Maternal Resveratrol Supplementation and its Effects on Cardiac Hypertrophy, Mitochondrial Metabolism, and Calcium Flux in Isolated Fetal Cardiomyocytes

By

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Contribution of Authors

Marcelo Ninalaya designed experiments (specifically Fluo-4 calcium tracking, seahorse glycolysis stress and fatty acid oxidation), performed the experiments, monitored the animals, generated and analyzed the data, and was responsible for writing and editing the manuscript. Gabriel Brawerman played a role in conceptualizing the studies (specifically the incorporation of Resveratrol in the maternal diet). Dr. Mateusz Tomczyk assisted with performing early experiments (cardiomyocyte isolation and seahorse mitostress test) and early data analysis and data visualization. Bo Xiang performed the animal work (sacrifice, echocardiography, and harvesting of the fetal pups). Dr. Stephanie Kereliuk played a role in conceptualizing the studies (specifically GDM exposure on rat offspring cardiovascular health and mitochondrial dysfunction) and designing the earlier experimental procedures (cardiomyocyte Isolation and seahorse Mitostress test). Dr. Vernon Dolinsky played an advisor role and contributed to conceptualizing the studies.

Abstract

Gestational diabetes mellitus (GDM) is a condition that manifests in pregnancy that is characterized by insulin resistance, glucose intolerance, and hyperglycemia. Medications have shown effectiveness but have associated risk of adverse pregnancy outcomes. Our previous studies in rats found cardiomyocytes of GDM-offspring exhibit hypertrophy, mitochondrial dysfunction, and impaired calcium flux. We hypothesize that administration of resveratrol (RESV) to maternal GDM diet will mitigate mitochondrial dysfunction, cardiac hypertrophy and improve calcium flux in GDM-offspring. A diet-induced model was implemented on female Sprague-Dawley rats six weeks before mating to induce GDM. A subgroup of GDM dams were switched to a diet containing RESV. Fetal echocardiography was performed to assess cardiac structure. Measurements of mitochondrial respiration and calcium flux were performed using the Agilent-Seahorse XF 24 and Cytation 5. Maternal RESV supplementation improved mitochondrial respiration and improved calcium flux upon angiotensin II stimulation. Importantly, maternal RESV supplementation attenuated GDM-induced cardiac hypertrophy in GDM-offspring.

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List Of Abbreviations

ACC – Acetyl-CoA Carboxylase

ADP – Adenosine Diphosphate

AKT – Protein Kinase B

AMPK – Adenosine Monophosphate-activated Protein Kinase

ANOVA – Analysis of Variance

ARAC – Cytosine β-D-arabinofuranoside

ATP – Adenosine Triphosphate

BMI – Body mass Index

BSA – Bovine Serum Albumin

CHD – Coronary Heart Disease

CHRIM – Children's Hospital Research Institute of Manitoba

CPT1 – Carnitine Palmitoyl Transferase 1

CVD – Cardiovascular Disease

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DNAse – Deoxyribonuclease

DOHaD – Developmental Origins of the Health and Disease

db/+ - Leptin receptor-deficient

ECAR – Extracellular Acidification Rate

EF – Ejection Fraction

F-12 – Ham's F-12 Nutrient Mixture

FCCP – Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone

FBS – Fetal Bovine Serum

FS – Fractional Shortening

GDM – Gestational Diabetes Mellitus

GLUT1 – Glucose Transporter 1

GLUT2 – Glucose Transporter 2

GLUT4 – Glucose Transporter 4

GMC – Genetic Models Center

GSIS – Glucose Stimulated Insulin Secretion

HBSS – Hank's Balanced Salt Solution

HFS – High-Fat and High-Sucrose

IVS – Intraventricular Septal Thickness

LF – Low Fat Diet

LV – Left Ventricle

LVH – Left Ventricular Hypertrophy

LVID – Left Ventricular Interior Diameter

LVPW – Left Ventricular Posterior Wall Thickness

MiG – Metformin in Gestational Diabetes

MiTy – Metformin in Women with Type 2 Diabetes in Pregnancy

NPH – Neutral Protamine Hagedorn

OCR – Oxygen Consumption Rate

OCT3 – Organic Cation Transporter 3

PBS – Phosphate-Buffered Saline

PDK1 – Phosphoinositide-dependant Kinase 1

PGC-1a – Peroxisome Proliferator-activated Receptor-Gamma coactivator 1-alpha

PI3K – Phosphatidylinositol 3-kinase

PIP3 – Phosphatidylinositol-3,4,5-trisphosphate (PIP3)

Prlr – Prolactin Receptor

RESV – Resveratrol

ROI – Region of Interest

SIRT1 - Sirtuin 1

SREBP-1 – Sterol Regulatory Element-Binding Protein 1

STZ – Streptozotocin

T2D – Type 2 Diabetes Mellitus

XF – Extracellular Flux

XFe24 – Extracellular Flux e24

Chapter 1: Introduction

1.1 Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) is a metabolic condition that is characterized as glucose intolerance first observed during the late second and third trimester of pregnancy, resulting in varying severity of hyperglycemia (Kim et al., 2019). In a healthy pregnancy, complex endocrine and metabolic changes are necessary to meet the energy requirements to support both the mother and fetus (Di Cianni et al., 2003). During the early stages of pregnancy, glucose tolerance and insulin sensitivity remain relatively normal. As pregnancy progresses, insulin resistance begins to set in leading to a reduction in insulin sensitivity (Catalano et al., 1999). This dynamic change allows for excess nutrient supply to remain in maternal circulation that can be spared to the placenta to ensure proper fetal development (Di Cianni et al., 2003). Previous longitudinal studies found that in the later stages of gestation, pregnant women with GDM had decreased insulin sensitivity and reduced suppression of hepatic glucose production compared to non-GDM pregnant women (Catalano et al., 1999). A major contributing factor for these changes in insulin secretion stems from alterations in hormones during pregnancy, such as human placental growth hormone, placental lactogen and maternal cortisol that can induce insulin resistance in both in vitro and in vivo models (Brelje et al., 1993; Barbour et al., 2002). In a healthy pregnancy, reduced insulin sensitivity is compensated by increased insulin production from pancreatic beta cells and enhanced glucose-stimulated insulin secretion (GSIS) (Plows et al., 2018). However, in GDM, the secretion of insulin is insufficient to compensate for elevated insulin resistance.

1.1.1 Incidence and Prevalence Globally and in Canada

With a global prevalence of 14.0%, GDM is often considered one of the most common complications in pregnancy. According to Diabetes Canada, depending on the risk factors,

between 3-20% of pregnant women develop GDM (Feig et al., 2018). In Manitoba, the rate of gestational diabetes affects 9.0% of pregnancies as of 2017 (Public Health Agency of Canada, 2020). With the incidence of GDM continuing to rise in Canada from 7.2% in 2005 to 14.7% in 2019, there is a growing need to develop strategies that can treat potential complications that are associated with GDM pregnancies for both the mother and the child (Nethery et al., 2023).

1.1.2 GDM Effects on Mothers and Offspring

GDM can have significant impacts on maternal cardiovascular health both during pregnancy and in the long term. Various studies have highlighted the complications associated with GDM. Mao et al. (2022) found that women with a previous GDM diagnosis had higher risks associated with coronary heart disease (CHD), heart failure, and stroke (Mao et al., 2022). Furthermore, women with a history of GDM had an increased risk of developing type 2 diabetes (T2D) later in life, which can in turn increase the risk of developing these cardiovascular complications in women later in life (Vounzoulaki et al., 2020; Sun et al., 2021). Studies from Retnakaran and Shah (2016) found an increased risk of developing cardiovascular complications in women who had GDM (Retnakaran & Shah, 2016). Interestingly, women previously diagnosed with GDM who had developed T2D had an increased risk of renal dialysis, vitrectomy/photocoagulation and hospitalization for foot infections (Retnakaran & Shah, 2016). Cohort studies from Denmark by Yu et al. (2021) revealed that women with a history of GDM had a 40% higher overall risk of cardiovascular disease (CVD) when compared to women without a history of GDM (Yu et al., 2021). These women also showed a 65% increased risk of stroke and a two-fold elevated risk of myocardial infarction, heart failure, and peripheral artery disease (Yu et al., 2021).

Several studies examined the impact of maternal GDM and offspring cardiovascular health. Large follow-up cohort studies from Yu et al. (2019) found that children of mothers with diabetes had increased rates of early-onset CVD that spanned from childhood up to early adulthood (Yu et al., 2019). They also found that children of women with GDM or pregestational diabetes had an increased risk of heart failure, hypertensive disease, pulmonary embolism, and deep vein thrombosis compared to children from unexposed mothers (Yu et al., 2019). Additionally, several studies found that children born to mothers with GDM tend to have higher systolic blood pressure, which could be predictive of adult hypertension (Pathirana et al., 2020). Using data from the MySweetHeart cohort, Di Bernardo et al. (2023) reported thicker posterior left ventricular walls in children born from mothers with GDM compared to children from mothers without GDM (Di Bernardo et al., 2023).

1.2 GDM Animal Model

There are a variety of animal models that are used to examine the impact of maternal obesity and diabetes during pregnancy on both maternal and offspring health outcomes.

1.2.1 Previous Animal Models used to study GDM

One of the more common models utilizes a chemical induction of streptozotocin (STZ), an antibiotic extract from streptomycin that is highly toxic to pancreatic beta cells (He et al., 2020). STZ enters the beta cells via glucose transporter 2 (GLUT2), where it acts as an alkylating agent to cause DNA damage, resulting in the destruction of the beta cells (Pereira et al., 2015). This highly selective toxic effect on the beta cells results in insulin deficiency and hyperglycemia that mimic key characteristics of GDM. Although useful in establishing a model, this approach does bring limitations. For instance, STZ can cross the placenta, potentially affecting fetal development (Jawerbaum & White, 2010). Various studies have found that STZ has been

associated with more low-birth-weight pups, which contrasts with the large gestational-weight offspring often observed in pregnancy (Pereira et al., 2015). It should also be noted that some studies have also reported a reduction in litter number in STZ-induced GDM rodent models (Takahashi et al., 2024).

Alternatively, genetic models have also been used to study GDM, as numerous knockout models have been described throughout literature that aim to resemble the phenotype of GDM. One frequently used model seen in literature is the leptin receptor-deficient (*db/+*) mouse model. These mice have a heterozygous mutation in the leptin receptor gene and develop moderate glucose intolerance, insulin resistance, and increased weight gain during gestation in a manner that resembles GDM-like characteristics (Grupe & Scherneck, 2023). However, recent studies have reported inconsistent results using this model, suggesting it no longer reliably produces these GDM-like phenotypes in some colonies (Plows et al., 2017). Similar genetic models have also been proposed; the obese *ob/ob* mice model utilizes mutations in the obese gene, which is responsible for encoding the hormone leptin. This mutation causes impairments in functional leptin production, resulting in disruptions in regulating food intake and appetite (Suriano et al., 2021). As a result, these mice develop obesity, insulin resistance, and mild hyperglycemia, mimicking some aspects of T2D. However, the mutation causes infertility in female mice, which makes it unsuitable for studying pregnancy-related conditions such as GDM (Pereira et al., 2015). Alternatively, the nonobese diabetic and Akita mouse models better reflect characteristics of insulin-deficient diabetes, and although they are primarily used in type 1 diabetes studies, they do offer some insight into certain aspects of the disease condition, such as the effects of hyperglycemia during pregnancy and pregnancy-related insight on fetal development (Jawerbaum & White, 2010). Other genetic models utilize deficiencies in the prolactin receptor

to study GDM. Studies from Huang et al. (2009) demonstrated that the absence of the prolactin receptor (*Prlr*) impacts insulin secretion during pregnancy. Heterozygous *Prlr* +/- mice were observed to have decreased insulin secretion and impaired GSIS when compared to control mice. (Huang et al., 2009). Although these models are useful in contributing to further our understanding of GDM, it is crucial to consider that most women who are diagnosed with GDM do not exhibit profound insulin deficiency. Therefore, such studies may not be translatable to the majority of GDM patients (Pereira et al., 2015).

1.2.2 Previously Established Animal Model from Dolinsky Lab

A diet-induced model is an important approach for studying GDM and has been used to investigate the effects of maternal overnutrition during pregnancy as this approach closely mimics the weight gain, metabolic, and physiological changes that occur in GDM patients (Pereira et al., 2015). One of the key driving factors for the development of GDM is obesity. Excessive high fat intake in female rodents promotes glucose intolerance during pregnancy (Holemans et al., 2004). The most common approach involves feeding female rodents a high-fat and high-sucrose (HFS) diet before and during pregnancy, with females generally being fed several weeks before mating (Holemans et al., 2004). Studies utilizing this model observed an increase in gestational weight gain, hyperglycemia, and the development of insulin resistance in a manner that resembles the disease manifestation of GDM seen in clinical patients (Mishra et al., 2022). Similarly, Mishra et al. (2022) demonstrated that modified diets consisting of HFS that are introduced before pregnancy resulted in the development of insulin resistance and beta cell dysfunction in mice (Mishra et al., 2022). Similar findings are consistent with studies from our lab. Pereira et al. (2015) demonstrated a diet-induced model consisting of female Sprague-Dawley rats that were fed HFS six weeks before mating. Findings from this study observed that

pregnant rats under HFS exhibited excessive gestational weight gain, hyperinsulinemia and hyperglycemia (Pereira et al., 2015).

1.3 Developmental Origins of Health and Disease

The Developmental Origins of the Health and Disease (DOHaD) theory proposed by Dr. Barker hypothesizes that changes in environmental exposures, particularly during the in-utero period, can permanently alter the body's structure, function and metabolism, thus increasing the risk of developing cardiovascular complications in later life (Barker, 2007). Changes in maternal nutrition, such as under or over-nutrition during pregnancy, can influence CVD outcomes of the offspring in later life. Experimental animal studies have investigated the relationship between poor maternal nutrition and poorer outcomes concerning cardiovascular health in offspring. For instance, pregnant rats that were fed a low-protein diet resulted in elevated blood pressure in the offspring following adulthood (Langley & Jackson, 1994). Further animal studies using pregnant Wistar rats following maternal nutrient restriction showed a significant decrease in proteins involved in muscle contraction and glycolysis among offspring, thus increasing susceptibility to poor cardiovascular health outcomes (Zouridis et al., 2021). Similarly, undernutrition studies on sheep during pregnancy showed increased susceptibility to growth restriction and CVD in the offspring (Vonnahme et al., 2003). Early studies from Dr. Barker have found that maternal undernutrition caused low birthweights and higher rates of cardiovascular complications like ischemic heart disease and coronary artery disease (Barker et al., 1993). Other cohort studies that followed infants born with growth restriction observed changes in cardiac morphology and reduced stroke volume at follow-up in childhood, which could explain the increased susceptibility to cardiovascular complications in adult life (Crispi et al., 2010). In extreme cases, starvation can also be detrimental to the reprogramming of metabolic diseases, including T2D,

obesity, dyslipidemia, and hypertension, all of which are risk factors for coronary artery disease (Vaiserman, 2015).

Similar experimental studies in both animals and humans have found that at the opposite end of the spectrum, maternal overnutrition during pregnancy also increases the risk of CVD later in the life of the offspring. Human cohort studies have also examined the relationship between maternal nutrition and offspring cardiovascular health. Swedish population-based cohort studies using records from the Swedish medical birth register found positive associations between maternal obesity and CVD in offspring (Razaz et al., 2020). Studies from Brite et al. (2014) have also illustrated that maternal obesity was associated with an increase in offspring developing congenital heart defects (Brite et al., 2014). Similarly, studies from Madsen et al. (2013) have also drawn similar associations between infants with congenital heart defects and maternal obesity (Madsen et al., 2013) Studies from maternal obesity rat models using a high-fat diet during pregnancy programmed an increase in offspring adiposity and elevated systolic blood pressure (Desai et al., 2014; Khan et al., 2005; Inzani & Ozanne, 2022). Similar studies done in mice have also found elevated systolic blood pressure, poorer glucose tolerance and insulin sensitivity in offspring from mothers who were fed a high-fat diet (Masuyama & Hiramatsu, 2012).

1.4 Cardiometabolic Disease

The DOHaD theory proposed by Dr. Barker suggests that adverse conditions during fetal development, such as those caused by GDM, can often predispose offspring to diseases in later life. Among adults, the leading cause of morbidity and mortality is cardiometabolic disease. Cardiometabolic disease is an umbrella term encompassing interconnected disorders, including metabolic dysregulation, CVD, and diabetes risk factors (Howell et al., 2024; Eroglu et al.,

2024). These symptoms are generally characterized by impairments in glucose tolerance, dyslipidemia, hypertension, and obesity, which act as triggers for metabolic disturbances (Khan et al., 2023). However, at its core, cardiometabolic disease begins with insulin resistance, a phenotypic trait that involves the body's tissue becoming less responsive to the effects of insulin (Howell et al., 2024). These defects initiate a cascade of metabolic perturbations in glucoregulation, dyslipidemia, and inflammation that initiate and exacerbate a cluster of interrelated metabolic abnormalities known as cardiometabolic syndrome (Howell et al., 2024). As insulin resistance progresses, these cardiometabolic syndromes manifest into higher risk forms of cardiovascular and diabetes risk factors such as prediabetes and prehypertension, which can further progress into T2D, CVD and heart failure (Guo et al., 2014).

1.5 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a condition where there is myocardial metabolic dysfunction without the presence of coronary atherosclerosis and hypertension (Huo et al., 2023). Cardiometabolic disease and diabetic cardiomyopathy are closely intertwined, sharing common pathophysiological mechanisms such as insulin resistance, hyperglycemia, dyslipidemia, and inflammation (De Rosa et al., 2018). Diabetic cardiomyopathy is caused by a variety of factors including left ventricular hypertrophy (LVH), metabolic dysfunction and apoptosis (Falco-Pires & Leite-Moreira, 2012). However, one of the driving factors behind the development of diabetic cardiomyopathy is hyperglycemia, as it can increase the levels of growth factors and free fatty acids, leading to abnormalities in substrate utilization, lipid and calcium homeostasis (Falco-Pires & Leite-Moreira, 2012). Additionally, hyperglycemia can also impact the mitochondria function as the presence of high glucose levels can impair mitochondrial oxidative phosphorylation processes leading to a reduction in ATP generation and increased production of

reactive oxygen species (Pan et al., 2024; Jia et al., 2018). The reduction in ATP production and increased oxidative stress can subsequently affect the function of the myocardial cells and, if they persist, can lead to decreased myocardial contractile force and abnormalities in cardiac structure and function that can lead to the development of cardiac hypertrophy and heart failure (Pan et al., 2024).

1.6 Cardiac Hypertrophy

Cardiac hypertrophy is characterized as an increase in cardiomyocyte size and thickening of the ventricular walls, often initiated as an adaptive compensatory response to preserve cardiac function in instances of cardiac stress or injury. However, in instances when the stressor persists, the heart is unable to compensate, and as time progresses, these changes become maladaptive and ultimately result in heart failure (Tham et al., 2015). Cardiac hypertrophy can be classed into two types: physiological and pathological, and while both types develop in response to cardiac stress, the underlying molecular mechanisms and general phenotypes are vastly different (Nakamura & Sadoshima, 2018). Physiological hypertrophy can be characterized as a moderate increase in cardiac mass and cardiomyocyte growth with preserved or increased contractile function. Whereas, pathological hypertrophy is better characterized by concentric growth of the heart that progresses to wall thinning and dilatation of the ventricular chamber, which will ultimately develop into contractile dysfunction and eventually heart failure (Nakamura & Sadoshima, 2018). Unlike the physiological form, pathological hypertrophy can not be reversed and, over time, can progress to serious complications such as heart failure.

1.7 Structure and Function of Cardiomyocyte

Within the myocardium, there exists a plethora of various cell types like fibroblasts, smooth muscle cells, endothelial cells and cardiomyocytes. Among these cell types,

cardiomyocytes are the fundamental contractile cells of the myocardium. The primary function of the heart is to pump blood in a manner that ensures efficient circulation across various organs and tissues (Woodcock & Matkovich, 2005). To ensure adequate blood profusion across the body, cardiomyocytes are comprised of several key components that help regulate the contraction-relaxation cycles of the heart.

Each cardiomyocyte is a tubular structure comprised of chains of myofibrils. These rod-like units consist of repeating sections of sarcomeres and are the fundamental contractile units of the muscle cells (Golob et al., 2014). The sarcomeres are comprised of an intracellular anchor protein known as titin alongside repeating units of thick and thin filaments that play a role in generating the contractile force. The thick filaments contain myosin, which consists of rod-like domains and globular heads, which comprise the backbone and the cross-bridge, respectively (Golob et al., 2014). Thin filaments, on the other hand, are comprised of globular proteins known as actin that are polymerized into tropomyosin and troponin.

Within the cardiomyocytes exists the sarcolemma, a specialized structure that is composed of a lipid bilayer that surrounds individual cardiomyocytes, forming a barrier between extracellular and intracellular compartments (Walker & Spinale, 1999). Within the sarcolemma, there are distinct microdomains, such as transverse tubules, caveolae and intercalated discs, that play unique roles in facilitating an array of cellular processes (Kitmitto et al., 2019).

Transverse tubules, otherwise known as t-tubules, are invaginations of the sarcolemma that seep into the myocyte. Within this specialized region exists a rich concentration of ion channels that play an important role in propagating action potential, maintaining resting membrane potential, and signalling transduction (Hong & Shaw, 2016). However, the most crucial function of the t-tubules is regulating the process of excitation-contraction coupling,

which is made possible through the rich concentration of voltage-sensitive calcium channels located around the t-tubules (Lyon et al., 2009). Caveolae are small invaginations in the sarcolemma that are highly enriched in cholesterol and sphingolipids and play a critical role in various cellular processes. Located on the poles of adjacent myocyte cells exist the intercalated discs, a complex structure comprised of desmosomes fascia adherens and gap junctions that play an essential role in facilitating the synchronized contraction of the heart (Kitmitto et al., 2019). Desmosomes are highly specialized cell structures that tether intermediate filaments to the plasma membrane and are responsible for providing strong adhesion between cells (Delva et al., 2009). When combined, all components within the sarcolemma help in facilitating a plethora of cellular functions.

