6-9-2022 1:15 PM

The Molecular Mechanisms of Hepatic Mitochondrial Dysfunction in Growth-Restricted Offspring with Hyperlipidemia

Shelby L. Oke, *The University of Western Ontario*

Supervisor: Hardy, Daniel B., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology and Pharmacology

Abstract

Intrauterine growth restriction (IUGR) is a pathological condition characterized by low birth weight and poor organ development. Growth of the fetal liver is often compromised at the expense of other vital organs, thereby leading to hepatic hyperlipidemia in the affected offspring. As such, epidemiological studies suggest a relationship between birth weight and long-term metabolic health, while the occurrence of postnatal catch-up growth can exacerbate this relationship. Animal studies have shown that IUGR offspring exhibit impaired mitochondrial function, which likely contributes to the later development of metabolic pathologies. That said, the molecular mechanisms by which mitochondria are affected remain unknown. In this thesis, I aimed to characterize the molecular mechanisms by which hepatic mitochondrial dysfunction occurs in growth-restricted offspring with catch-up growth. To do this, we utilized two different rodent models of IUGR: (1) the maternal protein restriction (MPR) model of undernutrition, and (2) gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC). Offspring from both models underwent hepatic catch-up growth by three weeks of age, while adult male offspring from both models exhibited hepatic hyperlipidemia. I demonstrated that male offspring further display aberrant markers of oxidative stress and mitochondrial dysfunction, including elevated p66Shc, 4-hydroxynonenol, and various antioxidant enzymes. These changes occurred exclusively following catch-up growth, suggesting that rapid postnatal weight gain is detrimental to mitochondrial metabolism and long-term metabolic health. I further found that the expression of microRNA-29 was significantly altered in the livers of adult male IUGR offspring from both models. My *in vitro* studies determined that miR-29 may be regulated by mitochondrial-induced oxidative stress, as treatment of HepG2 cells with rotenone and thapsigargin led to increased transcript abundance of miR-29. In addition, mRNA levels of fatty acid translocase (CD36), a membrane transporter protein involved in fatty acid uptake that is also a target of miR-29, was increased alongside miR-29. Overall, our data suggest that hepatic catch-up growth has great impact on mitochondrial function in growth-restricted offspring, and that this may occur in a sex-specific manner.

Keywords

Cannabis, developmental origins of health and disease (DOHaD), hyperlipidemia, endoplasmic reticulum stress, epigenetics, intrauterine growth restriction (IUGR), liver, metabolism, microRNA, mitochondria, nutrition, oxidative stress, pregnancy

Summary for Lay Audience

Intrauterine growth restriction (IUGR) is a condition that affects 4–7% of all pregnancies. IUGR often occurs due to a poor maternal environment, and it results in low birth weight and poor fetal development. This can lead to increased fat storage within the liver, resulting in high risk for obesity, diabetes, and cardiovascular disease. This risk is worsened through a process called catch-up growth, whereby IUGR babies gain weight quickly during infancy and early childhood. Studies have found that the function of mitochondria, the major energy producers of the cell, becomes impaired with IUGR and contributes to the progression of disease. However, the role of catch-up growth in this process is not well understood. In this study, we used two different rodent models to study the effects of catch-up growth on mitochondria in the IUGR rat liver. In our first model, pregnant rats were fed a low protein diet, and followed by induction of catch-up growth after birth. In our second model, pregnant rats were injected with Δ9-tetrahydrocannabinol (Δ9-THC), the major component of cannabis that affects the brain. Both models resulted in IUGR, as offspring had low birth weight and small livers. Following catch-up growth, adult offspring from both models had elevated levels of fat in the liver. We believe that this occurs due to changes in (1) proteins responsible for fat production, and (2) mitochondrial proteins that control energy production and the production of toxic molecules. We also found that poor mitochondrial function leads to altered regulatory mechanisms of gene expression. Overall, these studies provide insight into the molecular causes of IUGR, and they may contribute to the development of therapies used in clinical practice during prenatal and postnatal life.

Co-Authorship Statement

Chapter I is adapted from the following two manuscripts:

Oke, S.L. and Hardy, D.B. (2021). The role of cellular stress in intrauterine growth restriction and postnatal dysmetabolism. International Journal of Molecular Sciences, 22 (13), 6986. Ownership of copyright is assigned to the authors of the manuscript. SL Oke was the primary author in writing the manuscript, while DB Hardy assisted in preparation of the manuscript.

Oke, S.L., and Hardy, D.B. (2017). Effects of protein deficiency on perinatal and postnatal health outcomes. The Handbook of Famine, Starvation, and Nutrient Deprivation, 1–23. Text and figures are reproduced with permission from Springer Nature. SL Oke was the primary author in writing the manuscript, while DB Hardy assisted in preparation of the manuscript.

Chapter II is adapted from **Oke, S.L.**, Sohi, G., Hardy, D.B. (2020) Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. *Reproduction*. 159:27–39. Ownership of copyright is assigned to the authors of the manuscript. SL Oke was responsible for designing experiments with assistance from G Sohi and DB Hardy. All experiments were performed by SL Oke. All data was generated and analyzed by SL Oke in the laboratory of DB Hardy. SL Oke was the primary author in writing the manuscript, while DB Hardy assisted in preparation of the manuscript.

Chapter III is adapted from **Oke S.L.**, Lee, K., Papp, R., Laviolette, S.R., Hardy, D.B. (2021) In utero exposure to Δ9-tetrahydrocannabinol leads to postnatal catch-up growth and dysmetabolism in the adult rat liver. *The International Journal of Molecular Sciences*. 22(14): 2502. Ownership of copyright is assigned to the authors of the manuscript. SL Oke designed, performed, and interpreted all experiments with assistance from R Papp and DB Hardy. K Lee implemented the live animal model. SR Laviolette contributed to the design of animal experiments. SL Oke wrote the manuscript, while all other authors were involved in editing and approving the manuscript.

Chapter IV is entitled “Oxidative stress and endoplasmic reticulum stress promote increased expression of hepatic miR-29 and fatty acid translocase *in vitro*”. SL Oke performed all experiments and generated all data presented. All figures and tables were generated by SL Oke.

Preliminary data presented in **Chapter V** was generated by SL Oke. All figures and tables were generated by SL Oke.

Acknowledgments

First and foremost, I would like to thank my partner, Kane, for being my primary support system and best friend the last three years. None of this would have been possible without you by my side. You came into my life when I least expected it, but I'm SO glad that you did. You're the most patient person I've ever met, which is a blessing since you've had to deal with my high-strung personality as I finish this thesis (sorry!). It's crazy to think that most of our relationship has been overshadowed by COVD-19, but if we can get through a global pandemic, we can get through anything. While the last few months have been a bit chaotic, from buying our first home and getting a puppy, I wouldn't have it any other way. I love you and the life that we've created together, and I look forward to all the amazing things that are yet to come for us.

To my son, Oliver— Mom's finally not a student anymore, buddy! This thesis is as much yours as it is mine. You've dealt with more hardship than any person should ever have to, but you have become such a resilient person and handled things better than most adults would. I'm so happy that you share my curiosity and love for science, plus I'm glad that you got to spend some days in the lab during the pre-COVID era. The jury's still out as to whether I want to see you follow in my footsteps as a scientist, but just know that I will support you no matter what you do. I'm so proud of you, and I know that you will achieve great things.

Thank you to my family, especially my mom, for your constant support throughout my studies. It's been a tough few years for all of us, but it seems that we may finally be seeing the light at the end of the tunnel.

Thank you to Drs. Angela Beye and Anita Woods— the 'big sisters' that I never had. You both have made a tremendous impact on my professional and personal growth the last ten (!!) years. Your continued support and guidance will never be forgotten.

I would like to collectively thank all past and present members of the DDTA lab, who have become more like family than friends. Thank you to my advisory committee

members, Drs. Edith Arany, Dean Betts, and Timothy Regnault. I hope you all know how grateful I am to have you all in my corner.

To Alex, Zach, and Allyson— the original members of 'The Pity Party". You three deserve your own separate thank-you, as you've been there for me through absolutely everything. I will never forget the numerous hours spent with you in and out of the lab, and I am grateful for your everlasting friendship.

Last, and certainly not least, thank you to my amazing supervisor, Dr. Dan Hardy. I really cannot put into words how much your mentorship and guidance has meant to me. You took me in as a work-study student before starting my fourth year of undergraduate studies; little did I know, I would end up spending a total of seven years working under your supervision. You've taught me so many valuable life lessons that I will forever remember, including the importance of work-life balance, the names of WWE wrestlers by their place of birth, and that "it's not a party without a Hardy" (to anyone reading this— you can probably tell that we like to have fun in our lab). As much as I have been focused on my own academic and professional development the last few years, it has been an absolute pleasure to see your career blossom as well. Kendrick, Sebastian, and I have always referred to you as 'the G.O.A.T' (Greatest of all Time), and it's for good reason. Without your endless support, I would not be the scientist and person I am today.

Dedication

To my son, Oliver:

You are my biggest motivation and my proudest accomplishment.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACCα	Acetyl Co-A carboxylase alpha
ACL	ATP citrate synthase
ACS	Acyl Co-A synthetase
AGA	Appropriate for gestational age
AMPK	AMP-activated protein kinase
APGAT	1-acyl glycerol-3-phosphate acyltransferase
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
CD36	Fatty acid translocase
cDNA	Complementary deoxyribonucleic acid
C/EBPα	CCAAT/enhancer-binding protein alpha
ChIP	Chromatin immunoprecipitation
CPT	Carnitine palmitoyltransferase
CRP	C-reactive protein
Cyp	Cytochrome P450
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DNMT	DNA methyltransferase
DOHaD	Developmental origins of health and disease

ECAR	Extracellular acidification rate
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ETC	Electron transport chain
FABP	Fatty acid binding protein
$FADH_2$	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FATP	Fatty acid transporter protein
FGF	Fibroblast growth factor
GD	Gestational day
GH	Growth hormone
GLUT	Glucose transporter
GPAT	Glycerol-3-phosphate acyltransferase
GTP	Guanosine triphosphate
HDL	High density lipoprotein
HNF	Hepatocyte nuclear factor
HSC	Hepatic stellate cell
IMM	Inner mitochondrial membrane
IUGR	Intrauterine growth restriction
LDL	Low density lipoprotein

LPA	Lysophosphatidic acid
LXR	Liver receptor X
MAM	Mitochondrial-associated ER membrane
miRNA	MicroRNA
MNE	Maternal nicotine exposure
MNR	Maternal nutrient restriction
MPR	Maternal protein restriction
mRNA	messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide + hydrogen
NAFLD	Non-alcoholic fatty liver disease
NAS	N-acetyl-serotonin
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase

PDH	Pyruvate dehydrogenase
PND	Postnatal day
PUFA	Polyunsaturated fatty acid
PVM	Portal vein mesenchyme
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SEM	Standard error of the mean
SERCA	sarcoplasmic/endoplasmic reticulum bound Ca^{2+}-ATPase
SGA	Small for gestational age
Shc	Src homology 2 domain-containing
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor beta
TUDCA	Tauroursodeoxycholic acid
UTR	Untranslated region
VDAC	Voltage-dependent anion channel
VLDL	Very low density lipoprotein
11β-HSD1	11β-hydroxysteroid dehydrogenase type I
Δ9-THC	Δ9-tetrahydrocannabinol

Chapter I

1 Introduction

Excerpts of this chapter have been previously published in the following works:

Oke, Shelby L. and Hardy, Daniel B. (2017). Effects of protein deficiency on perinatal and postnatal health outcomes. The Handbook of Famine, Starvation, and Nutrient Deprivation, 1–23.

Reproduced here with permission from Springer Nature.

Oke, Shelby L. and Hardy, Daniel B. (2021). The role of cellular stress in intrauterine growth restriction and postnatal dysmetabolism. International Journal of Molecular Sciences, 22 (13), 6986.

1.1 Overview

1.1.1 Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is a pathological condition that affects approximately 4–7% of all pregnancies (Romo *et al.*, 2009; Creasy *et al.*). IUGR is defined as the decreased rate of fetal growth relative to that of the general population, culminating in fetal weight below the 10th percentile for gestational age (Suhag and Berghella, 2013; Sharma *et al.*, 2016). Although IUGR infants are defined as being small for gestational age (SGA), there is a distinction between pathological IUGR and healthy SGA fetuses (Sharma *et al.*, 2016). SGA fetuses are not exposed to an adverse uterine environment, but rather are genetically programmed to be small based on parental genotype (Vandenbosche and Kirchner, 1998; Sharma *et al.*, 2016). These fetuses exhibit proportional organ growth *in utero*, and they have normal organ function in postnatal life (Brites, 2002; Baschat, 2004). Conversely, the diminished growth of IUGR fetuses occurs as a consequence of the uterine environment (Baschat, 2004; Suhag and Berghella, 2013; Sharma *et al.*, 2016). In cases of symmetrical IUGR, cell numbers become decreased and fetal organ growth is reduced in a proportional manner (Suhag and Berghella, 2013; Sharma *et al.*, 2016). Alternatively, in cases of asymmetrical IUGR, the growth of select fetal organs is compromised at the benefit of others (*i.e.,* there is a brain-sparing effect; Barker *et al.*, 1993a). Both symmetrical and asymmetrical IUGR can occur due to utero-placental insufficiency, whereby the placenta cannot meet metabolic requirements set by the fetal genome (Baschat, 2004). Utero-placental insufficiency is often secondary to insults of maternal origin, including maternal malnutrition, drug use, and infection among others; therefore, maternal lifestyle has significant influence on offspring health (Baschat, 2004; Sharma *et al.*, 2016). Importantly, the postnatal environment has also been demonstrated to have an indirect role in provoking long-term metabolic dysfunction, as offspring born into an environment that is 'mismatched' from that *in utero* are subject to maladaptive changes in fetal programming (Hales and Barker, 2001). Despite the divergent phenotypes associated with symmetrical and asymmetrical IUGR, infants in both categories have increased risk for perinatal complications and the metabolic

syndrome in later life (Romo *et al.*, 2009; Suhag and Berghella, 2013; Sharma *et al.*, 2016).

Aside from fetal growth and weight, IUGR can be screened for and clinically diagnosed during pregnancy via placental ultrasound and umbilical artery (*i.e.,* feto-placental) doppler ultrasound. Placental ultrasound is used to determine maximal placental length and thickness, as placental insufficiency results in small placental size (Baschat and Hecher, 2004). Umbilical doppler ultrasound is further used to detect changes in blood flow and vascular resistance in the uterine artery and umbilical vessels, because feto-placental blood flow becomes reduced in IUGR pregnancies (Baschat and Hecher, 2004). This leads to, in part, decreased nutrient and oxygen supply to the fetus, accounting for poor gestational growth and development. Nutrient availability is also diminished via downregulation of placental nutrient transporters, particularly those that mediate uptake of amino acids. IUGR placentas have decreased activity of both system L (Jansson *et al.*, 1998) and system A amino acid transporters (Glazier *et al.*, 1997; Jansson *et al.*, 2002), along with reduced activity of the taurine transporter (Norberg *et al.*, 1998; Roos *et al.*, 2004). Consequences of these reduced transporter activities are twofold, because amino acids stimulate the production and release of fetal insulin to promote growth and development. There is evidence to show that activity of placental lipases are also decreased in IUGR placentas, particularly that of lipoprotein lipase (Magnusson *et al.*, 2004; Tabano *et al.*, 2006; Gauster *et al.*, 2007) and endothelial lipase (Gauster *et al.*, 2007). With respect to placental glucose transport, changes in glucose transporter (GLUT) expression and activity are controversial. GLUT1 is the primary glucose transporter present in the placenta, while GLUT3 and GLUT4 are also present at lower levels but higher affinity for glucose. Many studies have found that placental GLUTs remain unchanged with IUGR (Kainulainen *et al.*, 1997; Jansson *et al.*, 2002); however, Janzen *et al.* have reported increased protein abundance of GLUT3 in human IUGR placenta samples (Janzen *et al.*, 2013). Importantly, this study did not assess functional capacity of GLUT3, but it was hypothesized that placental oxidative stress may be involved in this process.

1.1.2 The Developmental Origins of Health and Disease (DOHaD)

The metabolic syndrome refers to a group of physiological symptoms that increase an individual's risk for cardiovascular disease and type II diabetes. These symptoms, including dyslipidemia, obesity, hyperglycemia, and hypertension, are often assessed independent of each other; however, their simultaneous occurrence is synergistic toward onset of the metabolic syndrome. It is well known that these symptoms are influenced by factors such as genetics and lifestyle, but the role of developmental priming is often overlooked. The developmental origins of health and disease (DOHaD) posits that there is a relationship between birth weight and long-term metabolic health, as adverse events in utero may permanently influence the function of metabolic organs. This is in alignment with the "Predictive Adaptive Response" hypothesis, which states that environmental cues during periods of developmental plasticity will induce a specific phenotype in anticipation of the postnatal environment (Bateson *et al.*, 2014). As such, a mismatch between the pre- and postnatal environment results in short-term adaptations that have adverse consequences later in life (Hales and Barker, 2001; Bateson *et al.*, 2014). Infants affected by IUGR exhibit impaired organ growth with metabolic disease in adulthood, as early epidemiological studies by Sir David Barker and colleagues determined that low birth weight individuals have high rates of obesity, glucose intolerance, and coronary artery disease (Ravelli *et al.*, 1976, 1998, 1999). These studies have since led to widespread investigation of the underlying causes of IUGR, as well as the metabolic pathologies that arise in response to impaired organ development.

1.1.2.1 Human Evidence of DOHaD

Evidence for DOHaD has been revealed by numerous human studies, many of which are focused on the role of famine and other nutritional insults during pregnancy. Barker's investigations of the Hertfordshire birth cohort are perhaps the best described, as these studies were among the first to uncover the relationship between impeded fetal growth and adult offspring health (Osmond *et al.*, 1993). In combination with

epidemiological studies of famine, the data from this cohort indicate a strong relationship between birth weight and risk for the metabolic syndrome. In particular, men and women who were born with low birth weight had increased risk of death from cardiovascular disease in adulthood, while this was also true of men who were small at one year of age (Osmond *et al.*, 1993). These studies led to further investigation of the surviving individuals from the cohort, and it was found that low birth weight was associated with increased risk for insulin resistance (Barker *et al.*, 1993b), type II diabetes (Fall *et al.*, 1995), and coronary artery disease (Hales *et al.*, 1991). These men and women also had increased risk for osteoporosis (Cooper *et al.*, 1997) and sarcopenia (Sayer *et al.*, 1998), indicating that the effects of small size at birth were not limited to just metabolic organs. Additional birth cohorts from Brazil, South Africa, and the United States have also reported similar trends, further solidifying the role of the *in utero* environment in the development of metabolic disease (Victora *et al.*, 2003; Musa *et al.*, 2015; Perng *et al.*, 2016). Finally, rising rates of obesity can be partially explained by developmental programming, as there is an association between birth weight and adult body composition. This relationship is U-shaped such that infants born with either IUGR (low birth weight, <2500 g) or macrosomia (high birth weight, >3500 g) have increased risk for obesity in adult life compared to those born appropriate for gestational age (Eriksson *et al.*, 2001).

Barker's studies of the Dutch famine cohort have brought much attention to the importance of a high-quality maternal diet in determining offspring metabolic health. The data, which were collected from individuals born before, during and after the famine of 1944–1945, demonstrated that those exposed to famine during mid to late gestation were glucose intolerant as adults (Ravelli *et al.*, 1998). Later studies of this cohort found that exposure to famine at any point during pregnancy also contributed to increased likelihood of hypertension during adult life (Stein *et al.*, 2006). Furthermore, males exposed to famine during the first two trimesters were more likely to be obese during adulthood (Ravelli *et al.*, 1976). Studies of the Chinese famine of 1959–1961 have reported similar trends, as fetal exposure to famine resulted in elevated fasting plasma glucose in adulthood (Wang *et al.*, 2016) and high rates of obesity among female adults (Yang *et al.*,

2008). Additionally, a retrospective cohort study of Chinese individuals found that risk for hypertension was increased in offspring exposed to famine exclusively during the first trimester (Wang *et al.*, 2012). Many studies have determined that exposure during infancy or childhood appears to play a much greater role in this process (Huang *et al.*, 2010; Wang *et al.*, 2016), highlighting the importance of the postnatal environment on offspring metabolic health and the complex relationship between fetal development and metabolic disease.

Postnatal catch-up growth, or rapid postnatal weight gain, is known to exacerbate the relationship between birth weight and long-term metabolic health. Barker's 'thrifty phenotype' hypothesis states that this is because fetal programming adapts in anticipation of a similar postnatal environment (Hales and Barker, 2001). Of note, circulating and hepatic lipid levels are highly sensitive to catch-up growth, and there are many studies illustrating that low birth weight offspring with catch-up growth exhibit dyslipidemia. For example, preterm infants who undergo catch-up growth following consumption of nutrient-dense formula have high ratios of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) during adolescence (Singhal *et al.*, 2004). Cohort studies of Finnish and Italian infants have also identified an association between birth weight and risk for non-alcoholic fatty liver disease (NAFLD) during childhood and adult life (Nobili *et al.*, 2007; Suomela *et al.*, 2016). Alarmingly, obesity and dyslipidemia are risk factors for insulin resistance and type II diabetes (James *et al.*, 2004), so the increased risk for NAFLD following catch-up growth may contribute to later onset of insulin resistance and cardiovascular disease (Anstee *et al.*, 2013). The previously mentioned birth cohorts from Brazil, South Africa, and the United States have also outlined the role of postnatal catch-up growth in elevating the risk for obesity. In these studies, increased weight gain during childhood was associated with greater fat mass and weight gain during mid-childhood and adolescence (Victora *et al.*, 2003; Musa *et al.*, 2015; Perng *et al.*, 2016). Importantly, childhood obesity is further predictive of adult body mass index and body composition (Salgin *et al.*, 2015); therefore, the battle against obesity and the metabolic syndrome is lifelong. Overall, these studies warrant the need for clinical preventative strategies in mediating both fetal and postnatal growth.

In addition to excess visceral adiposity, impaired liver function contributes to dyslipidemia. The liver is central in managing systemic levels of nutrients in both the fed and fasted state, as it coordinates the various metabolic pathways that maintain appropriate levels of glucose and lipid molecules. Individuals with hepatic dysfunction exhibit dyslipidemia, which can give rise to fibrosis and eventual cirrhosis of the liver (Anstee *et al.*, 2013). Again, this may further lead to glucose intolerance and the development of type II diabetes, indicating the high potential for hepatic dysfunction in producing widespread metabolic disease (Anstee *et al.*, 2013). IUGR offspring have impaired liver growth as indicated by decreased abdominal circumference at birth, which is correlated with elevated levels of total cholesterol, triglycerides, and LDL in adult life (Barker *et al.*, 1993a; Roberts *et al.*, 1999; Skilton *et al.*, 2011). The strong correlation between these two variables suggests that long-term hepatic function becomes compromised following gestational insult. Again, IUGR infants undergo rapid catch-up growth in early postnatal life, and the undernourished liver stands to gain the most relative to other organs (Neerhof, 1995; Valsamakis *et al.*, 2006). This is evident in SGA infants who undergo hypersomatotropism by postnatal day (PND) four, which occurs due to increased levels of insulin growth factor 1 (IGF-1) in the liver and blood (Deiber *et al.*, 1989). Keeping with the DOHaD hypothesis, this rapid weight gain is detrimental to hepatic function and can further exacerbate risk for the metabolic syndrome (Hales and Barker, 2001).

While the clinical evidence in support of DOHaD is abundant, these studies are limited in that they provide little insight into the underlying mechanisms linking a poor *in utero* environment to postnatal metabolic disease. It is for this reason that animal models are essential in improving our understanding of how molecular mechanisms contribute to adverse metabolic outcomes of IUGR offspring. Some of these models will be discussed in detail in Section 1.2.5.

1.2 The Liver

1.2.1 Liver Development

Hepatogenesis is a highly complex process that begins during gastrulation. Briefly, the liver is derived from the anterior portion of the definitive endoderm (Douarin, 1975). Progenitor hepatoblast cells are in the posterior foregut region of this layer, which eventually closes to form the hepatic diverticulum (*i.e.,* the hepatic bud; Trefts *et al.*, 2017). The hepatic diverticulum folds such that it lies proximally to the lateral plate mesoderm, which secretes key signaling molecules and extracellular growth factors to induce differentiation of progenitor hepatoblasts (Si-Tayeb *et al.*, 2010). These signaling molecules include fibroblast growth factors (FGF), bone morphogenic proteins (BMPs), transforming growth factor beta (TGF-β), Wnt, and NOTCH, while important transcription factors include those belonging to the FoxA1 and GATA families (Trefts *et al.*, 2017). In particular, the gradient of TGF-β secreted from the portal vein mesenchyme (PVM) is necessary for differentiation of hepatoblasts into hepatocytes and cholangiocytes (Trefts *et al.*, 2017). That is, the fates of these cells are dependent on their proximity to the PVM. High concentrations of TGF-β pushes these cells towards a cholangiocyte fate, while lower concentrations of TGF-β promote differentiation of hepatoblasts into hepatocytes (Trefts *et al.*, 2017). TGF-β is also an inhibitory molecule of CCAAT/enhancer-binding protein alpha (C/EBPα), which regulates the expression of hepatocyte-specific co-activators such as hepatocyte nuclear factor 1 homeobox A (HNF-1α) and hepatocyte nuclear factor 4 alpha (HNF-4α; Trefts *et al.*, 2017). Specific loss of HNF-4α from fetal hepatoblasts appears to have significant impact on hepatocyte polarity and liver structure, indicating that it is essential for the progression of hepatogenesis (Battle *et al.*, 2006). Additionally, the presence of HNF-4α in the adult liver is important as it is maintains metabolic function of mature hepatocytes (Hayhurst *et al.*, 2001).

Again, hepatogenesis is highly elaborate and requires synchronized expression of all these signaling molecules along with numerous others. There appears to be a high degree

of similarity between human and rodent embryogenesis; however, there are key distinctions in timing that contribute to structural and functional differences between these species (Figure 1-1). In humans, terminal differentiation of the liver occurs prior to birth between gestational weeks 34 and 37 (Trefts *et al.*, 2017). Alternatively, the rodent liver continues to develop postnatally until days 18–21 of life (Gruppuso and Sanders, 2016; Trefts *et al.*, 2017). Function of the adult liver is also quite different from that of the fetal liver. Prior to the formation of bone marrow, the developing liver serves as the site of fetal hematopoiesis. In the final weeks of perinatal development, the liver shifts towards being a primarily metabolic organ (Trefts *et al.*, 2017). The adult liver is highly versatile in that it has many functions, which will be expanded upon below in Section 1.2.3.

