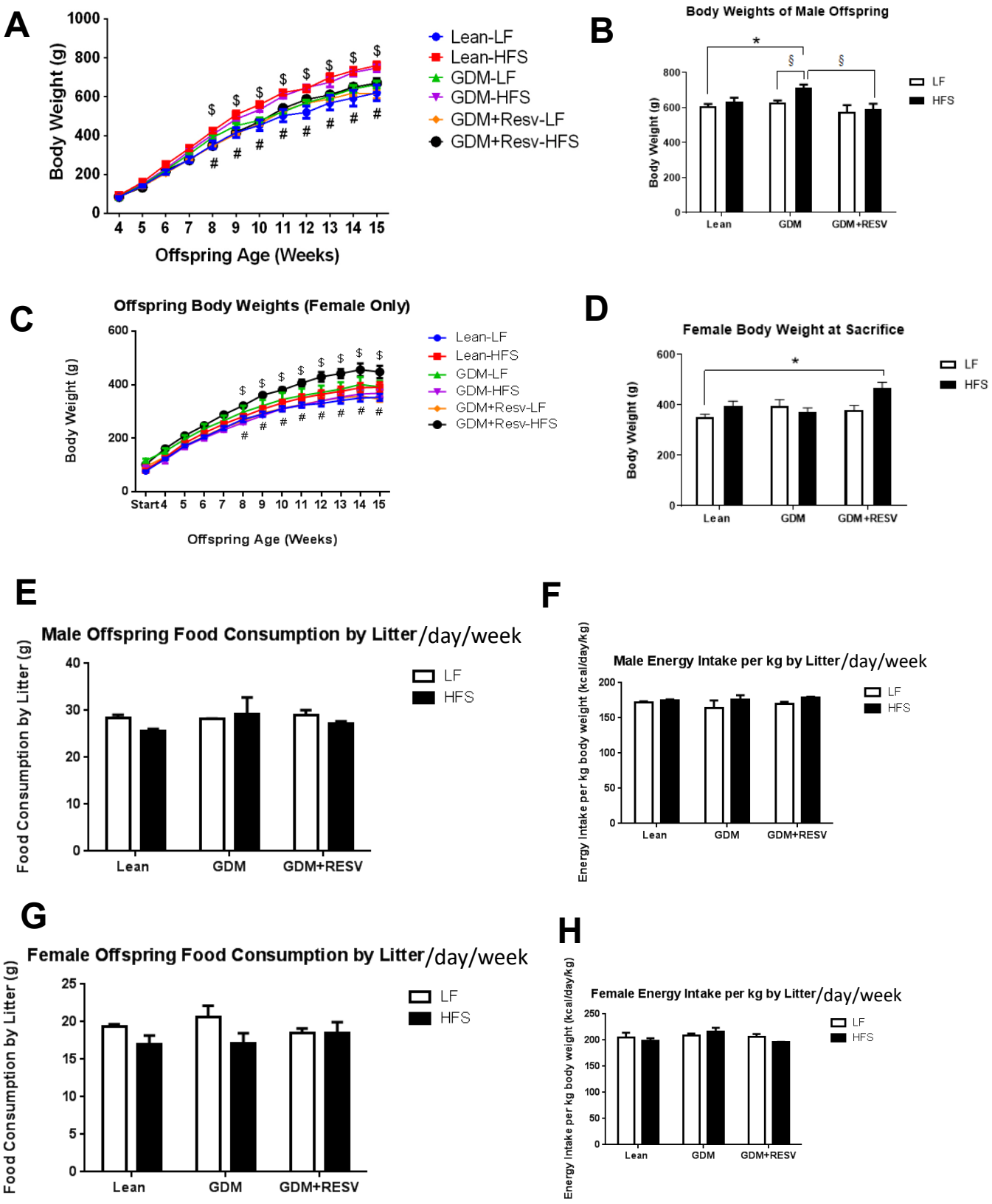
or the fat pad surrounding the kidneys, and gonadal white adipose tissue (gWAT), were all heavier in offspring that consumed the HFS diet postnatally when compared to offspring fed a LF diet (Fig. 9C). In fact, the GDM-HFS offspring had significant 2.12 and 1.78 fold increases in pWAT weights when compared to Lean-LF and GDM-LF offspring, respectively (Fig. 9C). Interestingly, the GDM+RESV-HFS offspring had significant 1.7 and 1.38 fold increases when compared to Lean-LF and GDM+RESV-LF offspring, respectively (Fig. 9C), showcasing that the HFS postnatal diet consumption, regardless of the maternal diet, resulted in increased fat accumulation. Moreover, the combination of GDM and postnatal HFS diet induced an additive increase in pWAT mass, but RESV decreased this effect by ~25% ($P>0.05$). Similarly, the GDM+RESV-HFS offspring had a significant 1.68 and 1.4 fold increases in gWAT weights when compared to Lean-LF and GDM+RESV-LF offspring, respectively, again highlighting the impact of a HFS postnatal diet (Fig. 9C). Therefore, this increase in visceral fat pad mass was directly associated with the percent body fat found in the offspring. The fat mass in these animals, which was another parameter reported in the DEXA scan, was also dependent on whether animals consumed a HFS or LF diet postnatally, as the GDM-HFS offspring had a significant 1.59 fold increase in fat mass when compared to the GDM-LF offspring (Fig. 9D).

When female offspring were assessed, the results were different from males. No changes were observed in the GDM offspring, however, the GDM+RESV-HFS offspring had a significant 2.33 and 1.92 fold increase in pWAT weights when compared to the Lean-LF and GDM+RESV-LF offspring, respectively (Fig. 9E). Similarly, the gWAT weight was significantly increased by a factor of 2.27 when comparing the GDM+RESV-HFS to the Lean-LF offspring (Fig. 9E). This suggests that in utero RESV exposure had additive effects on fat accumulation in female offspring

fed a postnatal HFS diet, and that GDM did not affect the female offspring. Again, this is another example of how sex specific differences are potentially affecting the results.

Lastly, perirenal adipocyte, or fat cell, diameter and number in male offspring were calculated based on H&E stained adipocytes looked under a microscope (Fig. 9F). As it was noted with the previous results, the offspring fed a HFS postnatal diet had adipocytes with the biggest diameter (Fig. 9G), suggesting increased fat accumulation, regardless of maternal diet. In addition, and as expected, the adipocyte diameter was inversely related to the adipocyte number per section (Fig. 9H). In other words, as the adipocyte diameter increased, fewer adipocytes were present per section.

Fig 8: Maternal RESV supplementation reduced obesity in the 15 week-old male rat offspring, but not in the female rat offspring



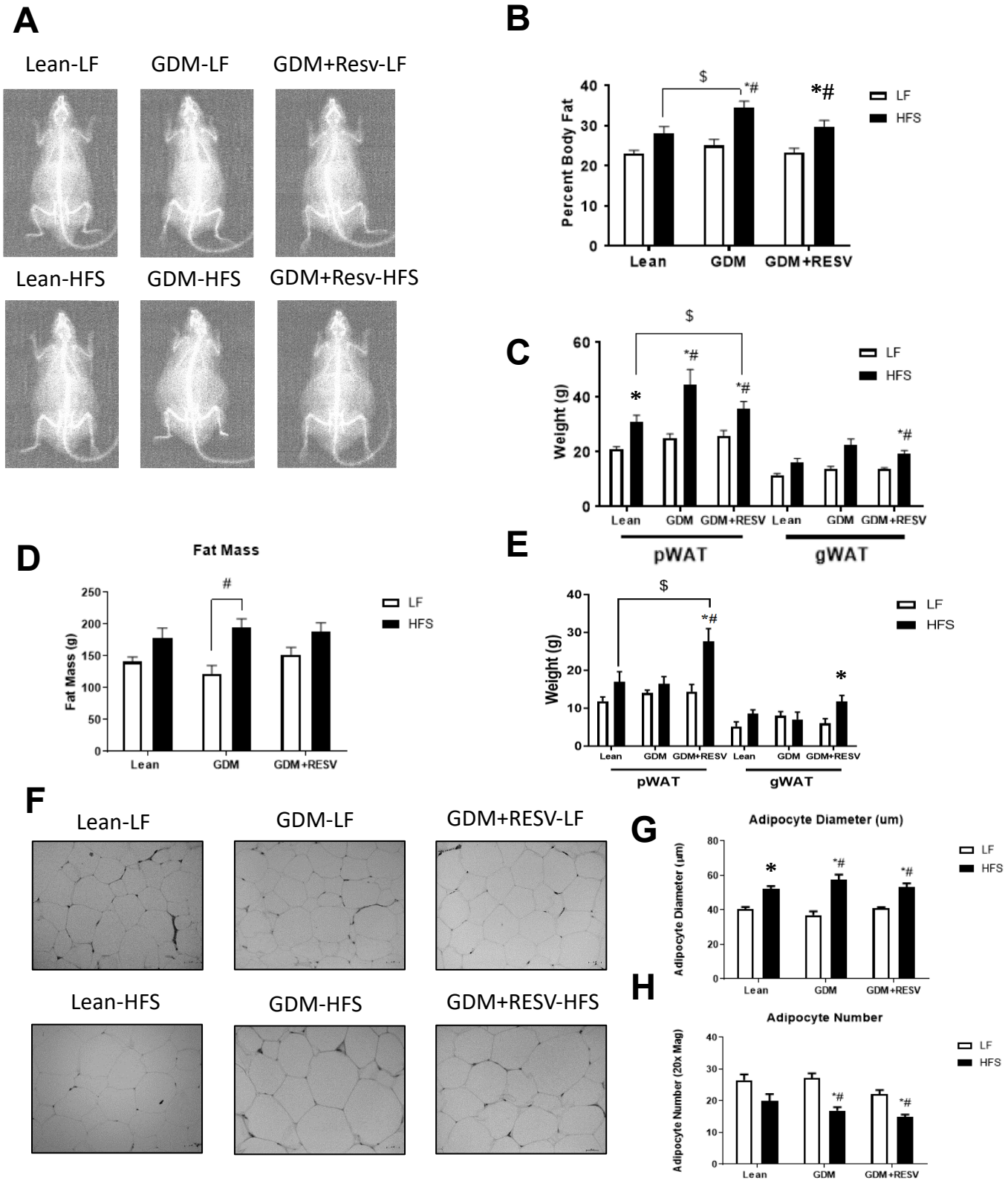
(A) Male body weight gain per week of Lean, GDM and GDM+RESV offspring, (B) Male body weight at sacrifice, (C) Female body weight gain per week of Lean, GDM, and GDM+RESV offspring, (D) Female body weight at sacrifice, (E) Male offspring food consumption by litter/day/week, (F) Male energy intake per litter/kg body weight/day/week, (G) Female offspring food consumption by litter/day/week, (H) Female energy intake per litter/kg body weight/day/week. Male Body Weights N=12-23, Female Body Weights N=3-8, Male Food Consumption/Energy Intake by Litter N=2-9, Female Food Consumption/Energy Intake by Litter N=2-4. p-values represent significance (<0.05) after Two-Way or Repeated Measures ANOVA with Bonferroni post-hoc tests. $p<0.05 postnatal LF vs. HFS diets, #p<0.05 GDM vs. Lean and GDM+RESV maternal diets, *p<0.05 vs. Lean-LF, §p<0.05 as indicated in the figure. LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

Table 5. Average daily food consumption/energy intake in the male and female offspring - grouped by litter

Average daily food consumption by litter							
		Lean		GDM		GDM+RESV	
Male Offspring		LF Diet (N=9)	HFS Diet (N=9)	LF Diet (N=2)	HFS Diet (N=3)	LF Diet (N=4)	HFS Diet (N=4)
	Food Consumption (g)	28.38 +/- 2.01	25.61 +/- 1.42	28.19 +/- 0.10	29.18 +/- 6.22	29.04 +/- 2.10	27.27 +/- 0.74
	Energy Intake (kcal/day)	109.25 +/- 7.73	121.1 +/- 6.74	108.5 +/- 0.26	138.02 +/- 29.42 *	111.8 +/- 8.10	129 +/- 3.51
	Energy Intake (kcal/day/kg body weight)	171.7 +/- 5.42	174.6 +/- 5.39	164.4 +/- 14.9	176.1 +/- 11.09	170.1 +/- 5.34	178.6 +/- 3.21
		Lean		GDM		GDM+RESV	
Female Offspring		LF Diet (N=4)	HFS Diet (N=4)	LF Diet (N=2)	HFS Diet (N=2)	LF Diet (N=4)	HFS Diet (N=2)
	Food Consumption (g)	19.35 +/- 0.62	16.94 +/- 2.42	20.67 +/- 2.06	17.14 +/- 1.89	18.5 +/- 1.22	18.5 +/- 2.05
	Energy Intake (kcal/day)	74.5 +/- 2.41	80.15 +/- 11.46	79.59 +/- 7.94	81.09 +/- 8.96	71.23 +/- 4.72	87.56 +/- 9.68
	Energy Intake (kcal/day/kg body weight)	205.6 +/- 17.38	199.1 +/- 8.83	209.3 +/- 4.60	216.6 +/- 9.82	206.3 +/- 10.28	195.7 +/- 1.27

Daily averages of food consumption and energy intake in male and female offspring grouped by litter. Male Offspring N=2-9, Female Offspring N=2-4. *p-values represent significance (<0.05) between GDM-HFS and Lean-LF after 2 Way ANOVA with Bonferroni post-hoc tests. LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol

Fig. 9: Consumption of a postnatal HFS diet resulted in increased fat accumulation in the male rat offspring, while maternal RESV consumption increased fat mass in the female rat offspring at 15 weeks of age



(A) Representative images of DEXA scans in male offspring at 15 weeks of age, (B) Percent Body Fat of male offspring, (C) pWAT and gWAT weights of male offspring, (D) Fat Mass of male offspring, (E) pWAT and gWAT weights of female offspring, (F) Representative images of male adipocytes in pWAT stained with H&E taken at 20x magnification, (G) Male Adipocyte Diameter, (H) Male Adipocyte Number per Section. DEXA N=7-13, male pWAT/gWAT N=5-8, female pWAT/gWAT N=3-8, Adipocytes N=4. p-values represent significance (<0.05) after Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, #p<0.05 Postnatal HFS diet vs. LF diet within the same maternal group, $p<0.05 as indicated in the figure. DEXA: Dual X-Ray Absorptiometry Scans, pWAT: Perirenal White Adipose Tissue, gWAT: Gonadal White Adipose Tissue, H&E: Hematoxylin & Eosin, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

3.4.3 Maternal RESV supplementation prevented hepatic steatosis development in the male rat offspring at 15 weeks of age:

Since RESV attenuated the GDM-induced obesity in the males, next we assessed whether maternal RESV supplementation also affected hyperlipidemia (i.e. circulating triglycerides and free fatty acids) as well as the accumulation of triglycerides in the liver (i.e. hepatic steatosis) that are associated with obesity. Male offspring fed the postnatal HFS diet had a ~50-60% reduction in serum triglycerides when compared to the LF fed offspring, while the maternal environment did not influence the values (Fig. 10A). Serum free fatty acids were significantly elevated (2.11 fold increase) in the GDM-HFS offspring when compared to the GDM-LF offspring, while all other groups did not differ, although the GDM-HFS offspring had a ~1.4 fold increase in serum free fatty acids when compared to GDM+RESV-LF and HFS offspring (P>0.05) (Fig. 10B). Finally, in order to determine whether hepatic steatosis was occurring, liver triglyceride levels were measured. Lean-HFS offspring exhibited a 1.7 and a 1.53 fold significant increase in hepatic steatosis when compared to Lean-LF and GDM+RESV-HFS offspring, respectively (Fig. 10C), while GDM-HFS offspring had a 1.92 and a 1.75 fold significant increase over Lean-LF and GDM+RESV-HFS offspring, respectively (Fig. 10C), suggesting that maternal RESV supplementation protected these offspring from accumulating abnormal levels of triglycerides in the liver.

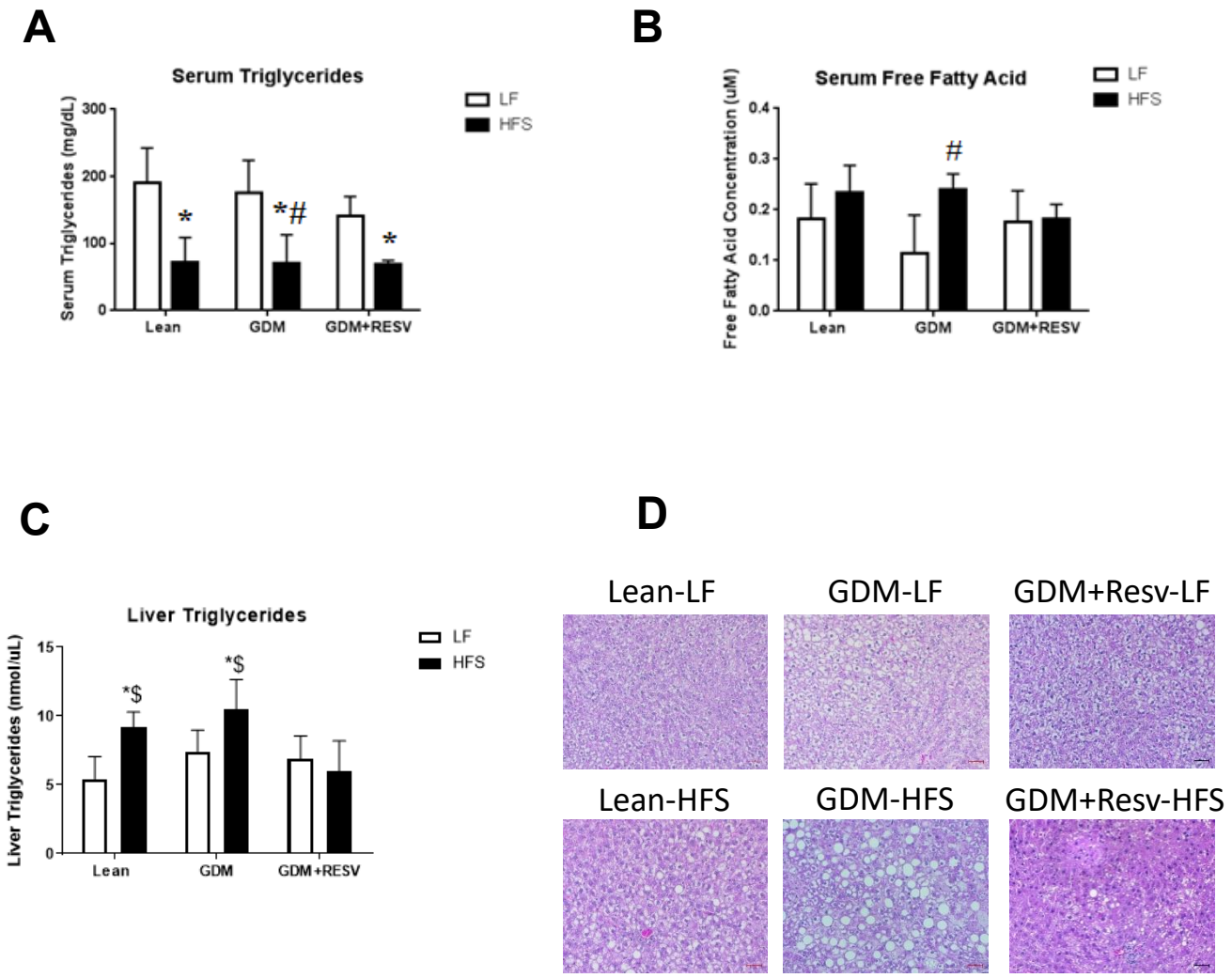
Histologically, hepatocytes stained with H&E, were observed under the microscope and the images clearly showed an increase in lipid droplets in both Lean-HFS and particularly in GDM-HFS offspring, while GDM+RESV-LF and GDM+RESV-HFS offspring had fewer lipid droplets accumulated (Fig. 10D), consistent with prevention of hepatic steatosis development. As hepatic steatosis is a risk factor for insulin resistance and T2D, Lean-HFS and GDM-HFS offspring may be at a greater risk for disease development. Interestingly, maternal RESV supplementation prevented the increase in hepatic triglyceride levels, and therefore could be lowering the risk for hepatic steatosis and insulin resistance.