1.8 Cardiac Energy Metabolism and the Role of Mitochondria

The heart requires a continuous supply of energy to support its contractile function, ion transport and Ca^{2+} homeostasis. However, the heart has a limited capacity to store energy (Nguyen et al., 2019). Therefore, to respond to increased demands for oxygen blood supply, the heart must rapidly produce energy in the form of adenosine triphosphate (ATP). The heart is one of the most metabolically active organs, with a very high content of mitochondria, comprising approximately 25-30% of cell volume (Brown et al., 2016). To meet these high-energy needs, mitochondrial oxidative phosphorylation plays a pivotal role in supplying the heart with the necessary energy to perform its function. Mitochondrial oxidative phosphorylation provides about 95% of the ATP required by the heart, with the remaining 5% provided by glycolysis (Lopaschuk et al., 2021). To maintain energy production, the heart can readily shift between different substrates such as fatty acids, glucose, ketones, lactate, and amino acids. This metabolic flexibility is vital to maintaining ATP production in the heart (Lopaschuk et al., 2021). Fatty

acids are the main substrates utilized in the heart. Approximately 40-60% of mitochondrial ATP production stems from the oxidation of fatty acids, with the remainder derived from the oxidation of other sources such as pyruvate, ketone bodies, and amino acids (Lopaschuk et al., 2021). Metabolic regulation is vital to cardiac function, such that drastic changes in energy metabolism can contribute to the development of diseases such as cardiac hypertrophy and heart failure (Huss & Kelly, 2005). The progression of heart failure is often associated with a gradual decline in the heart's ability to be metabolically flexible. As a result, the heart becomes energy deficient. Beer et al. (2002) observed that when compared to healthy hearts, end-stage failing hearts can have up to 30% less ATP content (Beer et al., 2002).

1.9 Cardiac Muscle Contraction

Regulating the changes in intracellular calcium concentration is vital in maintaining cardiac contractility. Under normal conditions, intracellular calcium concentration must be kept sufficiently high in periods of systole and low in periods of diastole to allow the heart to eject and refill with blood (Eisner et al., 2017). One of the key processes that is responsible for tightly regulating intracellular calcium concentration is known as the excitation-coupling contraction, a process that links electrical excitation caused by action potentials across the membrane of cardiac cells to their respective mechanical contraction (Eisner et al., 2017). As the action potential propagates along the sarcolemma and down into the t-tubules, it triggers the opening of voltage-sensitive calcium channels, which facilitate calcium entry into the cell. The influx of calcium triggers the release of calcium from intracellular stores in the sarcoplasmic reticulum, which is mediated by ryanodine receptors in a process known as calcium-induced calcium release (Klabunde, 2021). The increased levels of free calcium now in the cytoplasm bind to troponin C, this consequently results in tropomyosin detaching from the myosin-binding sites on

actin. The removal of this complex frees actin, which binds to myosin and forms a cross-bridge initiating contraction (Adams & Schwartz, 1980). Similar to skeletal muscle, cardiac muscle contraction occurs via the sliding filament model of contraction. Following the formation of the cross-bridge between actin and myosin, adenosine diphosphate (ADP) and inorganic phosphate are released from the myosin head, allowing myosin to utilize the energy stores for a conformational change (Adams & Schwartz, 1980). As the myosin head moves towards the M line, it pulls the actin along with it in a movement called the power stroke. As the actin gets pulled closer to the M line, the sarcomere shortens and the cardiac muscle contracts. As calcium ions are removed from troponin C and sequestered back into intracellular stores, ATP binds to myosin, causing it to detach from the actin, resulting in the dissociation of the actin-myosin complex and resting the contraction cycle (Adam & Schwartz, 1980).

1.10 GDM Treatment

There are various pharmacological treatment strategies that are implemented to combat GDM. These pharmacological agents primarily focus on managing and controlling maternal blood glucose levels through various mechanisms.

1.10.1 Insulin

Often considered the standard and first-line pharmacological treatment for GDM, insulin is a peptide hormone that plays a crucial role in the regulation of blood glucose in the body (Feghali et al., 2020). Insulin's mechanism of action follows a complex signalling cascade that first begins with insulin binding to the insulin receptor. This heterotetrameric protein consists of two extracellular alpha subunits and two transmembrane beta subunits (Petersen & Shulman, 2018). Insulin binds to the alpha subunits, which triggers the tyrosine kinase activity located in the beta subunit. This activation triggers a signalling cascade that stimulates downstream

metabolic responses that work in regulating glucose metabolism (Petersen & Shulman, 2018). One key signalling pathway is the phosphatidylinositol 3-kinase (PI3K) /protein kinase B (Akt) pathway, whereby insulin activates PI3K, which generates ĥosphatidylinositol-3,4,5-trisphosphate (PIP3) (Bertrand et al., 2008). PIP3 then activates Akt via phosphoinositide-dependent kinase 1 (PDK1), which inactivates AS160 (a 160-kDa substrate of Akt), a Rab GTPase-activating protein, downstream, promoting the translocation of glucose transporter 4 (GLUT4) to the cell membrane to facilitate glucose uptake (Bertrand et al., 2008).

Insulin therapy has been studied extensively and used clinically for the treatment of GDM. While it has been found to be a safe and effective method in mitigating hyperglycemia during pregnancy, care must be taken to avoid hypoglycemia (Bergel et al., 2016). It is critical to know that there are various types of insulin, ranging from fast-acting to intermediate/long-acting forms. Coupled with different regimens of administration, insulin therapy must be individualized to the patient (Bergel et al., 2016). Many insulin analogs from neutral protamine hagedorn (NPH), lispro, aspart, and detemir, although safe and effective, have their precautions that should be considered. NPH, for instance, is an intermediate-acting insulin analog with a 5–7-hour peak action duration. To maintain optimal glycemic control throughout the day, at least two to three injections must be provided. However, the use of NPH has been associated with a higher risk of hypoglycemia when compared to other long-acting analogs (Negrato et al., 2010). Insulin aspart is a short-acting insulin analog with a peak action of 30-70 minutes after injection. It was first demonstrated effective in improving glycemic control in women with GDM in a randomized trial consisting of 15 women with GDM who had inadequate diabetes controlled with diet alone. (Pettitt et al., 2003). Other studies found similar effectiveness with insulin aspart when compared to regular insulin with no significant difference in the risk of hypoglycemia (Deepaklal et al.,

2015). However, factors such as appropriate dosage monitoring must be considered, as patients using insulin aspart may need to adjust dosages as pregnancy progresses, thereby requiring close monitoring and communications with healthcare providers to avoid hypoglycemic events (Deepaklal et al., 2015).

1.10.2 Metformin

Metformin is a synthetic biguanide that has been shown to reduce hepatic glucose production and is mostly prescribed for the treatment of T2D. However, it has been increasingly used in GDM treatment (Rena et al., 2017). There are a variety of mechanisms that contribute to metformin's glucose-lowering properties. Metformin has been widely accepted to inhibit hepatic gluconeogenesis by inhibiting complex 1 in the mitochondria, resulting in a substantial decrease in energy in the form of ATP that would otherwise be required for carrying out ATP-dependent metabolic pathways like gluconeogenesis (Foretz et al., 2019). Activation of AMPK by metformin additionally suppresses the expression of sterol regulatory element-binding protein 1 (SREBP-1) and acetyl-CoA carboxylase (ACC), resulting in a decrease in the expression of lipogenic enzymes. Metformin can also improve hepatic insulin sensitivity through insulin receptor tyrosine phosphorylation (Gunton et al., 2003). Metformin also facilitates the translocation of glucose transporters, such as glucose transporter 1 (GLUT-1), to the plasma membrane, which enhances glucose uptake (Gunton et al., 2003).

Commonly considered a first-line antidiabetic medication, metformin has increasingly been used during pregnancy, often being prescribed to combat maternal obesity along with GDM (Verma & Mehendale, 2022). Various randomized trials have studied the benefits of metformin treatment in GDM. For instance, the metformin in gestational diabetes (MiG) trial found that metformin was as effective as insulin in achieving glycemic control. This is critical as, unlike

insulin, metformin does not directly cause maternal hypoglycemia (Tripathi et al., 2017). While metformin use during pregnancy can provide some benefits, there have been a few reported effects on maternal health. One of the most common side effects of metformin use during pregnancy is an increased risk of gastrointestinal side effects. A systemic review and meta-analysis by Tarry-Adkins et al. (2021), which included randomized trial data from 35 studies, reported that metformin use during pregnancy significantly increased the likelihood of experiencing gastrointestinal issues compared to other treatments or placebo (Tarry-Adkins et al., 2021).

In some cases, metformin alone can be inadequate in improving insulin sensitivity, resulting in the addition of insulin as a co-treatment. Data reported from the MiG trials, which followed 751 women with gestational diabetes, found that of the 363 women who were assigned metformin treatment, 46.3% required supplemental insulin (Rowan et al., 2008). Additionally, another randomized clinical study reported that 84.9% of patients with T2D in pregnancy who received metformin required an add-on insulin therapy by the end of the second trimester of pregnancy (Ainuddin et al., 2015). Various factors can influence the efficacy of metformin in regulating glycemic control. For instance, body mass index (BMI) appears to be a predictor for add-on insulin treatment as many of the patients that received insulin as an add-on had a BMI >30 (Ainuddin et al., 2015). It is also interesting to note that the requirement for insulin as an add-on increases during the later stages of pregnancy (around 26-28 weeks) when insulin resistance increases in pregnancy (Ainuddin et al., 2015).

Unlike insulin, metformin can cross the human placenta. Serum samples from umbilical cord, placental and fetal tissues collected from metformin-exposed pregnancies revealed that metformin can readily cross the placenta, exposing the fetus to similar concentrations seen in the

mother (Charles et al., 2006). This transplacental passage is facilitated by organic cation transporters, specifically organic cation transporter 3 (OCT3), which is localized at the syncytiotrophoblastic basal membrane and fetal capillaries (Owen et al., 2021). It has been demonstrated that OCT3 expression increases with gestational age, suggesting that higher concentrations of metformin can reach fetal tissues at the later stages of gestation, which brings concern to its use as an antidiabetic during pregnancy (Owen et al., 2021).

Recent studies collected from the metformin in women with type 2 diabetes in pregnancy (MiTy) trial showed that women with T2D during pregnancy who were treated with metformin had a higher prevalence of small for gestational-age infants compared to women receiving a placebo (Feig et al., 2020). Other studies found mixed results corresponding to the safety of metformin use during pregnancy. An administrative data cohort study performed in Finland tracked children who had maternal exposure to metformin, insulin or a combination of both. Findings from that cohort found that when assessing secondary outcomes, children exposed to maternal metformin during pregnancy showed no difference in small for gestational-age risk (Brand et al., 2022). A meta-analysis of 21 randomized control trials further demonstrated that when compared to insulin, metformin increased the risk for small for gestational age (He et al., 2022). It is important to take note that these risks may vary based on various factors. For instance, the MiTy trial found that women with comorbidity who received metformin had a higher risk of small for gestational-age infants (Feig et al., 2022).

1.10.3 Glyburide

Glyburide is an oral sulfonylurea that stimulates insulin secretion by binding to and inhibiting ATP-sensitive potassium channels located on pancreatic beta cells. The binding of glyburide causes the closure of the potassium channels, which leads to membrane depolarization

of the beta cells (Zeng et al., 2014). The depolarization triggers the opening of voltage-gated-calcium channels, allowing calcium ions to enter the cell. As the calcium concentration increases, it stimulates the release of insulin-containing granules from the beta cell (Boyd et al., 1990). Glyburide can also reduce insulin clearance in the liver, leading to increased circulating insulin concentrations (Hardin & Jacobs, 2019).

When considering glyburide versus insulin for treating GDM, factors such as the ease of use and lower cost of glyburide compared to insulin often make it more acceptable among patients (Hedderson et al., 2022). It is important to note that when compared to insulin in terms of regulating glycemic control, some studies have found that glyburide is as effective as insulin in regulating glycemic control (Miller, 2006). Randomized control trials performed by Langer et al. (2000) comparing glyburide and insulin in 404 women with GDM found that both groups achieved similar glycemic control (Langer et al., 2000). Retrospective cohort studies performed by Conway et al. (2004) found that 84% of GDM patients under glyburide treatment had achieved good glycemic control (Conway et al., 2004). While glyburide is associated with a lower risk of maternal hypoglycemia compared to insulin, the risks are still present, with some studies indicating that maternal hypoglycemia can occur, particularly if dosages are not carefully monitored (Affres et al., 2021). Although some evidence may suggest that glyburide can be an effective alternative, careful monitoring is still required as some patients may still require insulin to maintain optimal glycemic control.

One concern that studies have pointed out is the maternal transfer of glyburide to the fetus, hinting at potential offspring outcomes (Kirchner, 2001). The fetal-to-maternal ratio of 62% glyburide placental transfer raises questions about its use as a treatment for GDM (Bouchghoul et al., 2020). A retrospective cohort study showed that newborns from women

treated for GDM with glyburide had a higher risk for hypoglycemia, macrosomia, and neonatal intensive care unit admission compared to women who were treated with insulin (Castillo et al., 2015).

1.11 Natural Health Products: Resveratrol

Resveratrol (RESV) is a natural stilbenoid found in over 70 different plant species that is produced from a variety of plants as a defence mechanism against various pathogens and fungi (Salehi et al., 2018). It came to popular attention through studies by Renaud and De Lorgeril, which described the contradictory finding that French people had a relatively low incidence of CHD despite consuming a diet high in saturated fats, this observation formed the basis of what is now known as the "French paradox" (Renaud & De Lorgeril, 1992). Renaud and De Lorgeril argued that the paradox may be partly attributed to the moderate consumption of wine in France (Renaud & De Lorgeril, 1992). Since then, RESV received increased scientific attention, and further studies have shown RESV to act on multiple signalling pathways to improve health outcomes. Studies from Dolinsky et al. (2013) demonstrated that RESV inhibited the p70S6 kinase pro-hypertrophic signalling cascade while also enhancing the liver kinase B1-AMPK signalling pathway in spontaneously hypertensive rats (Dolinsky et al., 2013). Further studies from Bastin et al. (2011) have also demonstrated that RESV can enhance the protein expression of sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1a), key regulators that play a role in mitochondrial biogenesis and fatty acid metabolism (Bastin et al., 2011)

1.11.1 Maternal RESV Supplementation Effects in Animal Studies

The effects of RESV treatment in the context of GDM have been explored in various animal models. Zhang et al. (2021) investigated the effects of RESV on blood glucose and blood

lipids in female rats with GDM. Finding from the STZ-induced GDM model found that when compared with GDM dams, maternal blood glucose and body weight were significantly reduced in the RESV-treated group in a dose-dependent manner (Zhang et al., 2021). Additionally, when looking at blood lipids, the RESV-treated groups had significantly lower contents of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol and significantly increased high-density lipoprotein-cholesterol in a similar dose-dependent manner when compared to the GDM group (Zhang et al., 2021). Yao et al. (2015) conducted similar studies investigating the effects of RESV in the pregnant *db/+* mouse. Findings from their study reported that RESV treatment improved maternal glucose intolerance and improved insulin response in pregnant *db/+* female mice (Yao et al., 2015). Diet-induced GDM studies by Brawerman et al. (2019) found that administering RESV at the start of the third trimester in GDM dams led to significant improvements in glucose tolerance compared to GDM dams without RESV treatment (Brawerman et al., 2019). Additionally, GDM dams showed impaired GSIS when compared to lean dams. However, maternal RESV supplementation improved GSIS in GDM dams (Brawerman et al., 2019). Other studies have also focused on the effects of RESV on offspring health during treatment in pregnancy. In the same study, Brawerman et al. (2019) found that the administration of RESV in the third trimester of pregnancy prevented the development of GDM-induced hepatic steatosis in the offspring, as well as improved glucose tolerance and insulin sensitivity (Brawerman et al., 2019).

1.12 Thesis Rationale and Objectives

Worldwide, GDM occurs in up to 14% of pregnancies, in Manitoba alone, the incidence rate of GDM is 40 per 1,000 deliveries, making it often considered one of the most common medical complications of pregnancy (Yamamoto et al., 2018). Despite only occurring during the

later stages of pregnancy, the effects of GDM can bring forth both short- and long-term complications to both the health of the mother and child (Bianco & Josefson, 2019). Children exposed to GDM during pregnancy have an increased risk of developing cardiovascular complications as they age. Population-based cohort studies have shown that offspring exposed to GDM have a 29% increased risk of developing early-onset CVD (Yu et al., 2019). Furthermore, children exposed to GDM have increased risks of developing hypertensive disease, heart failure, and pulmonary embolism when compared to offspring of mothers with no diabetic complications (Yu et al., 2019). Similar cohort designs have also found that GDM-exposed offspring were associated with higher levels of blood pressure starting from the age of 10 (Miranda et al., 2019). GDM-exposed offspring also have an increased risk of morphologic changes in the heart during development, such as cardiac hypertrophy, with children from GDM mothers having thicker posterior left ventricular walls (Ghouse et al., 2024). Lifestyle changes are a good starting point for the treatment of GDM, with evidence from animal and clinical studies suggesting beneficial long-term health effects for both the mother and child, however, if the mother is unable to maintain adequate blood glucose levels, intervention strategies are needed.

The use of insulin as a form of treatment for GDM is extensively used as a first option. However, the choice of appropriate dosing can be challenging, especially with the persistent increase in insulin resistance throughout the later stages of pregnancy (Kelley et al., 2015). Glyburide, on the other hand, has been associated with neonatal hypoglycemia in addition to an increased risk of adverse effects such as macrosomia (Castillo et al., 2015). Although metformin has been associated with positive glycemic control in GDM, concerns of maternal gastrointestinal complications, increased risk of small-for-gestational-age, and its ability to readily cross the placenta often limits its use during pregnancy (Picón-César et al., 2021; Tarry-

Adkins et al., 2021; Feig et al., 2020). Given the limitations of existing therapies, there is a need for new therapeutic strategies to combat GDM while mitigating the harmful effects on offspring health. RESV is a natural polyphenol that is found in a variety of plant species ranging from grapes, peanuts, blueberries, and cranberries it has been shown in various human and animal models to show beneficial biological effects, including cardioprotective, anti-hypertensive, glucose lowering and anti-obesogenic (Gal et al., 2023). A previous study from our lab showed that maternal RESV supplementation restored glucose tolerance and improved insulin secretion in GDM pregnant rats and their offspring potentially making it a good therapeutic option (Brawerman et al., 2019). Since GDM is typically first diagnosed during the late 2nd trimester of pregnancy (Kim et al, 2019), this study was designed to determine the effects of RESV in a treatment aspect that is more clinically aligned with the utilization of therapeutics in GDM as opposed to other preventative strategies that have incorporated RESV either at the before or at the start of pregnancy (Yao et al., 2015; Roberts et al., 2014; Ros et al., 2018).

Previously data from our group have found that fetal rat offspring exposed to GDM in utero exhibited alterations in cardiac structure, specifically these offspring exhibited left ventricular hypertrophy (Kereliuk, 2022). Additionally, pervious data from our group also demonstrated that exposure to GDM also impaired calcium handling in fetal rat cardiomyocytes as they exhibited delayed and extended flux cycles (Kereliuk, 2022). Since RESV has been shown to ameliorate maternal hyperglycemia and has demonstrated cardioprotective effects in previous mouse models of cardiac hypertrophy, my first objective was to show what effects maternal RESV supplementation would have on the cardiac structure and calcium handling of GDM exposed offspring.

Studies from our lab have shown that fetal cardiomyocytes from GDM rat offspring exhibit mitochondrial dysfunction with alterations in energy metabolism (Kereliuk, 2022). This is critical as the mitochondria plays a major role in providing the heart with enough energy to maintain cardiac function. This dysfunction can subsequently impact cellular processes such as calcium handling as many of the membrane-bound pumps are energy dependant (Brown et al., 2016). Moreover, mitochondrial dysfunction can also contribute to the production of reactive oxygen species that can damage cardiac muscle tissue and if persist can further exacerbate functional impairments of myocardial cells (Pan et al., 2024). Previous studies have show that RESV can improve mitochondrial function through the activation of key regulators such as AMPK and SIRT 1 that are involved in cellular energy metabolism and mitochondrial biogenesis (Zheng et al., 2022). Therefore, my second objective was to show the effects of maternal RESV supplementation on mitochondrial metabolism in isolated fetal cardiomyocytes from GDM-exposed offspring.

1.13 General Hypothesis

I hypothesize that maternal RESV supplementation can alleviate GDM-induced cardiovascular outcomes such as cardiac hypertrophy seen in GDM-exposed offspring by improving cellular and mitochondrial function.

1.14 Specific Aims

Since Calcium is a universal intracellular molecule that plays a vital role in regulating cardiac muscle contraction, Firstly I will explore the effects of maternal RESV supplementation on calcium dynamics in GDM-exposed isolated fetal cardiomyocytes. I hypothesize that maternal RESV supplementation will improve cardiomyocyte calcium handling in GDM-exposed offspring.

The mitochondria play a major role in providing the energy required to shuttle calcium in order to facilitate contraction, therefore, the second aim of this project is to study the effects of maternal RESV supplementation on mitochondrial metabolism on isolated fetal cardiomyocytes from GDM-exposed offspring. I hypothesize that maternal RESV supplementation can improve mitochondrial metabolism in GDM-exposed fetal cardiomyocytes.

Chapter 2: Methods

2.1 Diet-Induced Animal Model

All experimental procedures conducted were approved by the University of Manitoba's Central Animal Care Committee. Four-week-old female Sprague-Dawley rats were received from the Genetic Models Center (GMC) core facility within the University of Manitoba and were housed 2 per cage within the animal facility at the Children's Hospital Research Institute of Manitoba (CHRIM). Female rats were randomly assigned to a low-fat (LF) diet (10% kcal fat, research diets, D12450B) or a high-fat and sucrose diet (HFS) (45% kcal, Research diets, D12451) for 6 weeks before mating to induce obesity and GDM. All rats were then mated with male rats (one male per two female rats) who were fed a regular chow diet (i.e. regular rodent food). Pregnancy was later confirmed 10 days after the separation of male breeders through echocardiography scans. Pregnant females were housed alone separately, and all assigned diets continued throughout pregnancy. At the start of the third trimester of pregnancy (day 14 of gestation) a subgroup of female rats that were under the high-fat and sucrose diet were switched to a high-fat and sucrose diet supplemented with RESV (45% kcal fat + 4g/kg body weight RESV, Research Diets, D10020402) which translates to a dosage of ~150 mg/kg/day, based on food consumption per rat per day. Thus began the creation of three experimental groups: lean dams consuming an LF diet (Lean), GDM dams consuming an HFS diet (GDM), and finally, GDM dams consuming an HFS diet supplemented with RESV (GDM+RESV). This dosage was previously demonstrated to improve insulin sensitivity and glucose response while being safe and non-teratogenic during pregnancy (Brawerman et al., 2019). Females fed the GDM+RESV diet were housed separately to ensure the appropriate dosage was met. Maternal food consumption and body weight were monitored and measured on a weekly basis.