1.2.2 Anatomical and Cellular Structure

The liver is the largest internal organ in the body, weighing approximately 1500 grams in fully grown adults (Ross and Pawlina, 2011). It is located underneath the ribcage in the upper right quadrant of the abdomen, and it is divided into two main lobes (*i.e.,* the left and right hepatic lobes) that are separated by the falciform ligament (Ross and Pawlina, 2011). The larger right lobe further contains two smaller lobes, the quadrate and caudate lobes. These lobes are separated into eight functional segments that correspond to blood supply and biliary drainage (Ross and Pawlina, 2011). Importantly, anatomical structure of the human liver differs from that of rodents in that it is not further lobated beyond the left and right lobes (*i.e.,* the rodent liver is a multi-lobated structure; Kogure *et al.*, 1999). Much like other organs, it is immediately surrounded by a fibrous connective tissue capsule layer called Glisson's capsule, which is encapsulated by the serous visceral peritoneum (Ross and Pawlina, 2011). Glisson's capsule is continuous with the internal connective tissue stroma, which contains the blood vessels, lymphatic vessels, nerves, and bile ducts that enter the liver parenchyma. The liver parenchyma is made up of stacks of hepatocytes organized into hexagonal-shaped liver lobules, which are the classic functional unit of the liver (Si-Tayeb *et al.*, 2010). These hepatocyte plates are separated by sinusoidal capillaries, which drain into the central vein of each lobule

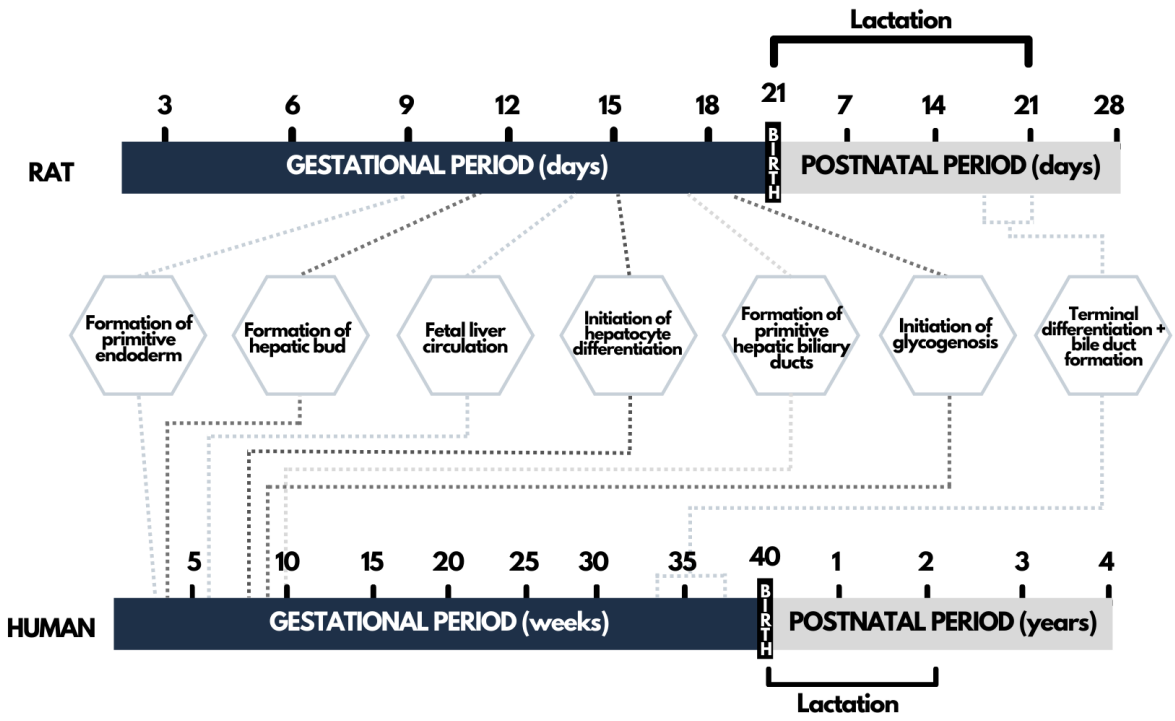

Figure 1-1. Timing of liver development in the rat and humans.

Major developmental events are marked on timelines representing the gestational and postnatal periods for rats (top) and humans (bottom). Given the differences in timing of pregnancy and lifespan, the scale for development in rats is in days, while the scale for development in humans is in weeks (gestational period) and years (postnatal period).

(Si-Tayeb *et al.*, 2010). Surrounding each lobule are multiple portal triads, which consist of a hepatic artery, portal vein, and bile duct (Si-Tayeb *et al.*, 2010). The hepatic artery and portal vein both open into the sinusoidal capillaries, while the bile ducts drain into interlobular bile ducts that eventually lead to the larger hepatic duct (Si-Tayeb *et al.*, 2010). This structural organization is strategic in that it allows for optimal nutrient and oxygen exchange within the liver, while simultaneously removing waste metabolites and releasing bile to the gallbladder.

The liver possesses a unique dual blood supply consisting of (1) the venous (portal) blood supply and (2) the arterial blood supply (Ross and Pawlina, 2011). These vessels enter the liver at a hilum between the quadrate and caudate lobes. This hilum is the same location at which the common bile duct and lymphatic vessels exit the liver; that is, blood travels in the opposite direction as bile and lymph fluid. The hepatic portal vein carries venous blood to the liver from the intestines, pancreas, and spleen, and it supplies the liver with approximately 75% of its major blood supply (Ross and Pawlina, 2011). Although this blood is deoxygenated, it is highly concentrated with nutrients that have been absorbed along the intestine (Trefts *et al.*, 2017). That said, the liver is also the first site exposed to toxic substances and metabolites that are absorbed. The hepatic artery is responsible for carrying oxygen-rich blood that makes up the remainder of the liver's blood supply (Ross and Pawlina, 2011). Both the hepatic portal vein and hepatic artery branch to form the smaller portal vein and arteries that surround the hepatic lobule, and the blood from both vessels mixes as it enters the sinusoidal capillaries (Trefts *et al.*, 2017). Because of this, hepatocytes are never exposed to blood that is fully oxygenated. As blood flows through the sinusoids, gas and nutrient exchange occurs with hepatocytes and the blood empties into the central vein of the lobule (Trefts *et al.*, 2017). These veins later join to form multiple sublobular veins, which eventually come together as the larger hepatic vein that exits the liver (Ross and Pawlina, 2011).

Being one of the most heterogenous tissues in the body, the liver is composed of numerous cell types that each play a critical role in hepatic physiology. As previously mentioned, hepatocytes are the main functional cell present in the liver. They make up

approximately 70–80% of the liver and are responsible for the liver's regenerative capacity (Si-Tayeb *et al.*, 2010; Ross and Pawlina, 2011). Hepatocytes are typically binucleate, and they contain high quantities of mitochondria, rough and smooth endoplasmic reticulum (ER), Golgi complexes, peroxisomes, and lysosomes (Ross and Pawlina, 2011). These abundant organelles account for the hepatocyte's ability to produce and secrete proteins, along with their proficient metabolism of various compounds (Si-Tayeb *et al.*, 2010). Additionally, hepatocytes are the major storage site of glucose and lipids in the liver as they express high levels of GLUT2 and various fatty acid transporters (Thorens, 1996). The remaining cells of the liver, including cholangiocytes, hepatic stellate cells, endothelial cells, Kupffer cells, and Pit cells, comprise a small overall percentage of the liver's cell population. Cholangiocytes, also known as bile duct cells, make up the walls of the hepatic bile duct to allow for transport of bile from the liver to the gallbladder (Si-Tayeb *et al.*, 2010; Trefts *et al.*, 2017). These cells control the composition and pH of bile as it leaves the liver, as they are capable of secreting water and bicarbonate (Si-Tayeb *et al.*, 2010). Hepatic stellate cells (HSC) are mesenchymal cells found within the perisinusoidal space that are responsible for maintaining extracellular matrix in the liver (Si-Tayeb *et al.*, 2010). These cells are the primary site of vitamin A storage within cytoplasmic lipid droplets, and they work alongside hepatocytes to promote hepatic regeneration (Si-Tayeb *et al.*, 2010). Endothelial cells make up the larger hepatic blood vessels, and are capable of controlling blood flow much like those found throughout the rest of the body (Si-Tayeb *et al.*, 2010). Alternatively, liver sinusoidal endothelial cells are specialized endothelial cells that form the sinusoidal capillaries of hepatic lobules. They allow for the transfer of various molecules between hepatocytes and blood, while simultaneously having a role in immune function and blood clotting in the liver (Si-Tayeb *et al.*, 2010). Finally, Kupffer cells and Pit cells are the immune cells of the liver. Kupffer cells exist within the lining of hepatic sinusoids, and they are the 'resident macrophages' due to their phagocytotic abilities (Si-Tayeb *et al.*, 2010). These cells are plentiful relative to Pit cells, which are rare natural killer (NK) cells possessing cytotoxic activity (Si-Tayeb *et al.*, 2010).

1.2.3 Physiological Function

As mentioned previously, the liver is arguably the most versatile organ in the body. It possesses both endocrine and exocrine function, making it essential in regulating metabolism, digestion, production of proteins, immune function, detoxification, and storage of macromolecules. With respect to exocrine function, the liver produces and delivers bile to the gallbladder via the hepatic duct. Bile is an aqueous secretion containing phospholipids, cholesterol, bile salts, bile pigments, and electrolytes (Ross and Pawlina, 2011). Many of these compounds are also recycled back to the liver via the hepatic portal circulation. Bile is primarily involved in the emulsification of lipids present in the duodenum, but it also contributes to maintaining cholesterol, bilirubin, and mineral (*e.g.,* iron and copper) levels (Si-Tayeb *et al.*, 2010). Moreover, the liver produces many plasma proteins present in circulation. These include albumins, glycoproteins, lipoproteins, clotting proteins (*e.g.,* prothrombin and fibrinogen), and non-immune globulin proteins (*e.g.,* α-globulins and β-globulins; Ross and Pawlina, 2011). Each of these plasma proteins are involved in either the regulation of fluid balance or the distribution of molecules in the bloodstream. For example, various vitamins and minerals (*e.g.,* vitamin A, vitamin D, vitamin K, and iron) require binding to plasma proteins in circulation before they are either stored at the liver or biochemically modified (Ross and Pawlina, 2011).

Aside from being the largest internal organ, the liver is also the largest endocrine gland in the body. It is capable of producing some hormones, including IGF-1 and angiotensinogen, along with modifying and metabolizing hormones produced by other organs (Rhyu and Yu, 2021). For example, thyroxine (T_4) and vitamin D both require conversion at the liver to their bioactive forms, while insulin and glucagon are pancreatic hormones that are primarily degraded at the liver (Rhyu and Yu, 2021). In addition to the metabolism of hormones, the liver is essential in managing systemic levels of xenobiotics and macromolecules (Corsini and Bortolini, 2013). Both phase I (oxidation) and phase II (conjugation) of drug metabolism occurs in the liver, whereby hepatocytes initiate biochemical reactions to convert drugs into their inactive forms. In phase I metabolism,

hepatic cytochrome P450 (Cyp) enzymes catalyze the carboxylation or hydroxylation of drugs at the smooth ER (Corsini and Bortolini, 2013). These modified compounds are made further water soluble through glucuronidation, acetylation, and sulfation reactions in phase II before their distribution to the kidney for excretion (Corsini and Bortolini, 2013).

Along with its endocrine and drug detoxification role, the liver controls the numerous molecular pathways involved in nutrient metabolism. With respect to glucose metabolism, the liver synthesizes and stores glucose via gluconeogenesis and glycogenosis. Alternatively, it can metabolize and release glucose by means of glycolysis and glycogenolysis. In the fasted state, the liver metabolizes glycogen stores in response to increased levels of plasma glucagon from the pancreas (Jones, 2016). The liver releases glucose into circulation so that it can be distributed to peripheral tissues, including skeletal muscle and the brain. In the fed state, glucagon levels subside and the pancreas releases insulin (Jones, 2016). Hepatic insulin receptors detect this increase in plasma insulin, thereby signaling the liver to decrease hepatic glucose output and increase glucose uptake. This allows for replenishment of hepatic glycogen stores such that glucose is available again later in the fasted state as previously described. Again, the liver is also a major site of protein synthesis, but it is also capable of digesting proteins and metabolizing amino acids (Trefts *et al.*, 2017). This process does not produce as much energy as glucose metabolism, and it requires the removal of nitrogenous waste via the urea cycle. That said, some amino acids can serve as a substrate for the tricarboxylic acid (TCA) cycle during gluconeogenesis in the fasted state. Finally, the liver plays a major role in lipid homeostasis through the synthesis and metabolism of lipids and lipoproteins (Jones, 2016). This will be discussed in greater detail below (Section 1.2.4).

1.2.4 Hepatic Lipid Biosynthesis and Metabolism

Management of homeostatic lipid levels is an intricate process involving numerous metabolic pathways. Many of these pathways are controlled by hepatocytes, which are capable of synthesizing and metabolizing lipids as an energy source (Si-Tayeb

et al., 2010). Lipids exist in three different forms, including phospholipids, steroids, and triglycerides. Phospholipids are amphipathic molecules that make up the lipid bilayer of cellular membranes; therefore, they have a role in cell signaling and the transport of various molecules into and out of the cell (Küllenberg *et al.*, 2012). Steroids are unique in that they have a fused ring structure, and they are key components of the endocrine system (Borah and Banik, 2020). Cholesterol, which is produced and metabolized in the liver, serves as a precursor to several steroid hormones (*e.g.,* estrogen and testosterone; Zampelas and Magriplis, 2019). Cholesterol also helps to maintain the fluidity and integrity of cell membranes through its presence in the phospholipid bilayer (Zampelas and Magriplis, 2019). Finally, triglycerides are esters comprised of three fatty acids bound to a single glycerol molecule (Bayly, 2014). Fatty acids can exist in circulation as their non-esterified form (*i.e.,* non-esterified fatty acids; NEFAs); however, they must be bound to albumin due to their hydrophobic nature (Bayly, 2014). NEFAs can be classified as either 'unsaturated' or 'saturated' depending on the number of carbon-carbon double bonds present in their chemical structure. Unsaturated fatty acids possess one (monounsaturated fatty acids; MUFAs) or more (polyunsaturated fatty acids; PUFAs) carbon-carbon double bonds, which further gives rise to *cis/trans* isomers (Tvrzicka *et al.*, 2011). Conversely, saturated fatty acids do not possess any carbon-carbon double bonds (Tvrzicka *et al.*, 2011). Importantly, NEFAs are derived from the breakdown of dietary triglycerides by lipase or thioesterase enzymes (Nguyen *et al.*, 2008). This enzyme frees NEFAs from the chylomicron structure, allowing them to be taken up by hepatocytes.

Lipid biosynthesis occurs in both the liver and adipose tissue, and it begins with the uptake of NEFAs from the bloodstream. In the liver, transmembrane NEFA transport occurs via passive diffusion or facilitated transport by fatty acid transporter proteins (FATPs) and fatty acid translocase (CD36; Nguyen *et al.*, 2008; Pepino *et al.*, 2014). Once inside the cell, NEFAs bind with fatty acid binding proteins (FABP) and acyl-CoA synthetases (ACS; Nguyen *et al.*, 2008). FABPs exist within hepatic microsomes and serve as cytosolic shuttles that mediate NEFA transport (Furuhashi and Hotamisligil, 2008). ACS uses NEFAs as a substrate in generating acyl-CoA, which is further

metabolized to generate acetyl-CoA (Furuhashi and Hotamisligil, 2008). Both NEFAs and acyl-CoA have a myriad of cellular fates, including *de novo* lipogenesis, synthesis of triglycerides and phospholipids, mitochondrial and peroxisomal β-oxidation, or transport to the nucleus to interact with various transcription factors (Nguyen *et al.*, 2008).

1.2.4.1 De novo Lipogenesis and Triglyceride Synthesis

De novo lipogenesis (DNL) is the synthesis of novel fatty acids from excess glucose. Because of this, *de novo* lipogenesis is highly sensitive to plasma insulin levels. Insulin resistance promotes increased *de novo* lipogenesis, often culminating in NAFLD (Smith *et al.*, 2020). Moreover, the expression of many lipogenic transcription factors are subsequently regulated by insulin. These include the sterol regulatory-element binding protein (SREBP) and carbohydrate response element binding protein (ChREBP) transcription factor families (Matsuzaka and Shimano, 2009). In short, *de novo* lipogenesis begins with hepatic glucose uptake at GLUT2 upon signaling from insulin (Figure 1-2). Following a series of glycolytic reactions in the cytosol, glucose is converted to pyruvate before its transport into mitochondria through the voltage-dependent anion channel (VDAC). Pyruvate undergoes decarboxylation by pyruvate dehydrogenase (PDH) to generate acetyl Co-A, which must enter the TCA cycle to produce citrate for export into the cytosol via the mitochondrial citrate transporter. ATP citrate synthase (ACL) converts citrate back into acetyl Co-A, which is converted to malonyl Co-A by acetyl-CoA carboxylase alpha (ACCα; Kim, 1997; Jensen-Urstad and Semenkovich, 2012). Fatty acid synthase (FAS) then uses malonyl Co-A as a substrate to generate long-chain saturated fatty acids such as palmitic acid (Kim, 1997; Jensen-Urstad and Semenkovich, 2012). Palmitic acid is further elongated by elongation of long-chain fatty acids family member 6 (ELOVL6) to produce stearic acid (Matsuzaka and Shimano, 2009), followed by action of stearoyl Co-A desaturase-1 (SCD-1) to generate MUFAs at the ER (Miyazaki and Ntambi, 2003). These MUFAs are subject to a series of elongation reactions, and acted on by ACS to produce acyl Co-A. From here, triglyceride synthesis begins with the acylation of acyl Co-A with glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to synthesize lysophosphatidic acid (LPA; Yu *et al.*,

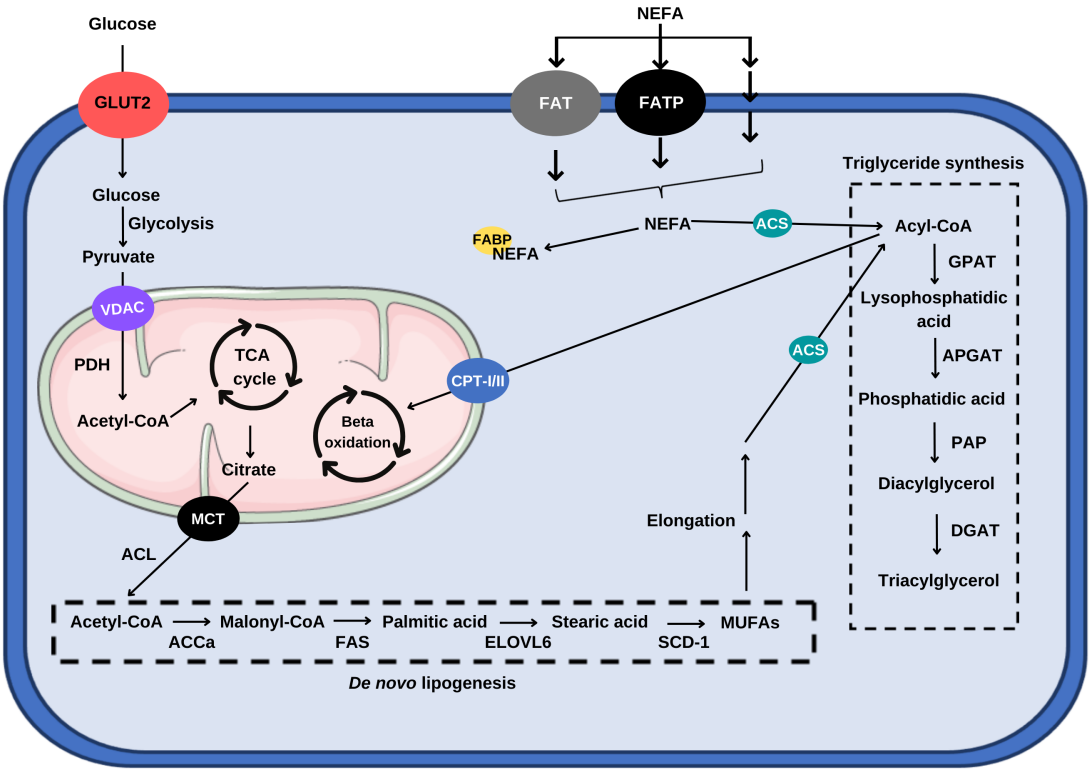

Figure 1-2. Overview of de novo lipogenesis and triglyceride synthesis in the liver.

Hepatic lipogenesis is an insulin-sensitive process requiring the coordination of fatty acid uptake, *de novo* lipogenesis, and triglyceride synthesis in the hepatocyte. Fatty acids can undergo cytosolic transport or be used to generate acyl-CoA for triglyceride synthesis or beta oxidation. NEFA, non-esterified fatty acid; FAT, fatty acid translocase; FATP, fatty acid transport protein; GLUT2, glucose transporter 2; VDAC, voltage-dependent anion channel; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; MCT, mitochondrial citrate transporter; ACL, ATP citrate synthase, ACCα, acetyl-CoA carboxylase alpha; FAS, fatty acid synthase; ELOVL6, elongation of long-chain fatty acids family member 6; SCD-1, stearoyl Co-A desaturase-1, MUFA, monounsaturated fatty acid; ACS, acyl-CoA synthetase; FABP, fatty acid binding protein; GPAT, glycerol-3-phosphate acyltransferase; APGAT, 1-acyl glycerol-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase.

2018). LPA is converted to phosphatidic acid (PA) by the 1-acyl glycerol-3-phosphate acyltransferase (APGAT) family of enzymes, and PA is dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG; Yu *et al.*, 2018). Importantly, diacylglycerol acyltransferase (DGAT) catalyzes the conversion of DAG to triglycerides as the terminal step of triglyceride synthesis (Cases *et al.*, 1998; Smith *et al.*, 2000). The resulting triglycerides can either be stored in the liver or secreted from hepatocytes to peripheral tissues as very low density lipoprotein (VLDL; Kawano and Cohen, 2013).

1.2.4.2 Mitochondrial and Peroxisomal β-Oxidation

Under low glucose conditions, hepatocytes can synthesize energy from lipids via β-oxidation in peroxisomes and mitochondria. While the goal of mitochondrial β-oxidation is to satisfy energy needs of the cell through coupling with ATP synthesis, peroxisomal β-oxidation metabolizes long-chain fatty acids that are too long for handling by mitochondria (Nguyen *et al.*, 2008). Peroxisomal β-oxidation ultimately produces hydrogen peroxide, which is broken down into water and oxygen by catalase (Nguyen *et al.*, 2008). Overall, β-oxidation is an efficient catabolic process that produces ATP from free fatty acids. Given that the liver stores fatty acids in the form of triglycerides, lipolysis of stored triglycerides must occur prior to β-oxidation to release fatty acids from glycerol chains. This reaction is catalyzed by the enzyme lipase, which is regulated by insulin and glucagon (Nguyen *et al.*, 2008; Trefts *et al.*, 2017). While free glycerol molecules remain in the liver as a substrate for gluconeogenesis, the resulting fatty acids either stay in the liver or are distributed to skeletal muscle (Trefts *et al.*, 2017). NEFAs present in circulation can also be imported into the liver as previously described. Regardless, all fatty acids must be converted into acyl-CoA by ACS such that they can undergo mitochondrial transport (Nguyen *et al.*, 2008). Acyl-CoA is further converted to acylcarnitine by carnitine palmitoyltransferase (CPT)-I at the outer mitochondrial membrane, allowing for transport into the mitochondrial matrix (Nguyen *et al.*, 2008). The function of CPT-I is inhibited by malonyl-CoA produced during fatty acid synthesis; therefore, the anabolism and catabolism of fatty acids cannot occur simultaneously.

Acylcarnitine is reconverted to acyl-CoA through action of CPT-II in the inner mitochondrial membrane, allowing for initiation of β-oxidation (Nguyen *et al.*, 2008). Alternatively, short- and medium-chain fatty acids pass through mitochondrial membranes freely and are converted to acyl-CoA in the matrix by ACS (Nguyen *et al.*, 2008). Ultimately, the end goal of reach round of β-oxidation is to produce one molecule of acetyl-CoA (Talley and Mohiuddin, 2022). Acetyl-CoA can feed into the TCA cycle to generate ATP, while it can also be used to produce ketone bodies via hepatic ketogenesis (Nguyen *et al.*, 2008). One round of β-oxidation removes two carbons from the end of a fatty acid chain, and this process occurs until the entire molecule is broken down. Because of this, fatty acids of different lengths will produce different amounts of ATP depending on the number of rounds of β-oxidation that occur. This breakdown is an oxidative process, and the corresponding reduction produces one molecule of $FADH_2$ (flavin adenine dinucleotide) and one molecule of NADH (nicotinamide adenine dinucleotide + hydrogen) per round (Talley and Mohiuddin, 2022). NADH and $FADH_2$ are electron carrier molecules; therefore, they consequently donate their electrons to the electron transport chain for ATP synthesis.

1.2.5 Animal Models Linking Intrauterine Growth Restriction and Hepatic Dyslipidemia

As outlined in Section 1.1.2.1, evidence in support of the relationship between birth weight and dyslipidemia is abundant. However, epidemiological studies are limited in that they provide no insight into the molecular mechanisms by which lipid handling is affected. It is for this reason that various animal models of IUGR have enhanced our knowledge of this relationship, along with distinguishing between maternal and fetal contributions toward metabolic dysfunction. These models include uterine ligation and ablation, maternal nutrient restriction, maternal protein restriction, and maternal drug exposure.

Uterine ligation and ablation are surgical interventions used to mimic the effects of idiopathic placental insufficiency. In this model, fetal nutrient and oxygen transfer

become reduced such that birth weight and liver to body weight ratio are decreased in the affected offspring (Yudkin and Stanner, 1998; Ross and Beall, 2009; Elias *et al.*, 2016; Sarr *et al.*, 2021). This is attributed to decreased expression of hepatic and circulating IGF-1 in the offspring, which is critical for both fetal growth and postnatal metabolism (Fu *et al.*, 2015). Offspring born from this model further demonstrate high occurrences of dyslipidemia, obesity, and type II diabetes in adult life, while these effects are also transgenerational as they arise in the F2 generation (Ogata *et al.*, 1986; Simmons *et al.*, 1992; Tran *et al.*, 2013; Goodspeed *et al.*, 2015; Sarr *et al.*, 2021). These metabolic defects occur due to impaired glucose and fatty acid metabolism in the liver, as demonstrated by increased expression and activity of hepatic glucocorticoid receptors, increased glucose production, insulin resistance, increased expression of hepatic GLUT1, and oxidative stress (Lane *et al.*, 1999, 2002; Baserga *et al.*, 2005; Raab *et al.*, 2009; Sarr *et al.*, 2021). Interestingly, neonatal administration of Exendin-4, a glucagon-like peptide-1 receptor agonist, improves both insulin resistance and oxidative stress in the livers of these offspring (Raab *et al.*, 2009).