3.4.4 Maternal RESV supplementation prevented glucose intolerance and attenuated the insulin resistance caused by a GDM pregnancy in the young-adult male rat offspring:

To determine how GDM and RESV influenced the insulin sensitivity of the male offspring, insulin tolerance tests (ITTs) and glucose tolerance tests (GTTs) were performed at 14 and 15 weeks of age respectively. Fasting blood glucose levels did not differ significantly among the offspring groups (Fig. 11A). Furthermore, the GDM+RESV-HFS offspring had a significant 2.75 fold increase in plasma insulin levels compared to the Lean-LF offspring (Fig. 11B). Similarly, the HOMA-IR index was 2.71 times higher in the GDM+RESV-HFS offspring when compared to the Lean-LF offspring (Fig. 11C), which could suggest increased insulin resistance in those offspring. However, the GTT showed that the GDM offspring fed a HFS postnatal diet were more glucose intolerant than the other groups fed the HFS diet, as they had higher glucose levels throughout the test (Fig. 11D). More importantly, the GDM+RESV offspring exhibited an even better response than the Lean offspring, suggesting it prevented glucose intolerance and improved glucose homeostasis (Fig. 11D). Based on the area under the curve graph, the GDM-HFS offspring were the most glucose intolerant as they had a significant 1.35, 1.17, and 1.28 fold increase when

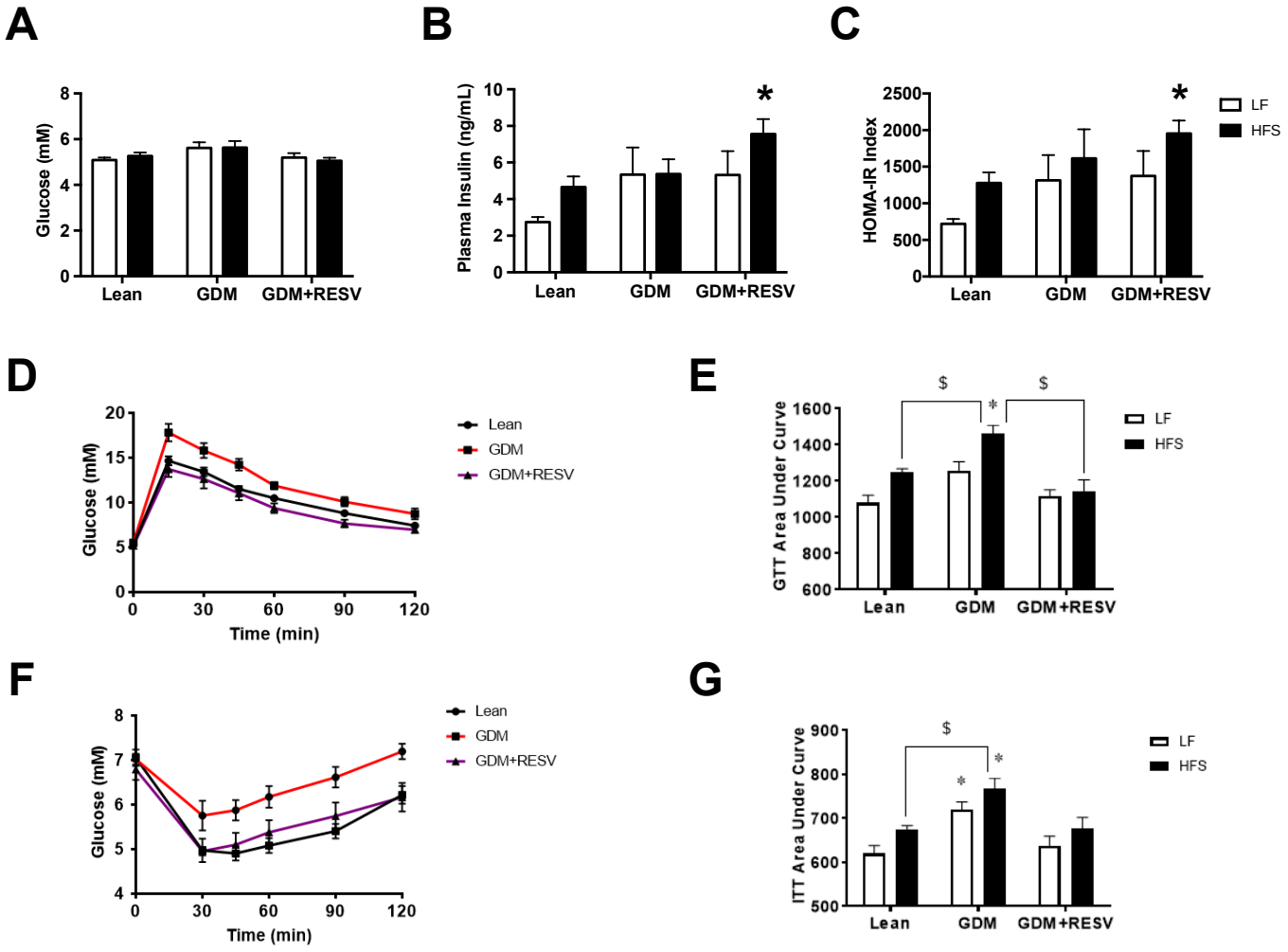
compared to Lean-LF, Lean-HFS, and GDM+RESV-HFS offspring, respectively (Fig. 11E), suggesting that maternal GDM induced glucose intolerance, while RESV supplementation prevented it. Lastly, the ITT indicated that the GDM offspring were the most insulin resistant, while the GDM+RESV offspring seemed to be similar to the Lean offspring, potentially having a better insulin response than the GDM offspring (Fig. 11F). Based on the area under the curve graph, the GDM-LF were more insulin resistant than the Lean-LF offspring by a significant fold change of 1.17, while the GDM-HFS offspring were significantly more insulin resistant than the Lean-LF and the Lean-HFS offspring by a fold change of 1.24 and 1.14, respectively (Fig. 11G), indicating that a GDM pregnancy predisposed these offspring to insulin resistance. Additionally, the GDM-HFS offspring were 1.14 times more insulin resistant than the GDM+RESV-HFS offspring ($p=0.0529$) (Fig. 11G), which shows that maternal RESV supplementation may have attenuated the insulin resistance response.

Fig. 10: Maternal RESV supplementation prevented hepatic steatosis development in the young-adult male rat offspring



(A) Serum Triglycerides, (B) Serum Free Fatty Acids, (C) Liver Triglycerides, (D) Representative images of H&E stained liver slides taken at 20x magnification. Serum Triglycerides N=5, Serum Free Fatty Acids N=4-6, Liver Triglycerides N=4-10, Liver Slides N=4-6. p-values represent significance (<0.05) after Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, #p<0.05 HFS postnatal diet vs. LF postnatal diet within the same maternal group, $p<0.05 vs. GDM+RESV-HFS. TG: Triglycerides, H&E: Hematoxylin & Eosin, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet

Fig. 11: Maternal RESV supplementation prevented glucose intolerance and attenuated the insulin resistance caused by GDM in the young adult male rat offspring



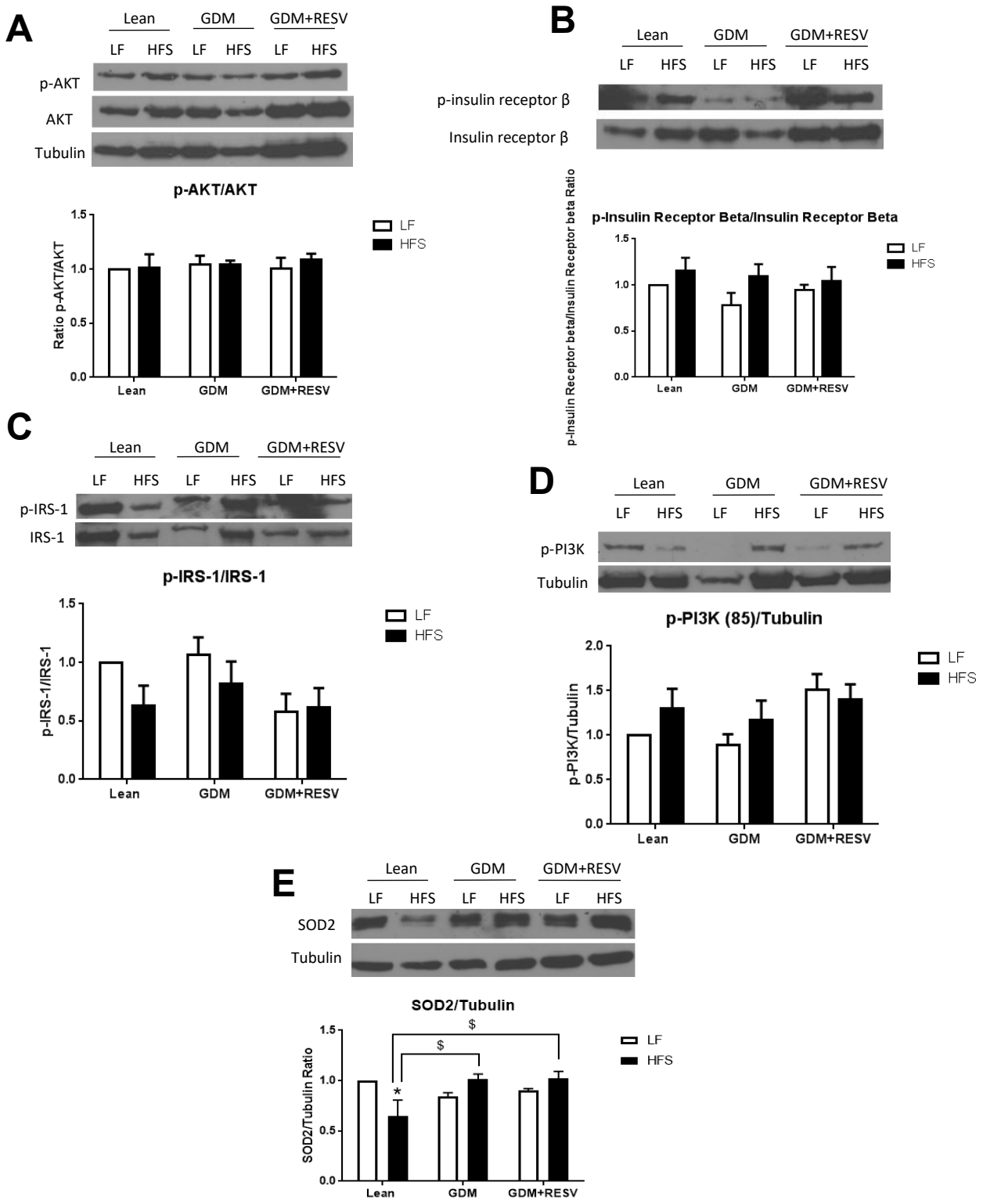
(A) Fasted blood glucose levels, (B) Plasma Insulin Concentration, (C) HOMA-IR Index, (D) GTT at 15 weeks of age in HFS postnatal diet groups, (E) GTT Area Under the Curve, (F) ITT at 14 weeks of age in HFS postnatal diet groups, (G) ITT Area Under the Curve. Glucose, plasma insulin and HOMA-IR N=4-10. GTT and ITT N=5-8. p-values represent significance (<0.05) after Two-Way ANOVA or Repeated Measures ANOVA where applicable with Bonferroni post-hoc tests. The litter was used as the unit of analyses. *p<0.05 vs. Lean-LF, $p<0.05 as indicated in the figure. HOMA-IR: Homeostatic Model of Assessment-Insulin Resistance, GTT: Glucose Tolerance Test, ITT: Insulin Tolerance Test, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

3.4.5 Maternal RESV supplementation did not affect the protein expression of proteins involved in the insulin signalling pathway in the 15 week old male rat offspring:

The insulin signalling pathway is a complex molecular pathway that controls liver lipid metabolism and gluconeogenesis, among other functions (Boucher, Kleinridder, and Kahn 2014). Thus, we determined the effect of maternal RESV supplementation on the insulin signaling pathway in the liver tissues of the 15 week-old male rat offspring. Liver tissue samples from insulin injected male rat offspring were prepared to run on western blots. Unfortunately, significant differences were not observed between neither the maternal diets (i.e. LF, HFS, HFS+RESV) nor the postnatal diets (i.e. LF or HFS). However, interesting trends were observed. Phosphorylated Protein Kinase B (AKT) (Ser473) was not affected by maternal diet nor by postnatal diet, as all offspring groups had the same level of protein expression when normalized to total AKT and Tubulin (Fig. 12A). Phosphorylated insulin receptor β (IRβ) (Tyr1150/1151) tended to have increased expression in the offspring fed HFS diet postnatally when normalized to total IRβ, regardless of in utero exposure to GDM, suggesting a potential increase in the receptor's activity (Fig. 12B). Interestingly, the phosphorylated insulin receptor substrate-1 (IRS-1) (Ser1101) expression tended to be increased in the GDM-HFS offspring by a factor of 1.32 when compared to the GDM+RESV-HFS offspring ($P>0.05$), while GDM and Lean offspring were similar when normalized to total IRS-1 protein (Fig. 12C). This specific phosphorylation site causes target inhibition, thus a reduction in phosphorylation actually represents an increase in activity. Next, the phosphorylated phosphatidyl-inositol-3-kinase (PI3K) (Tyr458) expression was 1.19 times higher in the GDM+RESV-HFS offspring when compared to the GDM-HFS offspring ($P>0.05$), and was 1.4 times higher than the Lean-LF offspring ($P>0.05$) after normalization to Tubulin (Fig. 12D). Finally, superoxide dismutase 2 (SOD2) expression was also assessed and it was found to be

significantly decreased in the Lean-HFS offspring when compared to both GDM-HFS and GDM+RESV-HFS offspring (1.57 fold reduction) after being normalized to Tubulin (Fig. 12E).

Fig. 12: Maternal RESV supplementation did not affect the protein expression of proteins involved in the insulin signalling pathway in the 15 week old male rat offspring



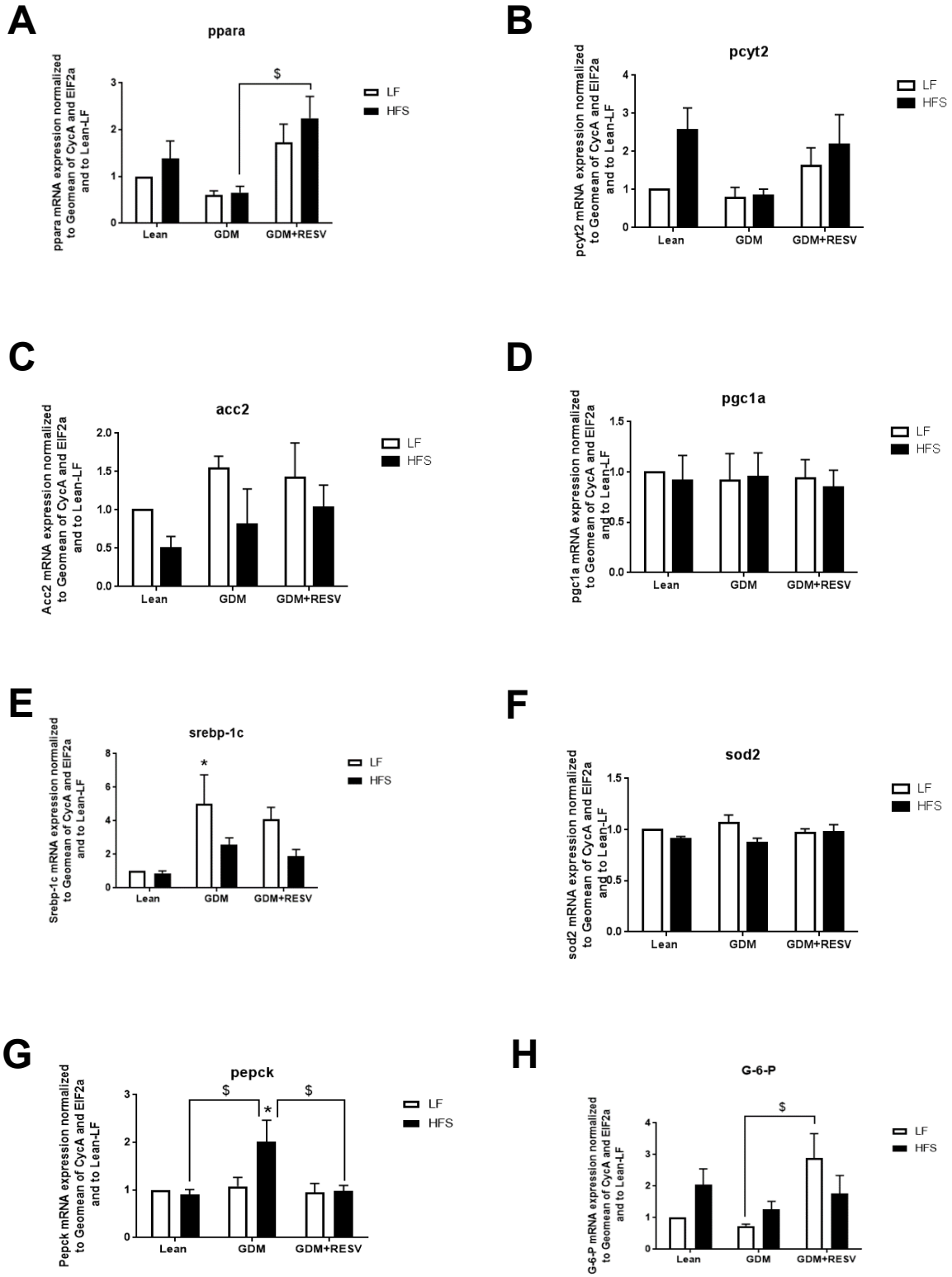
(A) p-AKT (Ser473) protein expression relative to total AKT protein normalized to Tubulin and Lean-LF, (B) p-IRβ (Tyr1150/1151) protein expression relative to total IRβ normalized to Lean-LF, (C) p-IRS-1 (Ser1101) protein expression relative to total IRS-1 normalized to Lean-LF, (D) p-PI3K (Tyr458) protein expression relative to Tubulin normalized to Lean-LF, (E) SOD2 protein expression relative to Tubulin and normalized to Lean-LF. N=4-6. p-values represent significance (<0.05) after Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, $p<0.05 as indicated in the figure. AKT: Protein Kinase B, IRβ: Insulin Receptor β, IRS-1: Insulin Receptor Substrate-1, PI3K: Phosphatydyl-inositol 3 Kinase, SOD2: Superoxide Dismutase 2, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