2.2 Fetal Echocardiography Scans

To assess cardiac structure in fetal offspring exposed to GDM and RESV, fetal echocardiography was performed in vivo using the Vevo 2100 ultrasound system provided by the central animal care small animal care core facility located at the University of Manitoba. Female Rats were imaged under mild anesthesia using 5% isoflurane and 1.0L/min oxygen (and maintained at 2-3% isoflurane and 1.0L/min oxygen) during the echocardiography procedure. Anesthetic levels were carefully monitored and modified as necessary to ensure a consistent heart rate among all groups. this precise control was critical as alterations in anesthesia-induced changes in heart rate can impact both cardiac structure and function, potentially impacting the accuracy of cardiac measures and assessments (Pachon et al., 2015; Roth et al., 2002). Hair was removed from the lower abdominal region of pregnant rats with Nair (3 in 1 hair removal lotion) prior to echocardiography. The MS550 transducer was used to capture fetal echocardiograms during the third trimester of pregnancy on embryonic day 18.5. Left ventricle (LV) wall thickness, LV chamber size, intraventricular septal thickness (IVS), Ejection Fraction (EF), and Fractional Shortening (FS) were measured and calculated during diastole and systole.

LV wall thickness, IVS and LV chamber size were used as markers to assess the changes in fetal cardiac morphology in Lean, GDM, and GDM+RESV fetal offspring. Each group consisted of litters from a maternal dam under LF, HFS, or HFS supplemented with RESV diet (dosage equivalent to ~150mg/kg/day, based on food consumption). All images captured from echocardiography consisted of all rats in utero at embryonic day 18.5 where n of 1 is represented as one entire dam litter.

2.3 Cardiomyocyte Isolation

Fetal ventricular cardiomyocytes from offspring of Lean, GDM, and GDM + RESV dams were isolated to study the differences between diet exposures on cells that play a role in the heart's contractility. Fetal hearts (taken at embryonic day 20.5) were isolated from the cesarian section after pregnant dams were euthanized. Fetal pups were euthanized by decapitation, and hearts were removed and briefly stored in 20 mL cold sterile 1X phosphate-buffered saline (PBS) in a 50 ml conical tube (to ensure adequate yield hearts were pooled from one dam litter). Fetal hearts were rinsed 3 times with 20 mL 1X PBS in a biosafety cabinet and extra debris (aorta/lung pieces) were aspirated and removed. Hearts were minced using sterile curved iris scissors in the lid of a sterile petri dish containing a minimum volume of cold sterile 1X PBS (~0.5 mL). Using a 3 mL sterile transfer pipette, minced hearts were then transferred to a non-vented T25 culture flask containing 17 mL of cold sterile 1X PBS, if multiple sets of hearts were isolated at the same time, hearts would be kept on ice until all sets of hearts were minced and ready to proceed to the next step.

Now in the T25 flask, 1 mL of filter-sterilized 0.5% Deoxyribonuclease 1 (DNAse)/ 2% Collagenase and 0.5 mL of filter-sterilized 2% Trypsin were added to the flask to begin the first enzymatic dissociation. DNAse, Collagenase, and Trypsin (Worthington Biochemical Corporation, Product #LS004176, LS002007 and LS003707 respectively) were weighed into a 15 mL conical tube. Enzymes were then diluted in cold, sterile 1X PBS and filter sterilized with a 0.22 μM syringe filter (VWR, Product# 28145-501) (Enzyme preparation occurred ~ 1 hour prior to excising the offspring hearts in a dark biosafety cabinet and kept on ice in the dark throughout the duration of the isolation)

The T25 flask (now containing both the minced hearts and the enzymes) was agitated on a rotary shaker set at 80 rpm at a temperature of 37°C for 20 minutes. Afterwards, the contents in the T25 flask were pipetted into a sterile 50 mL conical tube containing 20 mL of pre-warmed (37°C) DF20 media before being spun using a centrifuge set at 800 rpm at a temperature of 18°C for 2 minutes to form a cell pellet. After centrifugation, the supernatant was aspirated and discarded.

DF20 media was composed of modified DMEM/F-12 (1:1) media (Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture liquid media, containing 17.49 mM D-glucose; HyClone Product #SH30126). In a biosafety cabinet, DMEM/F-12 media was supplemented with 20% fetal bovine serum (FBS; ThermoFisher Scientific Product #12483020), 1% penicillin-streptomycin (HyClone Product #SV30010) and 50 µg/mL gentamicin (ThermoFisher Product #15750078) solution days prior to cardiomyocyte isolation and was pre-warmed in a water bath set at 37°C the day of isolation.

After discarding the supernatant 17 mL of pre-warmed (37°C) sterile 1X PBS was added to the pellet followed by 1 mL of 0.5% DNAse/2% Collagenase and 0.5 mL of 2% Trypsin before mixing the contents (disturbing the pellet) and transferring to a new non-vented T25 flask to begin the second dissociation. The T25 flask was then again agitated on a rotary shaker at a speed of 80rpm at 37°C for 20 minutes before pipetting the contents into a new sterile 50 mL conical tube containing 20 mL of DF20 media, which would then be centrifuged at a speed of 800rpm at 18°C for 1 minute to form a pellet.

The supernatant of the second dissociation was collected and transferred into a new sterile 50 mL conical tube. Now, 17 mL of warm sterile 1X PBS was added to the pellet before adding 1 mL of 0.5% DNAse/2% Collagenase and 0.5 mL of 2% Trypsin, the contents were

mixed before being transferred to a new sterile non-vented T25 flask and agitated on a rotary shaker set at 80 rpm at a temperature of 37°C for 20 minutes to begin the third dissociation. During the 20-minute enzymatic digestion, the supernatant collected from the second dissociation was centrifuged at 1700 rpm at 18°C for 7 minutes leaving a cell pellet. The supernatant was then aspirated and discarded before adding 20 mL of DF20 media. After the 20-minute enzymatic digestion, the contents of the T25 flask (which contains the third dissociation) would then be transferred to a tube containing the pellet breaking up the pellet in the process. The conical tube would then be centrifuged at 1700 rpm at 4°C for 7 minutes before aspirating and discarding the supernatant.

To the pellet, 12 mL of plating media (pre-warmed at 37°C) was added and triturated 20 times with a sterile 3 mL transfer pipette leaving the cells in suspension. Plating media was composed of modified DMEM/F-12 (1:1) media (HyClone, Product #SH30126.01). In a biosafety cabinet, the media was supplemented with 10% horse serum (Thermofisher, Product #16050122), 5% FBS (Thermofisher, product #12483020), 1% penicillin-Streptomycin (HyClone, Product #SV30010), and 50 µg/µL gentamicin (Thermofisher, Product #15750078) and pre-warmed in a water bath set at 37°C the day of isolation.

Cells now in suspension were filtered through a 100 µM nylon cell strainer (Falcon, Product #352360) into a vented T75 flask and placed in a cell culture incubator at 37°C with 5% CO^2 for 2 hours. To ensure a purer yield of cardiomyocytes, the T75 flask was removed from the incubator 1 hour into incubation. The sides of the flask were washed down with the suspension before transferring the contents to a new vented T75 flask and returned to the incubator for the final hour of incubation.

Afterwards, the cell suspension was then transferred to a sterile 50 mL conical tube and centrifuged at 100 rpm for 2 minutes at 18°C leaving a cell pellet. After centrifugation, the supernatant was removed, and cells were diluted with plating media at a ratio of 1 mL plating media/heart used in isolation. Cells were then mixed, and 20 µL of cell suspension was added to 80 µL of trypan blue (in a sterile 1.5 mL Eppendorf tube), resulting in a 1:5 dilution, of which 10 µL was added to each side of a hemocytometer for manual cell counting.

To find the number of cardiomyocytes per mL, we averaged the hemocytometer counts and multiplied the average by 10,000. The resulting value was further multiplied by the dilution factor of 5. Isolated cardiomyocytes were then plated at appropriate seeding densities, as described in Table 1, for subsequent experiments. For experiments regarding seahorse extracellular flux and measuring oxygen consumption a seeding density of 100,000 cells/well. To measure calcium flux in fetal cardiomyocyte cells a seeding density of 500,000 cells/well. To prevent replication of proliferative cells in the cardiomyocyte culture the DNA synthesis inhibitor cytosine β-D-arabinofuranoside (ARAC) was added to the plating media. plates containing cells were then placed in a cell culture incubator at 37°C and 5% CO^2. Cells were then checked the morning after for adherence, cell plates were washed 3 times with pre-warmed (37°C) sterile 1X PBS to remove dead cells before adding new plating media to the wells at the appropriate volume (as described in Table 1).

Table 1 Cardiomyocyte Plating Density

Plate Type	Cells/Well	Well Volume (µL) (Final*)	# of cells for concentration
6-well	2,000,000	2000	1,000,000
24-well	500,000	1000	500,000
Seahorse XF24	100,000	500*	1,000,000

* Cells were plated 100,000 cells/well (100 µL) and incubated for an hour before adding 400 of plating media containing ARAC.

2.4 Seahorse XF Extracellular Flux

Measurements of mitochondrial respiration were performed and captured using Agilent's Seahorse Extracellular Flux (XF) 24-well analyzer. Prior to cardiomyocyte isolation, XF24 plates were coated with fibronectin/gelatin. 0.1 g gelatin (Sigma, Product #G1393) was weighed into a 500 ml glass bottle containing 500 mL of distilled water and autoclaved to make a final concentration of 0.02% gelatin. 1 mL of fibronectin (1 mg/mL) (Sigma, Product #F1141) was diluted in 79 mL 0.02 sterile gelatin before being aliquoted and stored at -20°C.

The day before isolating cardiomyocytes an aliquot was thawed at 37° C and XF24 plates were coated, before being stored in the cell culture incubator overnight at 37° C with 5% CO^2. On the day of isolation, prior to plating cells, coated plates containing fibronectin/gelatin were aspirated from the plate and cells were plated at a density of 100,000 cells/well (as described in Table 1) leaving wells A1 and D6 as blanks. The plate was then placed in a cell culture incubator for 1 hour at 37° C with 5% CO^2. After 1 hour cells were topped up with plating media containing ARAC to a final volume of 500 µL before being placed back into the incubator. A day prior to any seahorse assay, an XF sensor cartridge would be hydrated in Seahorse XF calibrant and placed in a non-CO^2 incubator.

Bradford protein assay was performed to normalize the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) readings to total protein concentration. Several parameters of mitochondrial function were calculated from OCR and ECAR readings. Non-mitochondrial respiration, basal respiration, maximal respiration (maximum rate after FCCP – Non-mitochondrial respiration), and spare respiratory capacity (maximal respiration – Basal Respiration) were calculated from OCR readings respectively. Glycolysis rate (maximum rate after the addition of saturation of glucose – basal ECAR before the addition of glucose),

glycolytic capacity (maximum ECAR rate following the addition of oligomycin), and glycolytic reserve (difference between glycolytic capacity and glycolysis rate) were calculated from ECAR reading respectively. Three different assays were performed using isolated fetal cardiomyocytes from Lean, GDM, and GDM + RESV offspring.

2.4.1 Seahorse XF Mito Stress Test

Cardiomyocyte mitochondrial respiration in the presence of glucose was determined using the Cell Mito Stress test assay. Plating media was changed to XF assay media (modified DMEM without glucose, pH 7.4) supplemented with 1 mM sodium pyruvate and 25 mM glucose before incubating for 1 hour in a non-CO^2 incubator at 37C. Meanwhile, the hydrated XF sensor cartridge would be removed from the non-CO^2 incubator and Oligomycin, carbonyl cyanide-4 (FCCP), and a mixture of rotenone and antimycin A (Sigma, Product #O4876, Product #C2920, Product #R8875, Product #A8674 respectively) would be added to injector ports A, B and C respectively at a final concentration of 1 µM oligomycin, 2 µM FCCP, and 1 µM rotenone/antimycin A. Once the drugs are loaded to the appropriate injector ports the sensor cartridge would then be loaded into the seahorse XF analyzer for calibration (~20-30 minutes). after the calibration period is over the utility plate is removed from the machine and the assay is ready to begin. Once the 1-hour incubation is over the cell plate (containing the isolated cardiomyocytes) is removed from the non-CO^2 incubator and placed in the seahorse XF analyzer. Once in the machine, 4 measures of OCR are taken at intervals of 10 min before injection of the drugs in Port A, B and C respectively.

2.4.2 Seahorse XF Fatty Acid Oxidation Stress Test

Cardiomyocyte mitochondrial respiration in the presence of palmitate was determined using a palmitate fatty acid oxidation stress test. Plating media was changed with XF assay media (modified DMEM without glucose, pH 7.4) supplemented with 1 mM sodium pyruvate,

2.5 mM glucose, and 0.5 mM carnitine for 45 minutes in a non-CO^2 incubator. Following the 45 minutes, 40 μM etomoxir (Sigma, Product #E1905) or vehicle (water) control was added to the appropriate wells and the plate was incubated for another 15 minutes in the non-CO^2 incubator. Meanwhile, preparations such as loading the drugs into the ports located on the sensor cartridge, and calibrating the sensor cartridge (as previously described in 2.3.2) would be performed in between incubation steps. After the 15-minute incubation, immediately before loading the cell plate into the XF 24 analyzer, 0.17 mM bovine serum albumin (BSA) control or 0.175 mM palmitate-BSA substrate conjugate would be added to the respective well and loaded into seahorse XF 24 analyzer (as described in 2.3.2).

2.4.3 Seahorse XF Glycolysis Stress Test

Cardiomyocyte glycolytic activity was determined using a glycolysis stress test. Plating media was replaced with seahorse XF media (modified DMEM without glucose, pH 7.4) supplemented with 100 mM L-glutamine for 1 hour in a non-CO^2 incubator. Meanwhile, the sensor cartridge would be removed from the non-CO^2 incubator. At this time glucose, oligomycin, and 2-deoxy-D-glucose would be added to injector ports A, B and C at a final concentration of 20 mM, 2 μM, and 100 mM respectively before loading the sensor cartridge in the Seahorse XFe24 analyzer to begin the calibration. Once calibration is complete and the 1-hour incubation is over the utility plate would be removed from the seahorse XFe24 analyzer and the cell plate containing isolated cardiomyocytes would be loaded into the machine. Measurements of ECAR would be recorded and calculations of glycolytic rate, glycolytic capacity, and glycolytic reserve would be assessed (as described in 2.3.1).

2.5 Measurement of Cytosolic Ca^{2+} in Fetal Cardiomyocytes Using Fluo-4

Real-time measurements of cytosolic calcium oscillations on isolated fetal cardiomyocytes were performed using the Ca^{2+} sensitive dye, Fluo-4 AM (Thermofisher Product #F14201). Measurements were carried out on isolated cardiomyocytes from Lean, GDM, and GDM + RESV offspring, cardiomyocytes were isolated (as previously described in 2.2) and plated in a 24-well plate at a density of 500,000 cells/well. On the day of imaging experiments, cells were washed 3 times with pre-warmed (37°C) Hank's balanced salt solution (HBSS) (Thermofisher, Product #14025134) before being incubated in serum-free media containing Fluo-4 and 0.02% pluronic acid dissolved in dimethyl sulfoxide (DMSO) for 30 minutes in a cell culture incubator at 37° C with 5% CO^2. Serum-free media was composed of DMEM/F-12 (1:1) media (HyClone, Product #SH30126.01) supplemented with 1% penicillin-streptomycin solution (HyClone Product #SV30010) and 50 µg/µL gentamycin (Thermofisher, Product #15750078). Afterwards, cells were washed 1 time with pre-warmed HBSS before being incubated for 30 minutes in serum-free media at 37° C to allow for de-esterification. After de-esterification cells were washed 2 times in prewarmed HBSS before being imaged, imaging was done using Agilent's BioTek Cytation 5 (internal conditions were kept at 37° C with 5% CO^2 using the Cytation 5 software). Images were captured at a frame rate of 10 frames per second, and an initial baseline recording was recorded during the first 30 seconds. After baseline recording angiotensin 2 was injected using the auxiliary injector arms of the Cytation 5 at a final concentration of 20 µM and images were captured for 120 seconds. Raw images were exported from the Cytation 5 and analyzed in image J. Changes in fluorescence intensity were monitored over time and plotted as changes in fluorescence intensity over time. The time between calcium peaks was measured with each peak being analogous to one contractile cycle. Frequency was then calculated as the inverse of the time of one complete contraction cycle. The time for calcium

release to reach 50% of peak (from baseline to peak) was recorded. The time of decay (time it took for fluorescence intensity to 50% from peak to baseline) was also recorded. A set of region of interest (ROI) was used to capture our readings and assess our calcium flux parameters for each experimental run, however, the ROI between each experimental run was kept consistent between groups and experimental replicates.

2.6 Statistical Analysis

Data are presented as the mean +/- standard error of the mean. Graphad Prism 10 Software (La Jolla, CA, USA) was used for all statistical analysis. One-way Analysis of Variance (ANOVA) was used to assess significance between diets (i.e. Lean, GDM, GDM+RESV) with Tukey multiple comparisons post-hoc test. Statistical Significance with two variables (e.g., maternal and calcium flux baseline vs angiotensin II injection in chapter 3 or maternal diet and vehicle control vs. Etomoxir seen in chapter 4) were assessed using two-way ANOVA with Tukey multiple comparisons post-hoc test. p-values are given and a $p<0.05$ was considered statistically significant

Chapter 3: Effects of Maternal RESV Supplementation on Fetal Rat Cardiac Structure and Calcium Dynamics in GDM Offspring

3.1 Introduction

Maternal diabetes during pregnancy does not only impose complications on the mother but can also increase the risk for cardiometabolic disease and the development of CVD in the offspring (Kereliuk & Dolinsky, 2022). Echocardiography from Do et al. (2019) showed that infants of diabetic mothers had increased left ventricular wall and IVS thickness that persisted from early to late infancy (Do et al., 2019). Follow-up studies from Do et al. (2019) further showed that cardiac hypertrophy persisted into early childhood (Do et al., 2021). Population-based cohort studies from Tam et al. (2017) have found that maternal diabetes during pregnancy increases the risk of higher systolic blood pressure in early childhood (Tam et al., 2017). Another population-based cohort study by Yu et al. (2019) showed elevated rates of CVD in the offspring of mothers with pregestational or gestational diabetes persisting from early childhood (ages 0-9) through early adulthood (ages 15-19) (Yu et al., 2019).

Research using animal models provided additional insight into the changes in cardiac structure and function in the offspring. Lehtoranta et al. (2017) demonstrated that newborn rats born from maternal hyperglycemia had increased heart weight as well as expressed altered cardiac genes important for myocardial growth, function, and metabolism 2 weeks postnatally (Lehtoranta et al., 2017). Other studies found that offspring from mice given a high-fat diet-induced cardiac hypertrophy in embryos (Lin et al., 2017). Previous studies from our lab found that fetal offspring exposed to GDM exhibited an increase in the thickness of the left ventricular wall and IVS (Kereliuk, 2022). Additionally, our group demonstrated that exposure to GDM also impaired calcium handling in fetal rat cardiomyocytes, these cardiomyocytes from GDM offspring exhibited delayed and extended calcium flux cycles (Kereliuk, 2022).

RESV is a natural polyphenolic compound that has cardioprotective properties. RESV reduces oxidative stress and inflammation in cardiac tissue by increasing the expression of antioxidant enzymes and decreasing inflammatory mediators (Gal et al., 2021). RESV was also found to attenuate high blood pressure and cardiac hypertrophy in hypertensive rats and mice (Dolinsky et al., 2013). Furthermore, RESV attenuated cardiac fibrosis and dysfunction in models of cardiac hypertrophy (Ma et al., 2023).

Previously, our group showed that administration of RESV to GDM rats in the last week of pregnancy reduced maternal blood glucose levels and improved GSIS without altering body weight or litter number (Brawerman et al., 2019). Since our group had previously established that GDM-exposed offspring exhibit increased thickness of the left ventricular wall and intraventricular septum as young adults (Kereliuk, 2022) we wanted to assess the effects of RESV on GDM-exposed offspring heart. We hypothesized that RESV supplementation incorporated in the diet of GDM dams in the final week of pregnancy would prevent the development of cardiac hypertrophy in GDM-exposed offspring. Furthermore, since GDM exposure impaired calcium handling within the fetal offspring cardiomyocytes (Kereliuk, 2022), we hypothesized that maternal RESV supplementation would improve contractility in the GDM-exposed offspring by improving calcium flux cycles. In this chapter, we explore how maternal RESV supplementation during the third trimester of pregnancy within GDM mothers can impact left ventricular cardiac hypertrophy and calcium flux cycles in the fetal hearts of GDM-exposed offspring.