Maternal nutrient restriction (MNR) and maternal protein restriction (MPR) are models of maternal undernutrition that reduce gestational caloric or protein intake, respectively. Both models lead to decreased fetal nutrient availability; therefore, MNR and MPR are useful in studying the impact of maternal malnutrition and placental insufficiency. Importantly, MPR leads to amino acid deficiencies in the fetus despite no changes to maternal weight gain or food intake (Desai and Hales, 1997; Sohi *et al.*, 2015). Amino acids are essential for fetal growth and development (Crosby, 1991), so the MPR model provides insight into the consequences of fetal amino acid deficiencies specifically. Both MNR and MPR offspring exhibit asymmetrical IUGR, along with postnatal catch-up growth resulting in long-term hepatic hyperlipidemia, glucose intolerance, and insulin resistance. Studies have demonstrated that these effects occur in response to epigenetic regulation of genes encoding metabolic enzymes. For example, MNR offspring exhibit decreased methylation within the promoter region of phosphoenolpyruvate carboxylase (PEPCK), a rate limiting enzyme involved in hepatic gluconeogenesis (George *et al.*, 2012). Alternatively, MPR offspring with catch-up

growth have increased methylation and decreased acetylation of histone H3 lysine residues [K9,14] surrounding the promoter region of Cyp7a1, the major enzyme responsible for cholesterol catabolism (Sohi *et al.*, 2011). These two histone modifications result in chromatin silencing, leading to decreased expression of Cyp7a1 and elevated hepatic and circulating cholesterol (Sohi *et al.*, 2011).

Finally, our laboratory has previously implemented a rodent model of maternal nicotine exposure (MNE) to study the effects of nicotine on postnatal metabolism. With this model, pregnant dams receive daily subcutaneous injections of saline or nicotine bitartrate (1 mg/kg/day) up until the point of weaning (Ma *et al.*, 2013). At birth, MNE offspring are low birth weight, and undergo whole body and hepatic catch-up growth by three weeks of age (Ma *et al.*, 2013). In adult life, MNE offspring exhibit increased hepatic triglyceride levels, while male MNE offspring also display increased fasting serum triglycerides (Ma *et al.*, 2013). Further investigation of male MNE offspring showed increased transcript abundance of FAS and ACCα in the liver, while FAS protein abundance was also increased (Ma *et al.*, 2013). Moreover, these changes occurred alongside increased hepatic protein levels of liver X receptor alpha (LXRα), a transcription factor involved in the silencing of genes associated with glucose production. LXRα is known to regulate expression of enzymes that partake in lipid synthesis; therefore, it is possible that it contributes to the hyperlipidemia observed in MNE male offspring. Chromatin immunoprecipitation (ChIP) assays revealed an increase in the acetylation of histone H3 [K9,14] surrounding the LXR binding element (LXRE) on the proximal promoter of FAS, providing further evidence that lipid synthesis pathways are sensitive to altered epigenetic mechanisms (Ma *et al.*, 2013).

It's clear that each of the previously described IUGR animal models have replicated the metabolic outcomes observed among human cohorts of IUGR individuals. In addition, they have further provided great insight into the underlying molecular mechanisms that promote dyslipidemia due to a poor *in utero* environment and postnatal catch-up growth. These molecular mechanisms will be expanded upon below in Section 1.3.

1.3 Molecular Mechanisms Underlying Metabolic Disease in Growth-Restricted Offspring

Fetal growth and development consist of intricate cellular processes that are highly sensitive to intra- and extracellular stressors. Because of this, it is plausible that the presence of metabolic disease in adult IUGR offspring is attributed in part to cellular stress and programmed cell death. While events of cell stress and cell death are often protective, they can also be destructive and contribute to the development of metabolic disease. Studies have demonstrated that a suboptimal prenatal environment initiates cell stress and cell death in the placenta, giving rise to compromised fetal growth. This may further lead to different forms of cellular stress, including oxidative stress, mitochondrial dysfunction, and ER stress. Each of these forms of cell stress can lead to programmed cell death (*i.e.,* apoptosis and autophagy) when uncontrolled; however, for the sake of this thesis, I will only discuss the types of cell stress previously mentioned. Moreover, molecular mechanisms such as epigenetics have a role in this process, including DNA methylation, post-translational histone modifications, and microRNAs (miRNAs). Finally, the occurrence of rapid postnatal weight gain (*i.e*., catch-up growth) in low birth weight offspring can lead to cellular stress and metabolic disease in an indirect manner. As such, this section addresses the latest research findings on the various types of cellular stress and epigenetic mechanisms that are prevalent in IUGR offspring during postnatal life.

1.3.1 Oxidative Stress and Mitochondrial Dysfunction

Mitochondria are membrane-bound organelles present in most eukaryotic organisms. Each mitochondrion is composed of an inner and outer mitochondrial membrane, which separates the mitochondrion into two distinct compartments known as the intermembrane space and the matrix (McCarron *et al.*, 2013). While most cellular DNA is present within the nucleus, mitochondria also have their own genome (*i.e.,* mitochondrial DNA; mtDNA) that contains genes which are critical for mitochondrial function (Taanman, 1999). These include genes that control aerobic metabolism, as

mitochondria are key energy-producing organelles in the cell. Each cell contains an abundance of mitochondria; however, certain cell types possess greater numbers of mitochondria due to higher energy requirements. For example, hepatocytes can contain anywhere from 1000–2000 mitochondria per cell, as they are responsible for regulating various metabolic pathways (Wiesner *et al.*, 1992). In the presence of oxygen, mitochondria are capable of producing energy in the form of adenosine triphosphate (ATP) at much higher levels than anaerobic metabolic processes (*i.e.,* glycolysis) alone (Naifeh *et al.*, 2022). Aerobic metabolism consists of the TCA cycle and oxidative phosphorylation, which occur within the mitochondrial matrix (Naifeh *et al.*, 2022). Following the completion of glycolysis, pyruvate is transported into mitochondria through the voltage-dependent anion channel (VDAC) present in the outer mitochondrial membrane (OMM), followed by the mitochondrial pyruvate carrier (MPC) in the inner mitochondrial membrane (IMM; Zangari *et al.*, 2020). Once present in the matrix, pyruvate is converted to acetyl Co-A by PDH (Naifeh *et al.*, 2022). This acetyl-CoA molecule is used as a substrate by the TCA cycle, which consists of a series of oxidative reactions that produces two molecules of carbon dioxide, three molecules of NADH, one molecule of $FADH_2$, and one molecule of guanosine triphosphate (GTP) per cycle (Naifeh *et al.*, 2022). Together with the two NADH molecules generated during glycolysis, the NADH and $FADH_2$ molecules generated during the TCA cycle can donate their electrons to oxygen at the electron transport chain. This process is referred to as oxidative phosphorylation, which involves five cytochrome enzymes present in the inner mitochondrial membrane (Demirel and Gerbaud, 2019). These enzymes include complexes I–V, and they are present in series with each other such that they can pass electrons to each other in a stepwise manner (Demirel and Gerbaud, 2019). As a result, the complexes become reduced while NADH and $FADH_2$ are oxidized. As electrons are passed from electron carriers to the ETC, protons are also shuttled across the inner mitochondrial membrane from the matrix to the intermembrane space (Demirel and Gerbaud, 2019). This proton gradient is a source of potential energy that fuels complex V in generating ATP (Demirel and Gerbaud, 2019).

Pregnancy and early postnatal development are metabolically challenging periods of growth for both the mother and offspring. Accordingly, IUGR is often associated with increased stress within mitochondria. Mitochondria utilize oxygen as the terminal electron acceptor in aerobic respiration; however, the production of damaging reactive oxygen species (ROS) and ROS by-products is a negative side effect (Cadenas and Davies, 2000). While low levels of ROS are required for some enzymatic reactions and signaling pathways, they inflict oxidative damage on macromolecules when present in excess (Krumova and Cosa, 2016). Oxidative stress arises when there is an imbalance between ROS production and antioxidant enzymes, which are responsible for the transformation of ROS into less harmful molecules (Cadenas and Davies, 2000). The mitochondrial electron transport chain is both a source and target of ROS; therefore, mitochondrial dysfunction commonly coexists with unabated oxidative stress (Cadenas and Davies, 2000). Importantly, both oxidative stress and mitochondrial dysfunction are consistently associated with the IUGR-induced metabolic syndrome. Studies have revealed that mothers of growth-restricted offspring tend to have increased ROS and decreased levels of antioxidants in the blood (Karowicz-Bilinska *et al.*, 2002; Gupta *et al.*, 2004; Biri *et al.*, 2007; Saker *et al.*, 2008), while tissues taken from IUGR offspring also demonstrate oxidative stress. This includes not only an increase in ROS, but also the differential expression of antioxidant enzymes, increased lipid peroxidation, and compromised synthesis of ATP (Rodríguez-Rodríguez *et al.*, 2018) . Collectively, these trends indicate that oxidative stress is an important contributor to the aberrant metabolism seen in IUGR offspring.

The liver is highly subjected to oxidative stress given that it is abundant in mitochondria due to its critical role in nutrient metabolism. Many chronic forms of liver disease, including non-alcoholic steatohepatitis (NASH) and NAFLD, are characterized by increased markers of oxidative stress, which is often accompanied by an accumulation of damaged or dysfunctional mitochondria (Mantena *et al.*, 2008; Rolo *et al.*, 2012; Begriche *et al.*, 2013; Chen *et al.*, 2020). The IUGR liver is no exception to this, as numerous studies have found evidence of hepatic oxidative and mitochondrial stress in growth-restricted offspring. For example, in a porcine model of spontaneous IUGR,

growth-restricted neonates had increased levels of hepatic alpha-1-acid glycoprotein at birth, indicating hepatic and systemic oxidative stress (Wang *et al.*, 2008). These offspring also displayed increased protein levels of complex IV of the electron transport chain, suggesting that ATP availability may be reduced due to accelerated ATP hydrolysis (Wang *et al.*, 2008). In a rat model of caloric restriction, a 50% decrease in maternal caloric intake from gestational day 11 through PND 21 (*i.e.,* the point of weaning) resulted in increased lipid peroxidation marker 4-hydroxynonenol (4HNE) and decreased glutathione protein levels at three weeks (Devarajan *et al.*, 2019). Adult offspring had unaltered 4HNE and antioxidant levels after switching to a control diet at weaning, suggesting that the caloric restriction itself had caused the oxidative stress (Devarajan *et al.*, 2019). Many studies using the MPR rat model have also established that catch-up growth may be of greater detriment to hepatic mitochondrial function rather than the original in utero nutritional insult. Again, in this model, pregnant rat dams are fed a low protein diet throughout gestation, thereby inducing fetal protein deficiency and IUGR. Offspring that are maintained on a low protein diet postnatally (LP1 offspring) do not undergo catch-up growth and remain small relative to control offspring. These offspring are metabolically healthy in adult life, while they also exhibit decreased hepatic ROS production (Sohi *et al.*, 2011; Moraes *et al.*, 2014). Given that LP1 offspring do not experience a nutritional mismatch between the pre- and postnatal environments, it is possible that the maintenance of the low protein diet may be protective of mitochondrial function in the absence of catch-up growth. Alternatively, whole body and hepatic catch-up growth is induced by switching offspring to a normal protein diet at weaning (LP2 offspring) or at birth (LP3 offspring; Sohi *et al.*, 2011). We have shown that LP2 offspring exclusively develop hypercholesterolemia, glucose intolerance, and impaired drug metabolism at four months of age, while LP1 and LP3 offspring appear to be unaffected (Sohi *et al.*, 2011; Vo *et al.*, 2013). This occurs alongside ER stress, which is known to exist simultaneously with oxidative stress and mitochondrial dysfunction (Plaisance *et al.*, 2016). Other studies have also shown that catch-up growth after weaning results in increased hepatic oxygen consumption, altered antioxidant level and activity, and decreased mitochondrial DNA-encoded gene expression (Park *et al.*, 2003;

Theys *et al.*, 2009; Moraes *et al.*, 2014). Again, LP3 offspring do not exhibit these metabolic and mitochondrial deficits, so the timing of nutritional restoration and catch-up growth appear to be of importance in initiating oxidative stress as a driver of poor liver health. By introducing a normal protein diet prior to the completion of hepatic differentiation, the liver may be protected from the metabolic consequences of oxidative stress and poor mitochondrial function.

1.3.2 Endoplasmic Reticulum (ER) Stress

As mentioned previously, oxidative stress is also known to occur alongside ER stress. The mitochondrion and ER are physically connected at sites called the mitochondrial-associated ER membrane (MAM); these sites can indirectly influence the production of ATP, and they are responsive to ER signaling during instances of increased protein folding (Hayashi *et al.*, 2009). When the ER cannot facilitate proper protein folding, this leads to lumenal accumulation of misfolded or unfolded proteins in the ER (*i.e.*, ER stress). While ER stress is essential for embryonic development and the maintenance of pregnancy (Michalak and Gye, 2015), excessive or chronic ER stress during postnatal life can be triggered by numerous cellular insults. These may include oxidative stress, diminished calcium homeostasis, decreased supply of amino acids, viral infection, decreased formation of disulfide bonds, and decreased N-linked glycosylation (Xu *et al.*, 2005; Wong *et al.*, 2015). Furthermore, postnatal catch-up growth has been shown to trigger ER stress, specifically in the growth-restricted liver (Sohi *et al.*, 2013). In the unstressed cell, the chaperone protein Grp78 prevents the activation of ER transmembrane proteins IRE1, PERK, and Atf6 by binding their N-terminal domains in the ER lumen (Bertolotti *et al.*, 2000; Hong *et al.*, 2004). Under ER stress, the cell's unfolded protein response (UPR) becomes activated via release of these transmembrane proteins from Grp78 (Bertolotti *et al.*, 2000; Hong *et al.*, 2004). This allows for their oligomerization and subsequent activation of targets that promote an adaptive phenotype (Bertolotti *et al.*, 2000). This entails increased transcription of genes that strengthen the folding capacity of the ER, or protein degradation. If the ER cannot be successfully relieved of misfolded and unfolded proteins, ER-stress induced cell death is initiated

through the activation of C/EBP-homologous protein (CHOP; Xu *et al.*, 2005; Wong *et al.*, 2015). Certain ER stress pathways have been implicated in the onset of various metabolic diseases (Cnop *et al.*, 2012), so the presence of ER stress in IUGR tissues is not surprising.

Various animal models have found that hepatic ER stress may serve as an underlying mechanism for the dysregulated blood glucose and insulin resistance exhibited by IUGR offspring. Again, the liver regulates metabolic pathways that maintain blood glucose levels, including gluconeogenesis. Rodent models demonstrate that activation of the UPR either precedes or occurs simultaneously with accelerated hepatic gluconeogenesis, and these offspring later exhibit glucose intolerance and impaired hepatic glycogen storage (Sohi *et al.*, 2013; Vo *et al.*, 2013; Deodati *et al.*, 2018; Liu *et al.*, 2018). For example, one study showed that uteroplacental insufficiency due to uterine artery ligation resulted in increased markers of ER stress in the affected pups at birth, concomitant with increased transcript abundance of gluconeogenic enzymes Phosphoenolpyruvate Carboxykinase 1 (PCK1) and glucose-6-phosphatase catalytic subunit (G6Pc; Deodati *et al.*, 2018)) . At PND 105, these male IUGR offspring were glucose intolerant and still had active markers of ER stress (Deodati *et al.*, 2018). The UPR is also activated in protein-restricted offspring via upregulation of Atf2, Atf6, and phosphorylated eukaryotic transcription factor 2 alpha (p-eIF2α) during fetal life, followed by elevated fasting blood glucose and decreased Periodic acid-Schiff (PAS) stain (*i.e.,* decreased glycogen storage) at twelve weeks (Liu *et al.*, 2018). Alternatively, Sohi et al., demonstrated that ER stress occurs exclusively following catch-up growth in the protein-restricted liver (*i.e.,* LP2 offspring; Sohi *et al.*, 2013). These LP2 offspring exhibited increased hepatic Grp78, Grp94, p-eIF2α[Ser51], and spliced X box binding protein 1 (Xbp-1), along with increased protein levels of p85 and decreased phosphorylation of protein kinase B (Akt1) at serine residue 473 (p-Akt1[Ser473]; Sohi *et al.*, 2013). Increased p85 and decreased p-Akt1[Ser473] are commonly associated with impaired insulin signaling, while p-Akt [Ser473] and p-eIF2α[Ser51] are also inversely related and occur with insulin resistance (Mounir *et al.*, 2011; Sohi *et al.*, 2013). Of note, offspring that had not undergone catch-up growth (*i.e.,* LP1 offspring) did not exhibit

indices of ER stress or insulin resistance (Sohi *et al.*, 2013). Protein levels of Grp78 and Grp94 were also increased at embryonic day (ED) 19; therefore, it is possible that hepatic ER stress begins in the protein-restricted liver during fetal life and is worsened with postnatal catch-up growth (Sohi *et al.*, 2013).

1.3.3 Epigenetic Regulation of Gene Expression

Although there are many proposed definitions of epigenetics, it is generally defined as the study of heritable changes in phenotype without altering the underlying DNA sequence (Greally, 2018). Importantly, this definition is not meant to encompass all forms of transcriptional regulation, and rather describes three distinct mechanisms: (1) DNA methylation, (2) post-translational histone modification, and (3) non-coding RNAs (*e.g.,* microRNAs). Given that embryonic and fetal development require careful timing of changes to gene expression, and epigenetics is highly influenced by environmental changes, it is not surprising that aberrant epigenetic marks have been documented among IUGR offspring. That said, much attention has been given to the role of epigenetic mechanisms in implantation of the blastocyst and development of the placenta rather than metabolic organs. Here I will outline some of the evidence that does exist in support of epigenetics as a contributor to postnatal dysmetabolism among IUGR offspring.

DNA methylation is a covalent biochemical modification involving the transfer of a methyl group to cytosine residues (Schenkel *et al.*, 2017). It most commonly occurs at cytosine-guanidine (CpG)-rich regions of the genome, particularly at the 5’ end of promoter regions (Schenkel *et al.*, 2017; Goyal *et al.*, 2019). Methylation of promoter regions occurs via DNA methyltransferase (DNMT) enzymes, which catalyze the transfer of methyl groups from S-adenosyl-methionine to cytosine (Schenkel *et al.*, 2017). This causes the condensation of chromatic structure, thereby leading to transcriptional repression. Conversely, the removal of methyl groups by enzymes such as the ten-eleven translocation (TET) methylcytosine dioxygenase family can activate transcription and upregulate gene expression (Schenkel *et al.*, 2017). As mentioned previously, IUGR rodent offspring born to dams with uterine ligation or ablation exhibit decreased

expression of IGF-1; this is partially attributed to altered CpG methylation status, which is further seen in the F2 generation (Goodspeed *et al.*, 2015). This can be alleviated by weaning offspring onto an essential nutrient methyl-donor diet (Goodspeed *et al.*, 2015), which has been shown to reduce accumulation of hepatic triglycerides and NAFLD (Cordero *et al.*, 2013). In 2005, a study by Lillycrop *et al.* demonstrated that the CpG island methylation status of hepatic glucocorticoid receptor (*GR*) and *PPARα* promoters are significantly reduced in protein-restricted offspring, and this hypomethylated state is associated with increased expression of these genes (Lillycrop *et al.*, 2005). Interestingly, feeding of a low protein diet in combination with folic acid supplementation prevented these epigenetic changes, indicating that one-carbon metabolism is essential in preventing the effects of this maternal insult (Lillycrop et al, 2005). Further studies also confirmed that this alteration also exemplifies transgenerational effects, as methylation status is decreased in the F2 generation at postnatal day 80 (Burdge et al, 2007). This is characteristic of many epigenetic mechanisms, thereby illustrating relevance of perinatal insult to health outcomes of future generations.

In addition to DNA methylation, chromatic structure is greatly affected by post-translational histone modifications. Histones are proteins rich in positively charged lysine and arginine residues that provide structural support to chromosomes. Negatively charged DNA is wrapped around histone octamers, each consisting of two copies of the H2A, H2B, H3 and H4 histones (Hong *et al.*, 1993). This makes up the nucleosome core structure, which is linked to adjacent nucleosomes by stretches of DNA that are less than 100 bp long (Kornberg and Lorch, 1999). These DNA regions are associated with histone H1 to form higher level chromatic structures, which allows for formation of whole chromosomes (Kornberg and Lorch, 1999). Because of this structural arrangement, histones regulate DNA accessibility to replication and transcriptional machinery, and biochemical modifications to amino acid residues within histones can alter gene transcription. These modifications include histone acetylation, methylation, ubiquitination, sumoylation, phosphorylation, and ADP-ribosylation (Schenkel *et al.*, 2017). Notably, histone acetylation is associated with a euchromatic structure that permits gene transcription, while histone methylation is associated with heterochromatin and

transcriptional repression (Schenkel *et al.*, 2017). In addition to altering DNA methylation status, uterine artery ligation leads to changes in histone H3 acetylation and methylation at various lysine residues both surrounding the promoter region of IGF-1 and within the IGF-1 gene itself (Fu *et al.*, 2009). Interestingly, histone modifications were different between male and female IUGR offspring at specific lysine residues, suggesting that changes to the IGF-1 'histone code' are sex-specific (Fu *et al.*, 2009). In protein-restricted offspring, the long-term expression of gluconeogenic enzymes (*e.g.,* G6Pase and 11β-hydroxysteroid dehydrogenase type I; 11β-HSD1) is increased due to the histone-mediated silencing of LXRα at four months (Vo *et al*., 2013). Vo *et al.* demonstrated that there is a significant decrease in histone H3 acetylation [K9, 14] at the transcriptional start site of Lxrα in 4 month recuperated protein-restricted offspring (Vo *et al.*, 2013). This is concomitant with decreased association of LXRα at the LXR response element of G6Pase and 11β-HSD1, culminating in glucose intolerance (Vo *et al*., 2013). Again, these same offspring also exhibit decreased expression of hepatic Cyp7a1, leading to hypercholesterolemia in male offspring at three weeks and four months of age (Sohi *et al.*, 2011). This reduction in enzyme expression is due to epigenetic silencing at the Cyp7a1 promoter region, as there is increased tri-methylation and decreased acetylation of histone H3[K9, 14]. It is interesting that female protein-restricted offspring from the same cohort are protected from these histone modifications in adult life, as they show complete opposite trends in methylation and acetylation.

Lastly, non-coding RNA molecules have been demonstrated to influence long-term gene expression as an epigenetic mechanism. These include small interfering RNAs, long non-coding RNAs, and microRNAs, which will be discussed here. MicroRNAs (miRNAs) are short RNA molecules that can regulate gene expression by either targeting DNMTS or acting at specific target genes (Schenkel *et al.*, 2017). Much like other RNA molecules, miRNA biosynthesis begins with transcription by RNA polymerase II into a looped, hairpin primary-miRNA structure. This primary-miRNA is cleaved by the endonuclease Drosha, which results in a stem-loop precursor-miRNA molecule. This precursor-miRNA undergoes nuclear export through Exportin 5, followed by further enzymatic cleavage by the endonuclease Dicer. The resulting structure is a mature,

double-stranded miRNA molecule that binds with the RNA-induced silencing complex (RISC). The double-stranded miRNA is unwound while attached to RISC, with one strand being degraded while the other serves as a guide strand in finding target mRNA molecules. Should a miRNA have extensive complementarity between its 5' untranslated region (UTR) and the 3' UTR of an mRNA, degradation of the target mRNA molecule will occur. Alternatively, short regions of complementarity result in target transcriptional repression. Using a guinea pig model of uterine artery ablation, Sarr *et al.* demonstrated that growth-restricted offspring have decreased miR-146a transcript abundance in the liver at five months of age (Sarr *et al.*, 2016). It has been proposed that this miRNA modulates function of HSCs through targeting of SMAD4, and it is decreased in fibrotic liver tissues (He *et al.*, 2012). In 2016, Su *et al.* investigated the role of miR-15b in pancreatic beta cell proliferation of MPR mouse offspring. It was discovered that miR-15b is significantly increased in the pancreatic islets of MPR offspring, accompanied by reduced expression of cyclin D1 and D2 (Su *et al.*, 2016). Given the role of cyclins in cell cycle progression, it is believed that downregulation of these molecules contributes to impaired beta cell function and glucose intolerance. Our own studies of MPR during pregnancy and lactation have also shown that miR-29 is upregulated in MPR offspring with postnatal catch-up growth (Sohi *et al.*, 2015). Each isoform of miR-29 (miR-29a/b/c) was significantly increased in livers of three week and four month old offspring, further leading to reduced levels of Igf-1 mRNA (Sohi *et al.*, 2015). With that in mind, it is possible that the timing of nutritional restoration for IUGR offspring may play a role in long-term disease via modulation of miRs. Given that miRs also circulate in the blood, these animal studies could lead to novel therapeutic interventions with the use of miR inhibitors in neonatal treatment of the metabolic syndrome.

1.4 Rationale and Study Aims

It is widely accepted that growth of the IUGR liver becomes compromised at the expense of other fetal organs. Studies have established that IUGR offspring have poor long-term metabolic function of the liver, particularly involving hepatic lipid metabolism (Sohi *et al.*, 2011; Ma *et al.*, 2013). This is exacerbated with rapid postnatal catch-up

growth, and our laboratory has shown that various forms of cellular stress occur exclusively following catch-up growth (Sohi *et al.*, 2013; Barra *et al.*, 2017). That said, the role of mitochondria in this process remains elusive. Mitochondria are key regulators of cellular metabolism and oxidative stress, and mitochondrial dysfunction is known to occur with many metabolic pathologies. Not surprisingly, previous studies have found that IUGR offspring exhibit indices of mitochondrial dysfunction in metabolic tissues, including the liver (Park *et al.*, 2003; Moraes *et al.*, 2014; Barra *et al.*, 2017; Woodman *et al.*, 2018). Despite this, the molecular mechanisms leading to mitochondrial dysfunction in IUGR offspring remain largely unknown, along with impact of catch-up growth on mitochondrial function (Figure 1-3). Given that hepatic miRNA expression is altered in IUGR offspring with catch-up growth (Sohi *et al.*, 2015), it is conceivable that miRNAs also have a role in this process.

These findings give rise to my **overall hypothesis** that postnatal catch-up growth leads to hepatic mitochondrial dysfunction in IUGR offspring as an underlying mechanism of hyperlipidemia, independent of gestational insult. I further predict that aberrant expression of miRNAs contributes to hepatic mitochondrial dysfunction exclusively following catch-up growth. To address my hypothesis, the following specific aims are proposed:

1) Determination of the role of postnatal catch-up growth in mitochondrial dysfunction among maternal protein-restricted IUGR rat offspring.

2) Analysis and characterization of hepatic mitochondrial dysfunction in an alternative model of IUGR with postnatal catch-up growth.

3) Investigation of the relationship between hepatic cellular stress, miRNA expression, and hepatic hyperlipidemia using an *in vitro* model.

Significance: My proposed studies will provide novel insight into the mitochondrial mechanisms that underlie dyslipidemia in IUGR offspring. Overall, these studies will identify important targets that regulate mitochondrial dysfunction in response to insults

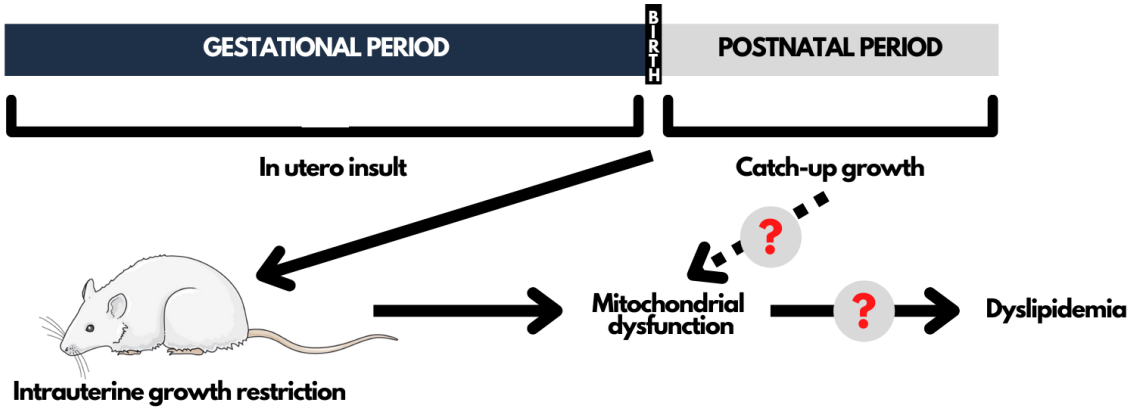

Figure 1-3. Intrauterine growth restriction (IUGR) leads to mitochondrial dysfunction and hyperlipidemia in the affected offspring.