3.4.6 GDM induced changes in hepatic gene expression of several genes involved in metabolism, but maternal RESV supplementation prevented them:

To further evaluate the gene expression of specific hepatic genes involved in fat metabolism and synthesis, mitochondrial biogenesis and anti-oxidants in the young adult male rat offspring, real-time quantitative polymerase chain reaction (qPCR) was used (Table 2). In order to study lipid metabolism, the expression of the peroxisome proliferator activated receptor *α* (*pparα),* which is a regulator of fatty acid oxidation, was measured in liver tissue of offspring. The GDM-HFS offspring had a significant reduction (3.42 fold) in the expression of *pparα* when compared to the GDM+RESV-HFS offspring (Fig. 13A). CTP:Phosphoethanolamine cytidylyltransferase 2 (*pcyt2*), which is the rate limiting enzyme of phosphatidylethanolamine (PE) synthesis, was reduced in the GDM-HFS offspring but increased in the Lean-HFS and GDM+RESV-HFS livers by a factor of 3.07 and 2.62, respectively (p>0.05) (Fig. 13B). Another important gene required for fatty acid production is acetyl-coenzyme A carboxylase 2 (*acc-2*). qPCR of this gene did not show any significant changes in gene expression, although the GDM-HFS offspring had a 1.61 fold change increase over Lean-HFS, while GDM+RESV-HFS had a 1.26 fold change increase over GDM-HFS offspring (P>0.05) (Fig. 13C). Furthermore, no changes were observed in the *ppar-γ* co-activator 1*α* (*pgc1α)* gene expression (Fig. 13D), which is important for mitochondrial biogenesis. The sterol response element binding protein 1c (*srebp-1c*) gene expression, which is required for cholesterol synthesis, had a significant 5 fold increase in the GDM-LF livers when

compared to the Lean-LF offspring, and had a 1.23 fold change when compared to GDM+RESV-LF offspring ($p>0.05$) (Fig. 13E). Additionally, the gene expression of *sod2* was not changed (Fig. 13F). Lastly, to determine whether gluconeogenesis and glycolysis were affected, phosphoenolpyruvate carboxykinase (*pepck*) and glucose-6-phosphate (*g-6-p*) gene expression were measured. *Pepck* gene expression was ~2 times higher in the GDM-HFS offspring livers when compared to the livers from Lean-LF, Lean-HFS, and GDM+RESV-HFS offspring (Fig. 13G), suggesting that the GDM-HFS offspring had increased hepatic glucose production. On the other hand, *g-6-p* gene expression was ~4 times higher in the GDM+RESV-LF livers when compared to the GDM-LF livers (Fig. 13H), suggesting that there was an increase in hepatic glycolysis and glucose utilization in those animals. Overall, these data suggest that GDM induced several changes in hepatic gene expression in the offspring and maternal RESV administration prevented many of these changes.

Fig. 13: GDM modified the expression of several hepatic genes involved in metabolism, but maternal RESV supplementation prevented some of those changes in the young adult male rat offspring



(A) *pparα* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (B) *pcyt2* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (C) *acc2* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (D) *pgc1α* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (E) *srebp-1c* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (F) *sod2* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (G) *pepck* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF, (H) *g-6-p* gene expression relative to geomean of *EIF2a* and *CycA* and normalized to Lean-LF. N=5-6. p-values represent significance (<0.05) after Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, $p<0.05 as indicated in the figure. *Pparα*: Peroxisome Proliferator Activated Receptor *α, eIF2a*: eukaryotic Translation Initiation Factor 2a, *CycA*: Cyclophilin A, *pcyt2*: CDP:Phosphoethanolamine Cytidylyltransferase-2, *acc2*: Acetyl-CoA Carboxylase-2, *pgc1α:* Peroxisome Proliferator Activated Receptor γ Co-Activator 1*α, srebp-1c*: Sterol Response Element Binding Protein-1c, *sod2*: Superoxide Dismutase 2, *pepck*: Phosphoenolpyruvate Carboxykinase, *g-6-p*: Glucose-6-Phosphate, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

3.5 Conclusion/Discussion

The objective of this study was to examine whether maternal RESV supplementation to a HFS diet during the third trimester of pregnancy, highlighted by GDM, and lactation would prevent the development of the metabolic syndrome in the 15 week-old young adult rat offspring. Further, we wanted to determine whether consumption of either a LF or a HFS diet postnatally would either protect or exacerbate the effects on offspring health outcomes. We found that the male and female offspring from GDM dams were macrosomic, while the male offspring developed obesity, hepatic steatosis (increased accumulation of triglycerides in the liver), insulin resistance and glucose intolerance and also had altered expression of several different genes involved in metabolism. Maternal RESV supplementation in combination with the postnatal diet prevented many of these effects in the offspring although sex specific differences were observed.

Based on the results described here, GDM induced macrosomia, or large for gestational age, in both male and female neonatal rat offspring. This is consistent with clinical and animal studies where GDM was found to predispose offspring to macrosomia (Hod et al. 1991; Yao et al. 2015), as well as previous findings from the Dolinsky lab (Troy J. Pereira et al. 2015). Offspring hyperinsulinemia and insulin resistance are associated with a pregnancy complicated by GDM, which is what we observed in the GDM male offspring, and since insulin is also a growth factor, this could explain why the GDM neonates were larger than both Lean and GDM+RESV male offspring (Arshad, Karim, and Hasan 2014; Scholl and Chen 2002). Even though insulin does not cross into the placenta, it can interact with the placenta by activating lipid carriers which will signal for increased fetal fat storage leading to increased fat accumulation and increased obesity and macrosomia (Ruiz-Palacios et al. 2017). Additionally, maternal hyperglycemia can result in increased insulin production by the fetal pancreas, which in turn, will raise fetal insulin secretion

resulting in increased fat storage and fetal obesity (Arshad, Karim, and Hasan 2014). However, our results showed that maternal RESV supplementation during pregnancy prevented the development of macrosomia in the male, but not in the female offspring. Other studies have also shown RESV treatment during pregnancy to reduce macrosomic risk in the offspring of a rat model of severe hypoxia in pregnancy (Bourque et al. 2012). Similarly, this study supplemented maternal diets with 4g RESV/kg of diet, which is a dosage equivalent to 146mg/kg/day, however, their timing of therapy was different, as the dams consumed RESV from gestational day 7 to day 21 (Bourque et al. 2012). Additionally, 10 mg/kg/day of RESV supplemented during a GDM pregnancy in db/+ mice showed a reduction in neonatal body weights (Yao et al. 2015). Importantly, these findings were consistent with the attenuated macrosomia we observed in the GDM+RESV neonates. Moreover, the GDM+RESV dams had reduced blood glucose levels when compared to the GDM dams (Fig. 6G), which may have potentially led to a reduction in fetal insulin production. Furthermore, RESV crosses the placenta and could have direct effects on the fetus (Bourque et al. 2012). But, this concept and its consequences require further investigation.

Interestingly, the male neonatal offspring from GDM+RESV dams had a more pronounced decrease in body weights versus the GDM offspring when compared to the females, suggesting that the decline in body weights could be sex dependent. The changes observed in male and female offspring response could be due to RESV interacting with sex-specific hormones, such as estrogen. In fact, RESV has been previously found to have affinity for the estrogen receptor and acting as an agonist (Gehm et al. 1997). Estrogen is a hormone which binds to nuclear receptors thereby affecting the expression of several different genes involved in various physiological functions, such as, tissue development, reproduction, cell growth, and is also involved during menopause and for the prevention of diseases such as cancer, coronary heart disease and T2D as it improves insulin

sensitivity (Murphy 2011; Velarde 2013). As females tend to have more estrogen than males, either activating or repressing the receptor could have an effect on the gene expression of key targets involved in signalling pathways which could determine the different sex specific responses that were observed in this study and others (Velarde 2013). However, this hypothesis is speculative as we did not measure hormone levels, therefore more research is needed to further elucidate the mechanisms that may be involved.

Our study also examined how maternal exposures interacted with postnatal LF or HFS diets to affect obesity development over 15 weeks of age in the offspring. As expected, the Lean-LF males gained the least weight, while the GDM-HFS offspring gained the most. Interestingly, the Lean-HFS males gained as much weight as the GDM-HFS males, suggesting that a mismatch in maternal and postnatal diet may have predisposed the offspring to increased obesity, consistent with the mismatch hypothesis (Bateson et al. 2004; P. D. Gluckman 2012). Importantly, the GDM+RESV male offspring, regardless of postnatal diet, had reduced obesity at 15 weeks of age, which resembled the Lean offspring body weights. Tanaka et al. have also shown a reduction in body weights in the male rat offspring after 20mg/kg maternal RESV supplementation during lactation only (Tanaka et al. 2017). Similarly, 200 mg/kg/day of RESV supplemented to pregnant mice from the beginning of pregnancy until lactation also decreased body weights in the male offspring (Zou et al. 2017). Thus, maternal RESV supplementation can efficiently lower the obesity risk in the male offspring.

Interestingly, female offspring exhibited a completely different response. In fact, there were no differences at 15 weeks of age when comparing body weights of GDM-LF female offspring to either Lean-LF or GDM-HFS offspring. In contrast, the GDM+RESV-HFS females were the most obese at 15 weeks of age when compared to all the other groups, suggesting that

RESV supplementation may not be beneficial for females. Even though most animal research involving RESV is currently being done on male offspring, one other study reported their findings on the effects of maternal RESV supplementation in female offspring. They found that 2.25 mg/kg/day of RESV supplemented during pregnancy and lactation resulted in a much greater reduction in body weights in female offspring rats that consumed a postnatal high fat diet when compared to male offspring consuming the same high fat postnatal diet (Ros et al. 2018). It is important to point out that this dosage was much lower than the dosage used in this thesis. Thus, these conflicting results highlight the potential differences that are found between males and females in terms of metabolism and how they may respond to the different diets. So, more research is needed to further elucidate the different responses and mechanisms that may occur in male and female offspring after maternal RESV consumption.

One of the strengths of this study is that we examined whole body fat composition using DEXA scans and we then compared those results with the amount of fat pads collected from the offspring to corroborate the results. We found that the male offspring that consumed a HFS postnatal diet exhibited increased abdominal fat deposition, increased percent body fat, increased fat mass, increased amounts of pWAT and gWAT and enlarged adipocytes when compared to male offspring that consumed a LF diet, regardless of maternal diets. These data suggests that the postnatal diet may have played a big role in determining fat deposition. To our knowledge, this is the first study which used DEXA scans to assess fat deposition in offspring exposed to GDM that were also supplemented with RESV during gestation. Other clinical and animal studies have used DEXA scans to assess the effects of RESV on body composition, fat deposition and bone densities in obese men with metabolic syndrome and in male rats with bone problems (Chen et al. 2012;

Durbin et al. 2012; Kjær et al. 2017; Ornstrup et al. 2014), but none were performed with pregnant women or pregnant rodents with GDM.

Interestingly, female offspring exhibited a different response again. Here, the GDM+RESV-HFS offspring had the highest accumulation of fat pads, while all other offspring groups were similar. This result further showcases that the different responses to the diets may depend on the sex of the animal.

Another important finding is that RESV supplementation prevented hepatic steatosis in the young adult male rat offspring. Hepatic steatosis is a common complication of obesity that results from increased triglyceride accumulation in the liver tissue and is closely associated with insulin resistance and T2D (Corey and Kaplan 2014; Kelley et al. 2003; Samuel and Shulman 2017; Vernon, Baranova, and Younossi 2011; B. A. Wicklow et al. 2012). In particular, overweight and obese young adults who have hepatic steatosis are at an increased risk of having risk factors associated with T2D, such as insulin resistance and glucose intolerance (B. A. Wicklow et al. 2012). Several studies have reported that offspring from high fat diet-fed dams have hepatic steatosis (Nguyen et al. 2018; Tanaka et al. 2017; Tiao et al. 2018). Consistent with previous findings from our group (Troy J. Pereira et al. 2015), we observed that both Lean-HFS and GDM-HFS male offspring had increased levels of hepatic triglycerides and histological evidence of hepatic steatosis. The GDM-induced hepatic steatosis was markedly reduced in the GDM+RESV offspring, in both postnatal diet groups. Other studies have also found that different dosages and timings of RESV supplementation reduced hepatic triglycerides in the offspring, as reviewed in (Aguirre et al. 2014), but the narrow window of RESV administration in our study suggests that this developmental period is very important for conditioning postnatal hepatic lipid levels in the offspring. Additionally, we observed a significant increase in the serum free fatty acid levels in

the GDM-HFS offspring when compared to the GDM-LF animals, while there was a ~1.4 fold increase in serum free fatty acids when GDM-HFS offspring were compared to GDM+RESV-LF and HFS offspring ($P>0.05$). The increase in serum free fatty acids in the GDM-HFS offspring may be due to a dysfunctional fatty acid oxidation in the mitochondria or an increase in fatty acid synthesis, thus, any excess that cannot be oxidized, will get secreted back into circulation (Sparks et al. 2005). The increased amounts of free fatty acids found in circulation in the GDM-HFS offspring may go to the liver where they get converted back into triglycerides to be stored (Aguirre et al. 2014). Another study also reported that 20mg/kg/day of maternal RESV supplementation only during lactation lowered fatty acid synthesis, which led to a reduction in hepatic steatosis in male rat offspring (Tanaka et al. 2017). Additionally, a recent study found that a maternal and postnatal high fat diet predisposed the male rat offspring to increased hepatic steatosis, but RESV (50mg/kg/day) supplementation from birth until 4 months of age resulted in prevention of hepatic steatosis development (Tiao et al. 2018). Even though this study did not supplement RESV during pregnancy, it does emphasize that post-partum RESV can prevent maternal high fat diet-induced hepatic steatosis in the offspring (Tiao et al. 2018). Thus, based on our data, RESV supplementation in a GDM pregnancy prevented the development of hepatic steatosis in the male offspring by decreasing free fatty acids in the circulation and preventing triglyceride accumulation in the liver.

We performed ITTs and GTTs to further elucidate glucose homeostasis and insulin sensitivity in the offspring. In agreement, with our previous report, the GDM offspring were more insulin resistant than the Lean offspring, regardless of postnatal diets (Troy J. Pereira et al. 2015). The GDM+RESV offspring exhibited an attenuation in insulin response and sensitivity when compared to the GDM offspring. Similar results were observed in another study where 200

mg/kg/day of RESV was supplemented from gestation to the end of pregnancy and improved insulin sensitivity in the male mouse offspring (Zou et al. 2017). In our study, the GDM-HFS offspring were more glucose intolerant than the other groups, but more importantly, the GDM+RESV offspring exhibited a better glucose response. This was observed as blood glucose levels decreased more rapidly in the GDM+RESV offspring after a glucose injection when compared to both Lean and GDM offspring. This improvement in glucose response was probably associated with the better insulin sensitivity, as well as a suppression in gluconeogenesis. Taken together, these results indicate that maternal RESV supplementation prevented glucose intolerance and attenuated the insulin resistance caused by a GDM pregnancy in male rat offspring, regardless of what they consumed postnatally.

Since maternal RESV supplementation during pregnancy improved insulin and glucose tolerance in the offspring we examined its effects on the hepatic insulin signalling pathway in the young adult male rat offspring. Interestingly, we did not find any significant changes in insulin signaling cascade in the liver, though we did observe some interesting trends. The signalling pathway starts when insulin binds to the insulin tyrosine kinase receptor β , which will trigger several auto-phosphorylation events (Boucher, Kleinridder, and Kahn 2014). We found that there was a trend for increased protein expression of p-IRβ in the offspring that consumed a HFS diet postnatally, suggesting that the increased in nutrient availability, namely fat and sugars, may have stimulated insulin secretion and insulin action in those animals when compared to the animals that consumed the LF diet instead. Further auto-phosphorylation of the IRβ results in the recruitment of the IRS-1 which interacts with the ligand-receptor complex and will itself become phosphorylated (Boucher, Kleinridder, and Kahn 2014). Our results showed that p-IRS-1 protein expression was increased by a factor of 1.32 in the GDM-HFS offspring when compared to the

GDM+RESV-HFS offspring (P>0.05). It is important to point out that the antibody we used was specific for a serine residue (Ser1101) which causes target inhibition when phosphorylated. Thus, the reduction in phosphorylation in the GDM+RESV offspring may actually indicate an increase in receptor activation. Similar results were obtained in a study where Streptozotocin was used to induce diabetes in male rats. These rats exhibited a reduction in hepatic IRS-1 activity, however after 20mg/kg/day of RESV supplementation the protein expression was restored (Sadi et al. 2015). Using a high fat diet induced obesity model in male mice, obesity resulted in a reduction in the phosphorylated IRS-1, but 30 mg/kg/day of RESV was enough to restore its expression (Hong et al. 2014). Upon IRS-1 phosphorylation and activation, the recruitment of PI3K occurs (Boucher, Kleinridder, and Kahn 2014). Our analysis showed that the protein expression of p-PI3K was slightly increased by a factor of 1.19 in the GDM+RESV-HFS offspring when compared to the GDM-HFS offspring (P>0.05). Other studies have also found an increase in both total and phosphorylated PI3K protein expression after RESV consumption in males (Hong et al. 2014; Sadi et al. 2015). Finally, after activation of PI3K and phosphorylation of other downstream proteins, AKT gets phosphorylated and activated in order to recruit glucose transporters (GLUT) to the plasma membrane and take up glucose from circulation, thereby reducing blood glucose levels in response to insulin action (Boucher, Kleinridder, and Kahn 2014). Our results did not show any trends in p-AKT as the protein expression was the same among all different diets. However, other studies did find an increase in both total AKT and p-AKT protein expressions after RESV supplementation, suggesting an increase in GLUT translocation to increase glucose uptake (Hong et al. 2014; Sadi et al. 2015). Taken together, these results do seem to follow the logical progression of events that occur in the signalling pathway once insulin binds to its receptor in the presence of glucose. Even though we did not see any significant differences in insulin signalling in the liver,

it is possible that other tissues, such as the skeletal muscle, adipose tissue, or heart, may have had changes in insulin signalling which led to an enhanced insulin response that could have impacted whole body insulin sensitivity and glucose tolerance. That could explain why we observed improvements in insulin sensitivity and glucose tolerance after RESV supplementation in the offspring.