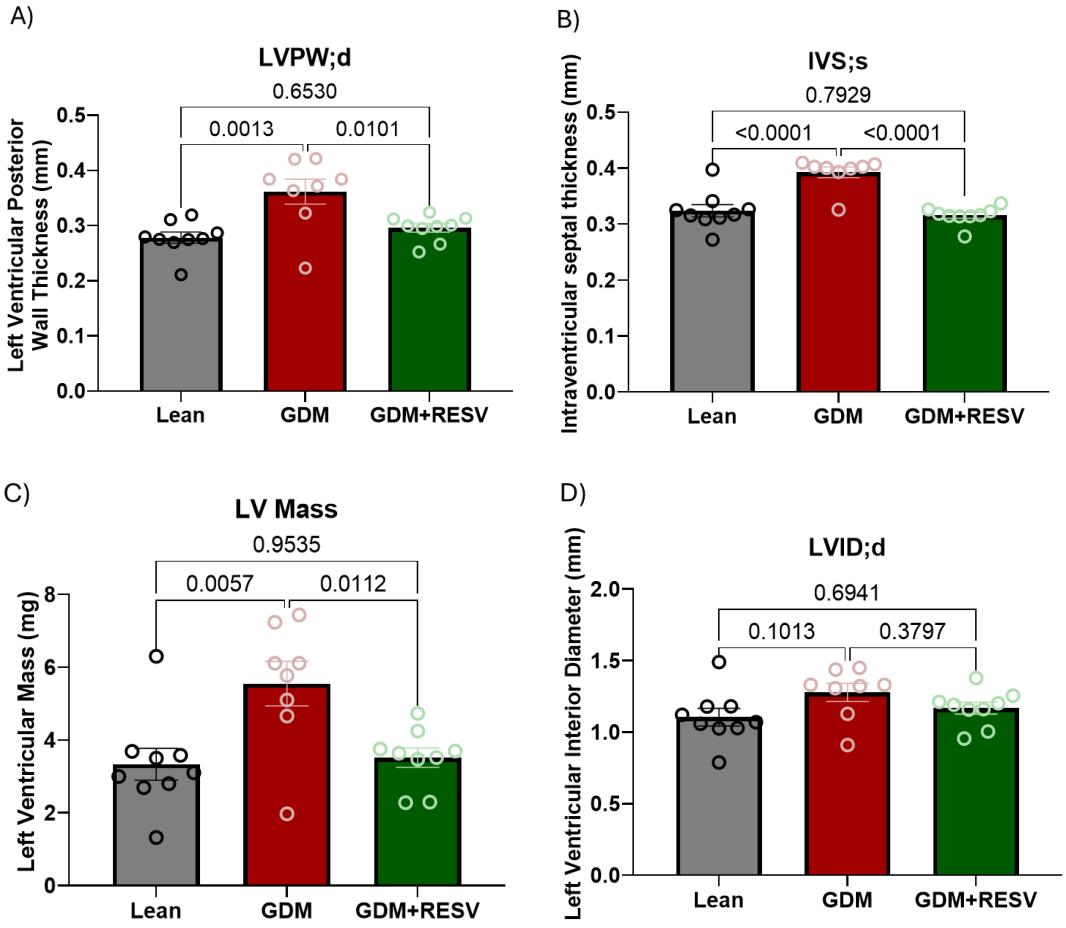
3.2 Methods

Refer Chapter 2 for material and methods

3.3 Assessments of Fetal Cardiac Structure via Echocardiography

Offspring cardiac morphology was evaluated via *in vivo* echocardiography in the fetal hearts that was performed on pregnant dams on embryonic day 18.5 (e18.5) (Fig 3.1). LVPW was increased 1.3-fold in GDM offspring, compared to Lean offspring ($p=0.0013$) (Fig 3.1A). Interestingly, the LVPW of GDM + RESV offspring was not significantly different compared to Lean offspring ($p=0.6530$). However, the LVPW of GDM + RESV offspring was ~20% lower compared to GDM offspring ($p=0.0101$) (Fig 3.1A). IVS was also assessed across all offspring from each diet. When compared to Lean, IVS was 1.2-fold greater in GDM-exposed offspring ($p<0.0001$) (Fig 3.1B). Interestingly, compared to Lean, we did not see an increase in the thickness of IVS in GDM + RESV offspring ($p=0.7929$). Compared to GDM offspring, the IVS of GDM + RESV offspring was 1.2-fold lower ($p<0.0001$) (Fig 3.1B). In agreement with our previous findings, when compared to Lean offspring, GDM offspring exhibited a significant 1.7-fold increase in LV mass ($p=0.0057$) (Fig 3.1C). When compared to GDM offspring, the GDM + RESV offspring's left ventricle mass was lower by a fold of 1.6 ($p=0.0112$) (Fig 3.1C). However, compared to Lean offspring, GDM + RESV offspring did not exhibit such an increase in left ventricle mass ($p=0.9535$) (Fig 3.1C). Lastly, when examining LVID, we found no significant differences across all groups (Fig 3.1D). Although we were unable to measure differences in sex between offspring, these findings suggest that GDM-exposed rats exhibit early markers of concentric left ventricular cardiac hypertrophy, and maternal RESV administration attenuated GDM-induced cardiac hypertrophy in fetal offspring.

Figure 3.1 Assessment of Cardiac Structure Using In vivo Echocardiography of Fetal Offspring (e.18.5)

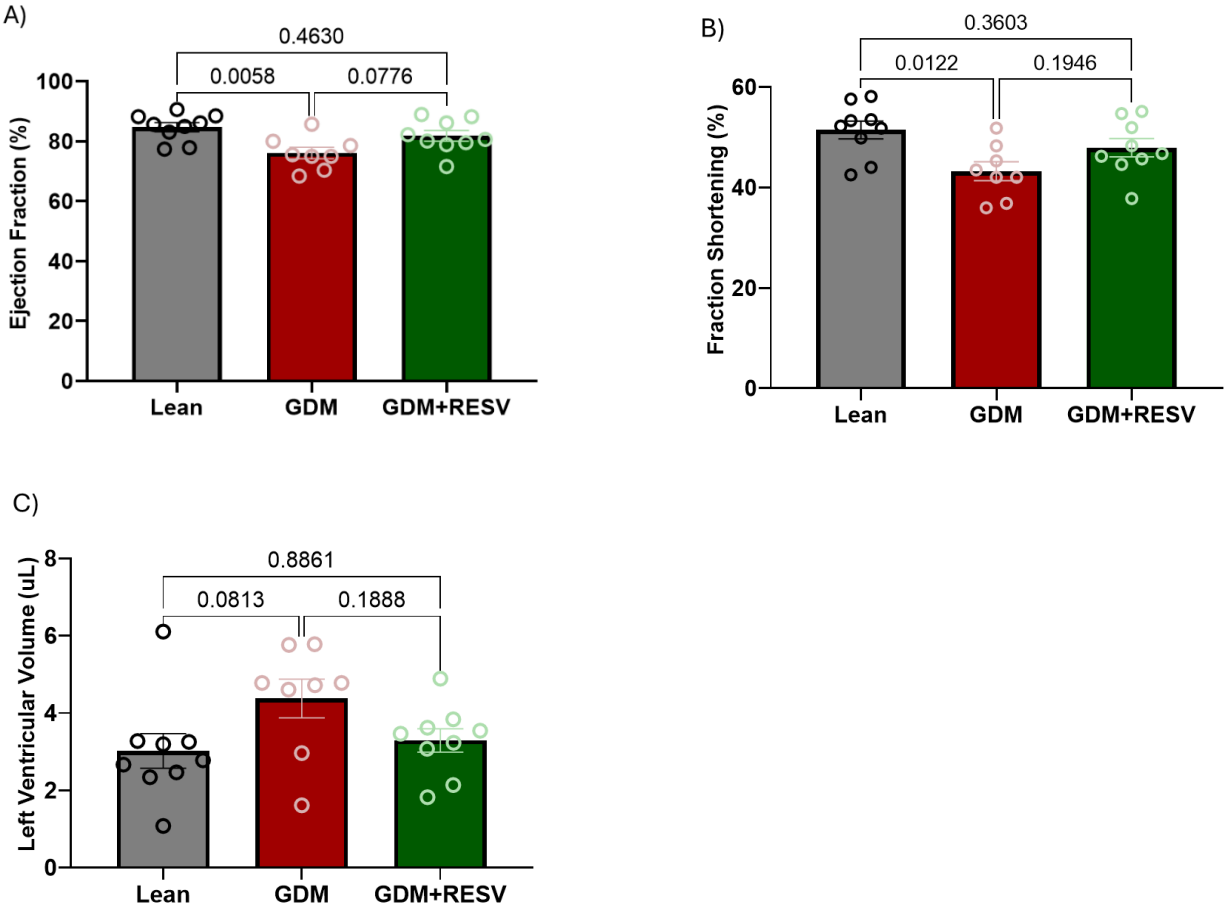


A) Left ventricular posterior wall (LVPW) of Lean (grey), GDM (red) and GDM + RESV (green) offspring taken at embryonic day 18.5 (e18.5) B) Intraventricular septal thickness (IVS) of Lean, GDM and GDM + RESV offspring C) Left ventricle (LV) mass of Lean, GDM, and GDM + RESV D) Left Ventricle Interior Diameter (LVID) of Lean, GDM and GDM+RESV offspring. Data presented as mean +/- S.E.M, (n= 9 Lean, 8 GDM, 9 GDM + RESV where n is represented as one dam litter). P-values represent significance (<0.05) after one-way ANOVA with multiple comparisons and Tukey post-hoc test.

3.4 Evaluation of Cardiac Function in Fetal Offspring via Fetal Echocardiography

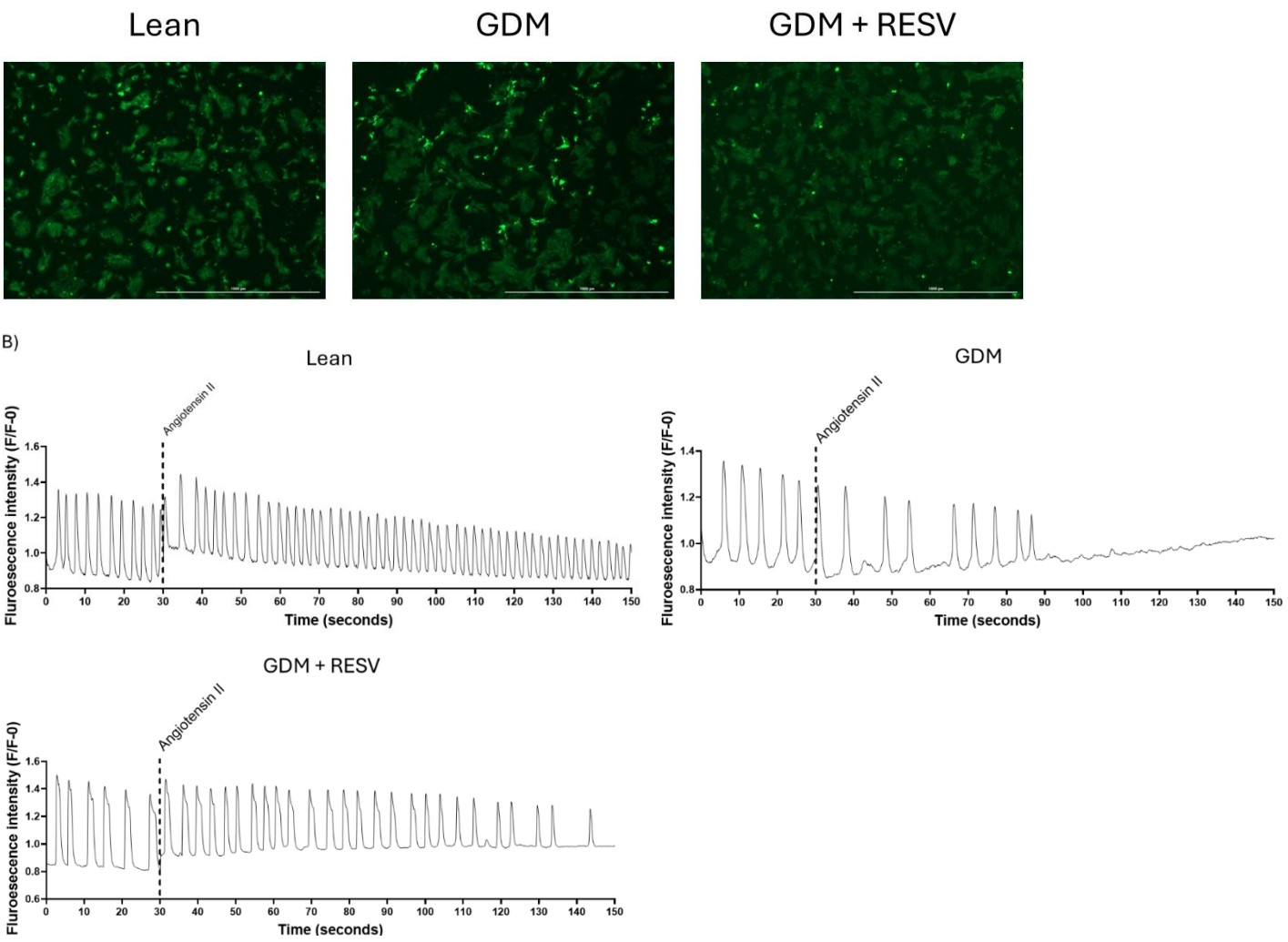
We evaluated cardiac function in e18.5 fetal offspring through in vivo echocardiography. To determine if GDM-exposed offspring suffered impacts on cardiac function, we measured EF, FS, and LV volume (Fig 3.2). When compared to Lean offspring, GDM offspring showed a significant 10% decrease in ejection fraction ($p=0.0058$) (Fig 3.2A). Ejection fraction was increased by 1.07-fold ($p=0.0776$) in GDM + RESV offspring compared to GDM, although this did not reach statistical significance (Fig 3.2A). GDM offspring showed a 1.2-fold reduced fraction shortening when compared to Lean offspring ($p=0.0122$) (Fig 3.2B). Fractional shortening was increased 1.1-fold in GDM + RESV compared to GDM offspring. However, it did not reach significance ($p=0.19$) (Fig 3.2B). Lastly, when evaluating LV volume, we noticed there were changes between Lean and GDM offspring however, these changes did not reach significance ($p=0.08$). Similarly, when comparing GDM + RESV offspring to GDM offspring, no significant difference was observed ($p=0.18$) (figure 3.2C). These findings, coupled with the preceding cardiac structure data (Fig 3.1), show that early-life exposure to GDM can significantly impact the cardiac structure in fetal offspring, leading to concentric left ventricular cardiac hypertrophy with potential early alterations to cardiac function as GDM offspring also exhibited decreased ejection fraction and fractional shortening (as seen in Fig 3.2). Importantly, maternal RESV treatment attenuated GDM-induced cardiac hypertrophy in fetal offspring.

Figure 3.2 Assessment of Cardiac Function Through In Vivo Fetal Echocardiography on Fetal (e18.5) Offspring



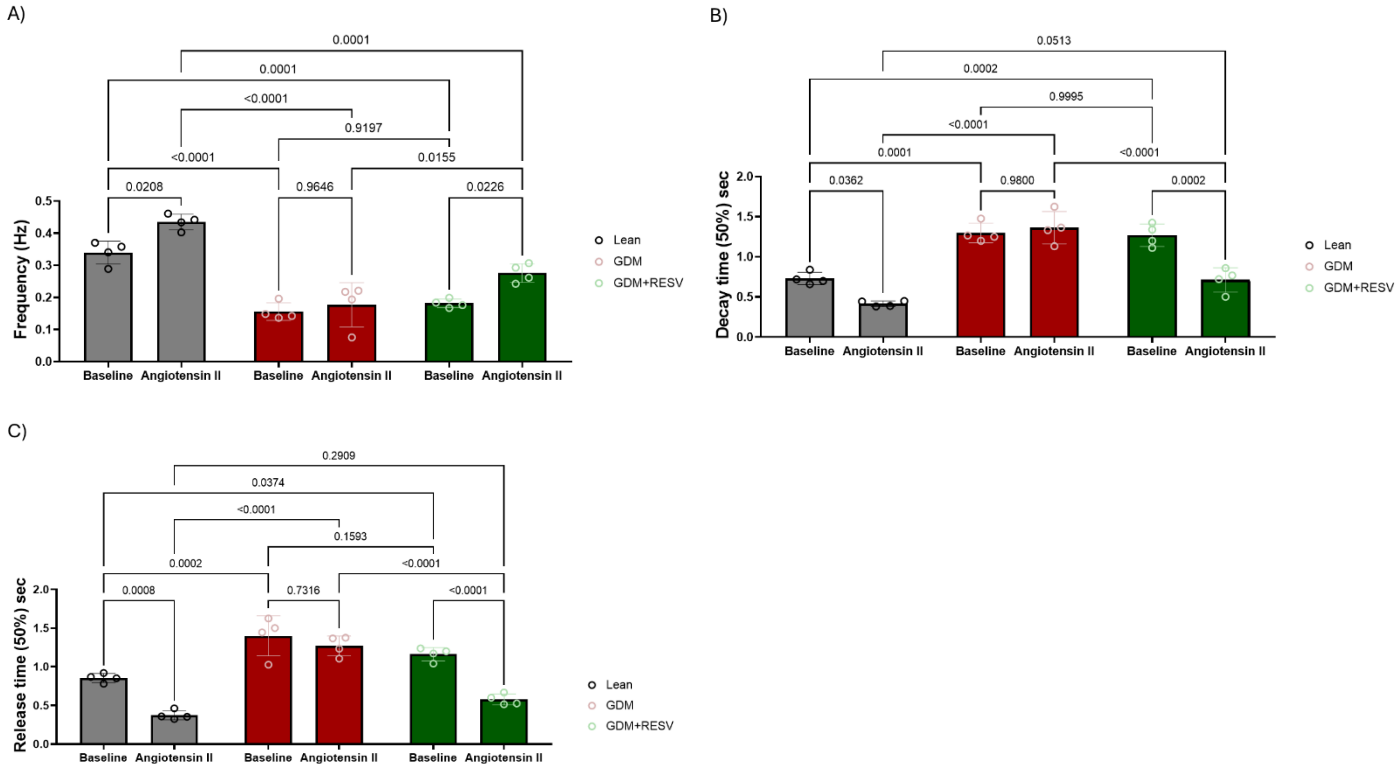
A) Ejection fraction of fetal offspring e18.5 from Lean (grey), GDM (red), and GDM + RESV (green) dams. B) Fractional shortening from e18.5 fetal offspring. C) Left ventricle volume of fetal offspring taken during diastole. Data presented as mean +/- S.E.M, (n= 9 Lean, 8 GDM, 9 GDM + RESV where n is represented as one dam litter). P-values represent significance (<0.05) after one-way ANOVA with multiple comparisons and Tukey post-hoc test.

Figure 3.3 Tracking Calcium Flux Using Fluo-4 in Isolated Fetal Cardiomyocytes



A) Representative calcium trace image from isolated cardiomyocytes from Lean, GDM, and GDM+RESV offspring (n= 4, n is represented as one entire dam litter). B) Representative calcium flux trace over time of isolated cardiomyocytes from Lean, GDM and GDM+RESV offspring before and after injection of angiotensin II (n= 4, n is represented as one entire dam litter). Calcium flux was measured using the cell-permeant Fluo-4. Calcium traces over time were analyzed from region of interest (ROI).

Figure 3.4 Assessment of Calcium Dynamics Using Fluo-4 in Isolated Fetal Cardiomyocytes



A) Average frequencies of cardiomyocytes isolated from Lean, GDM, and GDM+RESV offspring before and after angiotensin II injection. B) Average decay time (time for 50% fluorescence intensity) of fetal cardiomyocyte isolated from Lean, GDM and GDM + RESV offspring. C) Average release time (time to reach 50% fluorescence intensity) of fetal cardiomyocytes from Lean, GDM, and GDM + RESV offspring. Data presented as mean +/ S.E.M (n= 4, n is represented as one entire dam litter). Calcium flux was measured using the cell-permeant Fluo-4. P-values represent statistical significance after two-way ANOVA with Tukey post-hoc multiple comparison.

3.5 Calcium Dynamics in Isolated Fetal Rat Cardiomyocytes

Using a Ca^{2+} sensitive fluorescent dye Fluo-4 we performed real-time calcium imaging on isolated fetal cardiomyocytes of Lean, GDM, and GDM+RESV offspring (Fig 3.3A). We monitored the changes in release and reuptake cycles accordingly based on changes in fluorescence intensity over time, first gathering a baseline read before injecting angiotensin II to stimulate cardiomyocyte beat frequency (Fig 3.3B). Consistent with previous findings from our group, fetal cardiomyocytes of GDM offspring showed delayed flux cycles when compared to Lean offspring and quickly became unresponsive following angiotensin II injection (Fig 3.3B). While fetal cardiomyocytes isolated from GDM+RESV offspring had slower flux cycles at baseline recording, they were more responsive to the angiotensin II stressor (Fig 3.3B). This can be more clearly observed when we calculated and averaged the cardiomyocyte beat frequencies from these isolated fetal cardiomyocytes (Fig 3.4A). When comparing the beat frequencies of all groups, we noticed that both Lean and GDM+RESV were more responsive to the angiotensin II stressor, which was indicated by an increase in cardiomyocyte beat frequencies (1.59-fold and 1.74-fold, respectively) ($p=0.0208$ and $p=0.0226$) (Fig 3.4A). When we examined the average beat frequency of isolated fetal cardiomyocytes from GDM offspring, we found no significant changes in beat frequency after angiotensin II injection ($p=0.9646$) (Fig 3.4A). We also evaluated cardiomyocyte decay time, the time it takes for fluorescence intensity to drop by 50% from the peak, across all groups before or after angiotensin II injection (Fig 3.4B). We found that cardiomyocytes from GDM offspring had longer decay times when compared to Lean offspring. Specifically, the decay time recorded at baseline was 1.77-fold longer in GDM fetal cardiomyocytes when compared to Lean ($p=0.0001$) (Fig 3.4B). When we evaluated the decay time after angiotensin II, we found a decrease in decay time, specifically in cardiomyocytes from Lean and GDM + RESV offspring. Lean fetal cardiomyocytes showed a decrease in decay time

by 1.75-fold (p=0.0362) (Fig 3.4B). Similarly, GDM + RESV showed a decrease in decay time by a fold of 1.77 after angiotensin II injection (p=0.0002) (Fig 3.4B). We did not notice changes in decay time in GDM-isolated fetal cardiomyocytes after angiotensin II treatment (Fig 3.4B). We also evaluated release time (the time it takes for fluorescence intensity to reach 50% from baseline) before and after angiotensin II across all groups. Isolated cardiomyocytes from GDM offspring showed longer baseline release time by a fold of 1.63 when compared to cardiomyocytes from Lean offspring (p=0.0002) (Fig 3.4C). Similarly, GDM + RESV fetal cardiomyocytes also showed longer release time at baseline by a fold of 1.36 when compared to Lean (p=0.0374) (Fig 3.4C). However, after angiotensin II injection, both GDM + RESV and Lean fetal cardiomyocytes showed an increase in their release time. Specifically, release time was decreased by a fold of 2 (p<0.0001) and 2.3-fold (p=0.0008), respectively (Fig 3.4C). We did not notice significant changes in calcium release time in fetal cardiomyocytes from GDM offspring after angiotensin II (Fig 3.4C). These findings suggest that, at baseline, GDM fetal cardiomyocytes showed lower average beat frequency, increased decay time, and increased release time when compared to cardiomyocytes from Lean offspring. When we used angiotensin II to increase cardiomyocyte beat frequency, we noticed that GDM cardiomyocytes were unresponsive to the stressor, and no changes were observed. Although fetal cardiomyocytes from GDM + RESV offspring showed shorter beat frequency and longer baseline decay and release time when compared to Lean cardiomyocytes after angiotensin II was introduced, GDM + RESV cardiomyocytes showed more responsiveness towards the stressor through an increase in beat frequency, decay time, and release time.

3.6 Discussion and Conclusion.

The purpose of this research was to determine whether RESV supplementation in the maternal diet during the third trimester of pregnancy characterized by GDM would protect the offspring from cardiac hypertrophy and dysfunction. Previous work from our lab found that GDM-exposed offspring had increased thickness of the left ventricular wall and intraventricular septum (Kereliuk, 2022). In this thesis, we replicated our previous findings, but more importantly, we extended these findings to show that maternal RESV supplementation attenuated GDM-induced cardiac hypertrophy in the fetal offspring. Previous work from our lab has found that fetal cardiomyocytes of GDM offspring exhibited delayed and extended calcium flux using the calcium tracker Fura-2 (Kereliuk, 2022). Although we used a different calcium binding dye, we made similar observations using fluo-4. In this study, we found that isolated fetal cardiomyocytes from GDM offspring had lower beat frequency and longer decay and release time. Upon angiotensin-II stimulation, fetal cardiomyocytes from GDM offspring were not responsive to the stressor. While fetal cardiomyocytes from GDM+RESV offspring showed similar characteristics as GDM, they were more responsive to angiotensin II stimulation. To our knowledge, this is the first observation that RESV affects calcium flux in fetal cardiomyocytes.