Although it has been shown that IUGR offspring exhibit mitochondrial dysfunction in metabolic organs, the molecular mechanisms by which this occurs have not been studied. Additionally, the role of postnatal catch-up growth in this process remains unknown.

during the perinatal period. This research will further improve our understanding of DOHaD, and it has potential in leading to early life therapeutic strategies that could be employed to mitigate hepatic dysfunction in IUGR offspring.

1.5 References

Anstee QM, Targher G and Day CP (2013) Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nature Reviews Gastroenterology and Hepatology*.

Barker DJ, Martyn CN, Osmond C, Hales CN and Fall CH (1993a) Growth in utero and serum cholesterol concentrations in adult life. *BMJ (Clinical Research Ed.)* **307** 1524–1527.

Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K and Clark PM (1993b) Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* **36** 62–67.

Barra NG, Lisyansky M, Vanduzer TA, Raha S, Holloway AC and Hardy DB (2017) Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase and impaired mitochondrial function in male rat offspring. *Journal of Applied Toxicology* **37** 1517–1526.

Baschat AA (2004) Pathophysiology of fetal growth restriction: implications for diagnosis and surveillance. *Obstetrical & Gynecological Survey* **59** 617–627.

Baschat AA and Hecher K (2004) Fetal growth restriction due to placental disease. *Seminars in Perinatology* **28** 67–80.

Baserga M, Hale MA, McKnight RA, Yu X, Callaway CW and Lane RH (2005) Uteroplacental insufficiency alters hepatic expression, phosphorylation, and activity of the glucocorticoid receptor in fetal IUGR rats. *American Journal of*

Physiology. Regulatory, Integrative and Comparative Physiology **289** R1348-1353.

Bateson P, Gluckman P and Hanson M (2014) The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *Journal of Physiology* **592** 2357–2368.

Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM and Duncan SA (2006) Hepatocyte nuclear factor 4α orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. *Proceedings of the National Academy of Sciences* **103** 8419–8424.

Bayly GR (2014) CHAPTER 37 - Lipids and disorders of lipoprotein metabolism. In *Clinical Biochemistry: Metabolic and Clinical Aspects (Third Edition)*, pp 702–736. Eds WJ Marshall, M Lapsley, AP Day and RM Ayling. Churchill Livingstone.

Begriche K, Massart J, Robin MA, Bonnet F and Fromenty B (2013) Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. *Hepatology* **58** 1497–1507.

Bertolotti A, Zhang Y, Hendershot LM, Harding HP and Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biology*.

Biri A, Bozkurt N, Turp A, Kavutcu M, Himmetoglu Ö and Durak I (2007) Role of oxidative stress in intrauterine growth restriction. *Gynecologic and Obstetric Investigation* **64** 187–192.

Borah P and Banik BK (2020) 12 - Diverse synthesis of medicinally active steroids. In *Green Approaches in Medicinal Chemistry for Sustainable Drug Design*, pp 449–490. Ed BK Banik. Elsevier.

Brites D (2002) Intrahepatic cholestasis of pregnancy: changes in maternal-fetal bile acid balance and improvement by ursodeoxycholic acid. *Annals of Hepatology* **1** 20–28.

Burdge GC, Slater-Jefferies J, Torrens C, Phillips ES, Hanson MA and Lillycrop KA (2007) Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *The British Journal of Nutrition* **97** 435–439.

Cadenas E and Davies KJA (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biology and Medicine* **29** 222–230.

Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ *et al.* (1998) Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **95** 13018–13023.

Chen Z, Tian R, She Z, Cai J and Li H (2020) Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radical Biology and Medicine* **152** 116–141.

Cnop M, Foufelle F and Velloso LA (2012) Endoplasmic reticulum stress, obesity and diabetes. *Trends in Molecular Medicine* **18** 59–68.

Cooper C, Fall C, Egger P, Hobbs R, Eastell R and Barker D (1997) Growth in infancy and bone mass in later life. *Annals of the Rheumatic Diseases* **56** 17–21.

Cordero P, Gomez-Uriz AM, Campion J, Milagro FI and Martinez JA (2013) Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. *Genes & Nutrition* **8** 105–113.

Corsini A and Bortolini M (2013) Drug-Induced Liver Injury: The Role of Drug Metabolism and Transport. *The Journal of Clinical Pharmacology* **53** 463–474.

Creasy RK, Resnik R, Greene MF, Iams JD and Lockwood CJ *Creasy and Resnik's Maternal-Fetal Medicine : Principles and Practice*.

Crosby WM (1991) Studies in fetal malnutrition. *American Journal of Diseases of Children (1960)* **145** 871–876.

Deiber M, Chatelain P, Naville D, Putet G and Salle B (1989) FUNCTIONAL HYPERSOMATOTROPISM IN SMALL FOR GESTATIONAL AGE (SGA) NEWBORN INFANTS. *The Journal of Clinical Endocrinology & Metabolism* **68** 232–234.

Demirel Y and Gerbaud V (2019) Chapter 11 - Thermodynamics and Biological Systems. In *Nonequilibrium Thermodynamics (Fourth Edition)*, pp 489–571. Eds Y Demirel and V Gerbaud. Elsevier.

Deodati A, Argemí J, Germani D, Puglianiello A, Alisi A, De Stefanis C, Ferrero R, Nobili V, Aragón T and Cianfarani S (2018) The exposure to uteroplacental insufficiency is associated with activation of unfolded protein response in postnatal life. *PLoS ONE* **13**.

Desai M and Hales CN (1997) Role of fetal and infant growth in programming metabolism in later life. *Biological Reviews of the Cambridge Philosophical Society* **72** 329–348.

Devarajan A, Rajasekaran NS, Valburg C, Ganapathy E, Bindra S and Freije WA (2019) Maternal perinatal calorie restriction temporally regulates the hepatic autophagy and redox status in male rat. *Free Radical Biology and Medicine* **130** 592–600.

Douarin NM (1975) An experimental analysis of liver development. *Medical Biology* **53** 427–455.

Elias AA, Ghaly A, Matushewski B, Regnault TRH and Richardson BS (2016) Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reproductive Sciences (Thousand Oaks, Calif.)* **23** 219–227.

Eriksson J, Forsén T, Tuomilehto J, Osmond C and Barker D (2001) Size at birth, childhood growth and obesity in adult life. *International Journal of Obesity* **25** 735–740.

Fall CH, Osmond C, Barker DJ, Clark PM, Hales CN, Stirling Y and Meade TW (1995) Fetal and infant growth and cardiovascular risk factors in women. *BMJ (Clinical Research Ed.)* **310** 428–432.

Fu Q, Yu X, Callaway CW, Lane RH and McKnight RA (2009) Epigenetics: intrauterine growth retardation (IUGR) modifies the histone code along the rat hepatic IGF-1 gene. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* **23** 2438–2449.

Fu Q, McKnight RA, Callaway CW, Yu X, Lane RH and Majnik AV (2015) Intrauterine growth restriction disrupts developmental epigenetics around distal growth hormone response elements on the rat hepatic IGF-1 gene. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* **29** 1176–1184.

Furuhashi M and Hotamisligil GS (2008) Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nature Reviews Drug Discovery* **7** 489–503.

Gauster M, Hiden U, Blaschitz A, Frank S, Lang U, Alvino G, Cetin I, Desoye G and Wadsack C (2007) Dysregulation of Placental Endothelial Lipase and Lipoprotein Lipase in Intrauterine Growth-Restricted Pregnancies. *The Journal of Clinical Endocrinology & Metabolism* **92** 2256–2263.

George LA, Zhang L, Tuersunjiang N, Ma Y, Long NM, Uthlaut AB, Smith DT, Nathanielsz PW and Ford SP (2012) Early maternal undernutrition programs increased feed intake, altered glucose metabolism and insulin secretion, and liver function in aged female offspring. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **302** R795–R804.

Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM, Pardi G and Sibley CP (1997) Association between the activity of the system A amino acid transporter in the microvillous plasma membrane of the human placenta and severity of fetal compromise in intrauterine growth restriction. *Pediatric Research* **42** 514–519.

Goodspeed D, Seferovic MD, Holland W, Mcknight RA, Summers SA, Branch DW, Lane RH and Aagaard KM (2015) Essential nutrient supplementation prevents heritable metabolic disease in multigenerational intrauterine growth-restricted rats. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* **29** 807–819.

Goyal D, Limesand SW and Goyal R (2019) Epigenetic responses and the developmental origins of health and disease. *The Journal of Endocrinology* **242** T105–T119.

Greally JM (2018) A user's guide to the ambiguous word 'epigenetics'. *Nature Reviews Molecular Cell Biology* **19** 207–208.

Gruppuso PA and Sanders JA (2016) Regulation of liver development: implications for liver biology across the lifespan. *Journal of Molecular Endocrinology* **56** R115–R125.

Gupta P, Narang M, Banerjee BD and Basu S (2004) Oxidative stress in term small for gestational age neonates born to undernourished mothers: A case control study. *BMC Pediatrics* **4** 14–14.

Hales CN and Barker DJ (2001) The thrifty phenotype hypothesis. *British Medical Bulletin* **60** 5–20.

Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C and Winter PD (1991) Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ (Clinical Research Ed.)* **303** 1019–1022.

Hayashi T, Rizzuto R, Hajnoczky G and Su T-P (2009) MAM: more than just a housekeeper. *Trends in Cell Biology* **19** 81–88.

Hayhurst GP, Lee YH, Lambert G, Ward JM and Gonzalez FJ (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Molecular and Cellular Biology* **21** 1393–1403.

He Y, Huang C, Sun X, Long X, Lv X and Li J (2012) MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4. *Cellular Signalling* **24** 1923–1930.

Hong L, Schroth GP, Matthews HR, Yau P and Bradbury EM (1993) Studies of the DNA binding properties of histone H4 amino terminus. Thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 'tail' to DNA. *The Journal of Biological Chemistry* **268** 305–314.

Hong M, Luo S, Baumeister P, Huang JM, Gogia RK, Li M and Lee AS (2004) Underglycosylation of ATF6 as a Novel Sensing Mechanism for Activation of the Unfolded Protein Response. *Journal of Biological Chemistry*.

Huang C, Li Z, Wang M and Martorell R (2010) Early life exposure to the 1959-1961 Chinese famine has long-term health consequences. *Journal of Nutrition* **140** 1874–1878.

James PT, Rigby N and Leach R (2004) The obesity epidemic, metabolic syndrome and future prevention strategies. *European Journal of Cardiovascular Prevention & Rehabilitation* **11** 3–8.

Jansson T, Scholtbach V and Powell TL (1998) Placental transport of leucine and lysine is reduced in intrauterine growth restriction. *Pediatric Research* **44** 532–537.

Jansson T, Ylven K, Wennergren M and Powell TL (2002) Glucose transport and system A activity in syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth restriction. *Placenta* **23** 392–399.

Janzen C, Lei MYY, Cho J, Sullivan P, Shin B-C and Devaskar SU (2013) Placental Glucose transporter 3 (GLUT3) is Up-regulated in Human Pregnancies Complicated by Late-onset Intrauterine Growth Restriction. *Placenta* **34** 10.1016/j.placenta.2013.08.010.

Jensen-Urstad APL and Semenkovich CF (2012) Fatty acid synthase and liver triglyceride metabolism: Housekeeper or messenger? *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1821** 747–753.

Jones JG (2016) Hepatic glucose and lipid metabolism. *Diabetologia* **59** 1098–1103.

Kainulainen H, Järvinen T and Heinonen P (1997) Placental Glucose Transporters in Fetal Intrauterine Growth Retardation and Macrosomia. *Gynecologic and Obstetric Investigation* **44** 89–92.

Karowicz-Bilinska A, Suzin J and Sieroszewski P (2002) Evaluation of oxidative stress indices during treatment in pregnant women with intrauterine growth retardation. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research* **8** CR211-6.

Kawano Y and Cohen DE (2013) Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *Journal of Gastroenterology* **48** 434–441.

Kim KH (1997) Regulation of mammalian acetyl-coenzyme A carboxylase. *Annual Review of Nutrition* **17** 77–99.

Kogure K, Ishizaki M, Nemoto M, Kuwano H and Makuuchi M (1999) A comparative study of the anatomy of rat and human livers. *Journal of Hepato-Biliary-Pancreatic Surgery* **6** 171–175.

Kornberg RD and Lorch Y (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98** 285–294.

Krumova K and Cosa G (2016) Chapter 1 Overview of Reactive Oxygen Species. 1–21.

Küllenberg D, Taylor LA, Schneider M and Massing U (2012) Health effects of dietary phospholipids. *Lipids in Health and Disease* **11** 3.

Lane RH, Crawford SE, Flozak AS and Simmons RA (1999) Localization and quantification of glucose transporters in liver of growth-retarded fetal and neonatal rats. *The American Journal of Physiology* **276** E135-142.

Lane RH, MacLennan NK, Hsu JL, Janke SM and Pham TD (2002) Increased hepatic peroxisome proliferator-activated receptor-gamma coactivator-1 gene expression in a rat model of intrauterine growth retardation and subsequent insulin resistance. *Endocrinology* **143** 2486–2490.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA and Burdge GC (2005a) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition* **135** 1382–1386.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA and Burdge GC (2005b) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition* **135** 1382–1386.

Liu X, Wang J, Gao L, Jiao Y and Liu C (2018) Maternal Protein Restriction Induces Alterations in Hepatic Unfolded Protein Response-Related Molecules in Adult Rat Offspring. *Frontiers in Endocrinology* **9** 676–676.

Ma N, Nicholson CJ, Wong M, Holloway AC and Hardy DB (2013) Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicology and Applied Pharmacology* **275** 1–11.

Magnusson AL, Waterman IJ, Wennergren M, Jansson T and Powell TL (2004) Triglyceride Hydrolase Activities and Expression of Fatty Acid Binding Proteins in the Human Placenta in Pregnancies Complicated by Intrauterine Growth Restriction and Diabetes. *The Journal of Clinical Endocrinology & Metabolism* **89** 4607–4614.

Mantena SK, King AL, Andringa KK, Eccleston HB and Bailey SM (2008) Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases. *Free Radical Biology and Medicine* **44** 1259–1272.

Matsuzaka T and Shimano H (2009) Elovl6: a new player in fatty acid metabolism and insulin sensitivity. *Journal of Molecular Medicine* **87** 379–384.

McCarron JG, Wilson C, Sandison ME, Olson ML, Girkin JM, Saunter C and Chalmers S (2013) From Structure to Function: Mitochondrial Morphology, Motion and Shaping in Vascular Smooth Muscle. *Journal of Vascular Research* **50** 357–371.

Michalak M and Gye MC (2015) Endoplasmic reticulum stress in periimplantation embryos. *Clinical and Experimental Reproductive Medicine* **42** 1–7.

Miyazaki M and Ntambi JM (2003) Role of stearoyl-coenzyme A desaturase in lipid metabolism. *Prostaglandins Leukotrienes and Essential Fatty Acids* **68** 113–121.

Moraes C, Rebelato HJ, Amaral MEC, Resende TM, Silva EVC, Esquisatto MAM and Catisti R (2014) Effect of maternal protein restriction on liver metabolism in rat offspring. *The Journal of Physiological Sciences* **64** 347–355.

Mounir Z, Krishnamoorthy JL, Wang S, Papadopoulou B, Campbell S, Muller WJ, Hatzoglou M and Koromilas AE (2011) Akt determines cell fate through inhibition of the PERK-eIF2alpha phosphorylation pathway. *Science Signaling* **4** ra62–ra62.

Musa MG, Kagura J, Pisa PT and Norris SA (2015) Relationship between early growth and CVD risk factors in adolescents. *Journal of Developmental Origins of Health and Disease* **7** 132–143.

Naifeh J, Dimri M and Varacallo M (2022) *Biochemistry, Aerobic Glycolysis*. StatPearls Publishing.

Neerhof MG (1995) Causes of intrauterine growth restriction. *Clinics in Perinatology* **22** 375–385.

Nguyen P, Leray V, Diez M, Serisier S, Le Bloc'h J, Siliart B and Dumon H (2008) Liver lipid metabolism. *Journal of Animal Physiology and Animal Nutrition* **92** 272–283.

Nobili V, Marcellini M, Marchesini G, Vanni E, Manco M, Villani A and Bugianesi E (2007) Intrauterine Growth Retardation, Insulin Resistance, and Nonalcoholic Fatty Liver Disease in Children.

Norberg S, Powell TL and Jansson T (1998) Intrauterine growth restriction is associated with a reduced activity of placental taurine transporters. *Pediatric Research* **44** 233–238.

Ogata ES, Bussey ME and Finley S (1986) Altered gas exchange, limited glucose and branched chain amino acids, and hypoinsulinism retard fetal growth in the rat. *Metabolism: Clinical and Experimental* **35** 970–977.

Osmond C, Barker DJP, Winter PD, Fall CHD and Simmonds SJ (1993) Early growth and death from cardiovascular disease in women. *British Medical Journal* **307** 1519–1524.

Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K-U, Pak YK and Lee HK (2003) Fetal and Early Postnatal Protein Malnutrition Cause Long-Term Changes in Rat Liver and Muscle Mitochondria. *The Journal of Nutrition* **133** 3085–3090.

Pepino MY, Kuda O, Samovski D and Abumrad NA (2014) Structure-Function of CD36 and Importance of Fatty Acid Signal Transduction in Fat Metabolism. *Annual Review of Nutrition* **34** 281–303.

Perng W, Hajj H, Belfort MB, Rifas-Shiman SL, Kramer MS, Gillman MW and Oken E (2016) Birth size, early life weight gain, and midchildhood cardiometabolic health. *Journal of Pediatrics* **173** 122-130.e1.

Plaisance V, Brajkovic S, Tenenbaum M, Favre D, Ezanno H, Bonnefond A, Bonner C, Gmyr V, Kerr-Conte J, Gauthier BR *et al.* (2016) Endoplasmic Reticulum Stress Links Oxidative Stress to Impaired Pancreatic Beta-Cell Function Caused by Human Oxidized LDL. *PloS One* **11** e0163046.

Raab EL, Vuguin PM, Stoffers DA and Simmons RA (2009) Neonatal exendin-4 treatment reduces oxidative stress and prevents hepatic insulin resistance in intrauterine growth-retarded rats. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology* **297** R1785-94.

Ravelli GP, Stein ZA and Susser MW (1976) Obesity in young men after famine exposure in utero and early infancy. *The New England Journal of Medicine* **295** 349–353.

Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN and Bleker OP (1998) Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351** 173–177.

Ravelli AC, van der Meulen JH, Osmond C, Barker DJ and Bleker OP (1999) Obesity at the age of 50 y in men and women exposed to famine prenatally. *The American Journal of Clinical Nutrition* **70** 811–816.

Rhyu J and Yu R (2021) Newly discovered endocrine functions of the liver. *World Journal of Hepatology* **13** 1611–1628.

Roberts A, Nava S, Bocconi L, Salmona S and Nicolini U (1999) Liver function tests and glucose and lipid metabolism in growth-restricted fetuses. *Obstetrics and Gynecology* **94** 290–294.

Rodríguez-Rodríguez P, Ramiro-Cortijo D, Reyes-Hernández CG, López de Pablo AL, González MC and Arribas SM (2018) Implication of Oxidative Stress in Fetal Programming of Cardiovascular Disease. *Frontiers in Physiology* **9**.

Rolo AP, Teodoro JS and Palmeira CM (2012) Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radical Biology and Medicine* **52** 59–69.

Romo A, Carceller R and Tobajas J (2009) Intrauterine growth retardation (IUGR): epidemiology and etiology. *Pediatric Endocrinology Reviews : PER* **6 Suppl 3** 332–336.

Roos S, Powell TL and Jansson T (2004) Human placental taurine transporter in uncomplicated and IUGR pregnancies: cellular localization, protein expression, and regulation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **287** R886–R893.

Ross MG and Beall MH (2009) Prediction of preterm birth: nonsonographic cervical methods. *Seminars in Perinatology* **33** 312–316.

Ross M and Pawlina W (2011) *Histology: A Text and Atlas, with Correlated Cell and Molecular Biology*. Lippincott Williams & Wilkins.

Saker M, Soulimane Mokhtari N, Merzouk SA, Merzouk H, Belarbi B and Narce M (2008) Oxidant and antioxidant status in mothers and their newborns according to birthweight. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **141** 95–99.

Salgin B, Norris SA, Prentice P, Pettifor JM, Richter LM, Ong KK and Dunger DB (2015) Even transient rapid infancy weight gain is associated with higher BMI in young adults and earlier menarche. *International Journal of Obesity* **39** 939–944.

Sarr O, Blake A, Thompson JA, Zhao L, Rabicki K, Walsh JC, Welch I and Regnault TRH (2016) The differential effects of low birth weight and Western diet consumption upon early life hepatic fibrosis development in guinea pig. *The Journal of Physiology* **594** 1753–1772.

Sarr O, Mathers KE, Vanderboor C, Wiggers K, Devgan A, Hardy DB, Zhao L and Regnault TRH (2021) Sex-specific alterations in hepatic cholesterol metabolism in low birth weight adult guinea pigs. *Pediatric Research*.

Sayer AA, Cooper C, Evans JR, Rauf A, Wormald RPL, Osmond C and Barker DJP (1998) Are rates of ageing determined in utero? *Age and Ageing* **27** 579–583.

Schenkel LC, Rodenhiser D, Siu V, McCready E, Ainsworth P and Sadikovic B (2017) Constitutional Epi/Genetic Conditions: Genetic, Epigenetic, and Environmental Factors. *Journal of Pediatric Genetics* **6** 30–41.

Sharma D, Shastri S and Sharma P (2016) Intrauterine Growth Restriction: Antenatal and Postnatal Aspects. *Clinical Medicine Insights. Pediatrics* **10** 67–83.

Simmons RA, Gounis AS, Bangalore SA and Ogata ES (1992) Intrauterine growth retardation: fetal glucose transport is diminished in lung but spared in brain. *Pediatric Research* **31** 59–63.

Singhal A, Cole TJ, Fewtrell M and Lucas A (2004) Breastmilk feeding and lipoprotein profile in adolescents born preterm: follow-up of a prospective randomised study. *Lancet* **363** 1571–1578.

Si-Tayeb K, Lemaigre FP and Duncan SA (2010) Organogenesis and Development of the Liver. *Developmental Cell* **18** 175–189.

Skilton MR, Viikari JSA, Juonala M, Laitinen T, Lehtimäki T, Taittonen L, Kähönen M, Celermajer DS and Raitakari OT (2011) Fetal Growth and Preterm Birth Influence Cardiovascular Risk Factors and Arterial Health in Young Adults. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31** 2975–2981.

Smith SJ, Cases S, Jensen DR, Chen HC, Sande E, Tow B, Sanan DA, Raber J, Eckel RH and Farese RV (2000) Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nature Genetics* **25** 87–90.

Smith GI, Shankaran M, Yoshino M, Schweitzer GG, Chondronikola M, Beals JW, Okunade AL, Patterson BW, Nyangau E, Field T *et al.* (2020) Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *The Journal of Clinical Investigation* **130** 1453–1460.

Sohi G, Marchand K, Revesz A, Arany E and Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. *Molecular Endocrinology (Baltimore, Md.)* **25** 785–798.

Sohi G, Revesz A and Hardy DB (2013) Nutritional mismatch in postnatal life of low birth weight rat offspring leads to increased phosphorylation of hepatic eukaryotic initiation factor 2 alpha in adulthood. *Metabolism: Clinical and Experimental*.

Sohi G, Revesz A, Ramkumar J and Hardy DB (2015) Higher Hepatic miR-29 Expression in Undernourished Male Rats During the Postnatal Period Targets the Long-Term Repression of IGF-1. *Endocrinology* **156** 3069–3076.

Stein AD, Zybert PA, Van Der Pal-De Bruin K and Lumey LH (2006) Exposure to famine during gestation, size at birth, and blood pressure at age 59 y: Evidence from the dutch famine. *European Journal of Epidemiology* **21** 759–765.

Su Y, Jiang X, Li Y, Li F, Cheng Y, Peng Y, Song D, Hong J, Ning G, Cao Y *et al.* (2016) Maternal Low Protein Isocaloric Diet Suppresses Pancreatic β-Cell Proliferation in Mouse Offspring via miR-15b. *Endocrinology* **157** 4782–4793.

Suhag A and Berghella V (2013) Intrauterine Growth Restriction (IUGR): Etiology and Diagnosis. *Current Obstetrics and Gynecology Reports*.

Suomela E, Oikonen M, Pitkänen N, Ahola-Olli A, Virtanen J, Parkkola R, Jokinen E, Laitinen T, Hutri-Kähönen N, Kähönen M *et al.* (2016) Childhood predictors of adult fatty liver. The Cardiovascular Risk in Young Finns Study. *Journal of Hepatology* **65** 784–790.

Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. *Biochimica Et Biophysica Acta* **1410** 103–123.

Tabano S, Alvino G, Antonazzo P, Grati FR, Miozzo M and Cetin I (2006) Placental LPL Gene Expression Is Increased in Severe Intrauterine Growth-Restricted Pregnancies. *Pediatric Research* **59** 250–253.

Talley JT and Mohiuddin SS (2022) *Biochemistry, Fatty Acid Oxidation*. StatPearls Publishing.

Theys N, Clippe A, Bouckenooghe T, Reusens B and Remacle C (2009) Early Low Protein Diet Aggravates Unbalance between Antioxidant Enzymes Leading to Islet Dysfunction. *PLoS ONE* **4** e6110–e6110.

Thorens B (1996) Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. *The American Journal of Physiology* **270** G541-53.

Tran M, Gallo LA, Jefferies AJ, Moritz KM and Wlodek ME (2013) Transgenerational metabolic outcomes associated with uteroplacental insufficiency. *The Journal of Endocrinology* **217** 105–118.

Trefts E, Gannon M and Wasserman DH (2017) The liver. *Current Biology: CB* **27** R1147–R1151.

Tvrzicka E, Kremmyda L-S, Stankova B and Zak A (2011) FATTY ACIDS AS BIOCOMPOUNDS: THEIR ROLE IN HUMAN METABOLISM, HEALTH AND DISEASE - A REVIEW. PART 1: CLASSIFICATION, DIETARY SOURCES AND BIOLOGICAL FUNCTIONS. *Biomedical Papers* **155** 117–130.

Valsamakis G, Kanaka-Gantenbein C, Malamitsi-Puchner A and Mastorakos G (2006) Causes of intrauterine growth restriction and the postnatal development of the metabolic syndrome. *Annals of the New York Academy of Sciences* **1092** 138–147.