Since metabolism is a very complicated process involving a vast number of different pathways, we wanted to study the effect of maternal RESV supplementation and the postnatal diets on the expression of several different hepatic genes involved in lipid metabolism, phospholipid and fatty acid synthesis, sterol biosynthesis, gluconeogenesis and glycolysis in the young adult male rat offspring. Firstly, we looked at the expression of *pparα,* which is a nuclear transcription factor and a major regulator of lipid metabolism that gets activated under low energy status (Yoon 2009). We found that *pparα* expression was significantly reduced in the GDM-HFS offspring when compared to the GDM+RESV-HFS offspring, consistent with previous studies from our lab (Troy J. Pereira et al. 2015). Upon activation, this gene promotes the transcription of proteins involved in the uptake, utilization and transport of fatty acids, it also increases fatty acid β-oxidation in the mitochondria, and increases the expression of lipoprotein lipase to hydrolyze triglycerides into free fatty acids to provide more substrates for ATP generation (Yoon 2009). Thus, this finding further agrees with our preceding results (Fig. 10) showing that free fatty acids and hepatic triglycerides were lower in the GDM+RESV-HFS offspring, suggesting it could be due to increased *pparα* expression. On the other hand, as GDM offspring had reduced *pparα* expression, it is possible that it could have led to increased free fatty acid availability and increased hepatic steatosis.

Phosphatidylethanolamine (PE) is an important phospholipid found in the cell membrane and has a significant role during cell growth and development (Pavlovic and Bakovic 2013). Studies have found that a reduction in PE can result in increased hepatic steatosis and insulin resistance (Fullerton et al. 2009; Pavlovic and Bakovic 2013). *pcyt2*, is the rate limiting enzyme required for PE synthesis (Pavlovic and Bakovic 2013). We found that the expression of this gene was reduced in the GDM-HFS offspring, but increased in the Lean-HFS and GDM+RESV-HFS offspring by a factor of 3.07 and 2.62, respectively ($P>0.05$). Similar results were previously reported by (Troy J. Pereira et al. 2015). Thus, this result may further explain the reduction in hepatic steatosis and the improvement in insulin sensitivity observed in the GDM+RESV-HFS offspring. This is explained by the fact that Pcyt2 is a transferase involved in the CDP-ethanolamine Kennedy pathway which generates de novo PE from ethanolamine and diacylglycerol (DAG) through a series of different chemical reactions (Pavlovic and Bakovic 2013). In order to synthesize more PE, triglycerides may be broken down into DAG, thereby reducing the amount of triglycerides that may accumulate in the liver (Pavlovic and Bakovic 2013). Additionally, excess free fatty acids may also be esterified to produce PE (Pavlovic and Bakovic 2013; R. K. Singh et al. 2012). Importantly, as PE levels decline due to reduced Pcyt2 expression, as we observed in the GDM-HFS offspring, the availability of DAGs and free fatty acids will increase promoting the formation of triglycerides and thus the increase in hepatic steatosis as reviewed in (Pavlovic and Bakovic 2013; R. K. Singh et al. 2012). Therefore, it is possible that the increase in *pcyt2* in the GDM+RESV-HFS offspring could lead to increased PE synthesis which would then decrease the amount of available DAG and free fatty acids, thus preventing hepatic steatosis development.

We also studied the expression of *srebp-1c*, which is a transcription factor required for sterol and fatty acid biosynthesis (Yecies et al. 2011). Our results showed a significant increase in the expression of this gene in the GDM-LF offspring when compared to the Lean-LF offspring, while there was a 1.23 fold change between GDM-LF and GDM+RESV-LF offspring ($P>0.05$). These results may suggest that there could be a decline in both cholesterol and lipid synthesis in the GDM+RESV-LF offspring. This finding was similar to what was previously found by Pereira et al, although their GDM offspring that consumed the HFS diet postnatally had a higher expression than the ones that consumed the LF diet (Troy J. Pereira et al. 2015). Similarly, the effects of RESV on *srebp-1c* gene expression were also observed by (Tanaka et al. 2017). Thus, changes in *srebp-1c* could further explain the reduction in serum free fatty acid levels and the prevention of hepatic steatosis we observed in the GDM+RESV offspring (Fig. 10).

Finally, *pepck* and *g-6-p* gene expression were measured to assess whether RESV had any effect on hepatic gluconeogenesis or glycolysis respectively. We found that the GDM-HFS offspring hepatic gene expression of *pepck* was ~2 times higher than the Lean-LF, Lean-HFS and GDM+RESV-HFS offspring, suggesting that the GDM-HFS offspring were experiencing increased glucose production by their livers, which could explain their poor glucose tolerance. Additionally, the gene expression of *g-6-p* was reduced in the GDM-LF offspring by 4 times when compared to the GDM+RESV-LF offspring, indicating that these animals were not undergoing glycolysis, and therefore they may have had increased glycogen storage and poor glucose utilization. The opposite was observed in the GDM+RESV offspring where they had increased glycolysis and decreased gluconeogenesis, suggesting better glucose utilization. Similar results were also obtained in another study where they found that 10mg/kg/day of RESV supplementation throughout pregnancy reduced hepatic glucose production and improved glycolysis in their mice

(Yao et al. 2015). Thus, maternal RESV supplementation may inhibit hepatic gluconeogenesis and may promote glycolysis, thereby improving glucose utilization and energy generation.

In addition to the research findings, described above, adaptations by the endocrine pancreas of the offspring to GDM and maternal RESV administration could also influence offspring phenotypes. Using isolated islets, our lab has recently observed that GDM impaired glucose-stimulated insulin secretion (P. Agarwal et al. 2018). To further examine whether maternal RESV rescued the effects of GDM on the pancreas, islets were also isolated from the 15 week old offspring reported in this chapter of the thesis. All analyses of glucose stimulated insulin secretion were conducted in Dr. Christine Doucette's laboratory. Their results showed that insulin secretion from the islets were reduced in the GDM offspring when exposed to either basal or high glucose levels, suggesting an impairment in the secretion mechanism which could help explain the reduced plasma insulin levels and the adverse results we observed during the GTT in these offspring. Furthermore, in the absence of adequate insulin secretion when blood glucose levels were high, gluconeogenesis remained unchecked, since insulin would normally inhibit new glucose production, and this could explain why *pepck* gene expression was increased in the GDM rat offspring. On the other hand, the GDM+RESV offspring exhibited an increase in insulin secretion when exposed to either basal or high glucose levels and also had a higher insulin content than the GDM offspring. This may further explain the increase in plasma insulin levels and the improvements in glucose tolerance we observed in the GDM+RESV offspring. Additionally, β-cell area, but not number, was increased in the GDM+RESV animals which could also explain the increase in insulin content and secretion, suggesting that RESV may have increased β-cell size in utero to compensate for the hyperglycemic conditions caused by a GDM pregnancy. As opposed to the GDM offspring, the GDM+RESV offspring had a reduction in hepatic gluconeogenesis,

since *pepck* gene expression was reduced, and this could have been a consequence of higher insulin secretion from the β-cells of the pancreas which would then inhibit hepatic glucose production.

It is important to point out that we did not encounter any adverse effects with RESV supplementation in the short and long-term health outcome in the offspring. However, one study performed on macaques where they supplemented their diets with 0.37% RESV for 3 months prior to pregnancy and throughout gestation, did find a serious side effect where the fetal exocrine pancreas mass was increased by 42% compared to the controls (Roberts et al. 2014). They found that the insulin gene expression, and hence, β-cell mass, were not changed, however, the glucagon gene expression, which increases blood glucose levels, was increased. Additionally, they suggested that these changes were due to increased ki-67, which is a marker for proliferation, and decreased Bcl-2 which is a marker for cell death (Roberts et al. 2014). Nevertheless, other studies have shown RESV to be safe during pregnancy (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; Williams et al. 2009), meaning that the animal model as well as the timing of therapy may be critical in preventing adverse effects in the offspring (Fig. 3).

As it was mentioned before, timing of therapy and the onset of disease could determine whether the therapy will be successful or not. In fact, RESV supplementation did not have any effect on the offspring from dams that consumed a low fat diet (Ros et al. 2018). However, the offspring of dams that had a high fat and sucrose diet supplemented with 2.25mg/kg/day of RESV, had reduced body weight and adipose tissue content (Ros et al. 2018). These results suggest that RESV may not have a significant beneficial role in healthy populations (Ros et al. 2018). Thus, this highlights the importance of the need to supplement maternal diets with RESV at the onset of GDM and not before, which is what we did in our study and why our study is unique.

One important caveat is that more research is needed to further elucidate the effects of GDM and RESV supplementation on female offspring as we found significant differences between males and females in terms of obesity and fat distribution but we did not study the mechanisms that may be involved in the females. However, taken together, all of these findings seem to suggest that maternal RESV supplementation during the third trimester of a GDM pregnancy and lactation in rats is safe and it provides beneficial health outcomes which may prevent the development of the metabolic syndrome in the young adult offspring later in their lives.

Chapter 4:

Effects of Resveratrol Supplementation during Pregnancy on the 15-Week-Old Young-Adult Male Rat Offspring Heart

4.1 Introduction:

Heart failure is one of the leading causes of death and morbidity worldwide. Individuals with diabetes are at an increased risk for the development of cardiovascular disease (CVD) (Jia, Hill, and Sowers 2018; Levelt et al. 2018; Shindler et al. 1996). Diabetic cardiomyopathy (DC) is a complication of CVD as a consequence of diabetes and poorly controlled glycemia. In fact, almost 60% of people with type 2 diabetes (T2D) have DC (Mohammadshahi, Haidari, and Soufi 2014). DC is described as myocardial dysfunction with no coronary artery disease (CAD), high blood pressure, or dyslipidemia, as reviewed by (Jia, Hill, and Sowers 2018). DC is difficult to diagnose, however, echocardiography can detect several of the structural and functional changes that the heart may undergo (Negishi 2018). The etiology of DC is controversial, but hyperglycemia is believed to have an important role in the development of this condition (Falcão-Pires and Leite-Moreira 2012; Marwick et al. 2018). As a matter of fact, there is a 30% increased risk for heart failure for every 1% increase in glycated HbA1c levels in patients with T1D, while that risk decreases to 8% in patients with T2D for every 1% increase in HbA1c (Lind et al. 2011; Stratton et al. 2000).

One of the main features of DC and risk factors for heart failure is cardiac hypertrophy (Marwick et al. 2018). Cardiac hypertrophy, or thickening of the heart muscle, can either be physiological or pathological depending on how it occurs and can be described as being concentric or eccentric (Chung and Leinwand 2014; Chung, Yeung, and Leinwand 2012). Cardiac hypertrophy is initially adaptive in order to maintain cardiac output. Unfortunately, sustained hypertrophy can eventually become maladaptive and lead to the development of DC and ultimately heart failure (Marwick et al. 2018). Increased ROS production by the mitochondria is believed to be a key driving force behind the pathogenesis of cardiac hypertrophy (Marwick et al. 2018;

Mohammadshahi, Haidari, and Soufi 2014). This is because increased ROS can cause cardiomyocytes, or heart muscle cells, to become hypertrophied, fibrotic, stiff, and can eventually induce cell death (Mohammadshahi, Haidari, and Soufi 2014). Additionally, increased ROS in combination with hyperglycemia, have also been closely associated with the development of chronic low-grade inflammation which can further damage tissues resulting in apoptosis and disease (Jia, Hill, and Sowers 2018; Marwick et al. 2018).

Unfortunately, there are no specific treatments available for DC. The main strategy is to decrease blood glucose levels, whether it is through lifestyle therapy or by taking glucose lowering agents like metformin, as well as other pharmaceuticals to decrease hypertension, such as angiotensin-converting enzyme (ACE) inhibitors, or drugs used to lower lipids, like statins (Marwick et al. 2018). Furthermore, drugs currently used for heart failure may also be prescribed depending on the patients' situation (Marwick et al. 2018). Additionally, new promising research has been done on sodium glucose co-transporter 2 (SGLT2) inhibitors, such as Empagliflozin, where results show that it efficiently lowered blood glucose levels and reduced the risk for CVD and mortality in patients with T2D (Kaplan et al. 2018; Lipscombe et al. 2018; Lytvyn et al. 2017; Zinman et al. 2015).

Understanding the factors that contribute to the development of cardiac hypertrophy, DC and heart failure could lead to better treatment and management of patients at risk that could yield improvements in lifespan and quality of life. Gestational diabetes mellitus (GDM), one of the most common complications of pregnancy, has been found to be associated with an increased predisposition of mothers and their offspring to CVD and heart failure (American Diabetes Association 2015; Rodriguez et al. 2006). Previously in the Dolinsky lab, GDM exposed offspring exhibited increased hypertrophy development both at fetal and at young adult stages (T.J. Pereira

et al. 2013). However, it remains poorly understood how GDM conditions heart disease development in the offspring and the mechanisms that are involved. In addition, interventions administered during pregnancy could be key for combating the effects of GDM on cardiac health in the offspring.

Resveratrol (RESV) is a natural health product which gained popularity in CVD research ever since the "French Paradox" was described (Renaud and de Lorgeril 1992). Importantly, as RESV has been shown to increase the expression of anti-oxidant enzymes, lower the expression of inflammatory mediators as well as lower blood glucose levels, it could potentially be a good agent to use against DC (Coutinho et al. 2018; Mohammadshahi, Haidari, and Soufi 2014; Roberts et al. 2014; Turan, Tuncay, and Vassort 2012). Additionally, previous research has shown RESV to prevent the development of cardiac hypertrophy in an animal model of hypertension, as well as improve cardiac structure and function in males in a model of exercise training (V.W. Dolinsky et al. 2012; Vernon W Dolinsky et al. 2013). However, none of these studies were performed in a GDM model, so the effects of RESV supplementation during pregnancy on heart morphology and function are currently unknown.

Since our group previously reported that GDM-exposed offspring exhibited increased hypertrophy development both at fetal and at young adult stages (T.J. Pereira et al. 2013), this study was designed to determine whether RESV supplementation (4g/kg) during the third trimester of a GDM pregnancy and lactation would prevent the development of cardiac hypertrophy and improve cardiac function in the 15 week old young adult male rat offspring. Furthermore, to assess the role of postnatal diets, the offspring were fed either a low fat or a high fat and sucrose diet from the end of lactation up until 15 weeks of age (Fig. 5). Thus, we hypothesized that RESV supplementation during a GDM pregnancy would prevent left ventricular hypertrophy as well as

DC and heart failure as the offspring age. Our results indicate that RESV supplementation prevented the development of cardiac hypertrophy in fetal and young adult offspring. Additionally, we found that the GDM offspring exhibited an increase in cardiac steatosis, which was not observed in the GDM+RESV offspring. Maternal RESV supplementation also affected the protein expression of anti-oxidants and molecular regulators of the protein synthesis. Based on isolated cardiomyocyte experiments, RESV prevented the increase in mitochondrial ROS and reduced the mitochondrial membrane potential. Finally, no changes in cardiac function were observed at 15 weeks of age.

4.2 Materials and Methods – Refer to page 37.

4.3 Results:

4.3.1 Maternal RESV supplementation prevented the development of GDM-induced cardiac hypertrophy in fetal offspring:

Once dams gave birth, their litters were reduced to 8 pups, and the rest were sacrificed and their hearts, among other tissues, were collected. Neonatal pups from GDM-exposed dams exhibited a significant 14% and 16% larger hearts when compared to Lean and GDM+RESV pups, respectively (Fig. 14A). Additionally, the heart weight to body weight ratio was significantly decreased by 14% in the GDM+RESV neonates when compared to Lean neonates (Fig. 14B), suggesting that these offspring may have had a smaller heart due to having reduced obesity at birth. Furthermore, the GDM neonates had a significant 1.12 fold increase in heart weight to body length ratio when compared to GDM+RESV neonates (Fig. 14C), suggesting that their hearts could have been larger because the animal itself was bigger than the other group.