Calcium handling plays a crucial role in cardiac function, since calcium is a central component in the excitation-contraction coupling in cardiomyocytes, the timing of cardiac contraction and relaxation is important. During systole, the force of contraction is determined based on the amplitude of calcium transient in diastole rapid removal of cytosolic calcium is crucial for relaxation, allowing for the next round of contraction to occur (Gilbert et al., 2020; Sutanto et al., 2020). Impairments in calcium flux cycles can impact cardiac function as longer relaxation rates can result in impaired contractility of the heart (Law & Metzger., 2021). Similarly, prolonged calcium transient can also lead to a reduction in the generation of the force

of cardiac contraction that may shift the heart to its compensatory phase in order to maintain adequate cardiac output (Balke & Shorofsky, 1998; Schaub et al., 1998).

Concentric cardiac hypertrophy is a condition that is characterized by an increase in the thickness of the heart muscle without an overall change in the chamber size (Lorell & Carabello, 2000). The development of concentric cardiac hypertrophy in GDM may be a response to maternal hyperglycemia and hyperinsulinemia that may increase fetal cardiac size in utero (Al-Biltagi et al., 2021).

Previous studies have found that RESV attenuated left ventricular cardiac hypertrophy in hypertensive rats. Specifically, RESV was able to reduce left ventricle mass, LVPW thickness, and the ratio of heart weight/tibia length (Dolinsky et al., 2013). Studies from Ma et al. (2023) also found that RESV administration was able to reduce LV wall thickness in mouse models of cardiac hypertrophy (Ma et al., 2023). Based on our findings, we found that maternal RESV supplementation introduced in the final trimester of pregnancy prevented GDM-induced cardiac hypertrophy during the fetal stages, which may, in turn, protect the offspring from cardiac complications later in life.

Chapter 4: Effects of Maternal RESV on Mitochondrial Respiration and Glycolysis in GDM Offspring

4.1 Introduction

About 90% of cellular ATP is utilized for the contraction-relaxation cycles of the heart (Brown et al., 2016). To meet this high energy demand, cardiomyocytes have a high content of mitochondria compared to many other tissues (Brown et al., 2016). Mitochondrial function plays a crucial role in the development and progression of cardiac hypertrophy (Brown et al., 2016). In the preceding chapter, we used echocardiography to show that GDM caused fetal cardiac hypertrophy (Figure 3.1A). Moreover, we observed that GDM offspring exhibit cardiac hypertrophy that persists across the lifespan, impaired diastolic function in young adulthood and develop systolic dysfunction with advanced age (Kereliuk, 2022). During the early compensatory phase of cardiac hypertrophy, mitochondrial respiration adapts to meet the increased energy demands of the enlarged myocardium. However, as the heart transitions to the decompensatory phase, mitochondrial function begins to decline (Rosca et al., 2012). This is vital as a significant portion of the energy produced by the mitochondria is used for calcium handling within cardiomyocytes. When mitochondrial function declines, this can begin to impair cellular calcium handling as many of the membrane-bound pumps required for cytosolic calcium release and reuptake are energy-dependant, which can, in turn, lead to impairments in excitation-coupled contraction (Brown et al., 2016). Moreover, mitochondrial dysfunction contributes to the production of reactive oxygen species that can damage cardiac muscle tissues (Bhullar and Dhalla, 2023).

Previous studies from our lab found that fetal cardiomyocytes from GDM offspring exhibit mitochondrial dysfunction with alterations in energy metabolism (Kereliuk, 2022). We hypothesize that RESV may improve mitochondrial function, specifically through the activation

of various signalling pathways such as AMPK and SIRT1. Specifically, we hypothesize that RESV activates AMPK, a key regulator in cellular energy metabolism and mitochondrial biogenesis (Zheng et al., 2022). Similarly, the activation of the class III deacetylase SIRT1 also plays a role in promoting mitochondrial function and biogenesis through deacetylation and activation of PGC-1a, a master regulator that promotes mitochondrial biogenesis (Iside et al., 2020). The activation of AMPK, PGC-1a and SIRT1 can lead to a cascade of events, ultimately enhancing mitochondrial biogenesis and oxidative phosphorylation and improving mitochondrial function, which can lead to improvements in energy production within fetal cardiomyocytes (Fang et al., 2018). In this chapter, we explore how GDM induces impairments in cardiomyocyte mitochondrial function and energy production potential and investigate whether RESV mitigates cardiomyocyte mitochondrial dysfunction in GDM offspring.

4.2 Methods

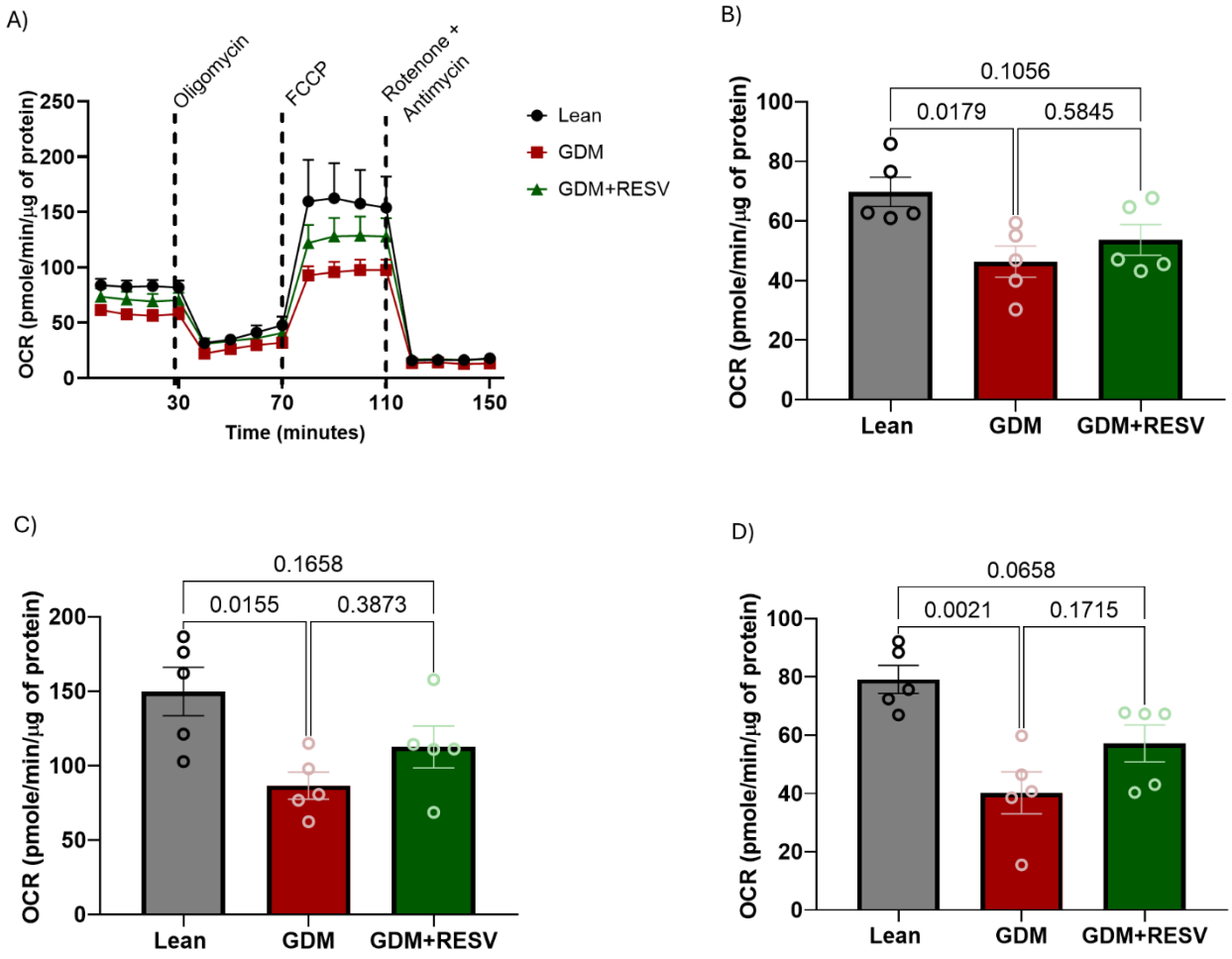
Refer to Chapter 2 for materials and methods

4.3 Cardiomyocyte Energy Metabolism in the Presence of Glucose

We isolated cardiomyocytes from fetal hearts (embryonic day 20.5) of rat offspring from Lean, GDM, and GDM + RESV dams. Then, we assessed mitochondrial bioenergetic function using a mitochondrial stress test performed on the Agilent XF24 Seahorse Extracellular Flux Analyzer in the presence of glucose as substrate (Fig. 4.1A). When compared to Lean cardiomyocytes, cardiomyocytes isolated from GDM offspring showed a 1.50-fold decrease towards calculated basal respiration in the presence of glucose ($p=0.0179$) (Fig 4.1B). Although cardiomyocytes isolated from GDM + RESV offspring showed a ~15.8% higher basal respiration when compared to GDM fetal cardiomyocytes, this did not reach significance ($p=0.5845$) (Fig 4.1B). When we calculated mitochondrial maximal respiration, we found that fetal

cardiomyocytes from GDM offspring showed a ~42.2% decrease in maximal respiration when compared to cardiomyocytes isolated from Lean offspring (p=0.0155) (Fig 4.1C). When compared to Lean cardiomyocytes, we noticed a ~21.2% decrease in maximal respiration in cardiomyocytes isolated from GDM + RESV offspring, which was not significant (p=0.1658). In addition, maximal respiration in cardiomyocytes in GDM + RESV offspring increased 1.42-fold when compared to GDM cardiomyocytes, though these findings did not reach significance (p=0.3873) (Fig 4.1C). Lastly, we assessed mitochondrial spare respiratory capacity from cardiomyocytes isolated from Lean, GDM and GDM + RESV offspring. When we compared the effects of GDM, we noticed that GDM cardiomyocytes exhibited a 2-fold decrease in mitochondrial spare respiratory capacity when compared to Lean cardiomyocytes (p=0.0021) (Fig 4.1D). When we examined the effects of RESV on cardiomyocyte spare respiratory capacity, we found a 29.6% increase when compared to GDM offspring. However, this did not reach significance (p=0.1715) (Fig 4.1D).

Figure 4.1 Cardiomyocyte Bioenergetics in the Presence of Glucose



A) Oxygen Consumption Rate (OCR) over time in Lean (black), GDM (red), and GDM + RESV cardiomyocytes (green) in the presence of glucose. B) Calculated parameters of basal mitochondrial respiration. C) Calculated parameters of maximal respiration. D) Calculated parameters of spare respiratory capacity. Readings normalized to protein concentration (ug). Data presented as mean +/- S.E.M, n=5 (where n is represented as 1 entire dam litter). P-values represent significance (<0.05) after one way ANOVA with Tukey multiple comparison.

4.4 Cardiomyocyte Energy Metabolism in the Presence of Palmitate

Fatty acids are the primary energy substrate used by the heart (Ardehali et al., 2012). Therefore, we investigated mitochondrial bioenergetics in the presence of fatty acids using palmitate, a 16-carbon saturated fatty acid. We repeated the mitochondrial stress test using palmitate with or without the addition of the carnitine palmitoyl transferase 1 (CPT1) inhibitor, etomoxir (with water being used as our vehicle control) (Fig 4.2 and 4.3). Similarly, we found that fetal cardiomyocytes isolated from GDM dams showed impaired oxidation of palmitate when vehicle control was administered (Fig 4.2A). Basal respiration of fatty acids (Fig 4.2B) by cardiomyocytes from GDM offspring exhibited a 37.7% decrease compared to Lean ($p=0.0751$) and a 32.3% lower basal respiration compared to GDM + RESV cardiomyocytes that did not reach significance ($p=0.1759$). Maximal respiration was evaluated across all groups, and GDM offspring exhibited a 40.8% decrease in maximal respiration when compared to Lean ($p=0.0005$) (Fig 4.2C). When examining cardiomyocytes from GDM + RESV offspring, maximal respiration was 34.5% higher than GDM ($p=0.0043$). Additionally, when compared to Lean cardiomyocytes, there were no significant differences in terms of maximal respiration between Lean and GDM + RESV cardiomyocytes (Fig 4.2C). Lastly, the spare respiratory capacity of isolated fetal cardiomyocytes from GDM offspring was 1.7-fold lower than in Lean cardiomyocytes ($P=0.0087$) (Fig 4.2D). Whereas spare respiratory capacity was 1.7-fold higher in the cardiomyocytes of GDM + RESV offspring compared to GDM fetal cardiomyocytes ($p=0.0082$) (Fig 4.2D).

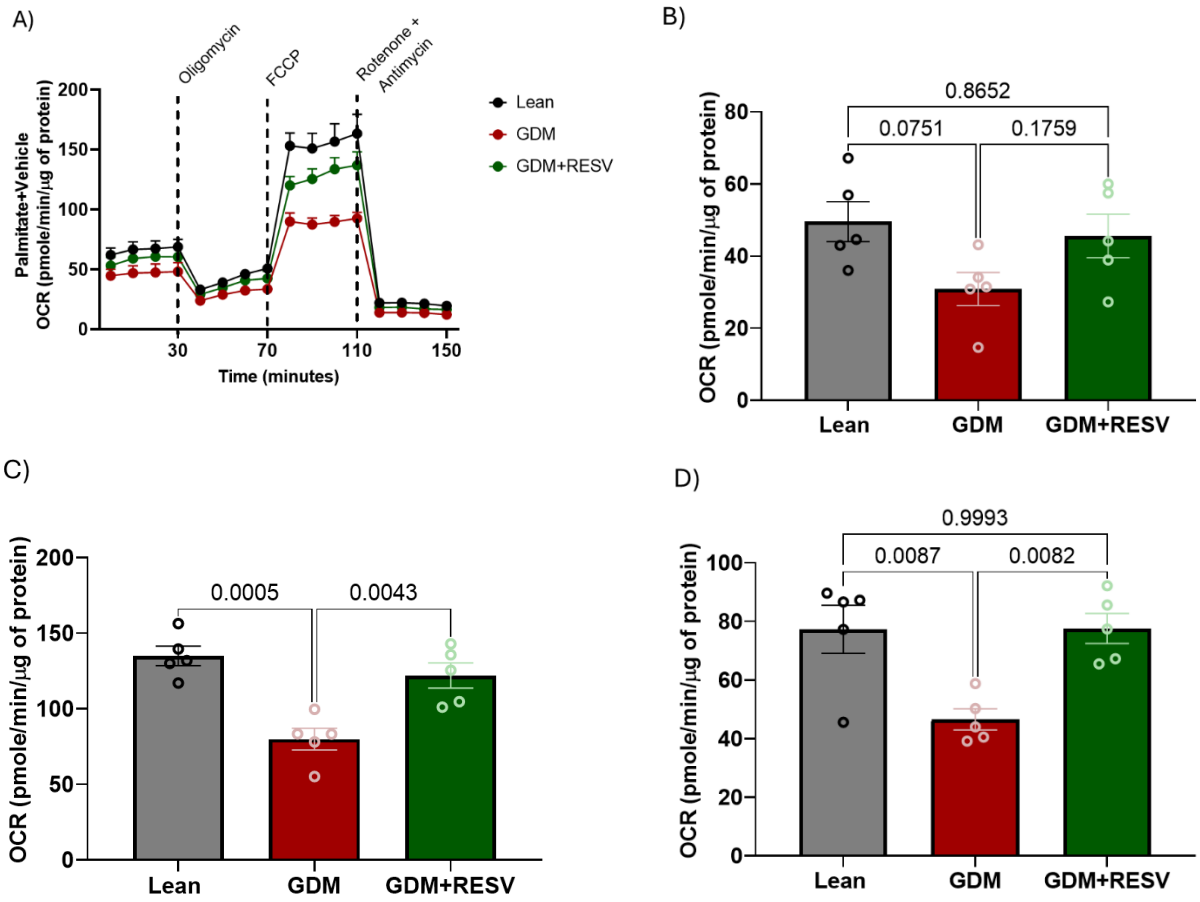
When cardiomyocytes were treated with the CPT1 inhibitor etomoxir, we noticed there was a decrease in substrate oxidation in the presence of palmitate in all groups (Fig 4.3A). When we calculated basal respiration in response to etomoxir, a decrease in OCR compared to our

vehicle control group was observed (compared to Fig 4.2). When treated with etomoxir, fetal cardiomyocytes from Lean, GDM and GDM + RESV offspring had reduced basal respiration (Fig 4.3B) however there were no significant differences across the groups as they expressed similar levels of basal OCR. Similarly, we also observed a reduction in maximal respiration within cardiomyocytes from Lean, GDM, and GDM + RESV treated with etomoxir (Fig 4.3C). Lastly, spare respiratory capacity was reduced in etomoxir-treated cardiomyocytes from Lean, GDM, and GDM + RESV offspring (Fig 4.3D). We compared the changes in OCR with or without the presence of etomoxir (Fig 4.4A-C). When we calculated basal respiration in response to etomoxir, we noticed that fetal cardiomyocytes from Lean offspring exhibited a reduction in basal respiration by ~2.1-fold when compared to our vehicle control ($p=0.002$) (Fig 4.4A). While not significant, we also noticed a small decrease in basal respiration in fetal cardiomyocytes from GDM offspring by a fold of 1.25 ($p=0.8865$) (Fig 4.4A). Fetal cardiomyocytes from GDM+RESV offspring, however, showed a reduction in basal OCR by a fold of 1.85 with the presence of etomoxir ($p=0.0154$) (Fig 4.4A). When comparing maximal respiration, we noticed that Lean cardiomyocytes treated with etomoxir exhibit a reduction in maximal OCR by 2.05-fold ($p<0.0001$) (Fig 4.4B). Meanwhile, GDM fetal cardiomyocytes only exhibit a 1.27-fold reduction toward its maximal OCR when treated with etomoxir ($p=0.3679$) (Fig 4.4B). Lastly, we examined changes within GDM + RESV fetal cardiomyocytes and found that the presence of etomoxir reduced maximal respiration by a fold of 1.88 ($p<0.0001$) (Fig 4.4B). We also evaluated changes in spare respiratory capacity, and we found that the presence of etomoxir reduced spare respiratory capacity in fetal cardiomyocytes from Lean, GDM and GDM + RESV offspring (Fig 4.4G). Specifically, spare respiratory was reduced by 1.80 ($p=0.0007$) and 1.92-fold ($p=0.0003$), respectively, in fetal cardiomyocytes from Lean and GDM + RESV offspring

(Fig 4.3G). Fetal cardiomyocytes from GDM offspring showed a 1.2-fold reduction in spare respiratory capacity ($p=0.878$) (Fig 4.4C).

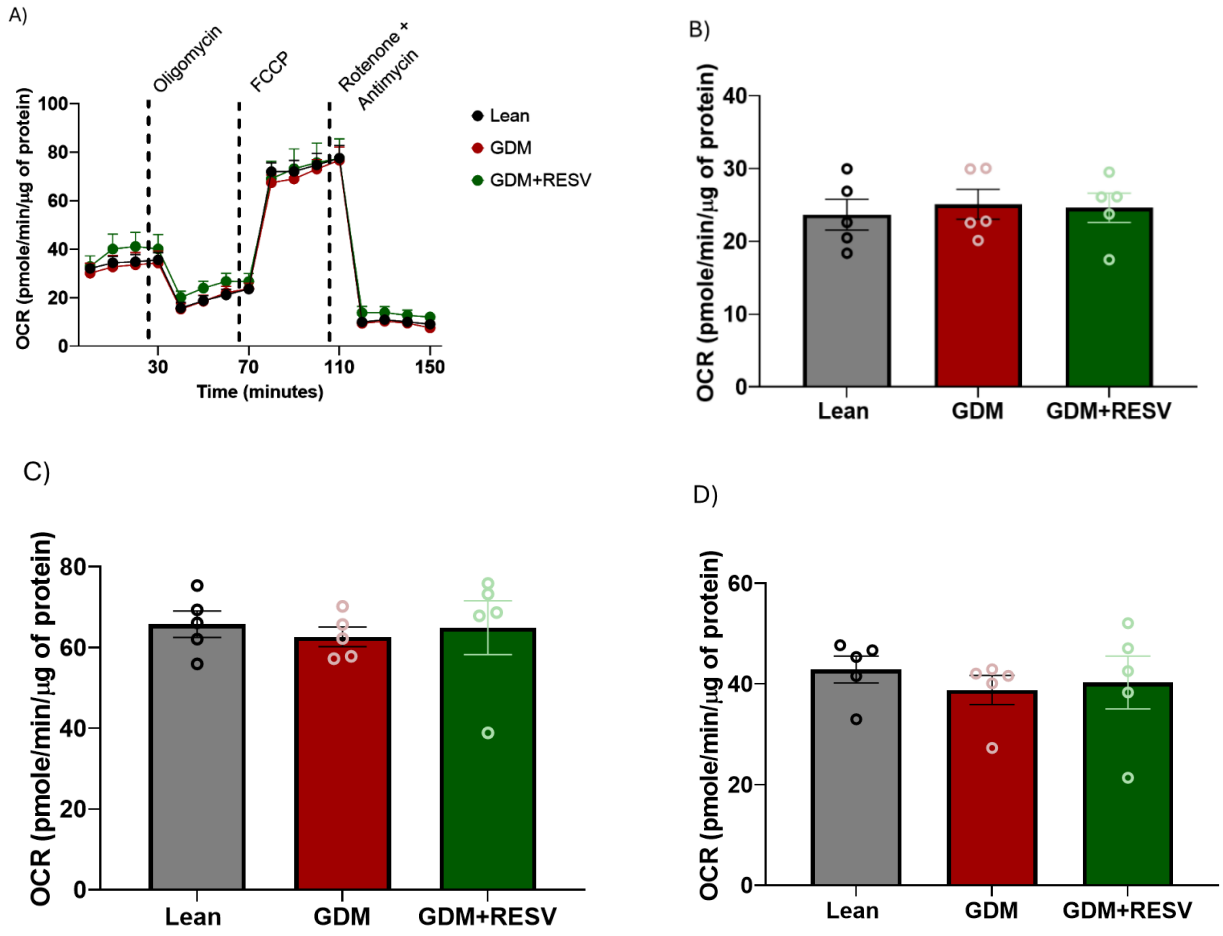
Together, these findings show that cardiomyocytes from Lean, GDM and GDM + RESV offspring exhibited decreased OCR traces when treated with etomoxir. This suggests that when CPT1 is inhibited, Lean and GDM + RESV cardiomyocytes exhibit a decreased maximal and basal respiration similar to calculated parameters from GDM cardiomyocytes when CPT1 is active (i.e., when cardiomyocytes are treated with vehicle control) suggesting that under the presence of fatty acids like palmitate, CPT1 could be a potential factor responsible for impaired mitochondrial function in cardiomyocytes isolated from GDM exposed offspring.