Vandenbosche RC and Kirchner JT (1998) Intrauterine growth retardation. *American Family Physician* **58** 1384–1390, 1393–1394.

Victora CG, Barros FC, Lima RC, Behague DP, Gon alves H, Horta BL, Gigante DP and Vaughan JP (2003) The Pelotas birth cohort study, Rio Grande do Sul, Brazil, 1982-2001. *Cadernos de Saúde Pública / Ministério Da Saúde, Fundação Oswaldo Cruz, Escola Nacional de Saúde Pública* **19** 1241–1256.

Vo TX, Revesz A, Ma N and Hardy DB (2013a) Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver x receptor. *Journal of Endocrinology* **218** 85–97.

Vo T, Revesz A, Ma N and Hardy DB (2013b) Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver x receptor. *Journal of Endocrinology* **218** 85–97.

Wang J, Chen L, Li D, Yin Y, Wang X, Li P, Dangott LJ, Hu W and Wu G (2008) Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *Journal of Nutrition* **138** 60–66.

Wang P-X, Wang J-J, Lei Y-X, Xiao L and Luo Z-C (2012) Impact of Fetal and Infant Exposure to the Chinese Great Famine on the Risk of Hypertension in Adulthood. *PLoS ONE* **7** e49720–e49720.

Wang J, Li Y, Han X, Liu B, Hu H, Wang F, Li X, Yang K, Yuan J, Yao P *et al.* (2016) Exposure to the Chinese famine in childhood increases type 2 diabetes risk in adults. *Journal of Nutrition* **146** 2289–2295.

Wiesner RJ, Rüegg JC and Morano I (1992) Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues. *Biochemical and Biophysical Research Communications* **183** 553–559.

Wong MK, Nicholson CJ, Holloway AC and Hardy DB (2015) Maternal nicotine exposure leads to impaired disulphide bond formation and augmented endoplasmic reticulum stress in the rat placenta. *PloS One* **in press**.

Woodman AG, Mah R, Keddie D, Noble RMN, Panahi S, Gragasin FS, Lemieux H and Bourque SL (2018) Prenatal iron deficiency causes sex-dependent mitochondrial dysfunction and oxidative stress in fetal rat kidneys and liver. *The FASEB Journal* **32** 3254–3263.

Xu C, Bailly-Maitre B and Reed JC (2005) Endoplasmic reticulum stress: Cell life and death decisions. *Journal of Clinical Investigation* **115** 2656–2664.

Yang Z, Zhao W, Zhang X, Mu R, Zhai Y, Kong L and Chen C (2008) Impact of famine during pregnancy and infancy on health in adulthood. In *Obesity Reviews*, pp 95–99. Obes Rev.

Yu J, Loh K, Song Z, Yang H, Zhang Y and Lin S (2018) Update on glycerol-3-phosphate acyltransferases: the roles in the development of insulin resistance. *Nutrition & Diabetes* **8** 1–10.

Yudkin JS and Stanner S (1998) Prenatal exposure to famine and health in later life. *Lancet* **351** 1361–1362.

Zampelas A and Magriplis E (2019) New Insights into Cholesterol Functions: A Friend or an Enemy? *Nutrients* **11** 1645.

Zangari J, Petrelli F, Maillot B and Martinou J-C (2020) The Multifaceted Pyruvate Metabolism: Role of the Mitochondrial Pyruvate Carrier. *Biomolecules* **10** 1068.

Chapter II

2 Perinatal protein restriction with postnatal catch-up growth leads to mitochondrial dysfunction in the adult rat liver

A version of this chapter has been previously published:

Oke, S.L., Sohi, G., Hardy, D.B. (2020) Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. *Reproduction*. 159: 27–39.

2.1 Introduction

Fetal undernutrition gives rise to intrauterine growth restriction (IUGR), a condition characterized by low birth weight and reduced organ growth. Epidemiological studies have established a relationship between birth weight and long-term metabolic health, as individuals exposed to undernutrition *in utero* exhibit high rates of the metabolic syndrome in adult life (Ravelli *et al.* 1998, Roseboom *et al.* 2001). This relationship is further exacerbated by postnatal catch-up growth, whereby the affected offspring undergo rapid weight gain during critical stages of growth and development. Collectively, this underlies Barker's 'thrifty phenotype' hypothesis, which states that an adverse intrauterine environment will cause permanent alterations to physiological processes in anticipation of a similarly hostile postnatal environment (Hales & Barker 2001). When a mismatch in nutrient availability occurs between the pre- and postnatal environments, these adaptations become maladaptive and pose risk for development of the metabolic syndrome (Hales & Barker 2001, Ozanne & Hales 2004, Bieswal *et al.* 2006, Sohi *et al.* 2011). While animal studies have revealed the contributions of postnatal catch-up growth to long-term dysmetabolism, the molecular basis of the relationship between birth weight and postpartum development remains poorly understood.

There is strong evidence to suggest that the composition of maternal diet during pregnancy plays a role in fetal health. Given that amino acids are essential for fetal growth and development (Battaglia & Meschia 1978, Crosby 1991), the maternal protein restriction (MPR) model of undernutrition has been widely utilized in rodents to investigate the role of protein availability on postnatal outcomes. We and others have demonstrated that MPR offspring are low birth weight and exhibit asymmetrical organ growth, with the fetal liver becoming selectively compromised at the expense of other organs such as the lungs and the brain (Desai & Hales 1997, Sohi *et al.* 2011, 2013). Not surprisingly, MPR offspring undergo hepatic and whole-body catch-up growth when introduced to a normal protein diet at birth or weaning (Ozanne & Hales 2004, Sohi *et al.* 2011). Our laboratory has established that these 'recuperated' offspring exhibit signs of impaired hepatic function at adulthood, including hypercholesterolemia, glucose

intolerance (*e.g.,* increased gluconeogenesis) and accelerated drug catabolism due to differential abundance of hepatic enzymes (Sohi *et al.* 2011, 2014, Vo *et al.* 2013). Conversely, MPR offspring without catch-up growth have normal cholesterol levels and drug metabolism later in life, but to date, the mechanisms underlying rapid catch-up growth and dysmetabolism are unclear (Sohi *et al.* 2011, 2014).

Mitochondria are intracellular energy producers that are largely responsible in regulating metabolism and oxidative stress. Moreover, impaired mitochondrial function is associated with various metabolic pathologies (Petersen *et al.* 2003, Nojiri *et al.* 2006, Ozgen *et al.* 2012), and a variety of maternal insults have been shown to compromise mitochondrial function in IUGR offspring (Park *et al.* 2003, Moraes *et al.* 2014, Barra *et al.* 2017, Woodman *et al.* 2018). While these studies demonstrate the importance of the maternal nutrition in mediating mitochondrial function, it remains unknown whether these abnormalities occur directly due to the maternal insult or due to postnatal catch-up growth. For example, using a model of maternal nicotine exposure (MNE), we have previously shown that IUGR offspring have both impeded mitochondrial function and cardiac dysfunction exclusively after catch-up growth has occurred (Barra *et al.* 2017). This may also occur in nutrition-induced IUGR offspring (*e.g.* MPR); however, the critical windows of nutrient deprivation leading to impaired mitochondrial function and metabolic disease remain elusive.

Several studies have implicated the p66Shc adaptor protein in processes contributing to mitochondrial dysfunction and oxidative stress (Migliaccio *et al.* 1999, Orsini *et al.* 2004, Giorgio *et al.* 2005, Pinton *et al.* 2007, Trinei *et al.* 2013). Upon cellular stress, cytosolic p66Shc becomes phosphorylated at serine residue 36 (Ser36), followed by a subsequent conformational change that is initiated by the cis/trans isomerase PIN1 (Giorgio *et al.* 2005, Pinton *et al.* 2007). After dephosphorylation of Ser36 by PP2A phosphatase, p66Shc undergoes mitochondrial translocation to permit binding with cytochrome C. This interaction promotes increased production of ROS, as well as accelerated rates of mitochondrial-induced apoptosis and cellular senescence (Migliaccio *et al.* 1999, Giorgio *et al.* 2005). Studies have shown that activation of p66Shc also leads to compromised aerobic metabolism, which may further contribute to

increased reactive oxygen species (ROS) production (Acin-Perez *et al.* 2010, Lone *et al.* 2018). While the mechanism by which this occurs is not yet clear, there is evidence to suggest that increased p66shc activation is linked to metabolic pathologies such as diabetes and coronary artery disease (Pagnin *et al.* 2005, Noda *et al.* 2010). Therefore, the overall objective of this study was to determine if MPR offspring exhibit hepatic mitochondrial dysfunction following postnatal catch-up growth, and to define if this was concomitant with elevated levels of p66Shc and oxidative stress in adulthood. Furthermore, given that increased p66Shc expression has been associated with endoplasmic reticulum (ER) stress (Zhang *et al.* 2013), and MPR offspring with catch-up growth exhibit hepatic ER stress (Sohi *et al.* 2013), we further anticipated that ER stress may mediate any increase in p66Shc expression.

2.2 Materials and Methods

2.2.1 Animals and Dietary Regimes

All procedures were performed according to guidelines set by the Canadian Council of Animal Care with approval from the Animal Care Committee at The University of Western Ontario. Male and female Wistar rats (250 g, 8–10 weeks old) were purchased from Charles River (La Salle, St. Constant, QC) and left to acclimatize to environmental conditions of the animal care facility for 3 weeks. Upon proestrus, female rats were placed in cages of male rats for mating, and pregnancy was confirmed the next morning via presence of sperm in the vaginal smear (gestational day 1). Pregnant dams were then fed either a normal (20% casein) protein diet or a low (8% casein, LP) protein diet for the remainder of gestation (Figure 2-1). Diets were made isocaloric with each other through the addition of carbohydrates (i.e. sucrose) to the LP diet as we have previously published (Sohi *et al.* 2015), and food was provided *ad libitum* throughout pregnancy. Despite the increased carbohydrate content, the LP diet is not considered to be high in carbohydrates as the addition of sucrose is modest (i.e., an increase of 13%) in comparison to decreased total protein (i.e., a decrease of 60%). At birth, litter size was

reduced to eight animals by selecting pups with birth weight closest to the litter mean. Offspring born to control diet-fed mothers continued to feed off a control diet for the remainder of life, while offspring born to LP dams were assigned to one of three LP groups (Figure 2-1): low protein 1 (LP1), low protein 2 (LP2) or low protein 3 (LP3). LP1 offspring were fed a LP diet throughout life, while LP2 offspring were introduced to a normal protein diet at weaning (postnatal day 21). Given that hepatic differentiation in rats is not complete at birth (Gruppuso & Sanders 2016), the LP2 group of offspring allows us to examine the effects of protein restriction during the entire perinatal period. LP3 offspring received a control diet at birth and continued to feed on this diet for the remainder of life. Necropsy was performed at postnatal days (PND) 21 and 130 to examine the direct and indirect effects of MPR, respectively. As demonstrated by our studies and others, PND 130 was chosen given it is the time at which poor metabolic outcomes (*i.e.,* glucose intolerance and hypercholesterolemia) are manifested in these low birth weight offspring (Chamson-Reig *et al.* 2009, Sohi *et al.* 2011). For the purposes of this study, male offspring were exclusively selected because female MPR offspring exhibit less metabolic deficits (*i.e.,* normal cholesterol levels) and to avoid confounding effects presented by the female estrus cycle (Sohi *et al.* 2011). The right medial hepatic lobe was collected and immediately flash-frozen in liquid nitrogen, followed by storage at −80°C until further use.

2.2.2 Cell Culture and Induction of ER stress

The HepG2 human hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (ATCC) and cultured in 5% CO_2/95% atmospheric air at 37°C. Cells were maintained in minimum essential medium (MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin solution (10,000 IU and 10,000 µg/mL, respectively; Fisher Scientific). For induction of ER stress, cells between passages 7 and 10 were seeded at a density of $2.0 \times \_10^5$ cells/well in six-well culture plates (Thermo Scientific) and allowed to proliferate for 24 h prior to treatment. Cells were then treated with 0.5, 1.0 and 2.0 µg/ mL tunicamycin

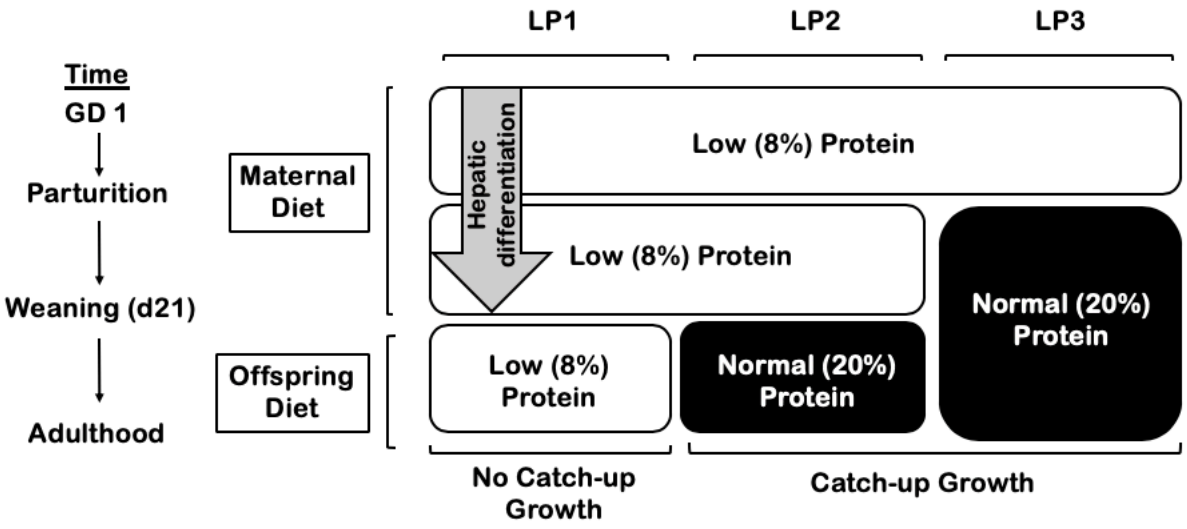

Figure 2-1. The maternal protein restriction (MPR) rodent model of undernutrition.

MPR diets differ according to percent protein composition and timing of nutritional intervention. Pregnant Wistar rat dams were subjected to an 8% protein diet during gestation, and pups were randomly assigned to one of three postnatal dietary regimens at birth: 8% protein diet throughout life (LP1), 8% protein diet until three weeks of age + 20% (control) diet after three weeks (LP2), or 20% protein diet throughout life (LP3). Importantly, liver development continues postnatally in the rat, with terminal hepatic differentiation occurring between postnatal days 18 and 21.

(BioShop Canada, Burlington, ON) for 1, 2 and 6 h. Additional cells were treated with a matching volume of DMSO as a vehicular control for 1, 2 and 6 h. Following treatment, cells were collected for protein isolation and western immunoblotting as described below.

2.2.3 RNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from PND 21 and 130 livers using the one-step method of Chomczynski and Sacchi (TRIzol; Invitrogen), followed by RT with a High-Capacity cDNA RT Kit (Applied Biosystems). Primer sets for gene targets of interest were designed using the National Center for Biotechnology Information and Ensembl genome browsers, followed by generation via Invitrogen Custom DNA Oligos (Table 2-1). Relative transcript abundance was determined via quantitative real-time PCR (qRT-PCR) as previously published (Barra *et al.* 2017). Values obtained for all gene targets of interest were normalized to geometric means of β-Actin and *GAPDH*, which were determined to be suitable housekeeping genes by using both the comparative ΔCt method and algorithms from geNorm, Normfinder and BestKeeper (Vandesompele *et al.* 2002, Pfaffl *et al.* 2004). Relative transcript abundance was calculated for each primer set as determined by the comparative ΔCt method.

2.2.4 Protein Extraction and Western Immunoblot

Whole-cell protein lysates were isolated from liver segments and HepG2 cells as previously published (Barra *et al.* 2017). Protein loading samples were prepared for western immunoblotting using sample lysates, NuPAGE reducing agent (10×; Invitrogen), NuPAGE LDS sample buffer (4×; Invitrogen), and deionized water. Loading samples were heated at 70°C to denature proteins, followed by loading into wells of 4–12% Bis-Tris gels (Invitrogen). For liver samples, 20 μg of protein was loaded per well, while cell samples were loaded at 10–12 μg per well. Following separation by gel electrophoresis, proteins were transferred onto PVDF membranes (Thermo Scientific) at 100 volts for 2 h. Membranes were blocked in 1× _Tris-buffered saline/Tween-20 (TBST) buffer with either 5% non-fat milk (Carnation) or 3% bovine serum albumin

(BSA; EMD Millipore), followed by probing with primary and secondary antibodies (Table 2-2). Immunoreactive bands were visualized using either Millipore Immobilon Forte Western HRP Substrate solution or BioRad Clarity Max Western ECL Substrate solution and imaged using a BioRad ChemiDoc XRS+ Imaging System. Resulting bands were analyzed using BioRad Image Lab™ Software, and band intensities of target hepatic mitochondrial proteins were normalized to those of β-Actin as previously published (Wei *et al.* 2009, Zhao *et al.* 2019). Note that while a sample size of 7–8 offspring per group was used for all experiments, representative immunoblots containing all experimental groups on one blot are presented with either 4 offspring (PND 130) or 5–6 offspring (PND 21) per group.

2.2.5 Statistical Analyses

All statistical analyses were performed using GraphPad Prism 8 software. Results were expressed as means of normalized values ± s.e.m., and the threshold for statistical significance was set as $P < 0.05$. As mentioned previously, a sample size of 7–8 offspring (i.e., litter is the statistical unit) was used for all *in vivo* experiments, as this provided enough statistical power to detect significant differences in outcome measures. Again, immunoblots presented are representative and do not contain all offspring included in statistical analyses, but are rather presented such that all groups are contained on the same blot. To ensure that trends were consistent within the control group on blots used for analysis, data were normalized such that the same control sample was used as an internal loading control (i.e., the first control sample, present in lane one). Results obtained from body weight or qRT-PCR were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Because we were interested solely in the differences between the control diet and each individual MPR treatment (i.e., the timing of nutritional intervention), individual Student's unpaired *t*-tests were performed on distinct blots comparing each MPR group to their respective control. All cell culture experiments were performed in biological replicates of 3, where each replicate represents an independent experiment using a different frozen cell stock or passage number. These *in vitro* data were analyzed at each time point using a Student's unpaired *t*-test, as the

Table 2-1. Forward and reverse sequences for primers used in analysis of mRNA targets via quantitative real-time PCR.

Gene	Forward Sequence	Reverse Sequence	GenBank/ Reference
pin1	CAGCTCAGGCCGTGTCTACTA	TCCGAGATTGGCTGTGCTTC	NM_001106701.2
p66Shc	TACTTGGTTCGGGTGAGTGC	GAGCAGGAAGTCCCGACAAA	NM_053517.2
nd1	CCGAGAACGCAACTCAGGTA	CCTAAGACACCACCAGCATGT	NM_001006972.1
β-Actin	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144
GAPDH	GGATACTGAGAGCAAGAGAGAGG	TCCTGTTGTTATGGGGTCTGG	NM_017008.4

Table 2-2. Western blot primary and secondary antibodies, dilutions, and company/catalog information.

Antibody Name	Source	Dilution	Company (Catalogue No.)
SHC1	Mouse monoclonal	1:1000	Acris Antibodies, Rockville, MD, USA (AM00143PU-N)
Pin1 (G-8)	Mouse monoclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-46660)
4-hydroxynonenal	Mouse monoclonal	1:1000	R&D Systems, Oakville, ON, Canada (MAB3249)
Superoxide dismutase (SOD)-1 (FL-154)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-11407)
Superoxide dismutase (SOD)-2 (FL-222)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-30080)
Catalase (H-300)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-50508)
pSer(232) pyruvate dehydrogenase	Rabbit polyclonal	1:1000	EMD Millipore, Etobicoke, ON, Canada (AP1063)
Pyruvate dehydrogenase	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (2784S)
LDHa	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (2012S)

Citrate Synthase	Rabbit polyclonal	1:1000	Provided by Dr. S. Raha, McMaster University
OXPHOS rodent cocktail	Mouse monoclonal	1:1000	Abcam Inc., Toronto, ON, Canada (ab110413)
TFAM (D5C8)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (8076S)
KDEL ER Marker (10C3)	Mouse monoclonal	1:250	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-58774)
β-Actin Peroxidase	Mouse monoclonal	1:25000	Sigma Aldrich Co., St. Louis, MO, USA (A3854-200UL)
Goat anti-rabbit IgG HRP-linked (H+L chain)	N/A	1:10000	Cell Signaling Technology Inc., Danvers, MA, USA (7074P2)
Horse anti-mouse IgG HRP-linked (H+L chain)	N/A	1:10000	Cell Signaling Technology Inc., Danvers, MA, USA (7076S)

purpose of this experiment was to determine if tunicamycin-induced ER stress led to elevated p66Shc abundance rather than the effects observed with time.

2.3 Results

2.3.1 Restoration of dietary proteins following maternal protein restriction results in rapid postnatal catch-up growth

We and others have previously shown that MPR causes decreased birth weight, while the restoration of dietary proteins produces rapid postnatal catch-up growth in the affected offspring (Hales *et al.* 1996, Desai & Hales 1997, Sohi *et al.* 2011). To determine if MPR offspring used in our study underwent whole body and/or liver catch-up growth, body weights and liver weights were recorded at birth and PND 21 and 130 (i.e., 3 weeks and 4 months of age). Using these weights, liver to body weight ratio was calculated for offspring at each time point. At postnatal day 21, LP1/LP2 offspring had significantly decreased average bodyweight and liver weight relative to control offspring (Table 2-3; $P < 0.0001$), while there were no differences in body weight and liver weight between LP3 and control offspring. Interestingly, liver to body weight ratios of both LP1/LP2 and LP3 offspring were significantly lower than those of control offspring at this time point (Table 2-3; $P < 0.01$ and $P < 0.05$, respectively). At 4 months of age, LP1 offspring weighed significantly less than control offspring (Table 2-3; $P < 0.05$), while body weights of LP2 and LP3 offspring were not significantly different from those of control offspring (i.e., postnatal catch-up growth had occurred). There were no significant differences between liver weights and liver to body weight ratios of control offspring compared to all groups of MPR offspring at 4 months of age (Table 2-3).

Table 2-3. Maternal protein restriction followed by restoration of dietary protein leads to rapid postnatal catch-up growth by four months of age.

		d21	d130
Body Weight (g)	**Control**	48.75 ± 1.06^{a}	544.25 ± 10.30^{bc}
	LP1	26 ± 0.53^{b}	467 ± 15.82^{a}
	LP2	N/A	504.38 ± 11.35^{ab}
	LP3	46.14 ± 2.97^{a}	602 ± 22.12^{c}
Liver Weight (g)	**Control**	1.91 ± 0.07^{a}	17.16 ± 1.84^{a}
	LP1	0.86 ± 0.001^{b}	15.60 ± 0.84^{a}
	LP2	N/A	16.94 ± 0.79^{a}
	LP3	1.65 ± 0.002^{a}	19.30 ± 1.54^{a}
Liver:Body Weight Ratio	**Control**	0.04 ± 0.001^{a}	0.031 ± 0.003^{a}
	LP1	0.03 ± 0.001^{b}	0.033 ± 0.001^{a}
	LP2	N/A	0.033 ± 0.001^{a}
	LP3	0.04 ± 0.001^{b}	0.032 ± 0.002^{a}

For offspring at both timepoints, liver growth was assessed by calculating liver to body weight ratio. All data are expressed as means ± SEM, and dietary effects were determined using one-way ANOVA followed by Tukey's multiple comparisons test. Groups labeled with different letters are significantly different from each other.

2.3.2 Maternal protein restriction during the perinatal period leads to increased hepatic p66Shc following postnatal catch-up growth

Given the strong association between p66Shc and impaired mitochondrial function (Migliaccio *et al.* 1999, Orsini *et al.* 2004, Giorgio *et al.* 2005, Pinton *et al.* 2007, Trinei *et al.* 2013), we first measured the expression levels of hepatic p66Shc in our MPR offspring. While the steady-state mRNA levels of *p66Shc* remained unaffected in all MPR offspring (Figure 2-2A), LP2 offspring demonstrated a significant increase in p66Shc protein abundance at 4 months of age compared to control offspring (Figure 2-2D; $P < 0.05$). Conversely, p66Shc protein levels were significantly decreased in LP1 and LP3 offspring when compared against that of control offspring (Figure 2-2D; $P < 0.05$). These data were consistent with changes in protein abundance of peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1), an isomerase that is essential for translocation of p66Shc into mitochondria. Transcript abundance of Pin1 remained unchanged across all groups (Figure 2-2B); however, PIN1 protein abundance was significantly increased in LP2 offspring at 4 months (Figure 2-2E; $P < 0.05$). PIN1 protein abundance was not significantly affected in LP1 or LP3 offspring at this time point (Figure 2-2E).

2.3.3 Maternal protein restriction causes increased oxidative stress following postnatal catch-up growth

Given that increased expression of p66Shc is indicative of oxidative stress, we next measured levels of markers of oxidative stress via Western immunoblotting. When cells exist in a state of oxidative stress, there is increased oxidation of polyunsaturated fatty acids (PUFAs) resulting in the formation of various lipid hydroperoxides. 4-Hydroxynonenal (4-HNE) is among the most bioactive of hydroperoxides produced, and it is recognized as an indirect marker of mitochondrial-induced oxidative stress when present at high levels (Liu *et al.* 2011). Using an antibody specific for 4-HNE adducts of histidine residues, we determined that all groups of 4-month old MPR offspring (LP1,

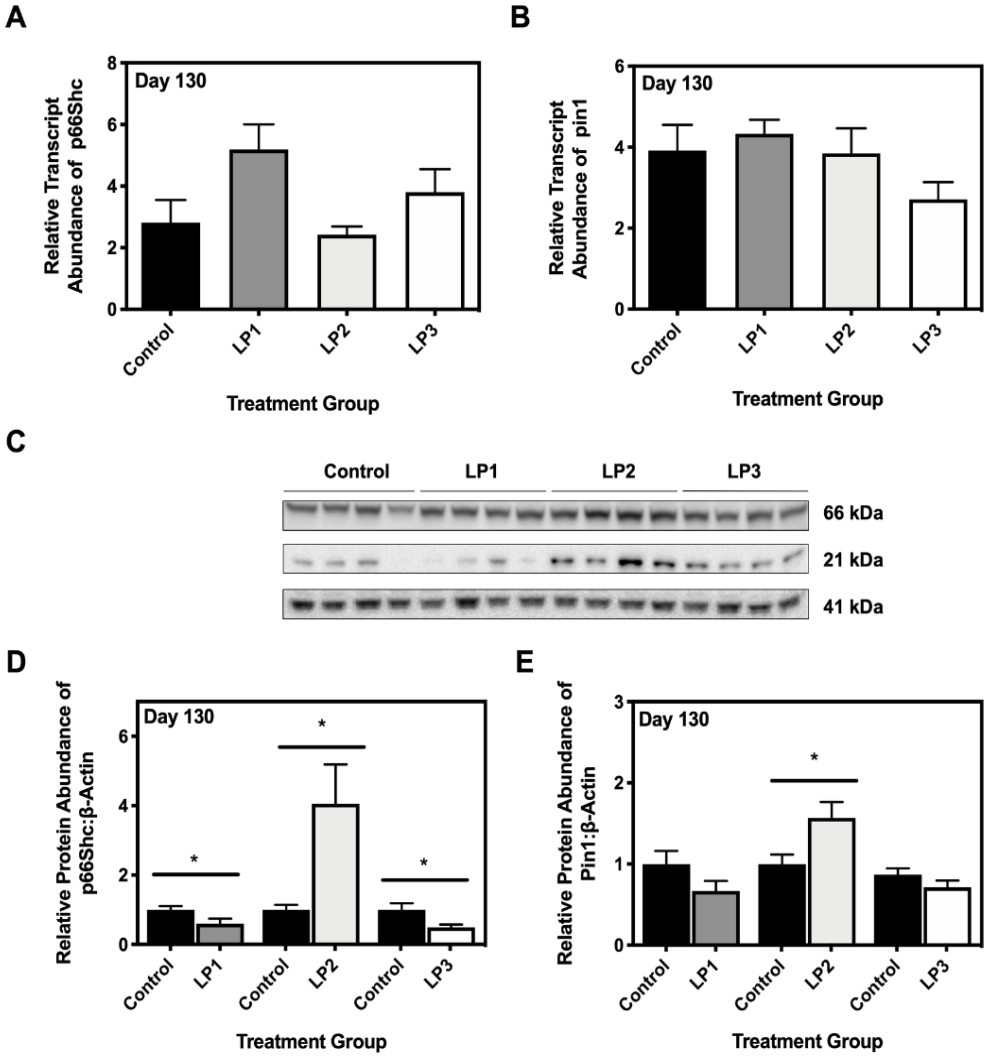

Figure 2-2. Maternal protein restriction (MPR) and postnatal catch-up growth together increase hepatic p66Shc and PIN1 in 4-month-old male offspring.