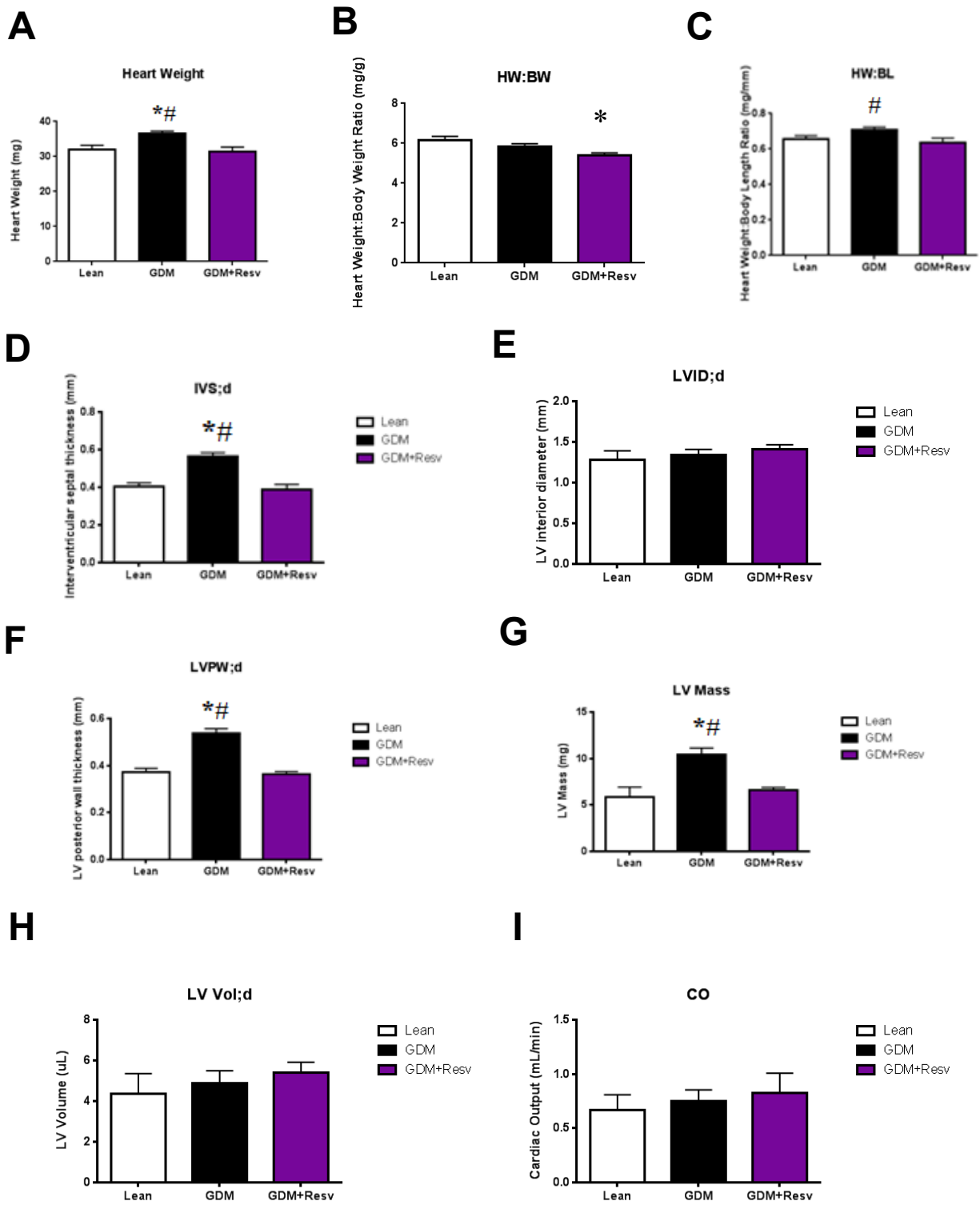
To further assess the effects of RESV on fetal cardiac structure and function, echocardiography was performed by a blinded and experienced technician on the anesthetized dams at ~e18.5. Interestingly, we found many different structural parameters to be affected by a GDM pregnancy, while maternal RESV consumption prevented those effects. The interventricular septal (IVS) thickness was ~1.4 times larger in the GDM fetal offspring when compared to both Lean and GDM+RESV offspring (Fig. 14D). The left ventricular interior diameter (LVID) did not differ among all three groups (Fig. 14E). However, the left ventricular posterior wall (LVPW) thickness, which is a marker for cardiac hypertrophy, was dramatically increased by a fold of 1.44 in the GDM fetal offspring when compared to the Lean, and by a fold of 1.48 when compared to the GDM+RESV fetal offspring (Fig. 14F). Accordingly, the left ventricular mass exhibited a 1.78 fold increase in the GDM fetal offspring when compared to the Lean, and a 1.58 fold increase when compared to the GDM+RESV offspring (Fig. 14G). There were no clear differences in left ventricular volume (Fig. 14H) or in cardiac output (Fig. 14I) at this developmental stage in these animals, suggesting that cardiac function was not altered.

4.3.2 GDM-induced cardiac hypertrophy persisted to 15 weeks of age in the male rat offspring, but was prevented by maternal RESV supplementation in the GDM+RESV-HFS offspring:

Upon evaluation of heart tissue at 15 weeks of age (i.e. at the end of the study), there were no significant changes in either male or female heart weights (Fig. 15A and 15B). To further determine whether GDM-induced cardiac hypertrophy persisted to 15 weeks of age, we performed echocardiography in the male rat offspring. Interestingly, we found that the LVPW thickness was still significantly enlarged in the GDM-HFS offspring by a ~1.2 fold when compared to the Lean-HFS and the GDM+RESV-HFS offspring, suggesting that a GDM pregnancy predisposed offspring to increased hypertrophy, while maternal RESV supplementation prevented it (Fig. 15C).

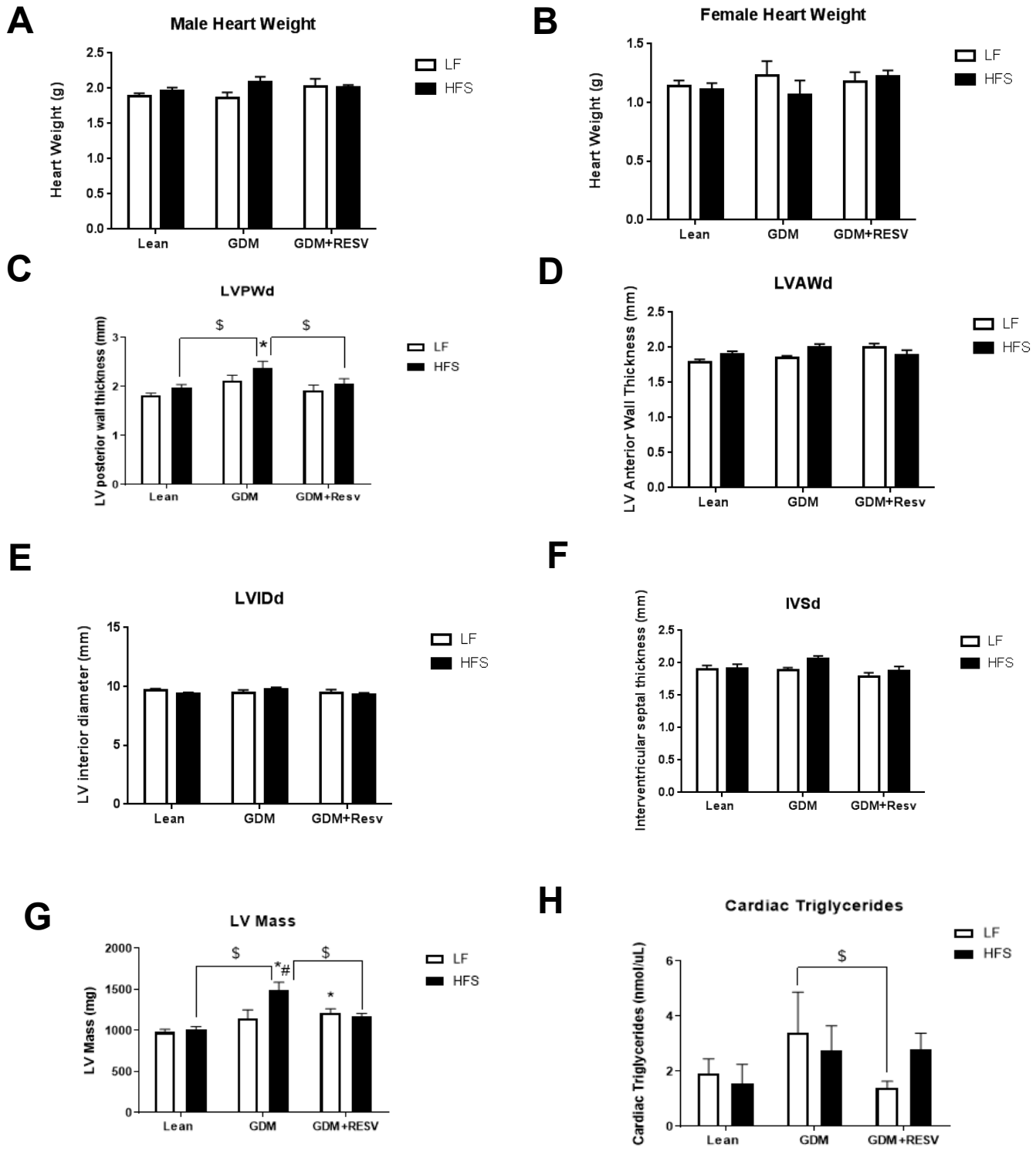
Additionally, the left ventricular anterior wall (LVAW) thickness was not different among the groups (Fig. 15D), neither was the LVID (Fig. 15E) nor the IVS thickness (Fig. 15F). Consistent with the development of cardiac hypertrophy and diabetic cardiomyopathy, the left ventricular mass was significantly increased in the GDM-HFS offspring by a 1.48 and a 1.28 fold increase when compared to the Lean-HFS and GDM+RESV-HFS offspring, respectively. Additionally, the GDM-HFS offspring had a 1.3 fold increase in left ventricular mass over the GDM-LF offspring, suggesting that the postnatal HFS diet exacerbated the effects of GDM. Interestingly, the GDM+RESV-LF offspring had a 1.24 fold increase in left ventricular mass when compared to the Lean-LF offspring (Fig. 15G).

Fig. 14: Maternal RESV supplementation prevented the development of cardiac hypertrophy caused by a GDM pregnancy in the fetal offspring, without affecting heart function



(A) Neonatal Heart Weights, (B) Neonatal Heart Weights to Body Weights Ratio, (C) Neonatal Heart Weights to Body Length Ratio, (D) Intraventricular Septal Thickness, (E) Left Ventricular Interior Diameter, (F) Left Ventricular Posterior Wall Thickness, (G) Left Ventricular Mass, (H) Left Ventricular Volume, (I) Cardiac Output. Fetal Heart Weights N=29-56, Fetal Echo N=5-10. p-values represent significance (<0.05) after One-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean, #p<0.05 vs. GDM+RESV. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, IVS: Intraventricular Septal Thickness, LVID: Left Ventricular Interior Diameter, LVPW: Left Ventricular Posterior Wall, LV: Left Ventricular, Vol: Volume, CO: Cardiac Output.

Fig. 15: Cardiac hypertrophy persisted at 15 weeks of age in the GDM-HFS male rat offspring, while it was prevented in the GDM+RESV-HFS male offspring



(A) 15-Week Old Male Heart Weights, (B) 15-Week Old Female Heart Weights, (C) Left Ventricular Posterior Wall Thickness, (D) Left Ventricular Anterior Wall Thickness, (E) Left Ventricular Interior Diameter, (F) Intraventricular Septal Thickness, (G) Left Ventricular Mass, (H) Male Cardiac Triglycerides Concentration. Male Heart Weight N=5-27, Female Heart Weight N=3-8, Cardiac Triglycerides N=3-5, Echo N=8-22. p-values represent significance (<0.05) after a Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, #p<0.05 HFS postnatal diet vs. LF postnatal diet within the same maternal group, $p<0.05 as indicated in the figure. LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, LVPW: Left Ventricular Posterior Wall, LVAW: Left Ventricular Anterior Wall, LVID: Left Ventricular Interior Diameter, IVS: Intraventricular Septal Thickness, LV: Left Ventricular.

4.3.3 Cardiac hypertrophy was not associated with a reduction in cardiac function:

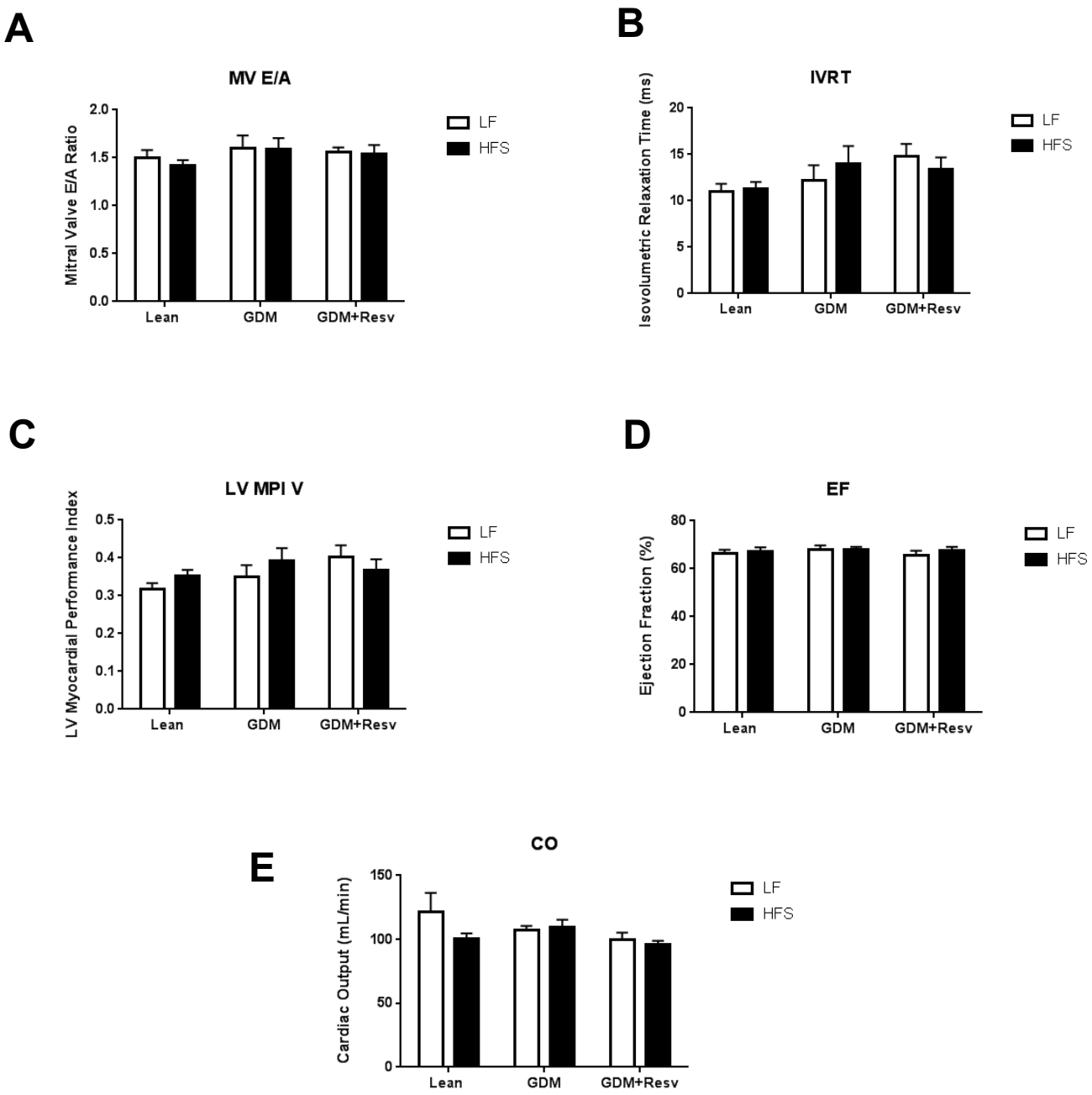
Even though a GDM pregnancy predisposed the offspring to increased cardiac hypertrophy development, neither diastolic nor systolic cardiac function parameters were changed among the groups at 15 weeks of age. For example, the mitral valve E/A ratio (MV E/A) (Fig. 16A), the isovolumetric relaxation time (IVRT) (Fig. 16B), the left ventricular myocardial performance index (LV MPI) (Fig. 16C), the ejection fraction (EF) (Fig, 16D), and the cardiac output (CO) (Fig. 16E) were similar in all groups.

4.3.4 GDM-LF male offspring exhibited cardiac steatosis, while this was prevented in the GDM+RESV-LF offspring at 15 weeks of age:

As previously shown in chapter 3 of this thesis (Fig. 10), Lean-HFS and GDM-HFS male offspring had an increased accumulation of hepatic triglycerides, while the development of hepatic steatosis in the GDM+RESV-HFS offspring was prevented. Thus, we wanted to determine whether there was any abnormal accumulation of triglycerides in the cardiac tissue of the GDM male offspring as well. This observation could be indicative of a maladaptive response as the heart is not known for storing fats, but rather prefers to utilize them for energy (Carley et al. 2007). Additionally, cardiac steatosis is also a feature of DC (Djouadi et al. 1998, 1999). Using a standard biochemical kit to measure triglyceride concentrations, it was calculated that the GDM-LF offspring hearts had a 2.4 fold increase in the accumulation of triglycerides when compared to the

GDM+RESV-LF offspring (Fig. 15H), suggesting that RESV prevented the abnormal cardiac steatosis development in those offspring. Interestingly, the GDM-HFS offspring had similar levels to the GDM+RESV-HFS offspring, suggesting that the HFS postnatal diet may have had a bigger impact than maternal diet alone in those groups of animals (Fig. 15H).

Fig. 16: Cardiac function was not affected by either maternal or postnatal diet at 15 weeks of age in the male rat offspring



(A) Mitral Valve E/A Ratio, (B) Isovolumetric Relaxation Time, (C) Left Ventricle Myocardial Performance Index, (D) Ejection Fraction, (E) Cardiac Output. N=8-22. No significant differences (<0.05) were observed after Two Way ANOVA with Bonferroni post-hoc tests. LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol, MV E/A: Mitral Valve E/A Ratio, IVRT: Isovolumetric Relaxation Time, LVMPI: Left Ventricle Myocardial Performance Index, EF: Ejection Fraction, CO: Cardiac Output.