Figure 4.2 Cardiomyocyte Bioenergetics in the Presence of Palmitate



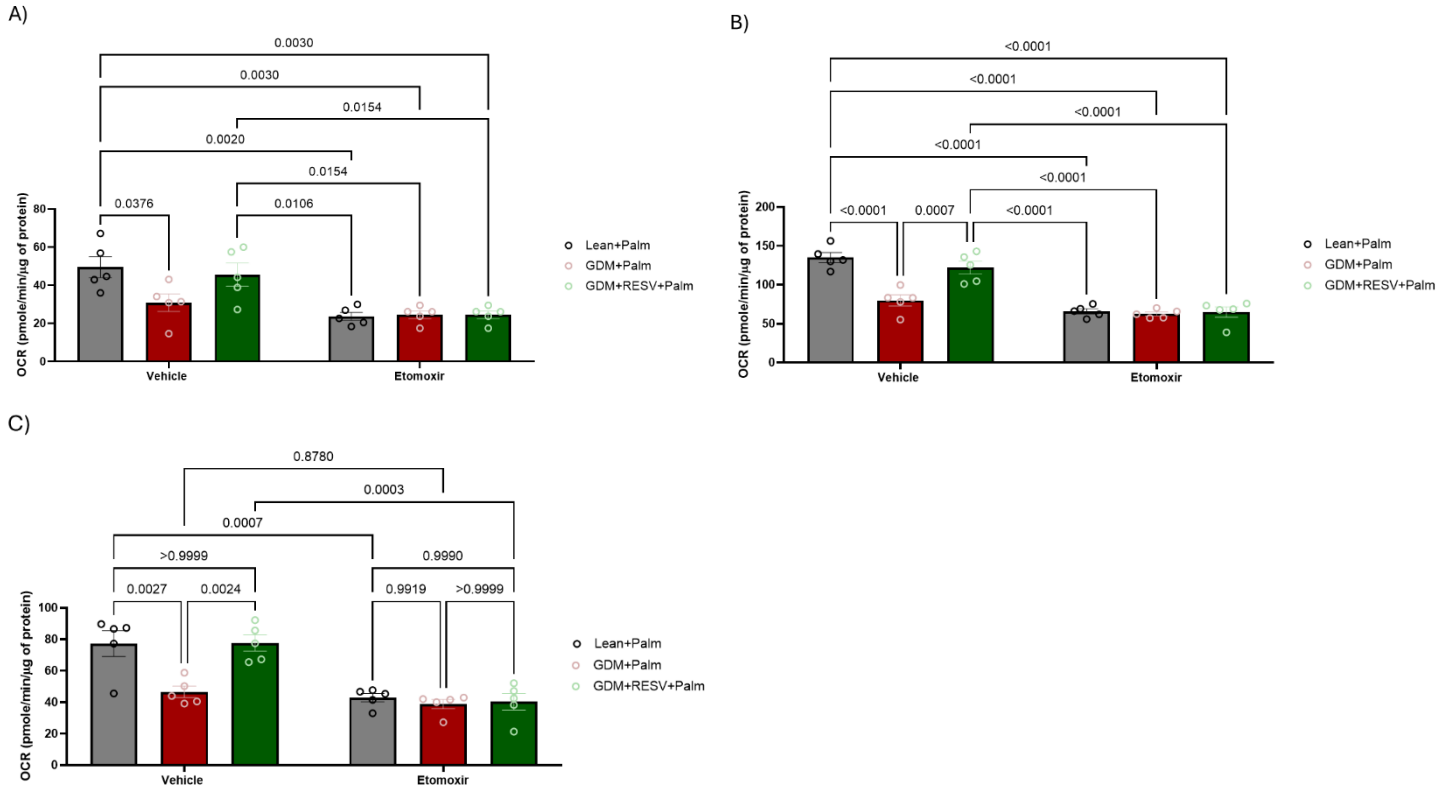
A) OCR over time in Lean (black), GDM (red), and GDM + RESV (green) cardiomyocytes in the presence of palmitate treated with vehicle. B) Calculated parameter of basal mitochondrial respiration. C) Calculated parameter of Maximal respiration. D) Calculated parameters of spare respiratory capacity. Readings normalized to protein concentration (ug). Data presented as mean +/- S.E.M, n=5 (where n is represented as 1 entire dam litter). P-values represent significance (<0.05) after one-way ANOVA with multiple comparisons.

Figure 4.3 Cardiomyocyte Bioenergetics in the Presence of Palmitate with Etomoxir



A) OCR over time in Lean (black), GDM (red), and GDM + RESV (green) cardiomyocytes in the presence of palmitate treated with etomoxir. B) Calculated parameter of basal mitochondrial respiration. C) Calculated parameter of Maximal respiration. Readings normalized to protein concentration (ug). D) Calculated parameters of spare respiratory capacity. Data presented as mean +/- S.E.M, n=5 (where n is represented as one entire dam litter). P-values represent significance (<0.05) after one-way ANOVA with multiple comparisons and Tukey post-Hoc test.

Figure 4.4 Comparison of Mitochondrial Respiration in the Presence or Absence of Etomoxir

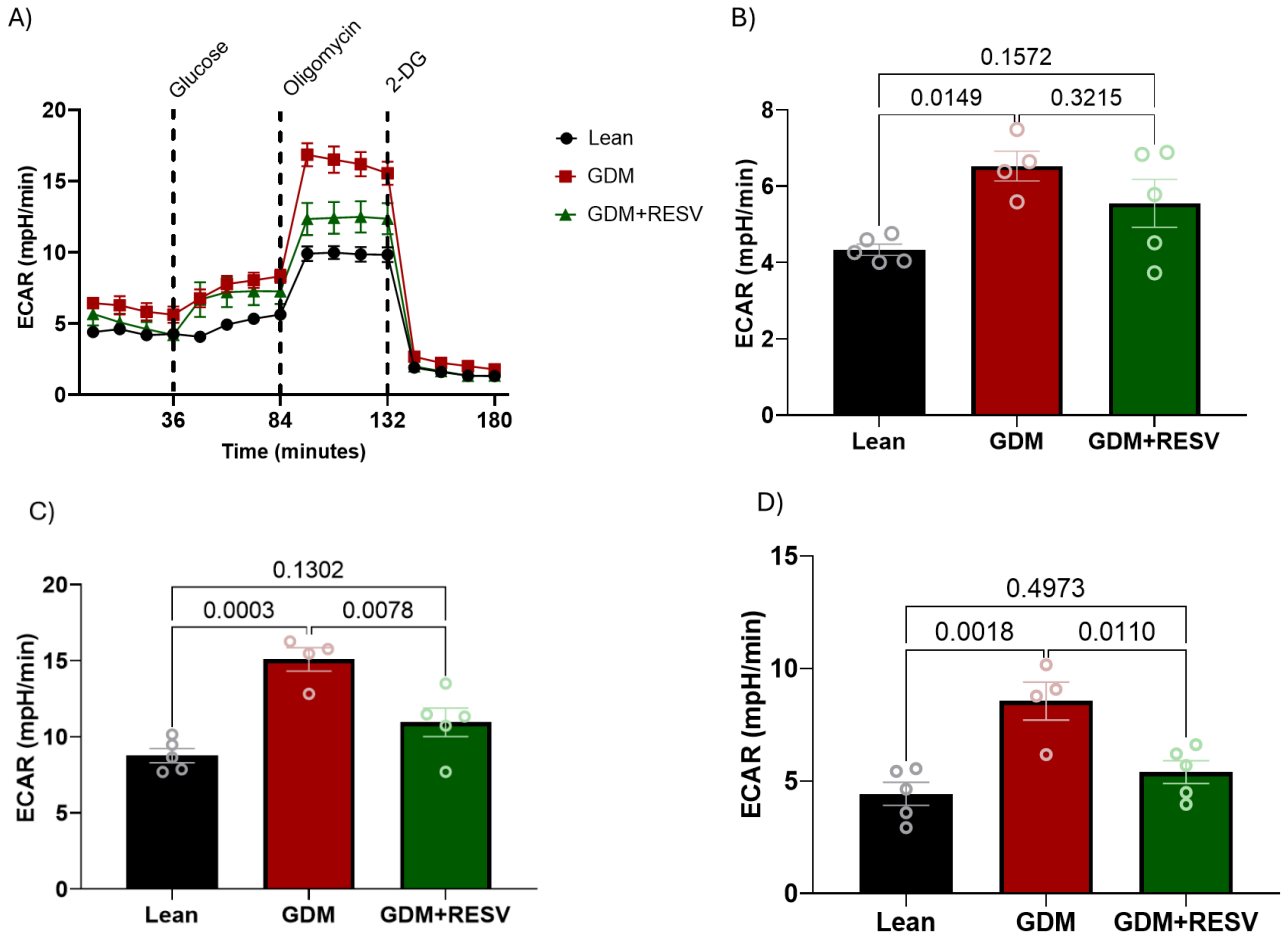


A) comparison of basal respiration between cardiomyocytes with or without the presence of etomoxir. B) comparison of maximal respiration between cardiomyocytes treated with or without etomoxir. C) comparison of spare respiratory capacity between cardiomyocytes with or without the presence of etomoxir. Readings normalized to protein concentration (ug). Data presented as mean +/- S.E.M, n=5 (where n is represented as one entire dam litter). P-values represent significance (<0.05) after two-way ANOVA with multiple comparisons and Tukey post-Hoc test.

4.5 Cardiomyocytes Energy Metabolism Under Glycolysis

To further investigate energy metabolism in fetal cardiomyocytes, we examined changes in glycolytic activity. To accomplish this, we isolated fetal cardiomyocytes from Lean, GDM, and GDM + RESV offspring and assessed glycolytic activity through a glycolysis stress test performed on the Agilent Seahorse XFe24 extracellular flux analyzer (XFe24). We examined the glycolytic activity of fetal cardiomyocytes in the presence of high glucose concentration. In the presence of a high glucose environment, fetal cardiomyocytes isolated from GDM offspring showed higher glycolytic activity (figure 4.5A). When compared to fetal cardiomyocytes from Lean offspring, GDM fetal cardiomyocytes showed a 1.5-fold higher glycolytic rate ($p=0.0149$) and a 1.2-fold higher glycolytic rate compared to GDM + RESV ($p=0.3215$) (figure 4.5B). When assessing glycolytic capacity, we found that when compared to Lean cardiomyocytes, GDM fetal cardiomyocytes displayed a 1.7-fold increase in glycolytic capacity ($p=0.0003$) and a 1.4-fold increase when compared to GDM + RESV cardiomyocytes ($p=0.0078$) (Fig 4.5C). Lastly, when evaluating glycolytic reserves, we found that GDM cardiomyocytes exhibited the highest glycolytic reserve, with a 1.9-fold higher glycolytic reserve compared to Lean ($p=0.0018$) and a 1.54-fold increase in glycolytic reserve compared to GDM + RESV ($p=0.0110$) (Fig 4.5D, $P<0.05$).

Figure 4.5 Cardiomyocyte Energy Metabolism Through Glycolysis



*(A) Extracellular Acidification Rate (ECAR) over time in fetal cardiomyocytes isolated from Lean (black), GDM (red), GDM + RESV (green). (B) Calculated parameter of glycolysis rate. (C) Calculated parameters of glycolytic capacity. (C) Calculated parameters of glycolytic reserve. Readings normalized to protein concentration (ug). Data presented as mean +/- S.E.M, (Lean n=5, GDM n=4, GDM+RESV n=5) (where n is represented as one entire dam litter). P-values represent significance (<0.05) after one-way ANOVA with multiple comparisons *p<0.05vs Lean, *p<0.05vs GDM+RESV.*

4.6 Discussion.

Mitochondria play a vital role in cellular energy production, which is especially important in the heart. Insufficient energy production can contribute to contractile dysfunction and eventually manifest as heart failure (Siasos et al., 2018). Using cardiomyocytes isolated from fetal offspring, we identified GDM-induced mitochondrial dysfunction through impaired glucose and fatty acid oxidation. Using the seahorse XFe24 analyzer, we were able to find that fetal GDM cardiomyocytes exhibited a decrease in mitochondrial oxygen consumption rate as evidence of lower oxidative phosphorylation and reduced ATP production. Moreover, the glycolysis stress test found that GDM fetal cardiomyocytes demonstrated an increase in glycolytic activity for ATP production. When combined with our mitostress test data, this indicates a metabolic shift from oxidative phosphorylation of substrates to an increase in glycolysis, which is less efficient for energy production (Bhullar & Dhalla, 2023). Alternatively, when we analyzed fetal cardiomyocytes from GDM dams receiving RESV supplementation, we noticed that, when compared to GDM, they exhibited higher oxidative phosphorylation and increased maximal and spare respiratory capacity in the presence of glucose and especially fatty acids substrate like palmitate. When analyzing the extracellular acidification through the glycolysis stress test, these cardiomyocytes were also less glycolytic active compared to cardiomyocytes from GDM offspring which could suggest that GDM + RESV cardiomyocytes would not rely on glycolysis for ATP production as mitochondrial oxidative phosphorylation is maintained.

These findings, alongside the findings in the previous chapter, demonstrate the effects of GDM exposure on offspring health. In our previous chapter, we showed that maternal GDM exposure altered offspring's cardiac structure as offspring exhibited left ventricular cardiac

hypertrophy. In this chapter, we demonstrated that GDM exposure altered cardiac energy metabolism and mitochondrial function within cardiomyocytes, which could serve as a driving factor towards cardiac dysfunction within the hearts of offspring.

Previous findings from our group have found similar GDM-induced alterations in fetal cardiac metabolism. Studies from Kereliuk. (2022) demonstrated that GDM-exposed fetal cardiomyocytes showed reduced glucose and fatty acid oxidation with reduced mitochondrial respiratory reserves (Kereliuk, 2022). Similarly, a study conducted by Mdaki et al. (2016) found that in their diabetes-exposed neonatal rat cardiomyocytes, there was a decrease in mitochondrial OCR, specifically, cardiomyocytes had lower basal, maximal and spare mitochondrial respiration (Mdaki et al., 2016).

During the early stages of pathological cardiac hypertrophy, the demand for ATP is increased in response to the increased mechanical stress on the heart (Rosca et al., 2012). Mitochondria have an important role here in providing sufficient energy for the larger cardiac muscle. To compensate mitochondrial biogenesis is upregulated to match the increased energy requirements of the hypertrophied heart (Rosca et al., 2012) additionally mitochondrial oxidative capacity can be preserved or even increased in the early stages of cardiac hypertrophy in order to reach the required energy demands (Rosca et al., 2012). As hypertrophy progresses to its decompensatory phase, mitochondrial function declines, and this decline is marked by a reduction in oxidative phosphorylation activity and impaired energy production that further contributes to the transition from compensated cardiac hypertrophy to decompensated heart failure (Rosca et al., 2012; Yang et al., 2022; Zhou & Tian, 2018). In this study, GDM may sensitize the cardiomyocytes of the offspring to the development of cardiac dysfunction through inadequate mitochondrial oxidative phosphorylation for ATP production.

In the previous chapter, we observed that maternal RESV supplementation during pregnancy prevented left ventricular cardiac hypertrophy in GDM offspring. Therefore, here we hypothesized that maternal RESV would improve mitochondrial oxidative phosphorylation and mitochondrial function in GDM-exposed fetal cardiomyocytes. Previous studies found that RESV improves mitochondrial function in the hearts of diabetic rats through the activation of the SIRT1-PGC-1a pathway, key regulators that are involved in mitochondrial function and energy metabolism (Fang et al., 2017). Additionally, RESV improved mitochondrial function in H9c2 cells cultured under high glucose conditions, which resulted from SIRT1 and PGC-1a activation promoting mitochondrial biogenesis and fatty acid metabolism (Fang et al., 2017). Studies from Bastin et al. (2011) have also demonstrated that RESV enhanced fatty acid oxidation in human fatty acid oxidation-deficient fibroblast through the activation of SIRT1 and PGC-1a pathways (Bastin et al., 2011). Our findings are consistent with these findings, illustrating that RESV increased glucose and fat oxidation in fetal cardiomyocytes.

To our knowledge, this is one of the first studies to examine RESV and its impact on glycolysis, especially within cardiomyocytes. However, based on our data and given that RESV enhanced more efficient oxidative phosphorylation, this would shift energy metabolism away from reliance on glycolysis and warrant further studies.

In this chapter, we noticed differences in mitochondrial energy metabolism and mitochondrial respiration. We first noticed a difference in oxidative phosphorylation within cardiomyocytes from GDM + RESV offspring, when compared to GDM, these cardiomyocytes had increased levels of mitochondrial respiration and increased maximal and spare respiratory reserves in the presence of substrates like glucose and, more importantly, fatty acids like palmitate. This maintained oxidative phosphorylation could potentially explain the decrease in

reliance for glycolysis that we see through our glycolysis stress test as GDM + RESV cardiomyocytes had lower levels of glycolytic capacity and glycolytic reserves than GDM cardiomyocytes.

Collectively, these findings showed that maternal RESV supplementation taken during the third trimester of GDM improved mitochondrial function within GDM-exposed fetal cardiomyocytes, thereby maintaining mitochondrial activity and ATP production that is sufficient to meet the required demands of the heart without the reliance on anaerobic pathways like glycolysis. This could potentially prevent early-onset cardiac hypertrophy in GDM-exposed offspring.

Chapter 5: Discussion

General Discussion and Conclusion

Previous studies have shown that GDM predisposes offspring to cardiovascular complications and elevates the risk of cardiovascular disease development (Benham et al., 2021; Guillemette et al., 2020). With the ongoing obesity epidemic, increasing numbers of women of childbearing age are overweight, and as a consequence of this major risk factor, the incidence of GDM will only continue to rise (Eades et al., 2024; Nelson et al., 2024). Therefore, it is important to identify new treatments for GDM and understand their implications for the long-term health of the offspring.

RESV is a natural polyphenol compound found in fruits and vegetables which has been extensively studied for its health benefits in various diseases and different animal models (Diaz-Gervinin et al., 2016; Berman et al., 2017; Pezzuto., 2018). Animal studies using RESV have been shown to be safe and well-tolerated during pregnancy (Williams et al., 2009). Studies utilizing pregnant C57BL/*KsJ-Lep*$^{db/+}$ mice have found that maternal RESV improved glucose metabolism and alleviated hyperglycemia in the mother (Yao et al., 2015), and while it has been reported that RESV can cross the placenta, it does not cause fetal loss or fetal malformations. (Bourque et al., 2012; Singh et al., 2013; Williams et al., 2009; Brawerman & Dolinsky, 2018).

In this study, we examined how supplementing the maternal diet at the start of the third trimester with RESV, which is the onset of hyperglycemia during pregnancy, affects cardiovascular health in the offspring. To accomplish this, we used a previously established diet-induced GDM animal model from our lab that mimics the phenotypic characteristics seen in mothers with GDM (Pereira et al., 2015). Previously, our lab and others showed that maternal RESV improved glucose tolerance and alleviated hyperglycemia within the context of diabetes

during pregnancy (Brawerman et al., 2019; Yao et al., 2015; Zheng et al., 2018). Previous work from our group have found that maternal RESV supplementation also attenuated GDM-induced hepatic steatosis and improved insulin secretion (Brawerman et al., 2019). One key thing to note based on previous studies is the timing and dosage of RESV varies across studies. Studies from Yao et al. (2015) incorporated RESV via oral gavage at a dose of 10mg/kg body weight/day 4 weeks prior to pregnancy and 20 days during pregnancy (Yao et al., 2015). Studies from Zou et al. (2016) supplemented RESV in high-fat diets of C57BL/6J mice at a dosage of 0.2% RESV, which was equivalent to 200mg/kg body weight/day (Zou et al., 2016). Studies by Roberts et al. (2014) using nonhuman primates utilized a Western-style diet supplemented with 0.37% RESV throughout pregnancy (Roberts et al., 2014). Ros et al. (2018) group utilized RESV in Wistar rats during pregnancy and lactation at a dosage of 2.0-2.5mg/kg body weight/day in drinking water (Ros et al., 2018). The study by Ros et al. supplemented the low-fat diet of pregnant and lactating dams with RESV and showed minimal effects on glucose homeostasis in the offspring (Ros et al., 2018). Therefore, in our study, we did not incorporate a pregnant low-fat diet group supplemented with RESV. Studies from our group conducted by Brawerman et al. (2019) introduced RESV in the maternal high fat and sucrose diet through supplementation at a dosage of ~147 mg/kg body weight/day only during the third trimester of pregnancy in female Sprague Dawley rats, and they were able to see changes within the offspring within a narrow window of treatment. (Brawerman et al., 2019). Likewise, findings from our study demonstrated that we were able to observe the effects of maternal RESV supplementation on fetal offspring within a small window of therapy (during the third trimester of pregnancy), which is critical within the context of therapeutics when designing an appropriate strategy that reduces chances of potential adverse effects. To our knowledge, this is the first study that incorporates RESV supplementation

in the maternal diet only during the final third of pregnancy and examines cardiovascular outcomes in the offspring.

In Chapter 3, we replicated our previous findings from our lab conducted by Kereliuk. (2022) that showed that GDM-exposed offspring exhibit left ventricular cardiac hypertrophy during the fetal stage of development (Kereliuk, 2022). Our studies replicate similar findings from our previous group, demonstrating a form of concentric cardiac hypertrophy developing within GDM-exposed offspring. LVH is characterized by the thickening of the walls of the left ventricle, including both the interventricular septum and the posterior wall. Although LVH can arise from various causes, research by Al-Biltagi et al. (2021) highlights maternal hyperglycemia during pregnancy as a significant risk factor for the subsequent development of LVH in the offspring (Al-Biltagi et al., 2021). This maladaptive form of hypertrophy is critical as it can significantly impair heart function, which can lead to several consequences and increase the risk of the development of further cardiovascular complications (Skovsgaard et al., 2024; Selvetella et al., 2004). Previous animal studies in the literature have demonstrated the potential of RESV in alleviating cardiac hypertrophy within the context of diabetes (Fang et al., 2018; Wang et al., 2018). In our study, we were able to demonstrate the protective effects of maternal RESV supplementation taken during the third trimester of pregnancy to alleviate GDM-induced cardiac hypertrophy seen in the offspring.