Transcript and protein abundances of p66Shc and Pin1 were determined via quantitative real-time PCR and Western immunoblotting, respectively. Relative transcript abundance of (A) p66Shc and (B) Pin1 were expressed as means normalized to the geometric mean of β-actin and GAPDH ± SEM (n = 7–8/group). (C) Representative western immunoblots for specific targeted protein bands of control, LP1, LP2 and LP3 offspring as detected by primary antibodies for p66Shc and PIN1. Relative protein abundances of (D) p66Shc and (E) PIN1 at 4 months of age were expressed as means normalized to β-Actin ± SEM (*n* =

7–8/group). All qRT-PCR data were analyzed using a one-way ANOVA and multiple comparisons test, while protein abundances were compared using a two-tailed unpaired Student's *t*-test. *Significant difference ($P < 0.05$).

LP2, and LP3) exhibited significantly increased 4-HNE relative to control offspring (Figure 2-3B; $P < 0.001$ [LP1] and $P < 0.01$ [LP2 and LP3]). To determine if antioxidant defenses were altered in response to this oxidative stress, we next examined protein abundance of hepatic superoxide dismutase (SOD) 1, SOD2 and catalase. Immunoblot analysis revealed that at four months of age, SOD1 and SOD2 were both significantly increased in LP2 offspring relative to control offspring (Figure 2-3C, D; $P < 0.05$ and $P < 0.01$). In contrast, SOD1 and SOD2 protein abundance remained unchanged in LP1 offspring at 4 months of age (Figure 2-3C, D; $P = 0.22$ and 0.20), as was the case for LP3 offspring (Figure 2-3C, D; $P = 0.73$ and 0.16). Interestingly, abundance of catalase was significantly reduced in both LP1 and LP2 offspring (Figure 2-3E; $P = 0.02$ and $P = 0.03$), while LP3 offspring were unaffected (Figure 2-3E; $P = 0.42$).

2.3.4 Maternal protein restriction in combination with postnatal catch-up growth leads to impaired aerobic respiration at 4 months of age

Because p66Shc has been suggested to mediate mitochondrial respiration through alterations in oxidative phosphorylation, we next investigated the expression of various enzymes involved in mitochondrial aerobic respiration (Nemoto *et al.* 2006, Acin-Perez *et al.* 2010, Lone *et al.* 2018). Following completion of glycolysis, pyruvate dehydrogenase (PDH) converts pyruvate to acetyl coenzyme-A (acetyl CoA) for use in the citric acid cycle (TCA). Increased phosphorylation of PDH results in inhibited PDH activity, thereby promoting increased glycolysis in the presence of oxygen (i.e., aerobic glycolysis). At 4 months of age, LP2 offspring displayed a significant increase in the ratio of phosphorylated PDH (p-PDH) to total PDH (Figure 2-4B; $P < 0.05$), as well as significantly increased levels of lactate dehydrogenase subunit A (LDHa; Figure 2-4C; $P = 0.03$). At this time point, the ratio of p-PDH to total PDH was unchanged in LP1 offspring (Figure 2-4B; $P = 0.25$), while it was significantly augmented in LP3 offspring (Figure 2-4B; $P < 0.05$). Abundance of LDHa was significantly decreased in LP1 and LP3 offspring (Figure 2-4C; $P = 0.02$ and $P = 0.04$).

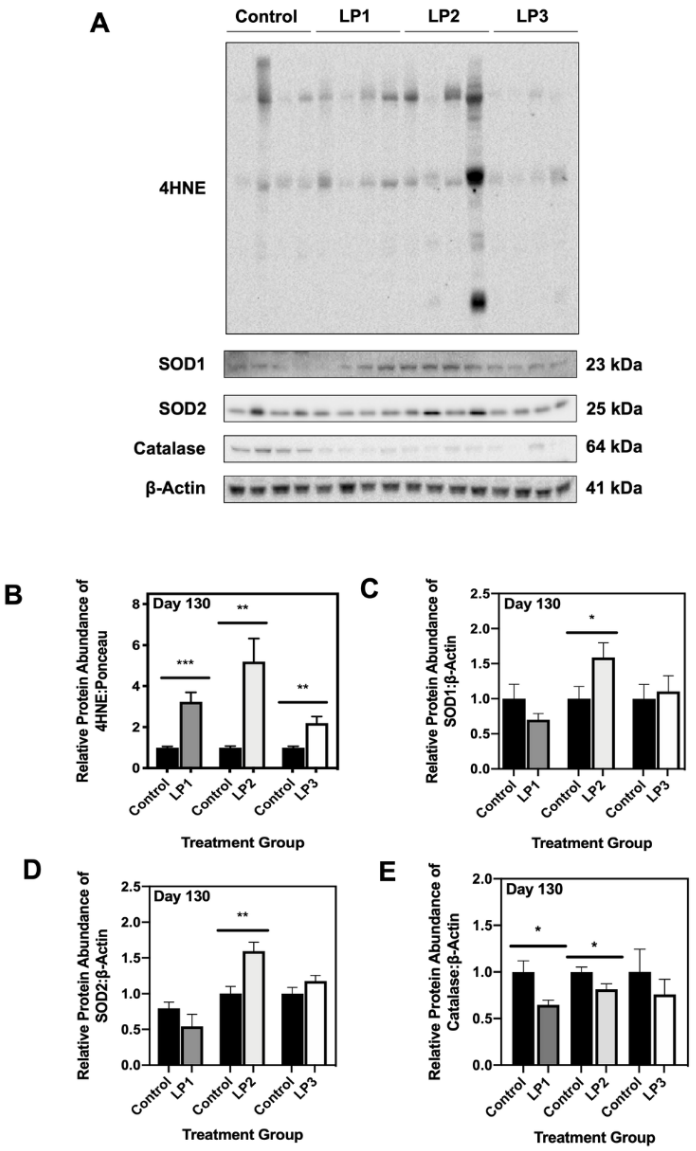

Figure 2-3. Maternal protein restriction leads to increased markers of hepatic oxidative stress at 4 months of age.

(A) Representative Western immunoblots illustrating expression of 4-hydroxynonenal, a marker of lipid peroxidation, superoxide dismutase (SOD) 1 and SOD2 in LP1, LP2 and LP3 offspring relative to control diet-fed offspring. Protein abundances of (B) 4HNE, (C) SOD1, (D) SOD2, and (E) catalase in each group of MPR offspring were each compared against control offspring. 4HNE abundance was expressed as means normalized to total

protein abundance ± SEM (n = 7–8/group), while SOD1, SOD2 and catalase abundances were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. *Significant difference ($P < 0.05$), **significant difference ($P < 0.01$), ***significant difference ($P < 0.001$).

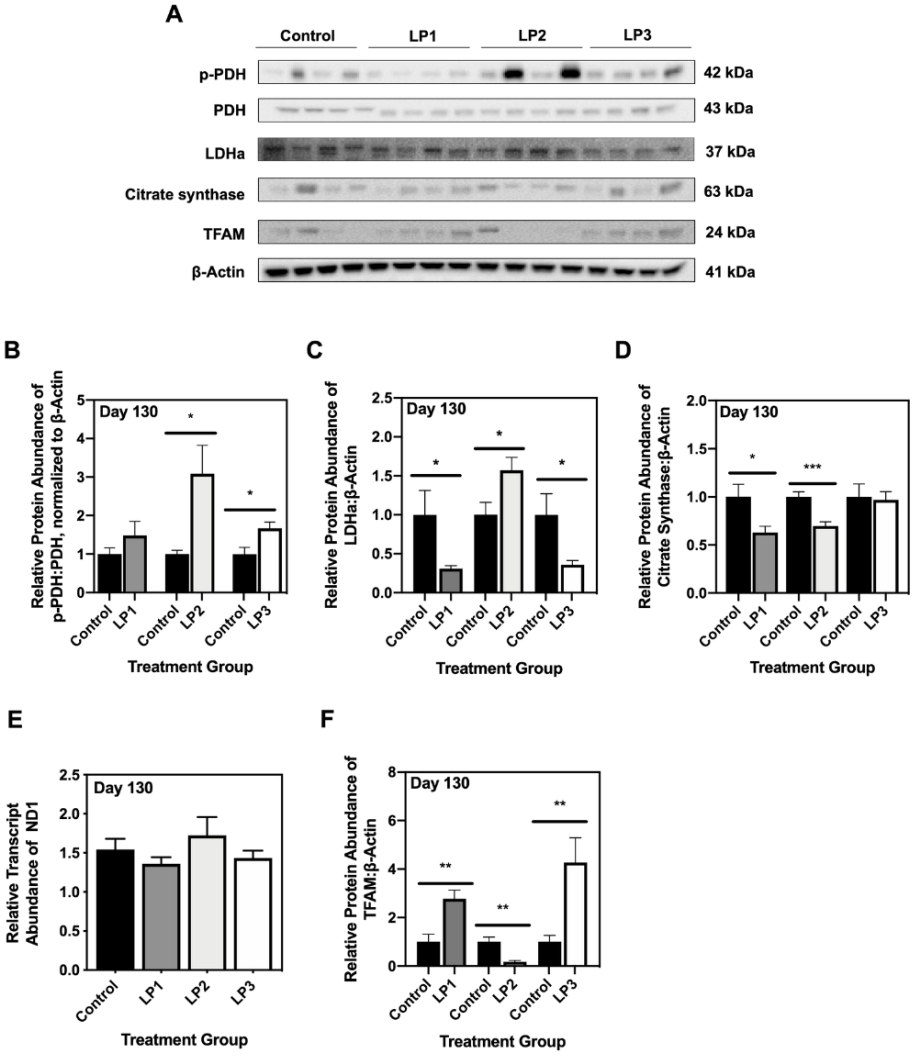

Figure 2-4. Maternal protein restriction and postnatal catch-up growth together lead to aberrant markers of aerobic metabolism at 4 months of age.

(A) Representative Western immunoblots for specific targeted protein bands for control, LP1, LP2 and LP3 offspring as detected by primary antibodies. Protein abundances of (B) phosphorylated pyruvate dehydrogenase (PDH) to total PDH, (C) lactate dehydrogenase subunit A (LDHa), (D) citrate synthase, and (F) mitochondrial transcription factor A (TFAM) in LP1, LP2 and LP3 offspring were compared against control diet-fed offspring and expressed as means normalized to β-Actin ± SEM (n = 7–8/group). (E) Transcript

abundance of NADH:ubiquinone oxidoreductase core subunit V1 (ND1), a marker of mitochondrial number, was expressed as means normalized to geometric means of β-Actin and GAPDH ± SEM (n = 7–8/group). All qRT-PCR data were analyzed using a one-way ANOVA and multiple comparisons test, while protein abundances were compared using a two-tailed unpaired Student's t-test. *Significant difference ($P < 0.05$), **significant difference ($P < 0.01$), ***significant difference ($P < 0.001$).

Considering the role of the electron transport chain (ETC) in oxidative phosphorylation, we measured levels of the five protein complexes within the ETC. While protein abundance of complexes I, III, IV and V were unaffected in LP2 offspring (Figure 2-5B, D–F), complex II was significantly decreased in LP2 offspring at 4 months of age (Figure 2-5C; $P < 0.0001$). In contrast, complex II protein abundance was unaffected in LP1 and LP3 offspring (Figure 2-5B). Moreover, the expression levels of mitochondrial complexes I, III, IV and V were also not altered in LP1 and LP3 offspring (Figure 2-5B, D–F).

Given the perinatal environment could have detrimental effects on mitochondrial size and abundance, we first assessed citrate synthase (CS) protein levels as a marker of mitochondrial mass and function. We observed a significant decrease in CS levels of LP2 offspring at 4 months of age relative to control offspring (Figure 2-4D; $P < 0.001$). Interestingly, LP1 offspring also displayed a significant decrease in CS levels at this time point when compared against control offspring (Figure 2-4D; $P < 0.05$). CS protein levels of LP3 offspring were unaffected compared to control offspring at 4 months of age (Figure 2-4D). Since we saw changes in the expression of CS and other mitochondrial metabolic enzymes, we next sought to determine if this was due to changes in mitochondrial number. Using qRT-PCR, we measured hepatic transcript abundance of NADH:ubiquinone oxidoreductase core subunit V1 (ND1) as a marker of mitochondrial number. At 4 months of age, ND1 mRNA abundance was unchanged in all groups of MPR offspring (Figure 2-4E). However, immunoblot analysis of mitochondrial transcription factor A (TFAM), which is critical in activating mitochondrial genome transcription, revealed a significant decrease in TFAM abundance in LP2 offspring (Figure 2-4F; $P = 0.004$). In contrast, TFAM abundance was significantly increased in LP1 and LP3 offspring (Figure 2-4F; $P = 0.002$ and 0.008).

2.3.5 Maternal protein restriction alone leads to decreased p66Shc protein abundance at 3 weeks of age

To distinguish between the direct effects of the MPR diet versus postnatal catch-up growth, we next evaluated the expression of p66Shc and previously examined

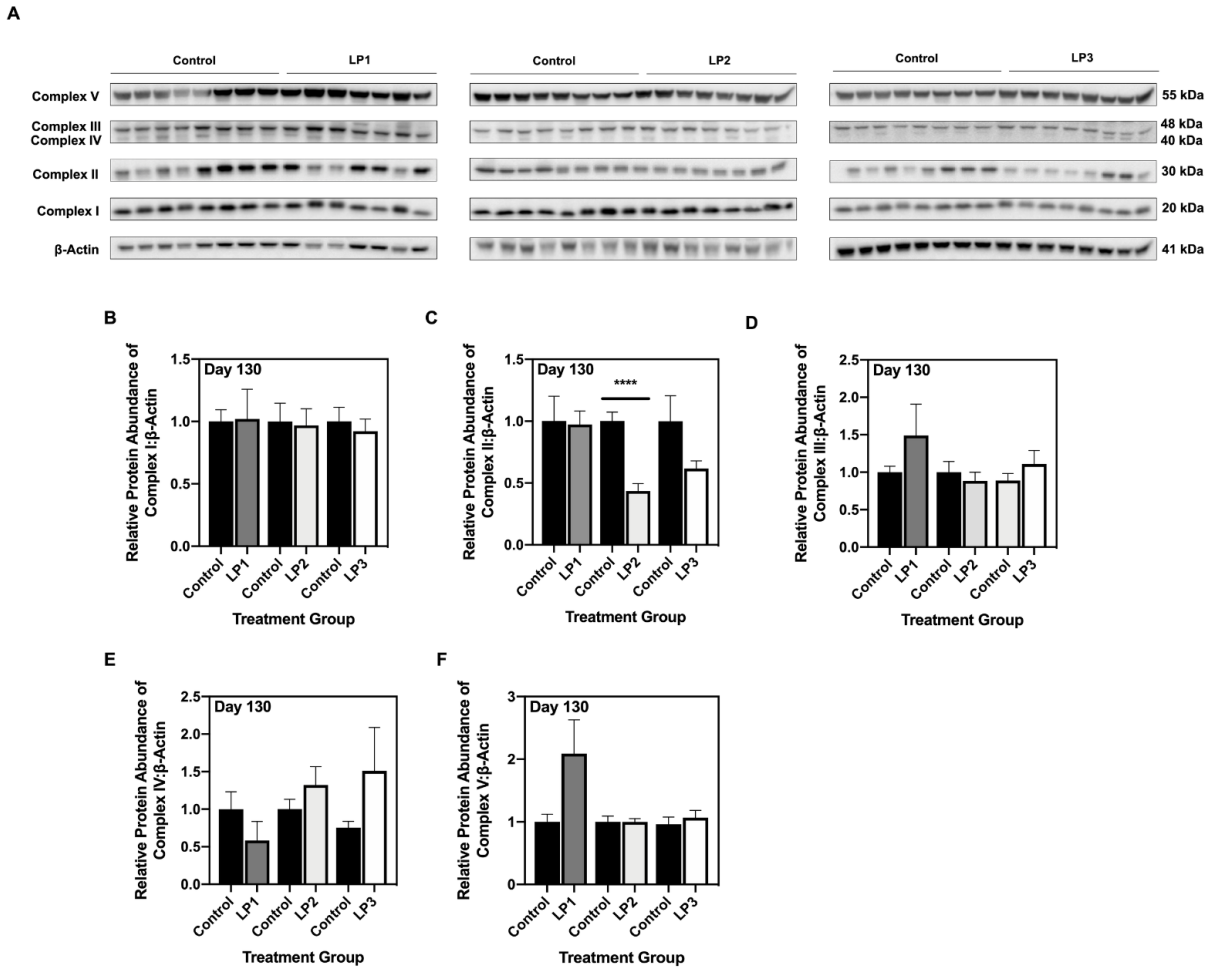

Figure 2-5. Maternal protein restriction leads to decreased protein levels of complex II of the mitochondrial electron transport chain at four months of age.

(A) Specific targeted protein bands of control, LP1, LP2, and LP3 offspring as detected by primary antibodies via western immunoblot. Protein abundances of (B) complex I, (C) complex II, (D) complex III, (E) complex IV, and (F) complex V protein abundances were compared in each group of MPR offspring against control offspring. Protein abundances of all targets were normalized to β-Actin ± SEM (n= 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test.

mitochondrial markers at three weeks (*i.e.,* postnatal day 21). Western immunoblots revealed a significant decrease in p66Shc protein levels in protein-restricted (*i.e.,* LP1/LP2) offspring compared to control offspring (Figure 2-6B; $P < 0.05$), while p66Shc was unchanged in LP3 offspring (Figure 2-6B). Levels of PIN1, 4HNE, citrate synthase, and all ETC complexes were unaltered in all MPR offspring relative to controls (Figure 2-6C, D, F and Figure 2-7B–F). While the ratio of p-PDH to total PDH was unchanged in LP1/LP2 offspring (Figure 2-6E; $P = 0.44$), LP3 offspring displayed a significant increase in p-PDH to total PDH (Figure 2-6E; $P < 0.01$).

2.3.6 Induction of ER stress in vitro promotes increased abundance of p66Shc

Given that our previous studies of these same MPR offspring revealed that only LP2 offspring exhibit ER stress (Sohi *et al.* 2013), and there is an association between increased ER stress and p66Shc (Zhang *et al.* 2013), we next investigated if induction of ER stress *in vitro* leads to augmented hepatic p66Shc protein levels in short-term cell culture. HepG2 cells were used for all *in vitro* experiments given their ability to secrete proteins that are found primarily in fetal hepatocytes (Maruyama *et al.* 2007). Cells were treated with various doses of tunicamycin, a potent inducer of ER stress in HepG2 cells (Lei *et al.* 2016), and collected at 1, 2 and 6 h to determine which dose was most effective in inducing ER stress. A dose of 0.5 μg/mL proved to be most consistent in inducing ER stress, as evidenced by a trending increase in Grp94 protein levels relative to cells treated with DMSO vehicular control (Figure 2-8B; $P = 0.08$ (1 h), 0.10 (2 h), 0.07 (6 h)). Interestingly, HepG2 cells treated with tunicamycin had a significant increase in p66Shc protein levels at 1 h and 2 h (Figure 2-8C; $P < 0.05$).

2.4 Discussion

In this study, we demonstrate that perinatal protein restriction with postnatal catch-up growth leads to hepatic oxidative stress and mitochondrial dysfunction in adult

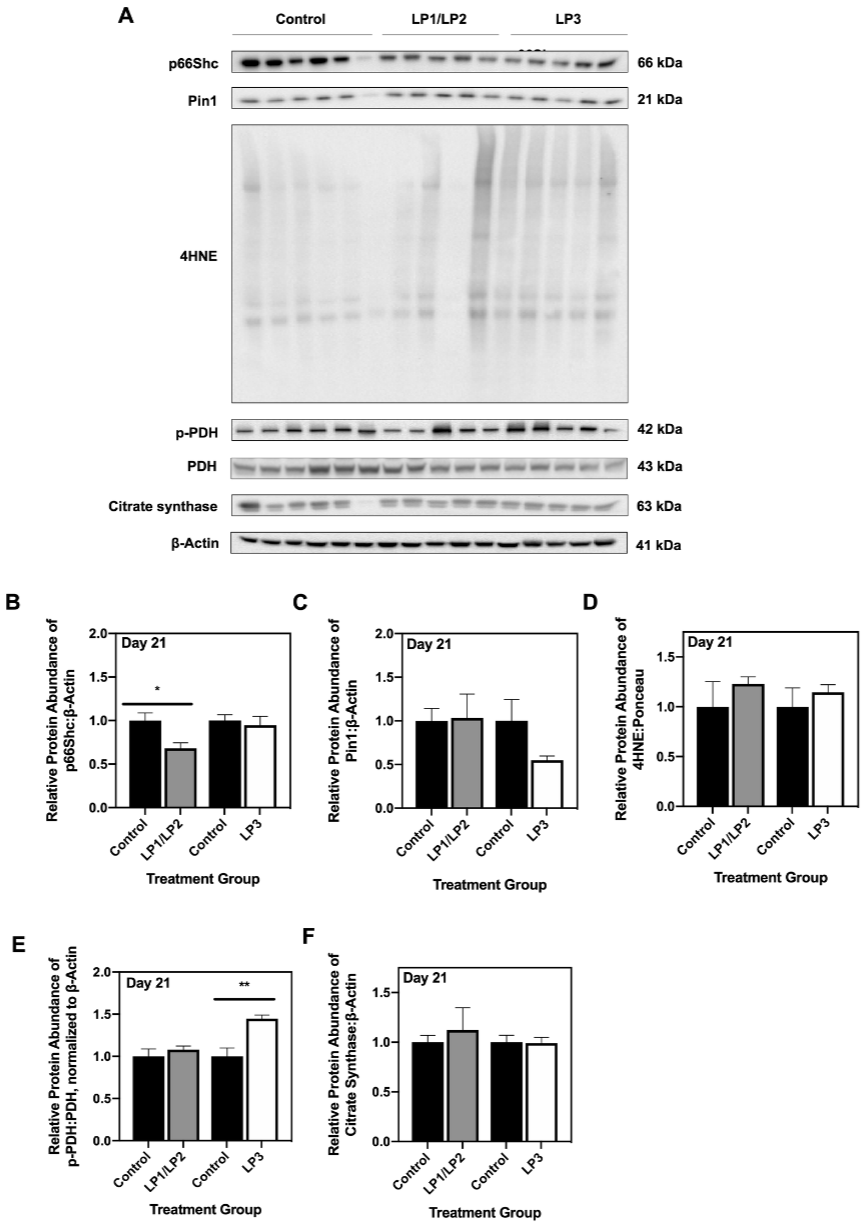

Figure 2-6. Maternal protein restriction does not independently contribute to increased p66Shc or oxidative stress at 3 weeks of age.

(A) Representative Western immunoblots of specific targeted protein bands for control, LP1/LP2, and LP3 offspring as detected by primary antibodies. Protein abundances of (B) p66Shc, (C) PIN1, (D) 4HNE, (E) p-PDH, and (F) citrate synthase were compared in each group of MPR offspring against control offspring. 4HNE abundance was expressed

as means normalized to total protein abundance ± SEM (n = 7–8/group), while abundances of all other targets were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. *Significant difference ($P < 0.05$), **significant difference ($P < 0.01$).

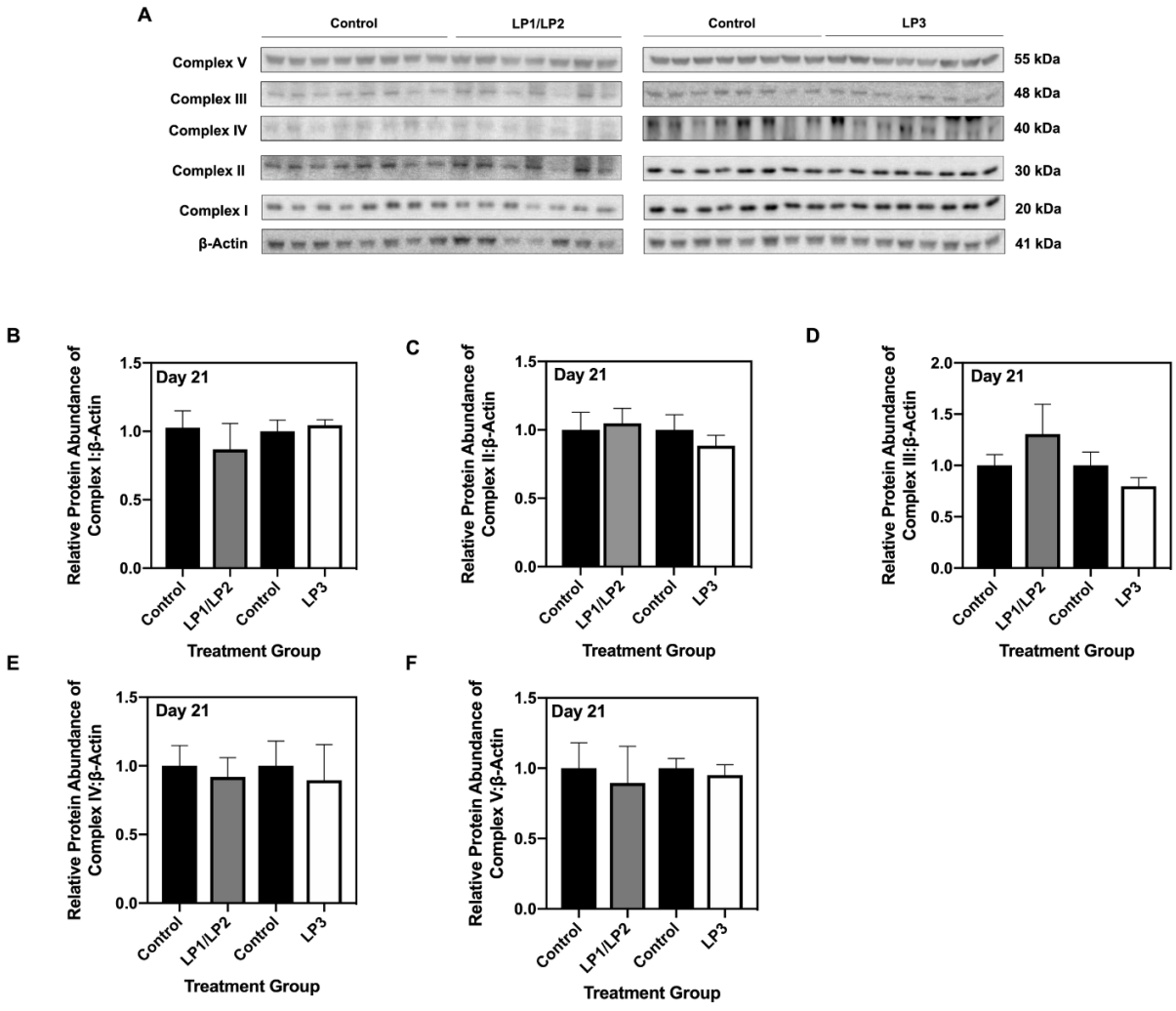

Figure 2-7. Maternal protein restriction does not independently contribute to altered complex proteins of the mitochondrial electron transport chain at three weeks of age.