4.3.5 Maternal RESV supplementation changed the protein expression of several proteins in the cardiac tissue of the 15 week old male rat offspring:

In order to better understand which molecular pathways might had been involved in protecting the heart from developing cardiac hypertrophy and cardiac steatosis in the GDM+RESV offspring, different molecular targets were assessed by western blotting of cardiac tissue from 15-week-old male rat offspring. The sarco/endoplasmic reticulum calcium ATPase (Serca2a) was found to be down-regulated after normalization to tubulin by a fold of ~1.3 in the GDM-LF offspring when compared to the Lean-LF and GDM+RESV-LF offspring. Similarly, the GDM-HFS offspring protein expression of Serca2a was also reduced by a fold of ~1.3 when compared to the Lean-LF, Lean-HFS, and GDM+RESV-HFS offspring (Fig. 17A). This suggests that there may be a dysfunctional calcium reuptake during the relaxation phase of the cardiac cycle in the GDM offspring, but with RESV supplementation that decrease in expression is prevented. Next, several different anti-oxidants were tested as high ROS levels are known to increase the risk for cardiac hypertrophy development, while RESV is known to have anti-oxidant characteristics (Mohammadshahi, Haidari, and Soufi 2014). Superoxide dismutase 2 (SOD2) was found to be increased in the GDM-LF offspring 1.38 fold when compared to the GDM+RESV-LF offspring, while the GDM-HFS offspring had a 1.54 fold increase in SOD2 protein expression when compared to GDM+RESV-HFS offspring after normalization to Tubulin (Fig. 17B), suggesting that there may be higher ROS levels in GDM and lower levels in GDM+RESV offspring. Additionally, a similar result was observed with catalase (Cat) expression where the Lean-HFS and GDM-HFS offspring exhibited a ~1.35 fold increase in Cat expression over the GDM+RESV-HFS offspring after normalization to Tubulin (Fig. 17C), suggesting that the GDM+RESV-HFS offspring may be producing less ROS than both Lean-HFS and GDM-HFS offspring. Furthermore, there was a trend for glutathione peroxidase 1 (GPX1) protein expression to be

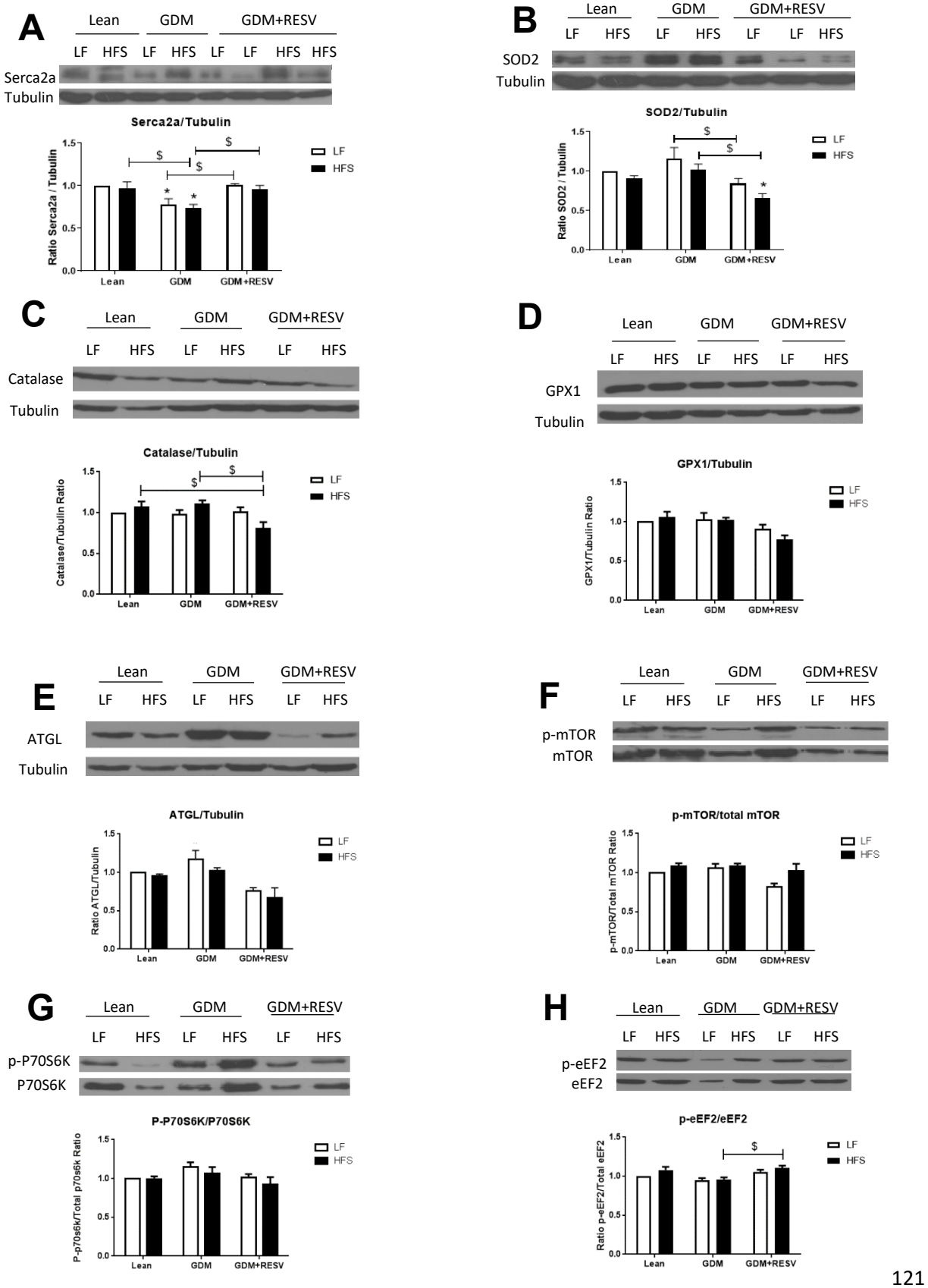
increased in the GDM-HFS offspring when compared to the GDM+RESV-HFS offspring and after normalizing to Tubulin (1.31 fold, P>0.05) (Fig. 17D), again suggesting that the GDM+RESV-HFS offspring may be dealing with fewer ROS than the GDM-HFS offspring.

In order to identify mechanisms responsible for alterations in cardiac steatosis in the GDM offspring, adipose triglyceride lipase (ATGL) levels were assessed. GDM-LF offspring had a 1.54 fold increase in ATGL protein expression when compared to the GDM+RESV-LF offspring after normalization to Tubulin (P=0.07), while GDM-HFS offspring had a 1.53 fold increase in ATGL protein expression when compared to the GDM+RESV-HFS offspring (P=0.08) (Fig. 17E), suggesting that the hearts of the GDM offspring may be undergoing increased triglyceride hydrolysis to compensate for the high triglyceride concentrations.

Lastly, to try to understand what could be the driving force behind the development of cardiac hypertrophy in GDM offspring and how RESV is preventing its development, we decided to study the mammalian target of rapamycin (mTOR) pathway which is involved in protein synthesis. We found no differences in phosphorylated mTOR (Ser2448) protein expression between GDM and Lean offspring, however there was a 1.29 fold increase in protein expression in the GDM-LF when compared to the GDM+RESV-LF offspring after normalization to total mTOR (P>0.05) (Fig. 17F), suggesting a potential inhibition of mTOR and its downstream effects in the GDM+RESV-LF offspring. The downstream effector, the phosphorylated ribosomal protein p70 S6 kinase (p70S6K) (Thr389), which is involved in protein synthesis, was found to be slightly increased by a factor of ~1.14 (P>0.05) in the GDM offspring when compared to the GDM+RESV offspring, regardless of postnatal diets and after normalization to total P70S6K (Fig. 17G). And finally, the phosphorylated form of the eukaryotic elongation factor 2 (eEF2) (Thr56), which is directly associated with protein synthesis when it is not phosphorylated, was found to be increased

in the GDM+RESV-HFS offspring by a factor of 1.15 when compared to the GDM-HFS offspring and after normalization to total eEF2 (Fig. 17H), indicating that the GDM-HFS offspring may have increased protein synthesis activity that could explain the increase in hypertrophy observed in those animals.

Fig. 17: Maternal RESV supplementation changed protein expression of several targets in the cardiac tissue of the 15 week-old young-adult male rat offspring



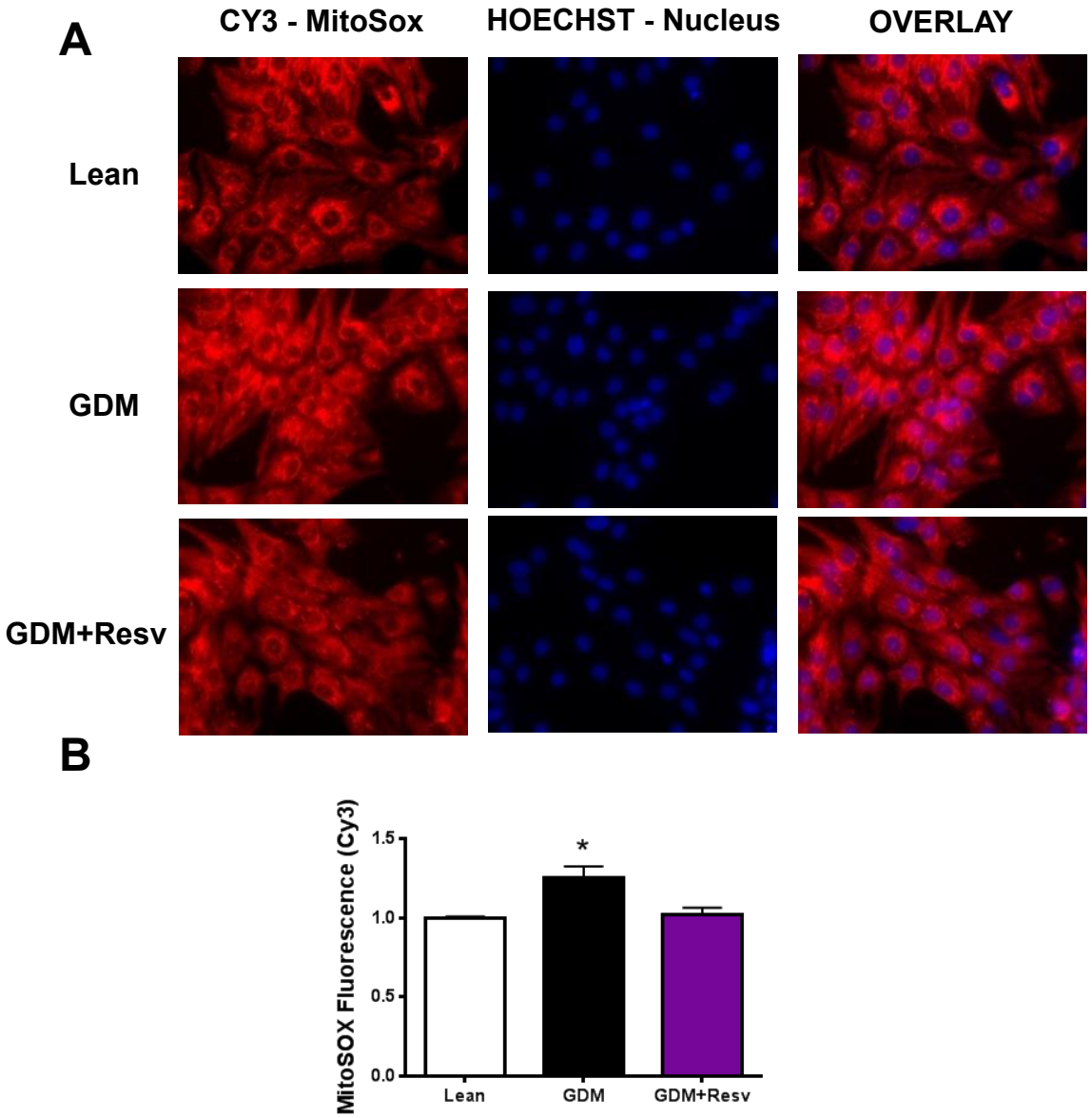
(A) Serca2a protein expression relative to Tubulin and normalized to Lean-LF, (B) SOD2 protein expression relative to Tubulin and normalized to Lean-LF, (C) Catalase protein expression relative to Tubulin and normalized to Lean-LF, (D) GPX1 protein expression relative to Tubulin and normalized to Lean-LF, (E) ATGL protein expression relative to Tubulin and normalized to Lean-LF, (F) p-mTOR (Ser2448) protein expression relative to total mTOR and normalized to Lean-LF, (G) p-P70S6K (Thr389) protein expression relative to total P70S6K and normalized to Lean-LF, (H) p-eEF2 (Thr56) protein expression relative to total eEF2 and normalized to Lean-LF. N=4-6 by group. P-values represent significance (<0.05) after Two-Way ANOVA with Bonferroni post-hoc tests. *p<0.05 vs. Lean-LF, $p<0.05 as indicated in the figure. Serca2a: Sarco/Endoplasmic Reticulum Calcium ATPase 2a, SOD2: Superoxide Dismutase 2, GPX1: Glutathione Peroxidase 1, ATGL: Adipose Triglyceride Lipase, mTOR: Mammalian Target of Rapamycin, P70S6K: Ribosomal Protein P70 S6 Kinase, eEF2: Eukaryotic Elongation Factor 2, LF: Low Fat Diet, HFS: High Fat and Sucrose Diet, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

4.3.6 GDM fetal cardiomyocytes exhibited increased reactive oxygen species, while maternal RESV supplementation attenuated the increase:

In addition to potentially having increased protein synthesis in the GDM offspring driving the development of cardiac hypertrophy, we investigated other plausible explanations about how GDM conditioned cardiomyocyte hypertrophy in these animals. We isolated fetal cardiomyocytes from fetal pups at ~e20.5 and applied different molecular dyes to test for mitochondrial ROS production, mitochondrial number, and mitochondrial membrane potential. As increased mitochondrial ROS production is known to affect cardiac hypertrophy development (Mohammadshahi, Haidari, and Soufi 2014), we applied MitoSOX dye to cardiomyocytes which fluoresces in the presence of ROS (Fig. 18A). The GDM-exposed fetal cardiomyocytes exhibited a significant 25% increase in fluorescence, indicating an increase in mitochondrial ROS production, when compared to the Lean cardiomyocytes, and a 22% increase in fluorescence when compared to the GDM+RESV cardiomyocytes (P=0.07) (Fig. 18B). To determine whether these changes were due to different amounts of mitochondria present in the wells, MitoTracker dye was used which fluoresces inside active mitochondria (Fig. 19A). There was a slight increase in fluorescence in the GDM fetal cardiomyocytes when compared to both Lean (1.12 fold change) and GDM+RESV (1.22 fold change) cardiomyocytes (P>0.05) (Fig. 19B), suggesting that

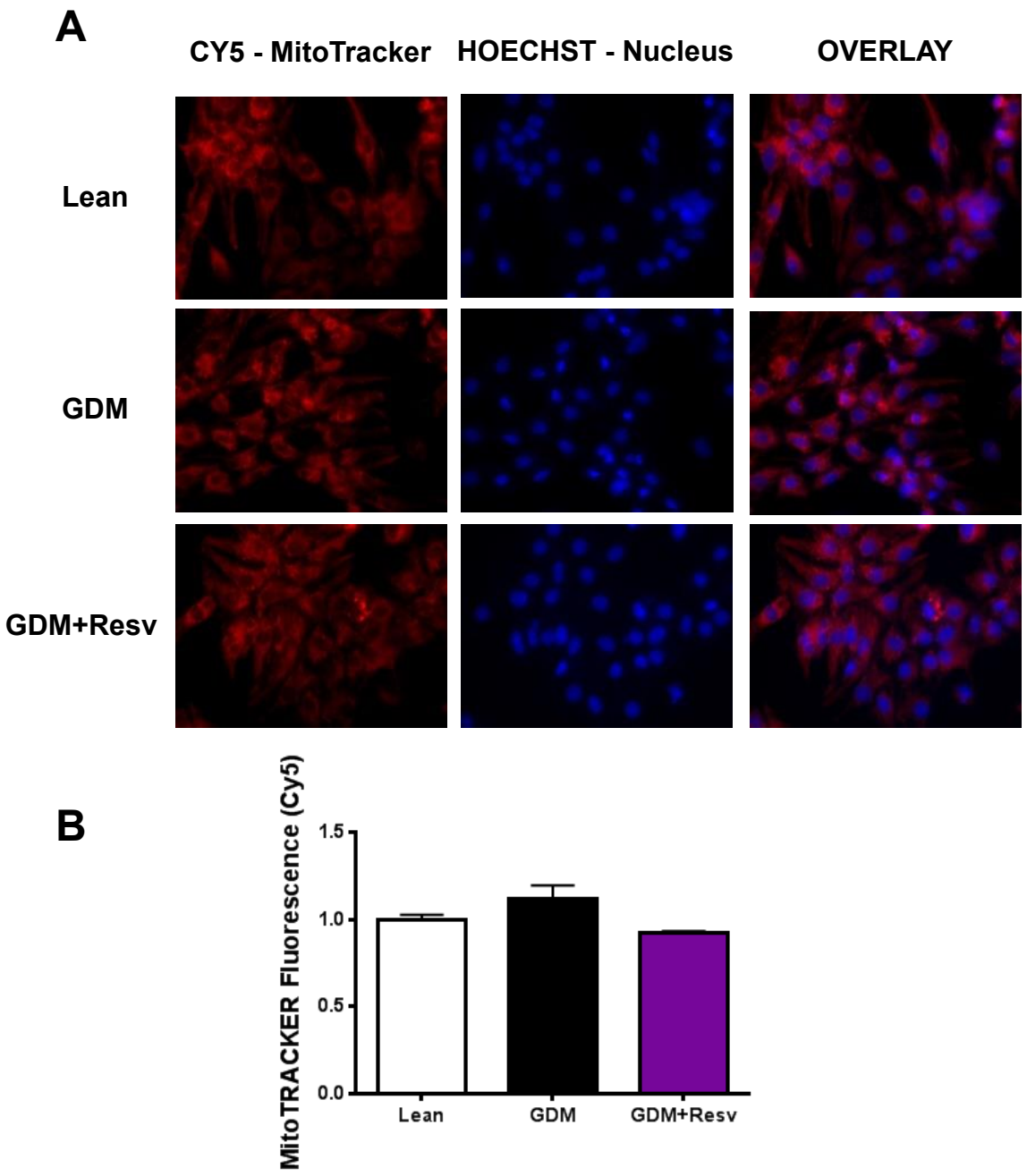
mitochondrial number may had been slightly increased in the GDM fetal cardiomyocytes. Finally, TMRM, which is a dye to look at mitochondrial membrane potential, was utilized (Fig. 20A). TMRM fluorescence was significantly increased in the GDM fetal cardiomyocytes by 10% and 17% when compared to both Lean and GDM+RESV cardiomyocytes, respectively (Fig. 20B), indicating that the GDM cardiomyocytes were more hyperpolarized and potentially more active than the cardiomyocytes from Lean and GDM+RESV pups.