Alterations in cardiac structure, such as concentric cardiac hypertrophy, can contribute towards impairments of calcium handling and calcium flux in the heart that can impact both contraction and relaxation processes (Balke & Shorofsky 1998). Previous findings from our group found that GDM-exposed fetal cardiomyocytes exhibit delayed and extended calcium flux cycles (Kereliuk, 2022). RESV has been reported to enhance the function of sarco-/endoplasmic

reticulum calcium ATPase (SERCA), a vital component in sequestering calcium back into the sarcoplasmic reticulum (SR), resulting in improved calcium dynamics and better contractile function in cardiomyocytes (Delucchi et al., 2012). Therefore, we wanted to examine alterations in calcium cycles within isolated cardiomyocytes from fetal offspring and determine whether RESV might have any effect on calcium flux. Findings from our calcium data demonstrated that cardiomyocytes from GDM offspring exhibit delayed basal flux cycles when compared to cardiomyocytes of Lean offspring. Decay time and release time were longer in GDM-exposed cardiomyocytes. When we examined cardiomyocytes from isolated fetal cardiomyocytes from GDM offspring under maternal RESV supplementation, we found no significant differences during our baseline basal recordings however, with the introduction to angiotensin II, a stressor used to increase cardiomyocyte beat frequency, we noticed that these fetal cardiomyocytes were more responsive to the stressor. It could be speculated that while we may not see alterations under basal conditions, maternal RESV supplementation may have protective effects on fetal cardiomyocytes by enhancing their ability to respond better to stressors like angiotensin II. However, further research is required. Previous work from our group demonstrated that exposure to GDM impaired calcium handling in fetal rat cardiomyocytes. Although previous work was performed using the ratiometric calcium dye Fura-2 on the Olympus IX-70, we were able to replicate these findings using a simpler experimental setup. Since Fluo-4 provides brighter fluorescence emission when excited at 488 nm, we can acquire stronger signal detection using lower concentrations of dye. Additionally, fluo-4 provides a better signal-to-background ratio compared to Fura-2, making it a viable candidate for detecting calcium changes in fetal cardiomyocytes. Since we also conducted our experiments using Agilent's BioTek Cytation 5, the single-wavelength indicator provided by Fluo-4 makes it easier to incorporate and implement

in the Cytation 5 system. Additionally, the use of the Cytation 5 allows us to capture images at 10 frames per second, allowing us to assess fast calcium kinetics. When coupled with the auxiliary injectors, we can immediately monitor changes in calcium kinetics in response to stressors like angiotensin II. However, some limitations to consider are unlike Fluo-4, the ratiometric nature of Fura-2 allows for more precise quantification of Ca^{2+} levels. It is also important to note that a preset ROI was used to capture our readings and assess our calcium flux parameters for each experimental run. However, the ROI between each experimental run was kept consistent between groups and experimental replicates.

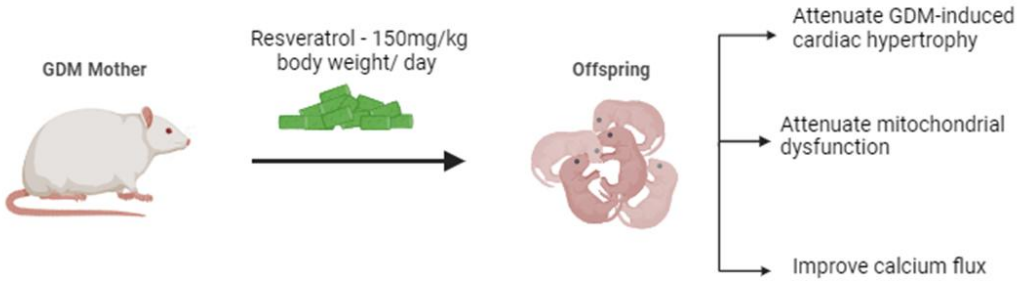
In Chapter 4, we found that maternal RESV supplementation improved mitochondrial respiration in isolated fetal cardiomyocytes exposed to GDM. GDM-exposed fetal cardiomyocytes demonstrated a decrease in fatty acid and glucose oxidation. These findings are consistent with previous findings generated by our group (Kereliuk, 2022). One study conducted by Yan et al. (2024) also demonstrated that short-term intrauterine hyperglycemia during late gestation caused a decrease in offspring mitochondrial oxygen consumption (Yan et al., 2024). However, what was interesting to note from our study was that the GDM+RESV offspring group showed improved glucose and fatty acid oxidation with a corresponding reduction in glycolysis. Previous studies using RESV found that it improved diabetes-induced mitochondrial dysfunction and biogenesis in diabetic rats (Fang et al., 2018). Studies from Csiszar et al. (2009) also demonstrated that RESV treatment normalized impaired mitochondrial biogenesis in the aortas of type 2 diabetic mice (Csiszar et al., 2009). While various studies examined the effects of RESV within the context of diabetes, our study provides new knowledge about the effects of RESV on offspring outcomes within the context of GDM. Through the use of the Seahorse XF analyzer, we were able to capture comprehensive measures of crucial parameters of

mitochondrial function such as basal respiration, maximal respiration and spare respiratory capacity. Next, we assessed mitochondrial respiration under substrates such as glucose and fatty acids, which is useful in understanding the metabolic characteristics of fetal cardiomyocytes. However, since we only assessed mitochondrial function on a mixed set of male and female isolated fetal cardiomyocytes, it is important to address our limitation in being able to evaluate sex-specific differences. The need for a substantial quantity of fetal rat hearts required to obtain a sufficient yield of cardiomyocytes for an experimental run (an entire litter typically consisting of 15-20 rat pups), coupled with the difficulty of determining sex in fetal rat pups, presents a challenge in the ability to incorporate sex as a variable in our experimental design. Previous studies from Louwagie et al. (2020) have demonstrated sex-based differences in mitochondrial bioenergetics within isolated cardiomyocytes from diabetes-exposed offspring (Louwagie et al., 2020) therefore, it warrants further studies to determine if the effects of maternal RESV supplementation are influenced by sex.

To our knowledge, this study was the first to investigate whether maternal RESV supplementation during the third trimester of pregnancy would help improve calcium flux cycles within cardiomyocytes isolated from the fetal offspring. Our central hypothesis was that maternal RESV supplementation introduced in the third trimester of gestational diabetes pregnancy would improve the offspring's cardiovascular outcomes (Fig 5.1). Specifically, we aimed: 1. To determine if the effects of maternal RESV supplementation GDM would attenuate GDM-induced cardiac hypertrophy and improve calcium flux cycles seen in the offspring; 2. Determine whether maternal RESV supplementation would improve mitochondrial function seen in GDM-exposed fetal cardiomyocytes. Findings from our experiments have shown that maternal RESV improved mitochondrial respiration slightly in glucose oxidation and, more importantly, fatty acid

oxidation and attenuated GDM-induced left ventricular cardiac hypertrophy. While we did not see any changes toward basal calcium flux, we did notice that fetal cardiomyocytes isolated from maternal RESV dams exhibited improved responsiveness to stressors like angiotensin II. Additionally, we found maternal RESV to be safe during pregnancy, as no alterations in litter size and litter numbers were observed. Since offspring exposed to GDM are at higher risk of cardiometabolic disease, our findings may suggest that RESV supplementation during the third trimester of pregnancy may help in preventing the development of cardiometabolic disorders.

Figure 5.1 Theoretical Framework of Maternal RESV Supplementation and Offspring Cardiovascular Outcomes



Exposure to GDM in utero can cause alterations in fetal cardiomyocyte metabolism that lead to impairments in mitochondrial function and cardiac energy metabolism this can result in impaired calcium flux and can contribute to the development of cardiac hypertrophy in fetal offspring. We propose that the incorporation of RESV through supplementation in the maternal diet during the third trimester of pregnancy will improve mitochondrial function and calcium flux within cardiomyocytes, thereby attenuating cardiac hypertrophy.

References

Adams, R. J., & Schwartz, A. (1980). Comparative mechanisms for contraction of cardiac and skeletal muscle. *Chest*, *78*(1), 123-139.

Ainuddin, J. A., Karim, N., Zaheer, S., Ali, S. S., & Hasan, A. A. (2015). Metformin treatment in type 2 diabetes in pregnancy: an actively controlled, parallel-group, randomized, open-label study in patients with type 2 diabetes in pregnancy. *Journal of diabetes research*, *2015*(1), 325851.

Al-Biltagi, M., & El Amrousy, D. (2021). Cardiac changes in infants of diabetic mothers. *World Journal of Diabetes*, *12*(8), 1233.

Balke, C. W., & Shorofsky, S. R. (1998). Alterations in calcium handling in cardiac hypertrophy and heart failure. *Cardiovascular research*, *37*(2), 290-299.

Barbour, L. A., Shao, J., Qiao, L., Pulawa, L. K., Jensen, D. R., Bartke, A., ... & Friedman, J. E. (2002). Human placental growth hormone causes severe insulin resistance in transgenic mice. *American journal of obstetrics and gynecology*, *186*(3), 512-517.

Barker, D. J., Godfrey, K. M., Gluckman, P. D., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *The Lancet*, *341*(8850), 938-941.

Barker, D. J. (2007). The origins of the developmental origins theory. *Journal of internal medicine*, *261*(5), 412-417.

Bastin, J., Lopes-Costa, A., & Djouadi, F. (2011). Exposure to resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient fibroblasts. *Human molecular genetics*, *20*(10), 2048-2057.

Beer, M., Seyfarth, T., Sandstede, J., Landschütz, W., Lipke, C., Köstler, H., ... & Neubauer, S. (2002). Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with 31P-SLOOP magnetic resonance spectroscopy. *Journal of the American College of Cardiology*, *40*(7), 1267-1274.

Benham, J. L., Booth, J. E., Donovan, L. E., Leung, A. A., Sigal, R. J., & Rabi, D. M. (2021). Prevalence of and risk factors for excess weight gain in pregnancy: a cross-sectional study using survey data. *Canadian Medical Association Open Access Journal*, *9*(4), E1168-E1174.

Bhullar, S. K., & Dhalla, N. S. (2023). Status of mitochondrial oxidative phosphorylation during the development of heart failure. *Antioxidants*, *12*(11), 1941.

Bergel, R., Hadar, E., Toledano, Y., & Hod, M. (2016). Pharmacological management of gestational diabetes mellitus. *Current diabetes reports*, *16*, 1-9.

Bertrand, L., Horman, S., Beauloye, C., & Vanoverschelde, J. L. (2008). Insulin signalling in the heart. *Cardiovascular research*, *79*(2), 238-248.

Bianco, M. E., & Josefson, J. L. (2019). Hyperglycemia during pregnancy and long-term offspring outcomes. *Current diabetes reports*, *19*, 1-8.

Bouchghoul, H., Alvarez, J. C., Verstuyft, C., Bouyer, J., & Senat, M. V. (2020). Transplacental transfer of glyburide in women with gestational diabetes and neonatal hypoglycemia risk. *Plos one*, *15*(5), e0232002.

Bourque, S. L., Dolinsky, V. W., Dyck, J. R. B., & Davidge, S. T. (2012). Maternal resveratrol treatment during pregnancy improves adverse fetal outcomes in a rat model of severe hypoxia. *Placenta*, *33*(5), 449-452.

Boyd III, A. E., Aguilar-Bryan, L., & Nelson, D. A. (1990). Molecular mechanisms of action of glyburide on the beta cell. *The American journal of medicine*, *89*(2), S3-S10.

Brand, K. M., Saarelainen, L., Sonajalg, J., Boutmy, E., Foch, C., Vääräsmäki, M., ... & CLUE Study Group. (2022). Metformin in pregnancy and risk of adverse long-term outcomes: a register-based cohort study. *BMJ Open Diabetes Research and Care*, *10*(1), e002363.

Brawerman, G. M., & Dolinsky, V. W. (2018). Therapies for gestational diabetes and their implications for maternal and offspring health: evidence from human and animal studies. *Pharmacological Research*, *130*, 52-73.

Brawerman, G. M., Kereliuk, S. M., Brar, N., Cole, L. K., Seshadri, N., Pereira, T. J., ... & Dolinsky, V. W. (2019). Maternal resveratrol administration protects against gestational diabetes-induced glucose intolerance and islet dysfunction in the rat offspring. *The Journal of physiology*, *597*(16), 4175-4192.

Brelje, T. C., Scharp, D. W., Lacy, P. E., Ogren, L., Talamantes, F. R. A. N. K., Robertson, M., ... & Sorenson, R. L. (1993). Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets:

implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology*, *132*(2), 879-887.

Brite, J., Laughon, S. K., Troendle, J., & Mills, J. (2014). Maternal overweight and obesity and risk of congenital heart defects in offspring. *International journal of obesity*, *38*(6), 878-882.

Brown, D. A., Perry, J. B., Allen, M. E., Sabbah, H. N., Stauffer, B. L., Shaikh, S. R., ... & Gheorghiade, M. (2016). Mitochondrial function as a therapeutic target in heart failure. *Nature Reviews Cardiology*, *14*(4), 238-250.

Castillo, W. C., Boggess, K., Stürmer, T., Brookhart, M. A., Benjamin, D. K., & Funk, M. J. (2015). Association of adverse pregnancy outcomes with glyburide vs insulin in women with gestational diabetes. *JAMA pediatrics*, *169*(5), 452-458.

Catalano, P. M., Huston, L., Amini, S. B., & Kalhan, S. C. (1999). Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus. *American journal of obstetrics and gynecology*, *180*(4), 903-916.

Charles, B., Norris, R., Xiao, X., & Hague, W. (2006). Population pharmacokinetics of metformin in late pregnancy. *Therapeutic drug monitoring*, *28*(1), 67-72.

Conway, D. L., Gonzales, O., & Skiver, D. (2004). Use of glyburide for the treatment of gestational diabetes: the San Antonio experience. *The Journal of Maternal-Fetal & Neonatal Medicine*, *15*(1), 51-55.

Crispi, F., Bijnens, B., Figueras, F., Bartrons, J., Eixarch, E., Le Noble, F., ... & Gratacós, E. (2010). Fetal growth restriction results in remodeled and less efficient hearts in children. *Circulation*, *121*(22), 2427-2436.

Csiszar, A., Labinskyy, N., Pinto, J. T., Ballabh, P., Zhang, H., Losonczy, G., ... & Ungvari, Z. (2009). Resveratrol induces mitochondrial biogenesis in endothelial cells. *American Journal of Physiology-Heart and Circulatory Physiology*, *297*(1), H13-H20.

Deepaklal, M. C., Joseph, K., Rekha, K., & Nandita, T. (2015). Insulin aspart in patients with gestational diabetes mellitus and pregestational diabetes mellitus. *Indian journal of endocrinology and metabolism*, *19*(5), 658-662.

Delucchi, F., Berni, R., Frati, C., Cavalli, S., Graiani, G., Sala, R., ... & Stilli, D. (2012). Resveratrol treatment reduces cardiac progenitor cell dysfunction and prevents morpho-functional ventricular remodeling in type-1 diabetic rats. *PloS one*, *7*(6), e39836.

Delva, E., Tucker, D. K., & Kowalczyk, A. P. (2009). The desmosome. *Cold Spring Harbor perspectives in biology*, *1*(2), a002543.

De Rosa, S., Arcidiacono, B., Chiefari, E., Brunetti, A., Indolfi, C., & Foti, D. P. (2018). Type 2 diabetes mellitus and cardiovascular disease: genetic and epigenetic links. *Frontiers in endocrinology*, *9*, 2.

Desai, M., Jellyman, J. K., Han, G., Beall, M., Lane, R. H., & Ross, M. G. (2014). Maternal obesity and high-fat diet program offspring metabolic syndrome. *American journal of obstetrics and gynecology*, *211*(3), 237-e1.

Diaz-Gerevini, G. T., Repossi, G., Dain, A., Tarres, M. C., Das, U. N., & Eynard, A. R. (2016). Beneficial action of resveratrol: How and why?. *Nutrition*, *32*(2), 174-178.

Di Bernardo, S. C., Lava, S. A., Epure, A. M., Younes, S. E., Chiolero, A., & Sekarski, N. (2023). Consequences of gestational diabetes mellitus on neonatal cardiovascular health: MySweetHeart Cohort study. *Pediatric research*, *94*(1), 231-238.

Di Cianni, G., Miccoli, R., Volpe, L., Lencioni, C., & Del Prato, S. (2003). Intermediate metabolism in normal pregnancy and in gestational diabetes. *Diabetes/metabolism research and reviews*, *19*(4), 259-270.

Do, V., Al-Hashmi, H., Ojala, T., Jain, V., Colen, T., Goncalvez-Alvarez, S., ... & Hornberger, L. K. (2019). Cardiovascular health of offspring of diabetic mothers from the fetal through late-infancy stages. *JACC: Cardiovascular Imaging*, *12*(5), 932-934.

Do, V., Eckersley, L., Lin, L., Davidge, S. T., Stickland, M. K., Ojala, T., ... & Hornberger, L. K. (2021). Persistent aortic stiffness and left ventricular hypertrophy in children of diabetic mothers. *CJC open*, *3*(3), 345-353.

Dolinsky, V. W., Chakrabarti, S., Pereira, T. J., Oka, T., Levasseur, J., Beker, D., ... & Dyck, J. R. (2013). Resveratrol prevents hypertension and cardiac hypertrophy in hypertensive rats and mice. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1832*(10), 1723-1733.

Eades, C. E., Burrows, K. A., Andreeva, R., Stansfield, D. R., & Evans, J. M. (2024). Prevalence of gestational diabetes in the United States and Canada: a systematic review and meta-analysis. *BMC Pregnancy and Childbirth*, *24*(1), 204.

Eisner, D. A., Caldwell, J. L., Kistamás, K., & Trafford, A. W. (2017). Calcium and excitation-contraction coupling in the heart. *Circulation research*, *121*(2), 181-195.

Eroglu, T., Capone, F., & Schiattarella, G. G. (2024). The evolving landscape of cardiometabolic diseases. *EBioMedicine*, *109,* 105447.

Falcão-Pires, I., & Leite-Moreira, A. F. (2012). Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. *Heart failure reviews*, *17*, 325-344.

Fang, W. J., Wang, C. J., He, Y., Zhou, Y. L., Peng, X. D., & Liu, S. K. (2018). Resveratrol alleviates diabetic cardiomyopathy in rats by improving mitochondrial function through PGC-1α deacetylation. *Acta Pharmacologica Sinica*, *39*(1), 59-73.

Feghali, M., Atlass, J., Abebe, K. Z., Comer, D., Catov, J., Caritis, S., ... & Scifres, C. (2020). Treatment of gestational diabetes mellitus and offspring early childhood growth. *The Journal of Clinical Endocrinology & Metabolism*, *106*(4), 1849-1858.

Feig, D. S., Berger, H., Donovan, L., Godbout, A., Kader, T., Keely, E., & Sanghera, R. (2018). Diabète et grossesse. *Can J Diabetes*, *42*(3), S255-82.

Feig, D. S., Donovan, L. E., Zinman, B., Sanchez, J. J., Asztalos, E., Ryan, E. A., ... & Newstead-Angel, J. (2020). Metformin in women with type 2 diabetes in pregnancy (MiTy): a multicentre, international, randomised, placebo-controlled trial. *The lancet Diabetes & endocrinology*, *8*(10), 834-844.

Feig, D. S., Zinman, B., Asztalos, E., Donovan, L. E., Shah, P. S., Sanchez, J. J., ... & Murphy, K. E. (2022). Determinants of small for gestational age in women with type 2 diabetes in pregnancy: who should receive metformin?. *Diabetes Care*, *45*(7), 1532-1539.

Foretz, M., Guigas, B., & Viollet, B. (2019). Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus. *Nature Reviews Endocrinology*, *15*(10), 569-589.

Gál, R., Halmosi, R., Gallyas Jr, F., Tschida, M., Mutirangura, P., Tóth, K., ... & Czopf, L. (2023). Resveratrol and beyond: The effect of natural polyphenols on the cardiovascular system: A narrative review. *Biomedicines*, *11*(11), 2888.

Gal, R., Deres, L., Toth, K., Halmosi, R., & Habon, T. (2021). The effect of resveratrol on the cardiovascular system from molecular mechanisms to clinical results. *International journal of molecular sciences*, *22*(18), 10152.

Ghouse, J., Hansson, M., Vøgg, R. O. B., Sillesen, A. S., Pærregaard, S., Raja, A. A., ... & Boyd, H. A. (2024). Maternal Diabetes and Cardiac Left Ventricular Structure and Function in the Infant: A Copenhagen Baby Heart Study. *Diabetes Care*, *47*(12), 2230-2238.

Gilbert, G., Demydenko, K., Dries, E., Puertas, R. D., Jin, X., Sipido, K., & Roderick, H. L. (2020). Calcium signaling in cardiomyocyte function. *Cold Spring Harbor perspectives in biology*, *12*(3), a035428.

Golob, M., Moss, R. L., & Chesler, N. C. (2014). Cardiac tissue structure, properties, and performance: a materials science perspective. *Annals of biomedical engineering*, *42*, 2003-2013.

Grupe, K., & Scherneck, S. (2023). Mouse models of gestational diabetes mellitus and its subtypes: recent insights and pitfalls. *International Journal of Molecular Sciences*, *24*(6), 5982.

Guillemette, L., Wicklow, B., Sellers, E. A., Dart, A., Shen, G. X., Dolinsky, V. W., ... & McGavock, J. (2020). Intrauterine exposure to diabetes and risk of cardiovascular disease in adolescence and early adulthood: a population-based birth cohort study. *CMAJ*, *192*(39), E1104-E1113.

Gunton, J. E., Delhanty, P. J., Takahashi, S. I., & Baxter, R. C. (2003). Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. *The Journal of Clinical Endocrinology & Metabolism*, *88*(3), 1323-1332.

Guo, F., Moellering, D. R., & Garvey, W. T. (2014). The progression of cardiometabolic disease: validation of a new cardiometabolic disease staging system applicable to obesity. *Obesity*, *22*(1), 110-118.

Hardin, M. D., & Jacobs, T. F. (2019). Glyburide.

He, Y., Wu, N., Yu, W., Li, L., OuYang, H., Liu, X., ... & Al-Mureish, A. (2020). Research progress on the experimental animal model of gestational diabetes mellitus. *Diabetes, Metabolic Syndrome and Obesity*, 4235-4247.

He, K., Guo, Q., Ge, J., Li, J., Li, C., & Jing, Z. (2022). The efficacy and safety of metformin alone or as an add-on therapy to insulin in pregnancy with GDM or T2DM: A systematic review and meta-analysis of 21 randomized controlled trials. *Journal of Clinical Pharmacy and Therapeutics*, *47*(2), 168-177.