(A) Specific targeted protein bands of control, LP1/LP2, and LP3 offspring as detected by primary antibodies via western immunoblot. (B) Complex I, (C) complex II, (D) complex III, (E) complex IV, and (F) complex V protein abundances were compared in each group of MPR offspring against control offspring. Protein abundances of all targets were normalized to β-Actin ± SEM (n= 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test.

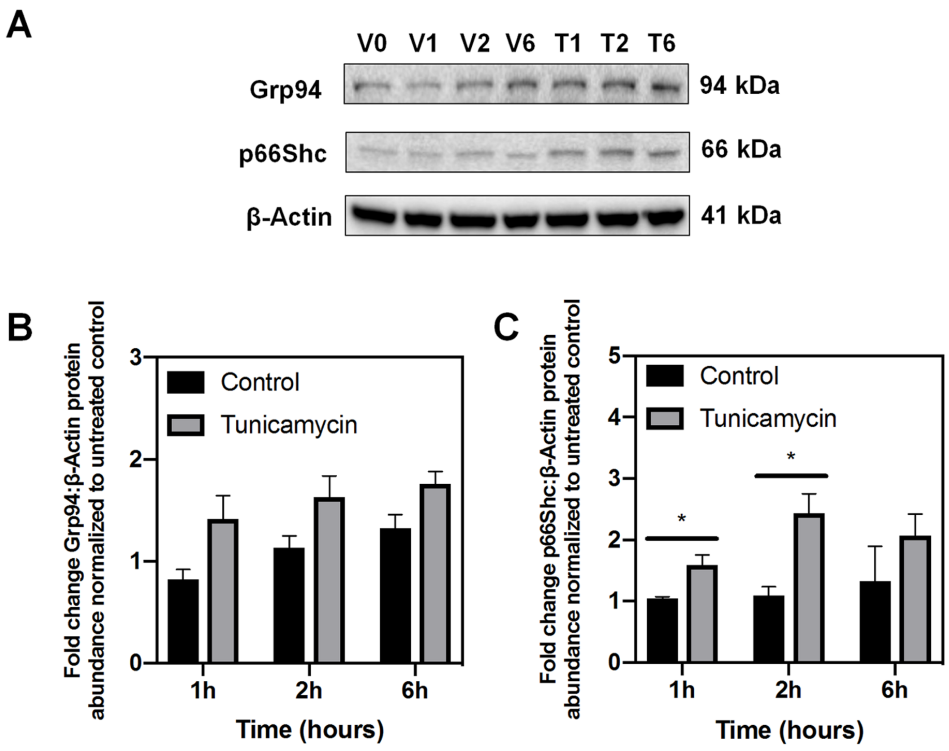

Figure 2-8. Induction of ER stress with tunicamycin results in increased protein abundance of p66Shc in the HepG2 cell line.

(A) Representative Western blots illustrating protein abundance of GRP94 and p66Shc in HepG2 cells treated with either DMSO vehicular control for 1 h (V1), 2 h (V2) or 6 h (V6), or 0.5 µg/mL tunicamycin for 1h (T1), 2h (T2) or 6h (T6). Fold change difference of (B) p66Shc and (C) GRP94 were expressed as means from three biological replicates normalized to β-Actin ± SEM, and again normalized to untreated control cells at 0h (V0). Protein abundances for each time point were analyzed using a two-tailed unpaired Student's *t*-test. *Significant difference ($P < 0.05$).

rat offspring. Our results suggest that catch-up growth, rather than the direct low protein diet, leads to increased expression of mitochondrial stress marker p66Shc and the cis/trans isomerase PIN1 at 4 months of age. These cellular stresses were exclusive to LP2 offspring, which experienced catch-up growth after completion of liver differentiation (i.e, after 3 weeks) in the rat (Gruppuso & Sanders 2016). It is noteworthy that LP2 offspring are also exposed to the longest period of protein restriction, including 22 days during gestation and 21 days in postnatal life. In addition, LP2 offspring exhibited long-term hepatic oxidative stress, as observed by the apparent changes in expression of 4-HNE, SOD1, SOD2, and catalase. Furthermore, these LP2 offspring display elevated levels of phospho-PDH(Ser232) and LDHa, as well as decreased abundance of CS and mitochondrial ETC complex II, collectively indicating impaired mitochondrial metabolism. Finally, we established a direct relationship between ER stress and hepatic p66Shc expression, thereby uncovering a potential mechanism for increased hepatic p66shc in these LP2 offspring. Taken together, our *in vivo* and *in vitro* results suggest that the dysmetabolism exhibited by MPR offspring is driven in part by mitochondrial dysfunction and oxidative stress (Figure 2-9).

While previous studies have verified the significance of oxidative stress and mitochondrial impairment in metabolic disease (Petersen *et al.* 2003, Nojiri *et al.* 2006, Ozgen *et al.* 2012), the role of developmental programming in this process remains largely unknown. In rat offspring, it has been suggested that MPR may be protective during the first month of life by decreasing production of ROS and promoting increased coupling between cellular respiration and oxidative phosphorylation (Moraes *et al.* 2014). Despite this, adult male offspring have been demonstrated to have increased hepatic oxygen consumption and markers of oxidative stress, as well as reduced expression of mitochondrial (mt)-DNA encoded genes (Park *et al.* 2003, They *et al.* 2009, Moraes *et al.* 2014). These data are consistent with human and rodent studies of obesity and cardiovascular disease, as mitochondrial defects arise with morbid obesity and insulin resistance (Dobrian *et al.* 2001, Koliaki *et al.* 2015).

In many cell types, increased oxidative injury is mediated, in part, by augmented expression of p66Shc (Orsini *et al.* 2004, Giorgio *et al.* 2005, Pinton *et al.* 2007). This is

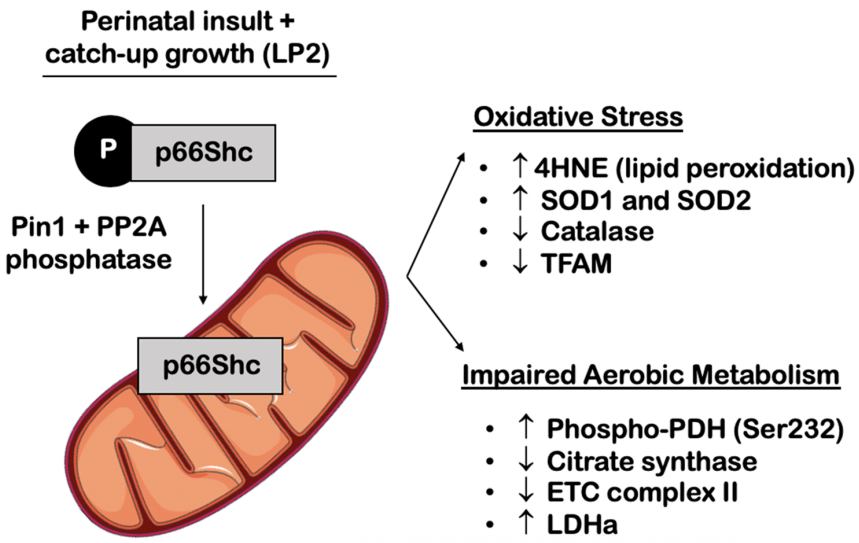

Figure 2-9. Proposed schematic illustrating the effects of perinatal protein restriction in combination with postnatal catch-up growth on hepatic mitochondrial health.

In summary, LP2 offspring were the only experimental group subject to MPR that exhibited increased protein abundance of p66Shc and PIN1. These offspring also displayed aberrant markers of oxidative stress and metabolism, indicating that mitochondrial health had become impaired in adulthood following catch-up growth in early life.

particularly evident in the diseased liver, which exhibits elevated expression of p66Shc (Haga *et al.* 2010, Perrini *et al.* 2015, Zhao *et al.* 2019). Using rodent models of various hepatic pathologies, knockdown of p66Shc prevents liver injury by ameliorating oxidative stress and mitochondrial dysfunction (Haga *et al.* 2010, Perrini *et al.* 2015, Zhao *et al.* 2019). Our expression analysis of p66Shc and PIN1 suggests that there may be increased stabilization of p66Shc in LP2 offspring, thereby accelerating hepatic mitochondrial ROS generation. Previous findings demonstrate that MPR offspring with catch-up growth display elevated renal p66Shc much later in life (*i.e.,* 6 months), but this is already a time of significant aging (Luyckx *et al.* 2009). In addition, a comparison to the effects of a low protein cohort (*i.e.*, LP1) were not determined. Conversely, our study highlights how the timing of postnatal nutritional intervention leads to differential expression of hepatic p66Shc expression in young adult offspring. Given that LP1 offspring have decreased expression of p66Shc and unchanged levels of PIN1, it is possible that maintenance of a LP diet in postnatal life may actually protect the adult liver from mitochondrial dysfunction in absence of catch-up growth. Moreover, decreased expression of p66Shc in adult LP3 offspring suggests that introduction of a normal protein diet during lactation, a period of hepatic plasticity, could 'rescue' MPR offspring by preventing mitochondrial stress (Gruppuso & Sanders 2016). LP3 offspring undergo catch-up growth prior to the completion of hepatic differentiation (Gruppuso & Sanders 2016); therefore, it is possible that earlier nutritional intervention (*i.e.*, <3 weeks postpartum) allows for recovery from any negative effects of the gestational MPR diet.

Since we observed elevated p66Shc and PIN1 expression in MPR offspring, we next investigated if this coincided with the presence of hepatic oxidative stress. Interestingly, our data revealed that LP2 offspring have increased 4-HNE and SODs, but decreased expression of catalase. Not only does this suggest that LP2 offspring exhibit the greatest degree of oxidative stress, but also that MPR with catch-up growth likely triggers the SOD antioxidant response over time. While this may occur as a compensatory mechanism in response to p66Shc-mediated oxidative stress, it is not sufficient in overcoming the cumulative oxidative stress that is brought about by other mechanisms. That said, decreased abundance of catalase in LP2 offspring may contribute

to the apparent oxidative stress via an inability to decompose hydrogen peroxide in the liver. Because 4-HNE, SOD1 and catalase are not specific to the mitochondria, the differential expression of these markers indicates the presence of general cellular oxidative stress as well. That said, SOD2 is localized to the mitochondria (Weisiger & Fridovich 1973); therefore, its upregulation, in combination with increased p66Shc and decreased TFAM, is suggestive of mitochondrial-induced oxidative stress. A previous report indicates that LP2 adult offspring have increased activity of hepatic SOD activity at three months of age; however, direct markers of oxidative stress were not measured (Theys *et al.* 2009). Furthermore, the authors did not examine how the timing of postnatal protein restoration may contribute to this antioxidant response (Theys *et al.* 2009). Collectively, given LP2 offspring are the only MPR group to exhibit abnormal expression of both p66Shc and PIN1 along with all examined markers of oxidative stress, it is conceivable that increased p66shc/PIN1 in these offspring is conducive to oxidative and mitochondrial damage in the liver.

Studies of aging have uncovered the relationship between oxidative stress and impaired mitochondrial metabolism, as oxygen-free radicals can cause inactivation of metabolic enzymes present within the mitochondrion (Cadenas & Davies 2000). More recently, p66Shc has been demonstrated to cause abnormal aerobic respiration (Acin-Perez *et al.* 2010, Lone *et al.* 2018); however, the mechanisms by which this occurs remain relatively unclear. Interestingly, p66Shc stabilization leads to reduced oxygen consumption rate and reduced production of ATP *in vitro* (Lone *et al.* 2018), while its deficiency can reduce basal and maximal oxygen consumption capacity and increase production of lactate by LDHa (Nemoto *et al.* 2006). Moreover, in cultured mouse embryos, greater total p66shc has been correlated with decreased cellular ATP production and increased superoxide production (Edwards *et al.* 2016). Our study determined that MPR and catch-up growth affects all stages of aerobic respiration, as we observed increased phosphorylation of PDH along with decreased abundance of citrate synthase and mitochondrial complex II proteins. Increased p-PDH is indicative of impaired mitochondrial function, as the cell begins to preferentially convert pyruvate to lactate rather than acetyl CoA (Lone *et al.* 2018). This is in agreement with our data concerning

LDHa, which was upregulated in adult LP2 offspring. With respect to the ETC, mitochondrial complex II was the only subunit to be influenced by MPR. This is of great interest considering that decreased activity of complex II can cause elevated ROS production within the mitochondria (Walker *et al.* 2006). Citrate synthase, the enzyme responsible for production of citrate from acetyl CoA and oxaloacetate in the TCA, is also a prominent marker of mitochondrial biomass. Decreased hepatic citrate synthase abundance and activity is associated with insulin resistance in male obese rats (Rector *et al.* 2010); therefore, the current decrease in hepatic citrate synthase abundance helps explain the decreased hepatic insulin sensitivity and enhanced gluconeogenesis of LP2 offspring observed in our previous studies (Sohi *et al.* 2013, Vo *et al.* 2013). Finally, the observed decrease in protein levels of TFAM are indicative of reduced mtDNA content. Suppression of TFAM has been previously associated with altered mitochondrial function, suggesting that it may have a role in mediating metabolic activity and disease (Vernochet *et al.* 2012).

To understand why MPR offspring with catch-up growth might exhibit exclusive increases in p66shc, we focused our attention of the role of ER stress on the mitochondria. The ER and mitochondrion are physically connected via sites known as the mitochondrial-associated ER membrane (MAMs), which are highly concentrated with calcium transporters and ion channels (Hayashi *et al.* 2009). These channels can indirectly alter ATP production in response to protein demands of the ER (Burton *et al.* 2017), and it has been demonstrated that their activity is influenced by nutrient availability (Theurey *et al.* 2016). Upon cellular stress, the presence of misfolded and unfolded proteins in the ER initiates the unfolded protein response (UPR), leading to activation of downstream targets that promote an adaptive or apoptotic phenotype (Xu *et al.* 2005). Under severe ER stress, the UPR is initiated to promote the transcription of genes that increase folding capacity of the ER (i.e., Grp94) or protein attenuation (i.e., phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) (Xu *et al.* 2005). We have previously demonstrated that the same LP2 offspring examined as part of this study exhibit elevated phosphorylated eIF2α _and higher expression of chaperone proteins in the liver (Sohi *et al.* 2013). Given that these findings are exclusive to LP2 offspring, it

appears that catch-up growth is implicated in onset of hepatic ER stress. Our *in vitro* studies in HepG2 liver cells directly linked ER stress with increased protein abundance of p66Shc, consequently implicating this process as a potential instigator of augmented hepatic p66Shc expression in LP2 offspring. It is noteworthy that in a model of intermittent hypoxia, p66Shc has been associated with increased ER stress in the rat testes; however, the direct role of ER stress on p66Shc was not investigated (Zhang *et al.* 2013). Because we saw an increase in p66Shc as early as 1 h after tunicamycin treatment, as well as an increase only in p66Shc protein but not mRNA *in vivo*, it is possible that ER stress may regulate p66Shc expression via post-transcriptional mechanisms. P66Shc has been demonstrated to be subject to post-transcriptional regulation (Kumar *et al.* 2017); therefore, future *in vitro* studies will investigate this relationship in efforts of elucidating the molecular mechanisms by which ER stress may regulate p66Shc *in vivo*.

In summary, our data indicate that postnatal catch-up growth plays a direct role in the mitochondrial function of adult MPR offspring. We are the first to demonstrate that the timing of nutritional insult can modify expression of hepatic p66Shc, an important modulator of mitochondrial-induced oxidative stress. Protein deficiency exclusively during the perinatal period appears to be most detrimental to hepatic mitochondrial function, while restoration of a normal protein diet at birth shows promise in ameliorating oxidative stress and mitochondrial defects. While these outcomes may be mediated by hepatic ER stress, future studies are warranted in determining the exact mechanism by which this occurs. Overall, our study provides further support for the main tenet of Barker's 'thrifty phenotype' hypothesis, as it highlights the importance of the nutritional environment in developing organ systems and risk for the adult metabolic syndrome.

2.5 References

Barra NG, Lisyansky M, Vanduzer TA, Raha S, Holloway AC and Hardy DB (2017) Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase and impaired mitochondrial function in male rat offspring. *Journal of Applied Toxicology* 37 1517–1526.

Battaglia FC and Meschia G (1978) Principal substrates of fetal metabolism. *Physiological Reviews* 58 499–527.

Bieswal F, Ahn MT, Reusens B, Holvoet P, Raes M, Rees WD and Remacle C (2006) The importance of catch-up growth after early malnutrition for the programming of obesity in male rat. *Obesity (Silver Spring, Md.)* 14 1330–1343.

Burton GJ, Yung HW and Murray AJ (2017) Mitochondrial – Endoplasmic reticulum interactions in the trophoblast: Stress and senescence. *Placenta* 52 146–155.

Cadenas E and Davies KJA (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biology and Medicine* 29 222–230.

Chamson-Reig A, Thyssen SM, Hill DJ and Arany E (2009) Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. *Experimental Biology and Medicine (Maywood, N.J.)* 234 1425–1436.

Crosby WM (1991) Studies in fetal malnutrition. *American Journal of Diseases of Children (1960)* 145 871–876.

Desai M and Hales CN (1997) Role of fetal and infant growth in programming metabolism in later life. *Biological Reviews of the Cambridge Philosophical Society* 72 329–348.

Dobrian AD, Davies MJ, Schriver SD, Lauterio TJ and Prewitt RL (2001) Oxidative stress in a rat model of obesity-induced hypertension. *Hypertension (Dallas, Tex. : 1979)* 37 554–560.

Edwards NA, Watson AJ and Betts DH (2016) P66Shc, a key regulator of metabolism and mitochondrial ROS production, is dysregulated by mouse embryo culture. *Molecular Human Reproduction*.

Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M *et al.* (2005) Electron Transfer between Cytochrome c and p66Shc Generates Reactive Oxygen Species that Trigger Mitochondrial Apoptosis. *Cell* 122 221–233.

Gruppuso PA and Sanders JA (2016) Regulation of liver development: implications for liver biology across the lifespan. *Journal of Molecular Endocrinology* 56 R115–R125.

Haga S, Morita N, Irani K, Fujiyoshi M, Ogino T, Ozawa T and Ozaki M (2010) p66Shc has a pivotal function in impaired liver regeneration in aged mice by a redox-dependent mechanism. *Laboratory Investigation* 90 1718–1726.

Hales CN and Barker DJ (2001) The thrifty phenotype hypothesis. *British Medical Bulletin* 60 5–20.

Hales CN, Desai M, Ozanne SE and Crowther NJ (1996) Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. *Biochemical Society Transactions* 24 341–350.

Hayashi T, Rizzuto R, Hajnoczky G and Su T-P (2009) MAM: more than just a housekeeper. *Trends in Cell Biology* 19 81–88.

Koliaki C, Szendroedi J, Kaul K, Jelenik T, Nowotny P, Jankowiak F, Herder C, Carstensen M, Krausch M, Knoefel WT *et al.* (2015) Adaptation of Hepatic Mitochondrial Function in Humans with Non-Alcoholic Fatty Liver Is Lost in Steatohepatitis. *Cell Metabolism*.

Kumar S, Kim Y, Vikram A, Naqvi A, Li Q, Kassan M, Kumar V, Bachschmid MM, Jacobs JS, Kumar A *et al.* (2017) Sirtuin1-regulated lysine acetylation of p66Shc governs diabetes-induced vascular oxidative stress and endothelial dysfunction. *PNAS* 114 1714–1719.

Lei L, Zhu Y, Gao W, Du X, Zhang M, Peng Z, Fu S, Li X, Zhe W, Li X *et al.* (2016) Alpha-lipoic acid attenuates endoplasmic reticulum stress-induced insulin resistance by improving mitochondrial function in HepG2 cells. *Cellular Signalling* 28 1441–1450.

Liu W, Porter NA, Schneider C, Brash AR and Yin H (2011) Formation of 4-hydroxynonenal from cardiolipin oxidation: Intramolecular peroxyl radical addition and decomposition. *Free Radical Biology and Medicine* 50 166–178.

Lone A, Harris RA, Singh O, Betts DH and Cumming RC (2018) p66Shc activation promotes increased oxidative phosphorylation and renders CNS cells more vulnerable to amyloid beta toxicity. *Scientific Reports* 8 17081.

Luyckx VA, Compston CA, Simmen T and Mueller TF (2009) Accelerated senescence in kidneys of low-birth-weight rats after catch-up growth. *American Journal of Physiology-Renal Physiology* 297 F1697–F1705.

Maruyama M, Matsunaga T, Harada E and Ohmori S (2007) Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biological & Pharmaceutical Bulletin* 30 2091–2097.

Migliaccio E, Giogio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L and Pelicci PG (1999) The p66(shc) adaptor protein controls oxidative stress response and life span in mammals. *Nature*.

Moraes C, Rebelato HJ, Amaral MEC, Resende TM, Silva EVC, Esquisatto MAM and Catisti R (2014) Effect of maternal protein restriction on liver metabolism in rat offspring. *The Journal of Physiological Sciences* 64 347–355.

Nemoto S, Combs CA, French S, Ahn B-H, Fergusson MM, Balaban RS and Finkel T (2006) The mammalian longevity-associated gene product p66shc regulates mitochondrial metabolism. *The Journal of Biological Chemistry* 281 10555–10560.

Noda Y, Yamagishi S, Matsui T, Ueda S, Ueda S, Jinnouchi Y, Hirai Y and Imaizumi T (2010) The p66shc Gene Expression in Peripheral Blood Monocytes Is Increased in Patients With Coronary Artery Disease. *Clinical Cardiology* 33 548–552.

Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S, Ohta Y, Sami M, Tachibana T, Ishikawa H *et al.* (2006) Oxidative stress causes heart failure with impaired mitochondrial respiration. *The Journal of Biological Chemistry* 281 33789–33801.

Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M *et al.* (2004) The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. *The Journal of Biological Chemistry* 279 25689–25695.

Ozanne SE and Hales CN (2004) Lifespan: catch-up growth and obesity in male mice. *Nature* 427 411–412.

Ozgen IT, Tascilar ME, Bilir P, Boyraz M, Guncikan MN, Akay C and Dundaroz R (2012) Oxidative stress in obese children and its relation with insulin resistance. *Journal of Pediatric Endocrinology and Metabolism* 25 261–266.

Pagnin E, Fadini G, De Toni R, Tiengo A, Calò L and Avogaro A (2005) Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: Relationship to oxidative. *Journal of Clinical Endocrinology and Metabolism*.

Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K-U, Pak YK and Lee HK (2003) Fetal and Early Postnatal Protein Malnutrition Cause Long-Term Changes in Rat Liver and Muscle Mitochondria. *The Journal of Nutrition* 133 3085–3090.

Perrini S, Tortosa F, Natalicchio A, Pacelli C, Cignarelli A, Palmieri VO, Caccioppoli C, De Stefano F, Porro S, Leonardini A *et al.* (2015) The p66 Shc

protein controls redox signaling and oxidation-dependent DNA damage in human liver cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 309 G826–G840.

Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW and Shulman GI (2003) Mitochondrial Dysfunction in the Elderly: Possible Role in Insulin Resistance. *Science* 300 1140–1142.

Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters* 26 509–515.

Pinton P, Rimessi A, Marchi S, Orsini F, Migliaccio E, Giorgio M, Contursi C, Minucci S, Mantovani F, Wieckowski MR *et al.* (2007) Protein kinase C beta and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66Shc. *Science (New York, N.Y.)* 315 659–663.

Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN and Bleker OP (1998) Glucose tolerance in adults after prenatal exposure to famine. *Lancet* 351 173–177.

Rector RS, Thyfault JP, Uptergrove GM, Morris EM, Naples SP, Borengasser SJ, Mikus CR, Laye MJ, Laughlin MH, Booth FW *et al.* (2010) Mitochondrial dysfunction precedes insulin resistance and hepatic steatosis and contributes to the natural history of non-alcoholic fatty liver disease in an obese rodent model. *Journal of Hepatology* 52 727–736.

Roseboom TJ, van der Meulen JH, van Montfrans GA, Ravelli AC, Osmond C, Barker DJ and Bleker OP (2001) Maternal nutrition during gestation and blood pressure in later life. *Journal of Hypertension* 19 29–34.

Sohi G, Marchand K, Revesz A, Arany E and Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. *Molecular Endocrinology (Baltimore, Md.)* 25 785–798.

Sohi G, Revesz A and Hardy DB (2013) Nutritional mismatch in postnatal life of low birth weight rat offspring leads to increased phosphorylation of hepatic eukaryotic initiation factor 2 alpha in adulthood. *Metabolism: Clinical and Experimental*.

Sohi G, Barry EJ, Velenosi TJ, Urquhart BL and Hardy DB (2014) Protein Restoration in Low-Birth-Weight Rat Offspring Derived from Maternal Low-Protein Diet Leads to Elevated Hepatic CYP3A and CYP2C11 Activity in Adulthood. *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 42 221–228.

Sohi G, Revesz A, Ramkumar J and Hardy DB (2015) Higher Hepatic miR-29 Expression in Undernourished Male Rats During the Postnatal Period Targets the Long-Term Repression of IGF-1. *Endocrinology* 156 3069–3076.

Theurey P, Tubbs E, Vial G, Jacquemetton J, Bendridi N, Chauvin MA, Alam MR, Le Romancer M, Vidal H and Rieusset J (2016) Mitochondria-associated endoplasmic reticulum membranes allow adaptation of mitochondrial metabolism to glucose availability in the liver. *J Mol Cell Biol* 8 129–143.