Fig. 18: GDM fetal cardiomyocytes exhibited increased production of reactive oxygen species, which was attenuated with maternal RESV supplementation



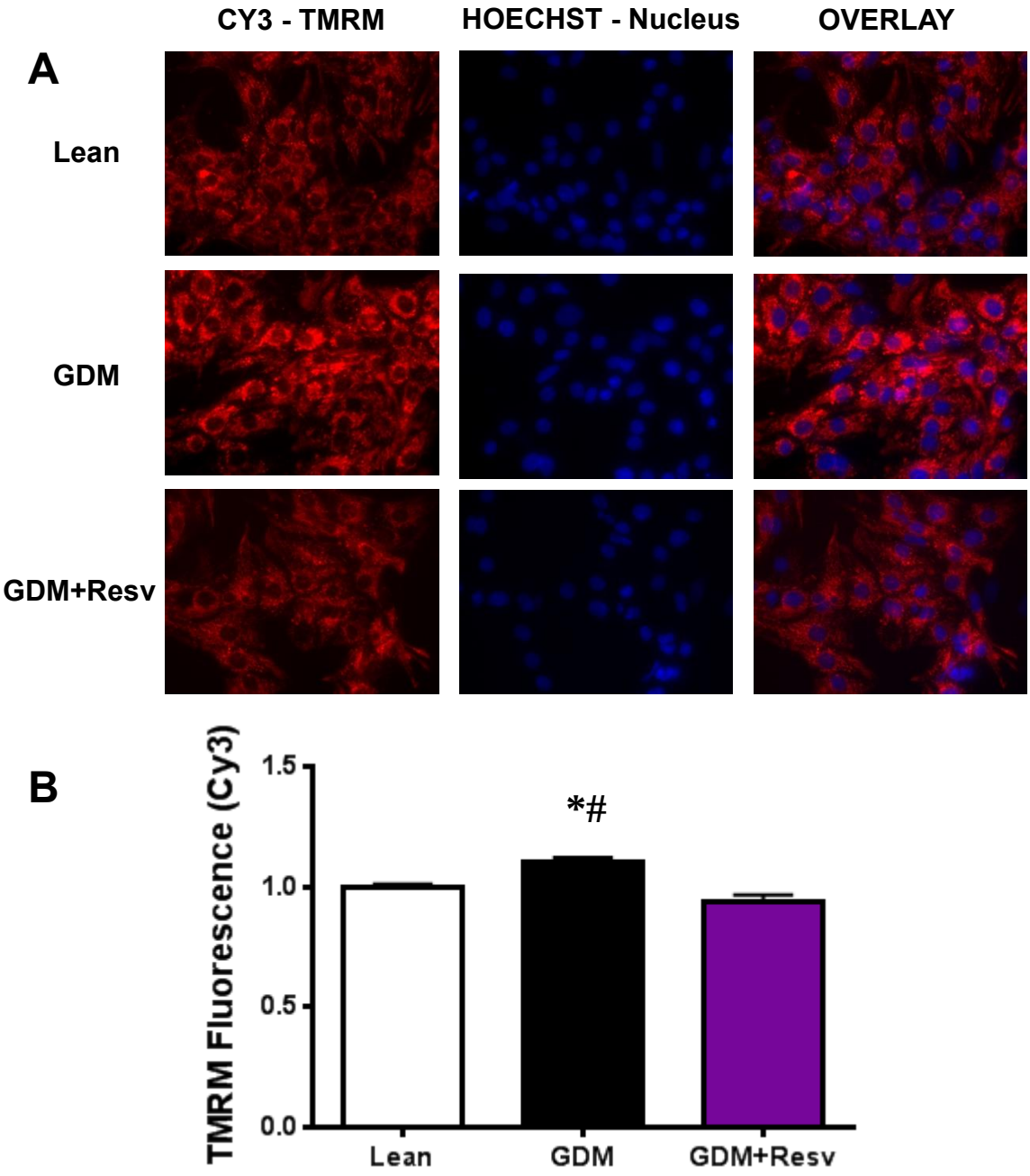
(A) Representative images of MitoSOX, Hoechst, and Overlay Staining with 40X magnification, (B) Relative MitoSOX Fluorescence normalized to Lean. N=4-8. *p values represent significant differences (<0.05) between GDM vs. Lean after One-Way ANOVA with Bonferroni post-hoc tests. p = 0.07 GDM vs. GDM+RESV. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

Fig. 19: GDM fetal cardiomyocytes had a slight increase in mitochondrial number when compared to Lean and GDM+RESV cardiomyocytes



(A) Representative images of MitoTracker, Hoechst, and Overlay staining with 40X magnification, (B) Relative MitoTracker Fluorescence normalized to Lean. N= 3-8. No significance (<0.05) was observed after One Way ANOVA with Bonferroni post-hoc tests. GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

Fig. 20: GDM fetal cardiomyocytes exhibited an increase in mitochondrial membrane potential when compared to both Lean and GDM+RESV fetal cardiomyocytes



(A) Representative images of TMRM, Hoechst and Overlay Staining with 40X magnification, (B) Relative TMRM Fluorescence normalized to Lean. N=4-16. *p<0.05 vs. Lean, #p<0.05 vs. GDM+RESV after One-Way ANOVA with Bonferroni post-hoc tests. TMRM: Tetramethylrhodamine, methyl ester perchlorate, GDM: Gestational Diabetes Mellitus, RESV: Resveratrol.

4.4 Conclusion/Discussion:

The purpose of this study was to determine whether maternal RESV supplementation (4g/kg) in addition to the postnatal diet would protect the 15 week-old young adult male rat offspring from developing cardiac hypertrophy and cardiac dysfunction. Currently, to our knowledge, this is the only study that has examined the effects of maternal RESV supplementation during a GDM pregnancy on offspring cardiac health. Previous work in the Dolinsky lab established that GDM-exposed young adult offspring experienced increased cardiac hypertrophy compared to Lean offspring (S. M. Kereliuk et al. 2016; T.J. Pereira et al. 2013). Thus, we were interested in determining whether RESV supplementation during the third trimester of pregnancy and lactation, at the onset of GDM, would provide any benefits. Our main finding was that maternal RESV supplementation prevented the GDM-induced cardiac hypertrophy by reducing protein synthesis and ROS production in the male offspring. Furthermore, we also found that RESV supplementation reduced the triglyceride accumulation in the hearts of the male rat offspring.

To begin with, we found that the neonatal hearts of the GDM offspring were larger than the hearts from both Lean and GDM+RESV pups. This increase in neonatal heart size and weight in the GDM-exposed pups was an early indicator of cardiac hypertrophy development and that these offspring could potentially be at a high risk of developing cardiac disease later on. At 15 weeks of age, the offspring were sacrificed and their heart tissue was analyzed. Even though heart weights did not differ at this time point in either male or female offspring, there was a trend for a larger heart in the GDM-HFS male offspring. Currently, there is a lack of research on the effects of supplementing RESV during pregnancy and its effects on the offspring heart. However, previous studies found that RESV supplementation of adult rodents with underlying cardiovascular

conditions, including hypertension (Vernon W Dolinsky et al. 2013), aortic constriction (Gupta, DiPette, and Supowit 2014; Juric et al. 2007), high salt diet (Rimbaud et al. 2011), and high fat diets (V. W. Dolinsky et al. 2011; Shah et al. 2017), prevented increases in heart weight and improved heart function. In fact, 146mg/kg/day of RESV reduced the heart weight to tibia length (HW:TL) ratio in hypertensive rats and mice (V. Dolinsky et al. 2015; Vernon W Dolinsky et al. 2013). In addition, using a SIRT1 deficient mice model, the authors found that these animals had increased heart weight to body weight (HW:BW) ratios, but 100mg/kg/day of RESV was sufficient to reduce heart weight to control levels, suggesting that these effects of RESV on the heart were SIRT1-independent (Sulaiman et al. 2010). However, one study did not find any improvements in heart weights when they supplemented their mice with 5mg/kg/day of RESV (Mohammadshahi, Haidari, and Soufi 2014). This may be due to the lower dosage of RESV that these authors used for their study. Thus, our data agrees with the literature showing that RESV supplementation prevents or attenuates cardiac hypertrophy; however, unlike these studies, our research shows that maternal supplementation with RESV during GDM has long-term effects on the offspring. A possible theory to explain how RESV might be protecting the offspring long-term is that RESV may be acting through SIRT1, a histone deacetylase, to change epigenetic markers and thus program different gene expression that is responsible for regulating heart size, cardiac metabolism, and anti-oxidative gene expression in the offspring. In fact, a previous study found that 10μM RESV treatment of cells that were exposed to oxidative stress or inflammation was enough to increase SIRT1 expression and restore methylation patterns (Maugeri et al. 2018), which suggests that RESV may be affecting the epigenetics of the cells. On the other hand, it is also possible that RESV supplementation during pregnancy affected maternal metabolism and the expression of anti-oxidative genes which may had provided a better intrauterine environment for fetal development

and thus protected the offspring from developing cardiac hypertrophy and disease later on. However, all of these hypotheses are highly speculative, and additional research is needed to prove or disprove these possibilities.

Since increased heart weight can be considered to be a sign for cardiac hypertrophy development, we performed echocardiography analyses at both fetal (i.e. ~e18.5) and young adult stages (i.e. 15 weeks of age) to determine cardiac morphometry and to test whether RESV supplementation would provide any benefits. At both stages of life, the LVPW thickness, which is a surrogate marker for cardiac hypertrophy, was much thicker in the GDM offspring, while RESV supplementation completely prevented the increase. Interestingly, the Lean-HFS offspring had a reduced LVPW thickness when compared to the GDM-HFS offspring, which implies that a GDM pregnancy predisposed the offspring to increased hypertrophy development. Importantly, this risk was prevented after RESV supplementation as the GDM+RESV-HFS offspring had reduced hypertrophy development, thereby potentially reducing their risk for DC. Accordingly, the left ventricular mass was increased at both stages of life in the GDM offspring when compared to the GDM+RESV offspring. As expected, the LVID was not different in any of the groups in either fetal stages or at 15 weeks of age. These findings show that the development of concentric hypertrophy, a condition where the left ventricular muscle wall is thickened but the chamber size remains the same, occurred early on before birth and was still present at 15 weeks of age in the GDM offspring. Concentric hypertrophy may be the result of a compensatory mechanism taking place during fetal development as a response to stress caused by increased ROS production and hyperglycemia, and is a key risk factor for the development of cardiac disease later on (Chung and Leinwand 2014; Chung, Yeung, and Leinwand 2012). Previous studies performed using a rat model of hypertension and using the same RESV dosage as this experiment found similar results

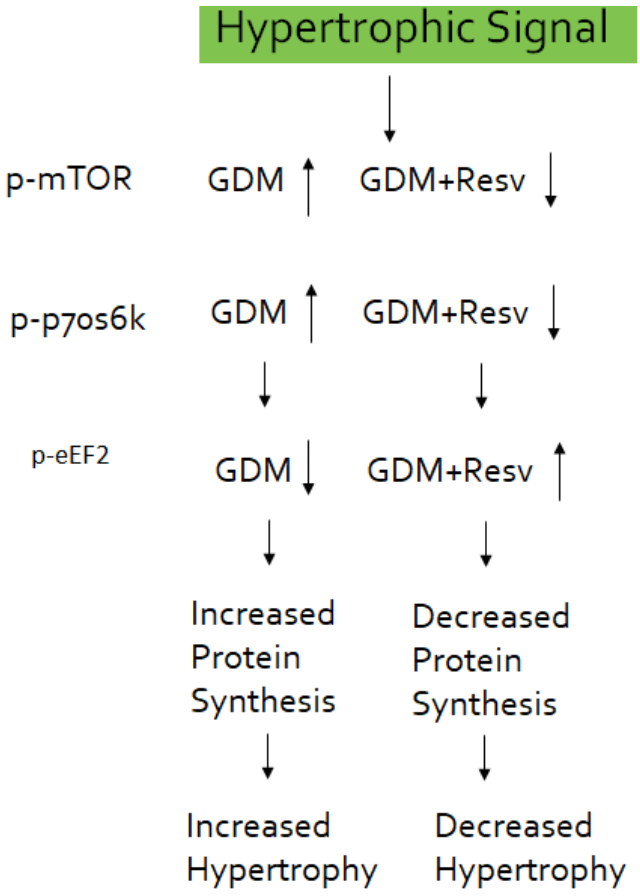
where the LVPW thickness, the left ventricular mass and the IVS thickness were all reduced after RESV supplementation, while the LVID remained unchanged (Vernon W. Dolinsky et al. 2009; Vernon W Dolinsky et al. 2013). Thus, based on these data, RESV supplementation during pregnancy prevented the GDM-induced cardiac hypertrophy in utero and into young adulthood, which may potentially protect the offspring from heart dysfunction and heart disease later on in life.

Since we observed that RESV supplementation during pregnancy prevented cardiac hypertrophy in the offspring, we hypothesized that cardiac function would also be improved. However, we did not observe any significant differences in either diastolic or systolic functional parameters when the different offspring groups were compared. The lack of changes could be explained by the fact that these offspring were not old enough to exhibit a decline in cardiac function since the GDM offspring group did not have impaired function at 15 weeks of age. We can speculate that if we continued to age the offspring, we would have observed a decline in cardiac function with the GDM offspring, while the GDM+RESV offspring would be protected. In a DC study, where diabetes was induced by Streptozotocin injection, mice exhibited a decline in diastolic cardiac function as shown by an increase in the left ventricular end diastolic diameter and a reduction in the fractional shortening at 3 months of age, but, 100 mg/kg/day of RESV supplementation completely prevented the cardiac dysfunction (Sulaiman et al. 2010). However, this was actually a T1D model, and no other studies have looked at the effects of RESV supplementation during a GDM pregnancy on offspring cardiac function.

Since GDM-induced cardiac hypertrophy was a major phenotypic characteristic of the offspring and cellular hypertrophy requires increased protein synthesis (A. Y. M. Chan et al. 2004), we investigated how GDM affected the mTOR/P70S6K/eEF2 pathway in the offspring. mTOR is

a serine/threonine protein kinase which is involved in cellular growth and proliferation, amino acid utilization and increased protein synthesis among other pathways (Capobianco et al. 2016; Mao and Zhang 2018). Our data showed that the GDM+RESV-LF offspring tended to have a reduced expression of the activated phosphorylated mTOR (Ser2448), while all the other groups were similar. The phosphorylation of the downstream effector P70S6K (Thr389) also tended to be slightly increased in the GDM offspring when compared to either Lean or GDM+RESV offspring, which may imply greater protein synthesis in the GDM-exposed animals. And finally, the phosphorylated eEF2 (Thr56) was decreased in the GDM-HFS offspring, but increased in the GDM+RESV-HFS offspring. It is important to point out that phosphorylation of eEF2 results in protein inhibition and a reduction in protein synthesis (A. Y. Chan et al. 2008). Therefore, the GDM-HFS offspring may be experiencing increased protein synthesis, which might explain the increased cardiac hypertrophy development observed in these animals. Similarly, other studies have shown that RESV supplementation protected against the development of hypertrophy by inhibiting the activities of mTOR, P70S6K and eEF2 via AMPK activation, thereby reducing protein synthesis (A. Y. Chan et al. 2008; Kovacic et al. 2003; Mao and Zhang 2018). Thus, our findings suggest that maternal RESV supplementation during a GDM pregnancy elicits sustained effects on molecular pathways that regulate protein synthesis in the offspring (Fig. 21).

Fig. 21: Proposed molecular mechanism for cardiac hypertrophy development in the young-adult male rat offspring.



In GDM offspring, as phosphorylated levels of mTOR increase due to some hypertrophic signal (ex. Glucose or ROS), so will phosphorylation of the downstream effector P70S6K. This will then result in phosphorylation of an eEF2 kinase, which will inactivate it, resulting in decreased phosphorylation of eEF2 and increased activity of the eEF2 leading to increased protein synthesis and hypertrophy development. The opposite occurs in the GDM+RESV offspring. mTOR: mammalian target of rapamycin; P70S6K: ribosomal protein P70 S6 kinase; eEF2: eukaryotic elongation factor 2; GDM: Gestational Diabetes Mellitus; RESV: Resveratrol, ROS: Reactive Oxygen Species.

Since cardiac steatosis is a major feature of DC, we measured the amount of triglycerides that were accumulated in the heart tissue. The GDM-LF offspring exhibited the greatest accumulation of triglycerides, while GDM-HFS and GDM+RESV-HFS offspring had similar concentrations. This finding is consistent with the mismatch hypothesis, where the prenatal and postnatal diets differed, thus potentially predisposing the offspring to disease (Bateson et al. 2004; P. D. Gluckman 2012; P. Gluckman and Hanson 2008). The GDM+RESV-LF offspring had the least accumulation among all groups. These findings were interesting because the heart has a low capacity for fat storage since fatty acids are typically utilized immediately to generate the energy required for contraction through oxidation (Carley et al. 2007). In diabetes, cardiac mitochondrial fatty acid oxidation is increased due to insulin resistance which prevents glucose uptake to generate energy (Djouadi et al. 1999; Fukushima and Lopaschuk 2016). This increase in oxidation may lead to increased oxygen consumption, a reduction in cardiac pumping, and eventually cardiac dysfunction (Fukushima and Lopaschuk 2016). Thus, it seems that the GDM-LF offspring were more prone to have increased triglyceride accumulation, indicating the presence of a maladaptive response by the heart, and poor energy utilization, potentially due to a dysfunctional mitochondria. Additionally, the HFS postnatal diet had a big effect as RESV supplementation alone was not able to cause a reduction in triglycerides in the GDM+RESV-HFS offspring. However, based on the data, RESV supplementation plus a healthy postnatal diet, i.e. LF diet, prevented the development of cardiac steatosis, suggesting that a healthy diet may also protect against the abnormal accumulation of triglycerides in the heart.

To better understand why the GDM heart had increased steatosis, we measured expression of ATGL. ATGL is an enzyme required to hydrolyze triglycerides into free fatty acids and diacylglycerol for energy production (Zimmermann et al. 2004). We found that the GDM

offspring, specifically the one fed a LF diet postnatally, tended to have an increased expression of ATGL protein when compared to the GDM+RESV-LF offspring (P=0.07). This could be due to a compensatory mechanism to deal with the increased triglyceride accumulation in the hearts of the GDM-LF offspring, thus, to generate more energy, ATGL protein expression may be increased to hydrolyze the extra triglycerides. In contrast, since the GDM+RESV-LF offspring had less triglyceride accumulation, ATGL expression may have been reduced (P=0.07).

In order to elaborate on potential mechanisms that may be affected by RESV supplementation, western blotting was used to study different molecular pathways and targets that may be protecting the heart from cardiac hypertrophy and diabetic cardiomyopathy in the 15 week-old male rat offspring. Serca2a is responsible for pumping calcium ions into the sarcoplasmic reticulum from the cytoplasm during diastolic relaxation to allow the heart to fill up with blood (Sulaiman et al. 2010). We found that in the GDM offspring Serca2a expression was down-regulated compared to both Lean and GDM+RESV offspring. However, the GDM+RESV offspring had similar levels as the Lean offspring, suggesting improved calcium transport during the cardiac relaxation phase, which would allow for more blood to be collected and later pumped. Studies have shown that animal models of T1D, T2D and DC have reduced levels of Serca2a expression leading to a greater risk for cardiac dysfunction development (Belke, Swanson, and Dillmann 2004; Choi et al. 2002; L. Pereira et al. 2006; Zhong et al. 2001). Previous work done in diabetic mice found that 100 mg/kg/day RESV supplementation increased SIRT1 activity, which is a Serca2a activator, thus increasing the expression of Serca2a, and improving cardiac function (Sulaiman et al. 2010). Therefore, RESV supplementation may rescue calcium transport allowing for proper cardiac contractility and potentially preventing cardiac dysfunction.