Hedderson, M. M., Badon, S. E., Pimentel, N., Xu, F., Regenstein, A., Ferrara, A., & Neugebauer, R. (2022). Association of glyburide and subcutaneous insulin with perinatal complications among women with gestational diabetes. *JAMA Network Open*, *5*(3), e225026-e225026.

Holemans, K., Caluwaerts, S., Verhaeghe, J., & Van Assche, F. A. (2004). Diet-induced obesity in the rat: A model for gestational diabetes mellitus. *Pediatric research*, *53*(6), 42..

Hong, T., & Shaw, R. M. (2017). Cardiac T-tubule microanatomy and function. *Physiological reviews*, *97*(1), 227-252.

Howell, C. R., Zhang, L., Mehta, T., Wilkinson, L., Carson, A. P., Levitan, E. B., ... & Garvey, W. T. (2024). Cardiometabolic disease staging and major adverse cardiovascular event prediction in 2 prospective cohorts. *JACC: Advances*, *3*(4), 100868.

Huang, C., Snider, F., & Cross, J. C. (2009). Prolactin receptor is required for normal glucose homeostasis and modulation of β-cell mass during pregnancy. *Endocrinology*, *150*(4), 1618-1626.

Huo, J. L., Feng, Q., Pan, S., Fu, W. J., Liu, Z., & Liu, Z. (2023). Diabetic cardiomyopathy: Early diagnostic biomarkers, pathogenetic mechanisms, and therapeutic interventions. *Cell death discovery*, *9*(1), 256.

Huss, J. M., & Kelly, D. P. (2005). Mitochondrial energy metabolism in heart failure: a question of balance. *The Journal of clinical investigation*, *115*(3), 547-555.

Inzani, I., & Ozanne, S. E. (2022). Programming by maternal obesity: a pathway to poor cardiometabolic health in the offspring. *Proceedings of the Nutrition Society*, *81*(3), 227-242.

Iside, C., Scafuro, M., Nebbioso, A., & Altucci, L. (2020). SIRT1 activation by natural phytochemicals: an overview. *Frontiers in pharmacology*, *11*, 1225.

Jawerbaum, A., & White, V. (2010). Animal models in diabetes and pregnancy. *Endocrine Reviews*, *31*(5), 680-701.

Jia, G., Hill, M. A., & Sowers, J. R. (2018). Diabetic cardiomyopathy: an update of mechanisms contributing to this clinical entity. *Circulation research*, *122*(4), 624-638.

Kelley, K. W., Carroll, D. G., & Meyer, A. (2015). A review of current treatment strategies for gestational diabetes mellitus. *Drugs in context*, *4,* 212282.

Kereliuk, S. M. (2022). Early life exposure to gestational diabetes mellitus induces cardiometabolic disease development in the rat offspring [Unpublished doctoral thesis].

Kereliuk, S. M., & Dolinsky, V. W. (2022). Recent experimental studies of maternal obesity, diabetes during pregnancy and the developmental origins of cardiovascular disease. *International Journal of Molecular Sciences*, *23*(8), 4467.

Khan, A. R., Salama, A. H., Aleem, Z., Alfakeer, H., Alnemr, L., & Shareef, A. M. M. (2023). The promising frontier of cardiometabolic syndrome: a new paradigm in cardiology. *Cureus*, *15*(9), e45542.

Khan, I. Y., Dekou, V., Douglas, G., Jensen, R., Hanson, M. A., Poston, L., & Taylor, P. D. (2005). A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *288*(1), R127-R133.

Kim, W., Park, S. K., & Kim, Y. L. (2019). Gestational diabetes mellitus diagnosed at 24 to 28 weeks of gestation in older and obese Women: Is it too late?. *PloS one*, *14*(12), e0225955.

Kitmitto, A., Baudoin, F., & Cartwright, E. J. (2019). Cardiomyocyte damage control in heart failure and the role of the sarcolemma. *Journal of muscle research and cell motility*, *40*(3), 319-333.

Klabunde, R. E. (2021). *Cardiovascular physiology concepts* (3rd ed.). Wolters Kluwer

Koren, G. (2001). Glyburide and fetal safety; transplacental pharmacokinetic considerations. *Reproductive Toxicology*, *15*(3), 227-229.

Langer, O., Conway, D. L., Berkus, M. D., Xenakis, E. M. J., & Gonzales, O. (2000). A comparison of glyburide and insulin in women with gestational diabetes mellitus. *New England Journal of Medicine*, *343*(16), 1134-1138.

Langley, S. C., & Jackson, A. A. (1994). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clinical science*, *86*(2), 217-222.

Law, M. L., & Metzger, J. M. (2021). Cardiac myocyte intrinsic contractility and calcium handling deficits underlie heart organ dysfunction in murine cancer cachexia. *Scientific reports*, *11*(1), 23627.

Lehtoranta, L., Koskinen, A., Vuolteenaho, O., Laine, J., Kytö, V., Soukka, H., ... & Räsänen, J. (2017). Gestational hyperglycemia reprograms cardiac gene expression in rat offspring. *Pediatric Research*, *82*(2), 356-361.

Lin, X., Yang, P., Reece, E. A., & Yang, P. (2017). Pregestational type 2 diabetes mellitus induces cardiac hypertrophy in the murine embryo through cardiac remodeling and fibrosis. *American journal of obstetrics and gynecology*, *217*(2), 216-e1.

Lopaschuk, G. D., Karwi, Q. G., Tian, R., Wende, A. R., & Abel, E. D. (2021). Cardiac energy metabolism in heart failure. *Circulation research*, *128*(10), 1487-1513.

Lorell, B. H., & Carabello, B. A. (2000). Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation*, *102*(4), 470-479.

Louwagie, E. J., Larsen, T. D., Wachal, A. L., Gandy, T. C., Eclov, J. A., Rideout, T. C., ... & Baack, M. L. (2020). Age and sex influence mitochondria and cardiac health in offspring exposed to maternal glucolipotoxicity. *Iscience*, *23*(11), 101746.

Madsen, N. L., Schwartz, S. M., Lewin, M. B., & Mueller, B. A. (2013). Prepregnancy body mass index and congenital heart defects among offspring: a population-based study. *Congenital heart disease*, *8*(2), 131-141.

Ma, E., Wu, C., Chen, J., Wo, D., Ren, D. N., Yan, H., ... & Zhu, W. (2023). Resveratrol prevents Ang II-induced cardiac hypertrophy by inhibition of NF-κB signaling. *Biomedicine & Pharmacotherapy*, *165*, 115275.

Mao, Y., Hu, W., Xia, B., Liu, L., Han, X., & Liu, Q. (2022). Association between gestational diabetes mellitus and the risks of type-specific cardiovascular diseases. *Frontiers in Public Health*, *10*, 940335.

Masuyama, H., & Hiramatsu, Y. (2012). Effects of a high-fat diet exposure in utero on the metabolic syndrome-like phenomenon in mouse offspring through epigenetic changes in adipocytokine gene expression. *Endocrinology*, *153*(6), 2823-2830.

Mdaki, K. S., Larsen, T. D., Wachal, A. L., Schimelpfenig, M. D., Weaver, L. J., Dooyema, S. D., ... & Baack, M. L. (2016). Maternal high-fat diet impairs cardiac function in offspring of diabetic pregnancy through metabolic stress and mitochondrial dysfunction. *American Journal of Physiology-Heart and Circulatory Physiology*, *310*(6), H681-H692.

Miller, K. E. (2006). Glyburide vs. Insulin Therapy in Women with Gestational Diabetes. *American Family Physician*, *73*(7), 1266.

Miranda, J. O., Cerqueira, R. J., Barros, H., & Areias, J. C. (2019). Maternal diabetes mellitus as a risk factor for high blood pressure in late childhood: a prospective birth cohort study. *Hypertension*, *73*(1), e1-e7.

Mishra, A., Ruano, S. H., Saha, P. K., & Pennington, K. A. (2022). A novel model of gestational diabetes: Acute high fat high sugar diet results in insulin resistance and beta cell dysfunction during pregnancy in mice. *PloS One*, *17*(12), e0279041.

Nakamura, M., & Sadoshima, J. (2018). Mechanisms of physiological and pathological cardiac hypertrophy. *Nature Reviews Cardiology*, *15*(7), 387-407.

Negrato, C. A., Rafacho, A., Negrato, G., Teixeira, M. F., Araújo, C. A., Vieira, L., ... & Gomes, M. B. (2010). Glargine vs. NPH insulin therapy in pregnancies complicated by diabetes: an observational cohort study. *diabetes research and clinical practice*, *89*(1), 46-51.

Nelson, C. R., Dzakpasu, S., Moore, A. M., Darling, E. K., Edwards, W., Murphy, P., ... & Ray, J. G. (2024). Diabetes mellitus in pregnancy across Canada. *BMC Pregnancy and Childbirth*, *24*(1), 349.

Nethery, E., Law, M. R., Kotaska, A., Janssen, P. A., & Hutcheon, J. A. (2023). The effect of changing screening practices and demographics on the incidence of gestational diabetes in British Columbia, 2005–2019. *CMAJ*, *195*(11), E396-E403.

Nguyen, B. Y., Ruiz-Velasco, A., Bui, T., Collins, L., Wang, X., & Liu, W. (2019). Mitochondrial function in the heart: the insight into mechanisms and therapeutic potentials. *British journal of pharmacology*, *176*(22), 4302-4318.

Owen, M. D., Baker, B. C., Scott, E. M., & Forbes, K. (2021). Interaction between metformin, folate and vitamin B12 and the potential impact on fetal growth and long-term metabolic health in diabetic pregnancies. *International journal of molecular sciences*, *22*(11), 5759

Pachon, R. E., Scharf, B. A., Vatner, D. E., & Vatner, S. F. (2015). Best anesthetics for assessing left ventricular systolic function by echocardiography in mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *308*(12), H1525-H1529.

Pan, X., Hao, E., Zhang, F., Wei, W., Du, Z., Yan, G., ... & Hou, X. (2024). Diabetes cardiomyopathy: targeted regulation of mitochondrial dysfunction and therapeutic potential of plant secondary metabolites. *Frontiers in Pharmacology*, *15*, 1401961.

Pathirana, M. M., Lassi, Z. S., Roberts, C. T., & Andraweera, P. H. (2020). Cardiovascular risk factors in offspring exposed to gestational diabetes mellitus in utero:

systematic review and meta-analysis. *Journal of developmental origins of health and disease*, *11*(6), 599-616.

Pereira, T. J., Fonseca, M. A., Campbell, K. E., Moyce, B. L., Cole, L. K., Hatch, G. M., ... & Dolinsky, V. W. (2015). Maternal obesity characterized by gestational diabetes increases the susceptibility of rat offspring to hepatic steatosis via a disrupted liver metabolome. *The Journal of physiology*, *593*(14), 3181-3197.

Petersen, M. C., & Shulman, G. I. (2018). Mechanisms of insulin action and insulin resistance. *Physiological reviews, 98,* 2133-2223.

Pettitt, D. J., Ospina, P., Kolaczynski, J. W., & Jovanovic, L. (2003). Comparison of an insulin analog, insulin aspart, and regular human insulin with no insulin in gestational diabetes mellitus. *Diabetes care*, *26*(1), 183-186.

Pezzuto, J. M. (2019). Resveratrol: twenty years of growth, development and controversy. *Biomolecules & therapeutics*, *27*(1), 1.

Plows, J. F., Stanley, J. L., Baker, P. N., Reynolds, C. M., & Vickers, M. H. (2018). The pathophysiology of gestational diabetes mellitus. *International journal of molecular sciences*, *19*(11), 3342.

Plows, J. F., Yu, X., Broadhurst, R., Vickers, M. H., Tong, C., Zhang, H., ... & Baker, P. N. (2017). Absence of a gestational diabetes phenotype in the LepRdb/+ mouse is independent of control strain, diet, misty allele, or parity. *Scientific reports*, *7*(1), 45130.

Public Health Agency of Canada. (2020). *Annual Statistics 2017-2018*. https://www.gov.mb.ca/health/annstats/as1718.pdf

Razaz, N., Villamor, E., Muraca, G. M., Bonamy, A. K. E., & Cnattingius, S. (2020). Maternal obesity and risk of cardiovascular diseases in offspring: a population-based cohort and sibling-controlled study. *The lancet Diabetes & endocrinology*, *8*(7), 572-581.

Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. Diabetologia, 60(9), 1577-1585.

Renaud, S. D., & de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet*, *339*(8808), 1523-1526.

Retnakaran, R., & Shah, B. R. (2016). Role of type 2 diabetes in determining retinal, renal, and cardiovascular outcomes in women with previous gestational diabetes mellitus. *Diabetes care*, *40*(1), 101-108.

Roberts, V. H., Pound, L. D., Thorn, S. R., Gillingham, M. B., Thornburg, K. L., Friedman, J. E., ... & Grove, K. L. (2014). Beneficial and cautionary outcomes of resveratrol supplementation in pregnant nonhuman primates. *The FASEB Journal*, *28*(6), 2466.

Rosca, M. G., Tandler, B., & Hoppel, C. L. (2013). Mitochondria in cardiac hypertrophy and heart failure. *Journal of molecular and cellular cardiology*, *55*, 31-41.

Ros, P., Diaz, F., Freire-Regatillo, A., Argente-Arizon, P., Barrios, V., Argente, J., & Chowen, J. A. (2018). Resveratrol intake during pregnancy and lactation modulates the early metabolic effects of maternal nutrition differently in male and female offspring. *Endocrinology*, *159*(2), 810-825.

Roth, D. M., Swaney, J. S., Dalton, N. D., Gilpin, E. A., & Ross Jr, J. (2002). Impact of anesthesia on cardiac function during echocardiography in mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *282*(6), H2134-H2140.

Rowan, J. A., Hague, W. M., Gao, W., Battin, M. R., & Moore, M. P. (2008). Metformin versus insulin for the treatment of gestational diabetes. *New England Journal of Medicine*, *358*(19), 2003-2015.

Salehi, B., Mishra, A. P., Nigam, M., Sener, B., Kilic, M., Sharifi-Rad, M., ... & Sharifi-Rad, J. (2018). Resveratrol: A double-edged sword in health benefits. *Biomedicines*, *6*(3), 91.

Schaub, M. C., Hefti, M. A., Zuellig, R. A., & Morano, I. (1998). Modulation of contractility in human cardiac hypertrophy by myosin essential light chain isoforms. *Cardiovascular research*, *37*(2), 381-404.

Siasos, G., Tsigkou, V., Kosmopoulos, M., Theodosiadis, D., Simantiris, S., Tagkou, N. M., ... & Papavassiliou, A. G. (2018). Mitochondria and cardiovascular diseases—from pathophysiology to treatment. *Annals of translational medicine*, *6*(12), 256.

Singh, C. K., Kumar, A., LaVoie, H. A., DiPette, D. J., & Singh, U. S. (2013). Diabetic complications in pregnancy: is resveratrol a solution?. *Experimental Biology and Medicine*, *238*(5), 482-490.

Skovsgaard, C. B., Møller, A., Bjerre, J. V., Kampmann, U., & Kyng, K. J. (2024). Diabetes in pregnancy and offspring cardiac function: a systematic review and meta-analysis. *Frontiers in Pediatrics*, *12*, 1404625.

Sutanto, H., Lyon, A., Lumens, J., Schotten, U., Dobrev, D., & Heijman, J. (2020). Cardiomyocyte calcium handling in health and disease: Insights from in vitro and in silico studies. *Progress in biophysics and molecular biology*, *157*, 54-75.

Sun, J., Kim, G. R., Lee, S. J., & Kim, H. C. (2021). Gestational diabetes mellitus and the role of intercurrent type 2 diabetes on long-term risk of cardiovascular events. *Scientific reports*, *11*(1), 21140.

Suriano, F., Vieira-Silva, S., Falony, G., Roumain, M., Paquot, A., Pelicaen, R., ... & Cani, P. D. (2021). Novel insights into the genetically obese (ob/ob) and diabetic (db/db) mice: two sides of the same coin. *Microbiome*, *9*, 1-20.

Takahashi, N., Ichii, O., Hiraishi, M., Namba, T., Otani, Y., Nakamura, T., & Kon, Y. (2024). Phenotypes of streptozotocin-induced gestational diabetes mellitus in mice. *Plos one*, *19*(4), e0302041.

Tam, W. H., Ma, R. C. W., Ozaki, R., Li, A. M., Chan, M. H. M., Yuen, L. Y., ... & Chan, J. C. N. (2017). In utero exposure to maternal hyperglycemia increases childhood cardiometabolic risk in offspring. *Diabetes care*, *40*(5), 679-686.

Tarry-Adkins, J. L., Ozanne, S. E., & Aiken, C. E. (2021). Impact of metformin treatment during pregnancy on maternal outcomes: a systematic review/meta-analysis. *Scientific reports*, *11*(1), 9240.

Tham, Y. K., Bernardo, B. C., Ooi, J. Y., Weeks, K. L., & McMullen, J. R. (2015). Pathophysiology of cardiac hypertrophy and heart failure: signaling pathways and novel therapeutic targets. *Archives of toxicology*, *89*, 1401-1438.

Tripathi, R., Tyagi, S., & Goel, V. (2017). Metformin in gestational diabetes mellitus. *Indian Journal of Medical Research*, *145*(5), 588-591.

Vaiserman, A. M. (2015). Epigenetic programming by early-life stress: Evidence from human populations. *Developmental Dynamics*, *244*(3), 254-265.

Verma, V., & Mehendale, A. M. (2022). A review on the use of metformin in pregnancy and its associated fetal outcomes. *Cureus*, *14*(10).

Vonnahme, K. A., Hess, B. W., Hansen, T. R., McCormick, R. J., Rule, D. C., Moss, G. E., ... & Ford, S. P. (2003). Maternal undernutrition from early-to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biology of reproduction*, *69*(1), 133-140.

Vounzoulaki, E., Khunti, K., Abner, S. C., Tan, B. K., Davies, M. J., & Gillies, C. L. (2020). Progression to type 2 diabetes in women with a known history of gestational diabetes: systematic review and meta-analysis. *bmj*, *369*.

Walker, C. A., & Spinale, F. G. (1999). The structure and function of the cardiac myocyte: a review of fundamental concepts. *The Journal of thoracic and cardiovascular surgery*, *118*(2), 375-382.

Williams, L. D., Burdock, G. A., Edwards, J. A., Beck, M., & Bausch, J. (2009). Safety studies conducted on high-purity trans-resveratrol in experimental animals. *Food and Chemical Toxicology*, *47*(9), 2170-2182.

Woodcock, E. A., & Matkovich, S. J. (2005). Cardiomyocytes structure, function and associated pathologies. *The international journal of biochemistry & cell biology*, *37*(9), 1746-1751.

Yamamoto, J. M., Kellett, J. E., Balsells, M., Garcia-Patterson, A., Hadar, E., Sola, I., ... & Corcoy, R. (2018). Gestational diabetes mellitus and diet: a systematic review and meta-analysis of randomized controlled trials examining the impact of modified dietary interventions on maternal glucose control and neonatal birth weight. *Diabetes care*, *41*(7), 1346-1361.

Yan, Y. S., Mo, J. Y., Huang, Y. T., Zhu, H., Wu, H. Y., Lin, Z. L., ... & Huang, H. F. (2024). Intrauterine hyperglycaemia during late gestation caused mitochondrial dysfunction in skeletal muscle of male offspring through CREB/PGC1A signaling. *Nutrition & Diabetes*, *14*(1), 56.

Yao, L., Wan, J., Li, H., Ding, J., Wang, Y., Wang, X., & Li, M. (2015). Resveratrol relieves gestational diabetes mellitus in mice through activating AMPK. *Reproductive Biology and Endocrinology*, *13*, 1-7.

Yu, Y., Arah, O. A., Liew, Z., Cnattingius, S., Olsen, J., Sørensen, H. T., ... & Li, J. (2019). Maternal diabetes during pregnancy and early onset of cardiovascular disease in offspring: population based cohort study with 40 years of follow-up. *Bmj*, *367,* 16398.

Yu, Y., Soohoo, M., Sørensen, H. T., Li, J., & Arah, O. A. (2021). Gestational diabetes mellitus and the risks of overall and type-specific cardiovascular diseases: a population-and sibling-matched cohort study. *Diabetes Care*, *45*(1), 151-159.

Zeng, Y. C., Li, M. J., Chen, Y., Jiang, L., Wang, S. M., Mo, X. L., & Li, B. Y. (2014). The use of glyburide in the management of gestational diabetes mellitus: a meta-analysis. *Advances in medical sciences*, *59*(1), 95-101.

Zhang, L. P., YIN, J. X., Liu, Z., Zhang, Y., WANG, Q. S., & Zhao, J. (2006). Effect of resveratrol on L-type calcium current in rat ventricular myocytes. *Acta Pharmacologica Sinica*, *27*(2), 179-183.

Zhang, W., Qian, S., Tang, B., Kang, P., Zhang, H., & Shi, C. (2023). Resveratrol inhibits ferroptosis and decelerates heart failure progression via Sirt1/p53 pathway activation. *Journal of cellular and molecular medicine*, *27*(20), 3075-3089.

Zhang, G., Wang, X., Ren, B., Zhao, Q., & Zhang, F. (2021). The effect of resveratrol on blood glucose and blood lipids in rats with gestational diabetes mellitus. *Evidence-Based Complementary and Alternative Medicine*, *2021*(1), 2956795.

Zheng, M., Bai, Y., Sun, X., Fu, R., Liu, L., Liu, M., ... & Huang, X. (2022). Resveratrol reestablishes mitochondrial quality control in myocardial ischemia/reperfusion injury through Sirt1/Sirt3-Mfn2-Parkin-PGC-1α pathway. *Molecules*, *27*(17), 5545.

Zheng, S., Feng, Q., Cheng, J., & Zheng, J. (2018). Maternal resveratrol consumption and its programming effects on metabolic health in offspring mechanisms and potential implications. *Bioscience reports*, *38*(2), BSR20171741.

Zou, T., Chen, D., Yang, Q., Wang, B., Zhu, M. J., Nathanielsz, P. W., & Du, M. (2016). Resveratrol supplementation of high-fat diet-fed pregnant mice promotes brown and beige adipocyte development and prevents obesity in male offspring. *The Journal of physiology*, *595*(5), 1547-1562.

Zouridis, A., Manousopoulou, A., Potiris, A., Sarli, P. M., Aravantinos, L., Pervanidou, P., ... & Eleftheriades, M. (2021). Impact of maternal food restriction on heart proteome in appropriately grown and growth-restricted wistar—rat offspring. *Nutrients*, *13*(2), 466.