Next, we assessed whether RESV supplementation during pregnancy conferred oxidative stress resistance in the cardiomyocytes of the offspring, as RESV is known to have anti-oxidant capabilities. We found that the GDM fetal cardiomyocytes had increased ROS production when compared to both Lean and GDM+RESV cells, based on the MitoSOX dye. This data seems to suggest that maternal RESV supplementation prevented the increase in ROS potentially by up-regulating the expression of endogenous anti-oxidants as other studies have shown (Mohammadshahi, Haidari, and Soufi 2014; Turan, Tuncay, and Vassort 2012). But, in this study, we found that SOD2, Cat and GPX1 protein expression were decreased in the 15 week old GDM+RESV male offspring when compared to the GDM offspring. These findings could imply that the GDM offspring may have increased ROS production, as it was observed prior in the cardiomyocytes, therefore requiring compensatory up-regulation of anti-oxidant mechanisms to deal with the increased oxidative stress. In contrast, the GDM+RESV offspring may have a reduction in antioxidant enzyme expression since there is less oxidative stress in this group of offspring at this age. Another study used fetal endothelial cells in utero that were exposed to a GDM pregnancy. These cells had increased mitochondrial ROS production and reduced anti-oxidant expression (Cheng et al. 2013), which is the opposite of what we observed in our GDM animals where both ROS and anti-oxidants were increased. However, there are no other studies showing the effects of RESV on cardiomyocytes exposed to a GDM pregnancy. Based on our findings, it is possible that the GDM+RESV offspring exhibited increased oxidative stress early during development as they were exposed to the HFS diet only, but upon RESV supplementation, those anti-oxidants could have been up-regulated in order to decrease ROS back to normal levels. Therefore, at 15 weeks of age, there was no need to have those enzymes up-regulated any longer as ROS levels declined to appropriate levels. In fact, several studies have found RESV to up-

regulate anti-oxidants to prevent ROS accumulation and prevent oxidative stress as reviewed in (Turan, Tuncay, and Vassort 2012). Also, using a rat model of DC, 5mg/kg of RESV prevented oxidative stress by up-regulating anti-oxidant expression (Mohammadshahi, Haidari, and Soufi 2014). However, no other studies have looked at the effects of RESV supplementation during a GDM pregnancy and its effects on anti-oxidant expression in the offspring.

Finally, the mitochondrial membrane potential, as shown using the TMRM dye, was increased in the GDM cardiomyocytes, and decreased in both Lean and GDM+RESV cells. The hyperpolarized mitochondria observed in the GDM cells may have resulted in increased mitochondrial activity leading to a potential increase in ATP generation, however, this could have increased ROS production and apoptosis as well, which could eventually cause heart disease (Gergely et al. 2002). Thus, this could further explain the increase in ROS observed in the GDM animals.

Taken together, these data showed that maternal RESV consumption during the third trimester of a GDM pregnancy and lactation prevented cardiac hypertrophy development in both fetal and young adult stages in the male rat offspring. We also identified several potential mechanisms that may be involved including reduced ROS production and inhibition of the mTOR signalling pathway thereby reducing protein synthesis. Therefore, RESV consumption during a GDM pregnancy may prevent the development of cardiac disease in the male offspring later on, although additional experiments are required to be able to reach that conclusion.

Chapter 5

General Discussion, Conclusions and Future Directions:

Since prescribing drug treatments during pregnancy is controversial and there is a low level of adherence to reducing calorie intake and/or initiating an exercise regimen during pregnancy, we examined how consumption of a natural health product, resveratrol, specifically during the third trimester of a GDM pregnancy and lactation, affected the health of both mother and offspring. For this work, we used a GDM animal model previously established in the Dolinsky lab which shared many of the same phenotypes as seen in mothers with GDM (Troy J. Pereira et al. 2015). Additionally, GDM induced adverse health consequences for the offspring, such as macrosomia, obesity, glucose intolerance, and insulin resistance (Troy J. Pereira et al. 2015) that are also observed clinically (Arshad, Karim, and Hasan 2014; Buchanan, Anny H. Xiang, and Page 2012; Hod et al. 1991; Kaaja and Rönnemaa 2008). Thus, we utilized an established experimental animal model and modified the protocol to test the effect of RESV supplementation on mother and offspring health.

As obesity levels continue to rise in the population due to unhealthy diets and sedentary lifestyles, more women of child-bearing age are beginning their pregnancies either obese or overweight, and as a result the incidence of GDM is also increasing (Corey and Kaplan 2014; Lehnert et al. 2013; Simmons 2011; Swinburn et al. 2011). This is problematic not only because GDM can predispose both mothers and their offspring to cardio-metabolic disease and CVD (Lehnert et al. 2013; Poston, Harthoorn, and Van Der Beek 2011), but also because this trend will undoubtedly reduce quality of life and increase the burden on the health care system (Cawley and Meyerhoefer 2012; Lehnert et al. 2013).

RESV is a natural health product which has been shown to have positive health outcomes in many different animal models and in many different diseases (Diaz-Gerevini et al. 2016). More importantly, in both clinical and animal studies, RESV has been shown to be safe during pregnancy, well tolerated and non-teratogenic to the offspring (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; B. Wicklow et al. 2015; Williams et al. 2009). RESV supplementation throughout pregnancy has been shown to improve maternal glucose homeostasis and insulin sensitivity in different diabetic animal models using nonhuman primates, rats, and mice (Roberts et al. 2014; C. K. Singh et al. 2011, 2013; Yao et al. 2015). One important caveat to consider is that current studies have used different dosages, timings and delivery methods of RESV to counteract the low bioavailability of the compound (Pangeni et al. 2014). Some studies, for example, fed RESV to female rats prior to pregnancy and then during gestation and lactation (Roberts et al. 2014; Yao et al. 2015), while others chose to give RESV at different time points during pregnancy (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; Zou et al. 2017), or only during lactation (Tanaka et al. 2017). To our knowledge, this study was the first to supplement maternal diets with RESV from the third trimester of pregnancy, at the onset of disease, until the end of lactation. In chapter 2, RESV supplementation also prevented maternal glucose intolerance, lowered blood glucose levels in the third trimester and improved pancreatic insulin secretion which were all altered by a GDM pregnancy. Importantly, we did not observe adverse events during the course of pregnancy in the dams and the litter sizes and newborn rat sizes were normal, consistent with the preceding reports. Since current therapeutics for GDM (e.g. insulin, glyburide and metformin) are associated with health risks for mothers and their offspring, (e.g. increased caesarean deliveries, macrosomia and hypoglycemia) as reviewed by (Brawerman and Dolinsky 2018), it is important to develop and bring to market new and safer treatments. Thus, our data is

an important contribution to the knowledge about GDM therapies because it suggests that a narrow window of RESV therapy merits research in primates and then human pregnancy.

In our study, we did not have a maternal group fed a low fat diet that was supplemented with RESV. This is because some studies have shown that a maternal low fat diet supplemented with RESV throughout pregnancy did not have any effect on offspring health (Ros et al. 2018). In contrast, maternal RESV supplementation in dams that consumed a high fat and sucrose diet resulted in a decrease in body weight in the offspring (Ros et al. 2018). Therefore, we suggest that RESV, in the context of pregnancy, should be utilized as a treatment rather than as a preventative intervention. This is because it may not have a significant beneficial effect in healthy populations since the data largely suggest that it has its greatest beneficial effect only once the disease is established.

Few studies have examined the long-term effects of RESV administration during a GDM pregnancy on the offspring. In chapter 3, we found that maternal RESV supplementation induced sustained effects on the metabolic health of the offspring, at least up to 15 weeks of age. These included a reduction in obesity and hepatic steatosis. Additionally, glucose homeostasis and insulin sensitivity were improved in the offspring, while several genes associated with metabolism had their gene expression modified by maternal RESV supplementation. Similarly, other animal studies reported RESV supplementation during pregnancy and lactation prevented obesity, decreased fat accumulation and glucose levels (Ros et al. 2018), increased insulin sensitivity (Zou et al. 2017) and decreased glucose production (Yao et al. 2015) in the offspring. Additionally, a study supplemented maternal diets with RESV during lactation only and found that the offspring had reduced body weights and reduced hepatic steatosis development (Tanaka et al. 2017). The previous studies fed the offspring either a chow or a high fat diet. However, our study is different

because we supplemented the maternal diet with RESV at the onset of GDM, at the third trimester, and we also analyzed the effect of postnatal diet on the offspring health and its interaction with biological sex. We found that the sex of the offspring played a significant role in determining health outcomes as males and females exhibited different responses to the diets. In fact, in our study, it seemed that RESV supplementation was beneficial for the male offspring, but detrimental for the female offspring. In contrast, only one study reported their findings comparing male and female offspring and also found different responses to maternal diet and RESV supplementation with female offspring being more affected than the males (Ros et al. 2018). Thus, due to the contradicting results, more research is required to assess the effects of RESV on female offspring. Nonetheless, our findings suggest that even a narrow window of RESV administration to the dams was sufficient to affect the health of the offspring. These findings are important because they suggest a strategy to reduce the potential adverse effects of RESV administration in pregnancy by reducing the duration of treatment. Moreover, in contrast to the preceding studies that utilized RESV as more of a GDM "prevention" strategy, the timing of RESV supplementation in the third trimester in our study was truly a RESV "treatment" for GDM and is more in line with the clinical utilization of therapeutics for GDM, as recently reviewed in (Brawerman and Dolinsky 2018). Furthermore, we emphasized that RESV administration in the third trimester could prevent the adverse effects of GDM on the offspring metabolic health.

Even though RESV has been shown to be safe for the offspring in most studies, one particular study described a concerning result. Japanese macaques were supplemented with 0.37% RESV for 3 months prior to pregnancy and then throughout gestation. Although they found beneficial health outcomes in the mothers, the offspring had a 42% increase in fetal pancreatic mass, suggesting a potential toxic effect of RESV (Roberts et al. 2014). Based on work by other

members of our lab and in collaboration with Dr. Christine Doucette's laboratory, we observed that in our model, RESV did not affect fetal β-cell mass and in fact prevented GDM-induced defects in insulin secretion by the isolated islets from the 15 week-old male offspring. Thus, the timing of therapy may be critical in determining the safety and efficacy of RESV treatments. However, since our study and other studies did not find any significant adverse effects, it is possible that the dosage, duration or the animal model, may have contributed to that outcome (Bourque et al. 2012; C. K. Singh et al. 2011, 2013; B. Wicklow et al. 2015; Williams et al. 2009).

The effects of maternal RESV supplementation on offspring metabolic health could have been a consequence of a better intrauterine environment (e.g. changes in circulating insulin and other hormones or reduced levels of excess nutrients) and better conditions for fetal growth and development (e.g. reduced placental oxidative stress or inflammation) when compared to the GDM environment. On the other hand, RESV could have affected epigenetic factors at the fetal stage, such as DNA methylation or protein acetylation, which may have long-term effects of programming gene expression in the offspring. Consistent with this concept, we observed that several genes were regulated in a GDM-dependent manner in the offspring and RESV attenuated these effects of GDM. For example, the gene expression of *pparα* was significantly reduced in the GDM offspring, but RESV supplementation completely prevented that reduction, which could have improved fatty acid β-oxidation and thus, better substrate utilization to generate energy. Additionally, *pepck* gene expression was increased and *g-6-p* gene expression was reduced in the GDM offspring, implying that they had increased hepatic gluconeogenesis and reduced glycolysis which could have affected their glucose sensitivity and response. However, the GDM+RESV offspring had reduced hepatic gluconeogenesis and increased *g-6-p* gene expression, suggesting better glucose utilization and better glucose homeostasis. A recent study used a human retinal

pigment epithelial cell line to research retinal neurodegenerative diseases. The authors found that when these cells were exposed to either oxidative stress or inflammation, the expression and activities of DNA methyltransferases and SIRT1 were reduced (Maugeri et al. 2018). In addition, the methylation of LINE-1, which is a marker for global methylation, was also reduced (Maugeri et al. 2018). They treated the cells with 10 μM RESV and found that this dosage did not affect cell viability, but it reduced ROS production. Additionally, these authors found that RESV restored the expression and activity of both SIRT1 and DNA methyltransferase as well as restored the methylation in LINE-1 in the cells exposed to both oxidative stress and increased inflammation (Maugeri et al. 2018). Thus, RESV supplementation may potentially affect epigenetic markers during fetal development, programming the offspring and providing protection against chronic disease in the future. Hence, as a future direction for this project, we will perform RNA sequencing of fetal and neonatal liver and heart tissues to identify specific genes in the offspring that may be modified upon GDM exposure and may be affected by RESV supplementation. Future work also needs to be performed in order to examine these epigenetic factors in relation to sex differences in the offspring.

Previously in the Dolinsky lab, it was identified that GDM predisposed offspring to have cardiac hypertrophy (S. M. Kereliuk et al. 2016; T.J. Pereira et al. 2013), which is a key risk factor for heart disease and heart failure. In fact, cardiac hypertrophy can lead to the development of DC which is one of the most common complications in people with T2D (Mohammadshahi, Haidari, and Soufi 2014). Previous studies have reported that RESV administration to adult rodents prevented cardiac hypertrophy and improved cardiac structure and function in models of hypertension and exercise training (V.W. Dolinsky et al. 2012; Vernon W Dolinsky et al. 2013). However, the effects of RESV supplementation during a GDM pregnancy on cardiac function and

structure in the offspring had not been previously reported. Therefore, in chapter 4 we chose to examine whether RESV supplementation during pregnancy could prevent cardiac hypertrophy in the offspring, thus protecting the offspring from cardiac dysfunction and disease later on. We found that the GDM male offspring exhibited cardiac hypertrophy at both neonatal and young adult stages, while RESV supplementation prevented myocardial hypertrophy. Interestingly, when we compared the GDM-LF to the GDM-HFS male offspring, we found that the GDM-HFS offspring had increased hypertrophy development. This finding implies that following GDM exposure, consumption of an unhealthy diet high in fats and sugars may accelerate the progression of heart disease beyond the levels observed in the offspring of lean mothers. This information has important public health implications because it suggests that following GDM pregnancies, children should be screened for potential cardiac disease development and dietary interventions could be effective in reducing heart disease risk.

Mechanistically, protein synthesis is important for cardiac hypertrophy development (A. Y. M. Chan et al. 2004). We determined that mTOR, P70S6K and eEF2 could be affected by RESV supplementation, indicating a potential reduction in protein synthesis in the male hearts. Other studies also found that RESV inhibited mTOR and its downstream effectors in neonatal cardiomyocytes resulting in reduced protein synthesis and the prevention of cardiac hypertrophy (A. Y. Chan et al. 2008; Kovacic et al. 2003; Mao and Zhang 2018). Since RESV crosses the placenta from the maternal circulation and as a result can enter the fetal circulation (Bourque et al. 2012), it may act directly on the cardiomyocytes to prevent GDM-induced cardiac hypertrophy.

Additionally, we observed that Serca2a expression, which is an important player for the heart's contractility and calcium homeostasis, was decreased in the GDM offspring, while RESV supplementation rescued the expression. Other studies have shown that Serca2a deficiency leads

to cardiac dysfunction and cardiac disease (Belke, Swanson, and Dillmann 2004; Choi et al. 2002; L. Pereira et al. 2006; Zhong et al. 2001), however RESV supplementation resulted in increased Serca2a expression via SIRT1 activation in another study (Sulaiman et al. 2010). Thus, based on these results, we hypothesized that RESV supplementation would improve cardiac function in these animals. However, we did not see any changes in the heart's functionality at this time point. This may be due to the fact that this is an early stage in heart disease progression and these rats have not begun to exhibit overt signs of heart disease. But, since we observed a prevention in cardiac hypertrophy development, we hypothesized that if we had continued with the experiment, we would have been able to see a protection later on in these animals, while the GDM offspring would have shown cardiac dysfunction earlier.

More research is needed to determine the optimal RESV dosage in pregnancy not only for animal studies, but also for human clinical trials. As described throughout this work, the GDM+RESV dams consumed 4g/kg of RESV, or 147.6 mg/kg/day of RESV which is a high dosage, but was consistent with other studies (Bourque et al. 2012; V. W. Dolinsky et al. 2011; V.W. Dolinsky et al. 2012; Vernon W Dolinsky et al. 2013). This high dose was chosen because RESV has low bioavailability, so to counteract the effect of first-pass metabolism, a greater concentration was used to ensure proper levels would reach the systemic circulation (Pangeni et al. 2014). However, RESV dosages in this thesis ranged from 2.5mg/kg/day to 200mg/kg/day in animal studies and up to 1g/kg/day in human studies, thus more research is needed to find the optimal RESV dose. This dose will have to be beneficial without causing serious side effects if the goal is to market RESV as a potential treatment for GDM. Accordingly, more clinical trials should be designed as there is a lack of research on the effects of RESV supplementation in women with GDM and their children.

Various studies have shown an association between specific genes (i.e. Myosin Heavy Chain α and β, and Brain Natriuretic Peptide) and increased cardiac hypertrophy development (V. Dolinsky et al. 2015; Nakagawa et al. 1995), therefore, future studies should focus on performing gene expression analyses of cardiac tissue to determine whether these hypertrophic genes are down-regulated in the GDM+RESV offspring, which could further aid in explaining why there is a decrease in hypertrophy development in those offspring. Furthermore, more cardiomyocyte experiments should be conducted to test the hypothesis that acute RESV supplementation will prevent hypertrophy in isoproterenol-treated cells, as well as rescue calcium signalling (especially given the effects of RESV on Serca2a expression in this model) and homeostasis in those cardiomyocytes preventing heart disease.

Taken together, this work was the first to investigate whether maternal diets supplemented with RESV during the third trimester of a GDM pregnancy and lactation would be beneficial to maternal and offspring short- and long-term health outcomes. We found RESV to be safe during pregnancy and to reduce obesity, improve glucose homeostasis, prevent hepatic steatosis and insulin resistance while also preventing cardiac steatosis and cardiac hypertrophy in the young adult male rat offspring. All of these results suggest that RESV supplementation during pregnancy may prevent the development of cardio-metabolic disease in the male offspring, although more research is required to assess the effects of RESV on female offspring.

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