Theys N, Clippe A, Bouckenooghe T, Reusens B and Remacle C (2009) Early Low Protein Diet Aggravates Unbalance between Antioxidant Enzymes Leading to Islet Dysfunction. *PLoS ONE* 4 e6110.

Trinei M, Migliaccio E, Bernardi P, Paolucci F, Pelicci P and Giorgio M (2013) p66Shc, Mitochondria, and the Generation of Reactive Oxygen Species. In *Methods in Enzymology*, pp 99–110.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3 RESEARCH0034.

Vernochet C, Mourier A, Bezy O, Macotela Y, Boucher J, Rardin MJ, An D, Lee KY, Ilkayeva OR, Zingaretti CM, Emanuelli B, Smyth G, Cinti S, Newgard CB, Gibson BW, Larsson N, Kahn CR (2012) Adipose-specific deletion of TFAM increases mitochondrial oxidation and protects mice against obesity and insulin resistance. *Cell Metabolism* 16 765–776.

Vo TX, Revesz A, Ma N and Hardy DB (2013) Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver x receptor. *Journal of Endocrinology* 218 85–97.

Walker DW, Hájek P, Muffat J, Knoepfle D, Cornelison S, Attardi G and Benzer S (2006) Hypersensitivity to oxygen and shortened lifespan in a Drosophila mitochondrial complex II mutant. *Proceedings of the National Academy of Sciences of the United States of America* 103 16382–16387.

Wei Y, Clark SE, Thyfault JP, Uptergrove GME, Li W, Whaley-Connell AT, Ferrario CM, Sowers JR and Ibdah JA (2009) Oxidative stress-mediated mitochondrial dysfunction contributes to angiotensin II-induced nonalcoholic fatty liver disease in transgenic Ren2 rats. *The American Journal of Pathology* 174 1329–1337.

Weisiger RA and Fridovich I (1973) Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. *The Journal of Biological Chemistry* 248 4793–4796.

Woodman AG, Mah R, Keddie D, Noble RMN, Panahi S, Gragasin FS, Lemieux H and Bourque SL (2018) Prenatal iron deficiency causes sex-dependent

mitochondrial dysfunction and oxidative stress in fetal rat kidneys and liver. *The FASEB Journal* 32 3254–3263.

Xu C, Bailly-Maitre B and Reed JC (2005) Endoplasmic reticulum stress: Cell life and death decisions. *Journal of Clinical Investigation* 115 2656–2664.

Zhang GL, Dai DZ, Zhang C and Dai Y (2013) Apocynin and raisanberine alleviate intermittent hypoxia induced abnormal StAR and 3β-HSD and low testosterone by suppressing endoplasmic reticulum stress and activated p66Shc in rat testes. *Reproductive Toxicology*.

Zhao Y, Wang Z, Feng D, Zhao H, Lin M, Hu Y, Zhang N, Lv L, Gao Z, Zhai X *et al.* (2019) p66Shc Contributes to Liver Fibrosis through the Regulation of Mitochondrial Reactive Oxygen Species. *Theranostics* 9 1510–1522.

Chapter III

3 Gestational exposure to ∆9-tetrahydrocannabinol leads to postnatal catch-up growth and hepatic mitochondrial dysfunction in the adult rat

A version of this chapter has been previously published:

Oke, S.L., Lee, K., Papp, R., Laviolette, S.R., Hardy, D.B. (2021). In utero exposure to ∆9-tetrahydrocannabinol leads to postnatal catch-up growth and dysmetabolism in the adult rat liver. *International Journal of Molecular Sciences.* 22: 7502.

3.1 Introduction

Cannabis is the most commonly used recreational drug among individuals of reproductive age. In 2019, the Canadian Cannabis Survey found that approximately 21% of female adolescents and young adults self-reported daily or almost daily use of cannabis, while 40% reported monthly use ('Canadian Cannabis Survey 2019 - Summary - Canada.ca'). The American College of Obstetricians and Gynecologists has further found that 2–5% of individuals use cannabis during pregnancy, and this was increased to 15–28% among young women who lived in urban settings and whom were socioeconomically disadvantaged ('Marijuana use during pregnancy and lactation', 2017). Along with the recent legalization of recreational cannabis in Canada and select American states, the use of cannabis during pregnancy has become increasingly popular despite limited evidence of its safety. Many pregnant individuals use cannabis to diminish nausea, anxiety and depression, as they believe it to be a 'safe' and natural alternative to prescription medications (Westfall *et al.*, 2006; Brown *et al.*, 2019; Chang *et al.*, 2019). Alarmingly, gestational exposure to cannabis can increase the risk for adverse neonatal outcomes, including low birth weight and preterm delivery (English *et al.*, 1997; Conner *et al.*, 2016; Gunn *et al.*, 2016). However, these studies are confounded by that fact that cannabis users are more likely to concurrently abuse other drugs, such as alcohol and tobacco (Michalski *et al.*, 2020), highlighting the need for animal studies that focus on the effects of specific constituents of cannabis (*i.e.,* Δ9-tetrahydrocannabinol [Δ9-THC] and cannabidiol [CBD]) on short- and long-term maternal-fetal outcomes (Michalski *et al.*, 2020).

Cannabis is composed of several distinct compounds that stimulate the endocannabinoid system via the interaction with cannabinoid receptor type 1 (CB1R) and CB2R. These receptors are highly expressed throughout the central nervous system (CNS). Therefore, their activation by cannabinoids has a profound effect on mood, pain, memory and appetite (Silvestri and Di Marzo, 2013). At the same time, CB1R and CB2R are localized in peripheral metabolic tissues, suggesting that the endocannabinoid system has additional roles outside of neurocognitive function (Bouchard *et al.*, 2003; Sun and

Dey, 2012; Malenczyk *et al.*, 2015; Ramírez-López *et al.*, 2016). Many cannabinoids are also lipophilic and able to cross the placental barrier, thereby entering fetal circulation and allowing for activation of CB1R and CB2R (Maia *et al.*, 2019). While the activation of the endocannabinoid system is essential for the maintenance of pregnancy, the presence of exogenous cannabinoids can also directly interfere with fetal growth and development through the disruption of endocannabinoid signaling. Gestational exposure to Δ9-THC, the principal psychoactive component of cannabis, has been demonstrated to promote placental insufficiency and symmetrical intrauterine growth restriction (IUGR) in rodents, characterized by low birth weight and reduced neonatal organ weight (Harbison and Mantilla-Plata, 1972; Fried, 1976; Hurd *et al.*, 2005; Benevenuto *et al.*, 2017; Chang *et al.*, 2017; Natale *et al.*, 2020). Notably, the liver-to-body weight ratio is reduced in Δ9-THC offspring, followed by rapid catch-up growth in the first three weeks of life (Natale *et al.*, 2020). While the developmental origins of health and disease (DOHaD) postulates that there is a relationship between birth weight and metabolic health, the role of gestational Δ9-THC exposure on postnatal hepatic function has not yet been investigated.

The liver has a critical role in controlling lipid metabolism, which involves the synthesis and degradation of structural and functional lipid molecules. Naturally, impaired liver function leads to dyslipidemia, whereby triglycerides and cholesterol become elevated in the liver and plasma. Dyslipidemia is a well-characterized attribute of the metabolic syndrome that often accompanies obesity. Therefore, individuals with either of these conditions exhibit an increased risk for developing diabetes and cardiovascular disease (James *et al.*, 2004). Moreover, epidemiological studies have identified that low birth weight individuals with decreased liver-to-body weight ratio are more likely to be obese and have non-alcoholic fatty liver disease (NAFLD) during childhood and adult life (Ravelli *et al.*, 1976; Barker *et al.*, 1993; Eriksson *et al.*, 2001; Nobili *et al.*, 2007; Yang *et al.*, 2008; Faienza *et al.*, 2013; Suomela *et al.*, 2016). Similar trends have been found in studies of rodent IUGR offspring. We previously shown that maternal nicotine exposure leads to elevated hepatic and circulating triglycerides in adult male offspring, while protein-restricted adult males have increased hepatic and

circulating cholesterol (Sohi *et al.*, 2011; Ma *et al.*, 2013). Hepatic hyperlipidemia often occurs due to divergent synthesis and metabolism of free fatty acids, which can be of dietary, circulating or de novo origin (Jensen-Urstad and Semenkovich, 2012). In particular, de novo lipogenesis is a highly regulated, multi-step process that involves the esterification of fatty acids to triglycerides through the action of numerous enzymes. Initially, acetyl-coA carboxylase (ACCα) catalyzes the carboxylation of acetyl-coA to malonyl-coA, which acts as a substrate for fatty acid synthase (FAS) in generating saturated fatty acids (Kim, 1997; Jensen-Urstad and Semenkovich, 2012). Stearoyl-coA desaturase-1 (SCD-1) then converts these saturated fatty acids into monounsaturated fatty acids (MUFAs), which undergo various elongation reactions to generate the long chain fatty acids that feed into triglyceride synthesis pathways (Miyazaki and Ntambi, 2003). The cytosolic transport of these long chain fatty acids is mediated by various fatty acid binding proteins (FABPs), which are highly expressed in tissues that control lipid metabolism. Importantly, the diacylglycerol acyltransferase (DGAT) enzyme catalyzes the terminal step of triglyceride synthesis (Cases *et al.*, 1998; Smith *et al.*, 2000). Studies of the growth-restricted liver demonstrate that many of these enzymes are particularly sensitive to developmental reprogramming, as their abundance and activity levels change with exposure to a variety of gestational insults leading to long-term dyslipidemia (Yamada *et al.*, 2011; Ma *et al.*, 2013; Deodati *et al.*, 2018; Cheng *et al.*, 2020). That said, the underlying mechanisms linking a poor in utero environment to these metabolic deficits remain elusive.

Oxidative stress and mitochondrial dysfunction occur with many metabolic pathologies, including hepatic hyperlipidemia. Many studies have shown that growth-restricted offspring exhibit oxidative stress and impaired mitochondrial metabolism in the liver (Park *et al.*, 2003; Wang *et al.*, 2008; Theys *et al.*, 2009; Moraes *et al.*, 2014; Devarajan *et al.*, 2019; Oke *et al.*, 2019). Therefore, it is possible that mitochondrial dysfunction precedes dyslipidemia in IUGR offspring. This may be mediated, in part, by the adaptor protein p66Shc, which is known to accelerate the mitochondrial production of ROS (Giorgio *et al.*, 2005). Human and animal studies indicate that the p66Shc-induced oxidative signal is involved in the accumulation of intracellular lipids, leading to obesity

and NAFLD (Berniakovich *et al.*, 2008; Tomita *et al.*, 2012). Not surprisingly, growth-restricted offspring display increased levels of p66Shc in the liver, kidney, and pancreas at birth and in postnatal life (Luyckx *et al.*, 2009; Oke *et al.*, 2019; Raez-Villanueva *et al.*, 2022). Furthermore, using a rodent model of maternal protein restriction, we have also shown that adult male offspring have increased hepatic protein abundance of p66Shc following postnatal catch-up growth (Oke *et al.*, 2019). These same offspring also have aberrant expression of various microRNAs (miRs) in the liver, so it is possible that the expression of genes involved in de novo lipogenesis and mitochondrial function may be regulated via epigenetic mechanisms (Sohi *et al.*, 2015). Based on the fact that gestational exposure to Δ9-THC leads to compromised liver growth, the present study investigates the effects of Δ9-THC on hepatic lipid metabolism in the exposed offspring. Given the role of mitochondria in metabolic disease and the growth-restricted liver, we hypothesized that Δ9-THC-exposed offspring would exhibit oxidative stress and mitochondrial dysfunction, along with aberrant expression of miRs that are known to result in dysmetabolism.

3.2 Materials and Methods

3.2.1 Animals and Experimental Handling

All procedures were performed according to guidelines set by the Canadian Council of Animal Care, and the animal use protocol was approved by the Animal Care Committee at The University of Western Ontario (AUP #2019-126, January 2019). All investigators understood and followed the ethical principles outlined by Grundy (Grundy, 2015), and the study design was informed by ARRIVE guidelines (Kilkenny *et al.*, 2010). Pregnant female Wistar rats were purchased from Charles River (La Salle, St. Constant, QC, Canada). Dams arrived at the animal care facility at gestational day 3 (GD3) and were left to acclimatize to environmental conditions for three days. All animals were maintained at 22 °C on a 12:12 h light-dark cycle in the animal care facility, while food and water were provided ad libitum for the entire duration of the experimental protocol.

Dams were randomly assigned to receive daily intraperitoneal (*i.p.*) injection of either vehicular control (1:18 cremophor:saline) or 3 mg/kg Δ9-THC (Sigma-Aldrich, St. Louis, MO, USA) from E6.5 to E22 (n = 14, where litter is the statistical unit) as previously performed (Natale *et al.*, 2020). This dose of Δ9-THC was selected as it results in rodent plasma concentrations (8.6–12.4 ng/mL) that are reflective of those found in human recreational cannabis smokers (using 6% Δ9-THC) 0–22h post-inhalation (13–63 ng/mL), as well as in the aborted fetal tissues of pregnant cannabis users (4–287 ng/mL; Klein *et al.*, 2011; Schwope *et al.*, 2011; Falcon *et al.*, 2012). This dose and method of delivery has also been demonstrated to have no impact on maternal or litter outcomes in rats (Mato *et al.*, 2004; Tortoriello *et al.*, 2014; Natale *et al.*, 2020). An oral route of administration was not chosen as to avoid the poor bioavailability and slowed adsorption of Δ9-THC when ingested with food, and as edibles are the least popular route of administration of cannabis among pregnant women (Dinieri and Hurd, 2012; Chang *et al.*, 2019). Injections were initiated at E6.5 because Δ9-THC can interfere with implantation of the blastocyst and induce spontaneous abortion (Dinieri and Hurd, 2012).

Maternal food intake and body weight were monitored daily throughout the entire gestational period. Dams were allowed to deliver normally, and all pups were weighed at birth. Litters were randomly culled to eight offspring per litter to ensure uniformity of litter size between treatment groups. As previously reported, we and others have found the selected dose of Δ9-THC has no impact on maternal or litter outcomes (Mato *et al.*, 2004; Tortoriello *et al.*, 2014; Chang *et al.*, 2017; Natale *et al.*, 2020). Liver weights of culled offspring were recorded and compared to body weight as a measure of fetal growth restriction and postnatal catch-up growth (Natale *et al.*, 2020). The food intake of these offspring was monitored by measuring their daily food consumption from PND50-60, as previously published (Sohi *et al.*, 2015). The remaining offspring were fasted for 24 h before being euthanized via i.p. injection of 100 mg/kg pentobarbital at either postnatal day (PND) 21 or six months of age (n = 8 males/8 females per group), followed by necropsy to examine the effects of Δ9-THC on metabolic and molecular outcomes. The right medial hepatic lobe was collected and immediately flash-frozen in liquid nitrogen, followed by storage at −80 °C until further use. Visceral fat was also weighed and

compared to body weight at six months as a measure of obesity. Blood was also collected, centrifuged, and stored at −80 °C.

3.2.2 Hepatic Triglyceride Measurements

Circulating and hepatic triglyceride and cholesterol measurements were detected using the Cobas® Mira S analyzer as previously published (Ma *et al.*, 2013). For triglyceride measurements, triglycerides were hydrolyzed by lipoprotein lipase to glycerol and fatty acids. Glycerol was then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate was catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 alters the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red-colored quinoneimine dye, which was measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample. For cholesterol measurements, cholesterol esterase cleaved cholesterol esters, which then were converted to choleste-4-en-3-one and H_2O_2 by cholesterol oxidase. Cholesterol levels were quantified using a colorimetric assay that measured the breakdown of H_2O_2 via the Trinder reaction as previously described (Sohi *et al.*, 2011).

3.2.3 RNA Isolation and Quantitative Real-Time PCR Analysis

MicroRNAs were isolated from frozen liver samples using a miRNeasy kit (QIAGEN Inc., Toronto, ON, Canada), followed by spectrophotometric analysis with a Nanodrop 2000. 0.5 µg of each miRNA sample was then reverse transcribed into cDNA using a miScript II RT kit (QIAGEN Canada) and stored at −20 °C. Forward sequence primers for miR-203a-3p, miR-29a, miR-29b, and miR-29c were purchased (QIAGEN Canada), while a universal reverse sequence primer for miRNAs was used as part of a miScript SYBR green PCR kit (QIAGEN Canada). MiRNA transcripts were amplified via quantitative real-time PCR (qRT-PCR) using the Bio-Rad CFX384 Real Time System, using cycling conditions as previously published (Sohi *et al.*, 2015). Cq values obtained for miRs of interest were normalized to that of a miRNA standard control

(Ctrl_miRTC_1; QIAGEN Canada), and relative transcript abundance was calculated for each target using the comparative ΔCt method.

3.2.4 Protein Extraction and Western Immunoblot

Whole-cell protein lysates were isolated using the protocol, previously described by Barra et al. (Barra et al., 2017) Prior to western immunoblotting, loading samples were prepared using sample lysates, NuPAGE reducing agent (10×; Invitrogen, Waltham, MA, USA), NuPAGE LDS sample buffer (4X; Invitrogen), and deionized water. Loading samples were heated at 70 °C to denature proteins, while a separate set of loading mixes were kept unheated for mitochondrial OXPHOS immunoblots. Samples were loaded into wells of 4–12% Bis-Tris gels (Invitrogen) at 20–30 μg per well (n = 8/group), followed by separation via gel electrophoresis. Proteins were transferred onto PVDF membranes (Thermo Scientific, Waltham, MA, USA) at 75 V for two hours, followed by one hour of blocking in 1X Tris-buffered saline/Tween-20 (TBST) buffer with either 5% non-fat milk (Carnation) or 3% bovine serum albumin (BSA; Fisher Scientific, Ottawa, ON, Canada). Membranes were probed with primary antibodies overnight, followed by probing with secondary antibodies for one hour (Table 3-1). To avoid the excessive use of PVDF membranes, multiple targets of interest were detected on each membrane by cutting the membrane according to molecular weight and probing with the appropriate primary antibodies in separate tubes. In the case that a membrane was reused, the primary antibody was removed with stripping buffer (ThermoFisher Scientific, Waltham, MA, USA), followed by incubation with secondary antibody and re-imaging to ensure that the initial antibody was removed. Immunoreactive bands were visualized using BioRad Clarity Max Western ECL Substrate solution (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada) and imaged using a BioRad ChemiDoc XRS+ Imaging System. Resulting bands were analyzed using BioRad Image Lab™ Software. Band intensities of target proteins were normalized to β-Actin, as previously published (Oke et al., 2019), with the exception of 4-hydroxynonenol which was normalized to total protein content detected with Ponceau staining (Hayes et al., 2012; Oke et al., 2019). Signals for

Table 3-1. Western blot primary and secondary antibodies, dilutions and company/catalogue information.

Antibody Name	Source	Dilution	Company (Catalogue No.)
ACCα (H-76)	Rabbit polyclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-30212)
FAS (C20G5)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3180)
SCD (H300)	Rabbit polyclonal	1:250	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-30081)
FABP1 (D2A3X)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#13368)
DGAT1	Rabbit polyclonal	1:1000	Novus Biologicals, Centennial, CO, USA (NB110-41487)
DGAT2 (4C1)	Mouse monoclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-293211)
SHC	Mouse monoclonal	1:1000	BD BioSciences, San Jose, CA, USA (610879)
TFAM (D5C8)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (8076)
pSer(232) pyruvate dehydrogenase	Rabbit polyclonal	1:1000	EMD Millipore, Etobicoke, ON, Canada (AP1063)
Pyruvate dehydrogenase	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (2784)
LDHa	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (2012)

Citrate Synthase	Rabbit polyclonal	1:1000	Provided by Dr. S. Raha, McMaster University
Catalase (H-300)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-50508)
Superoxide dismutase (SOD)-1 (FL-154)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-11407)
Superoxide dismutase (SOD)-2 (FL-222)	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-30080)
4-hydroxynonenal	Mouse monoclonal	1:1000	R&D Systems, Oakville, ON, Canada (MAB3249)
OXPHOS rodent cocktail	Mouse monoclonal	1:1000	Abcam Inc., Toronto, ON, Canada (ab110413)
β-Actin Peroxidase	Mouse monoclonal	1:25000	Sigma Aldrich Co., St. Louis, MO, USA (A3854-200UL)
Goat anti-rabbit IgG HRP-linked (H+L chain)	N/A	1:10000	Cell Signaling Technology Inc., Danvers, MA, USA (7074P2)
Horse anti-mouse IgG HRP-linked (H+L chain)	N/A	1:10000	Cell Signaling Technology Inc., Danvers, MA, USA (7076S)

proteins that were detected on the same membrane were normalized to that of β-Actin from the same membrane.

3.2.5 Statistical Analyses

At each timepoint, the selected offspring were taken from separate litters to avoid litter bias (*i.e.,* n = 1 represents pups from a single dam), where a sample size of n = 7–8 was used for each sex per group. This sample size was chosen based on our previous studies to achieve a statistically significant difference with an expected standard deviation of 15% or less (Beamish *et al.*, 2017; Gillies *et al.*, 2020). All statistical analyses were performed using GraphPad Prism 9 software. The results were expressed as means of normalized values ± SEM, and the threshold for significance was set as $p < 0.05$. Organ and body weight data were analyzed using Student's two-tailed unpaired *t*-test when examining both sexes together (*e.g.,* PND1), while sex-specific effects were analyzed by two-way ANOVA followed by a Holm-Sidak-corrected multiple comparisons test. All immunoblot data were analyzed using Student's two-tailed unpaired t-test. Triglyceride and cholesterol measurements obtained from lipid analyses were analyzed by two-way ANOVA, followed by Holm-Sidak-corrected multiple comparisons test between Δ9-THC offspring and their sex-matched controls. Grubbs' test was utilized to detect any statistical outliers.

3.3 Results

3.3.1 Gestational exposure to Δ9-THC leads to hepatic catch-up growth by three weeks of age

At birth (i.e., PND 1), Δ9-THC-exposed offspring exhibited decreased liver to body weight ratio compared to control offspring (Table 3-2; $p < 0.05$). It should be noted that in this same cohort of vehicle and Δ9-THC offspring, we have published that Δ9-THC exposure during pregnancy did not lead to changes in maternal food intake,

Table 3-2. Gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) leads to hepatic catch-up growth by three weeks and increased visceral adiposity in adult life.

	Treatment Group	Sex	Birth	Three Weeks	Six Months
Liver to Body Weight Ratio	Control	Male		$0.0458^{a} \pm 0.0026$	$0.0319^{a} \pm 0.0024$
		Female		$0.0445^{a} \pm 0.0017$	$0.0354^{a} \pm 0.0034$
		Both sexes	$0.0392^{\#} \pm 0.0039$	$0.0451^{\#} \pm 0.0011$	$0.0333^{\#} \pm 0.0020$
	Δ9-THC	Male		$0.0459^{a} \pm 0.0010$	$0.0350^{a} \pm 0.0017$
		Female		$0.0437^{a} \pm 0.0009$	$0.0351^{a} \pm 0.0022$
		Both sexes	$0.0293^{\blacklozenge} \pm 0.0018$	$0.0445^{\#} \pm 0.0020$	$0.0351^{\#} \pm 0.0014$
Adipose to Body Weight Ratio	Control	Male			$0.01685^{a} \pm 0.0015$
		Female			$0.0202^{a} \pm 0.0025$
		Both sexes			$0.01828^{\#} \pm 0.0014$
	Δ9-THC	Male			$0.0207^{a} \pm 0.0020$
		Female			$0.0234^{a} \pm 0.0022$
		Both sexes			$0.0223^{\blacklozenge} \pm 0.0015$

Liver growth and visceral adipose deposition were assessed for all offspring by calculating liver to body weight ratio and adipose to body weight ratio. All data are expressed as means ± SEM (n = 7–18/sex/group). The effects of Δ9-THC on liver to body weight ratio and visceral adipose to body weight ratio were determined via Student's two-tailed unpaired t-test. Sex-specific differences in liver to body weight ratio were assessed using a two-way ANOVA followed by a Holm-Sidak-corrected multiple comparisons test. Groups labelled with different letters or symbols are significantly different from each other.

maternal weight gain, litter size or gestational length (Natale et al., 2020). By three weeks of age, these offspring had exhibited hepatic catch-up growth, as there were no significant differences between liver to-body-weight ratios of Δ9-THC-exposed offspring and control offspring (Table 3-2). This is consistent with other rodent models of IUGR, including maternal nicotine exposure and maternal protein restriction, whereby growth-restricted offspring also undergo catch-up growth by three weeks (Sohi et al., 2011; Barra et al., 2017). At three weeks of age, there were also no differences in liver-to-body weight ratios between male and female offspring of either treatment group (Table 3-2), indicating that there were no sex-specific effects of gestational Δ9-THC with respect to hepatic catch-up growth. At six months of age, liver to body weight ratio remained equal between both groups and sexes (Table 3-2). It should also be noted that gestational exposure to Δ9-THC did not significantly affect postnatal food intake (PND50–60) in either males (vehicle, 19.3 ± 1.5 g food/day/offspring; Δ9-THC, 15.1 ± 2.1 g food/day/offspring) or females (vehicle, 17.7 ± 1.6 g food/day/offspring; Δ9-THC, 20.0 ± 1.9 g food/day/offspring.

3.3.2 Adult Δ9-THC-exposed offspring exhibit elevated visceral adiposity and hepatic hyperlipidemia

As mentioned previously, adult IUGR offspring are vulnerable to the development of obesity and hepatic pathologies involved in lipid storage and metabolism. Given that Δ9-THC induces symmetrical IUGR (Natale *et al.*, 2020), we were interested in comparing the levels of visceral adipose tissue and hepatic lipids between control and Δ9-THC-exposed offspring during adult life. At six months, offspring exposed to gestational Δ9-THC exhibited increased visceral adipose to body weight ratio (Table 3-2; $p < 0.05$), suggesting dyslipidemia in these animals. That said, this was true only when examining both sexes together, as there were differences in visceral adipose to body weight ratio when examining each sex individually. To explore this further, we measured hepatic and circulating triglyceride and cholesterol levels of each sex for both groups at six months of age. While both hepatic and circulating triglyceride and cholesterol levels of Δ9-THC-

exposed female offspring remained unchanged at six months (Figure 3-1A–D), hepatic triglycerides were elevated in Δ9-THC-exposed males in comparison to control males and all females (Figure 3-1A; $p < 0.05$). Interestingly, hepatic cholesterol was unaltered in six-month old male offspring exposed to gestational Δ9-THC, as were circulating triglycerides and cholesterol (Figure 3-1B–D). Since the differences in lipid content were observed exclusively in livers of male offspring, we further quantified hepatic triglycerides and cholesterol in male offspring at an earlier timepoint. At three weeks of age, hepatic triglycerides and cholesterol were not significantly altered between control and Δ9-THC-exposed male offspring (Figure 3-1E,F).

3.3.3 Elevated hepatic triglyceride levels coincide with increased diglycerol acyltransferase and p66Shc protein levels in the livers of adult male Δ9-THC-exposed offspring

To gain insight on possible mechanisms involved in the elevation of hepatic triglycerides among adult male Δ9-THC-exposed offspring, we performed western immunoblotting for enzymes involved in the triglyceride synthesis pathway. Δ9-THC-exposed males exhibited increased DGAT1 (Figure 3-2F; $p < 0.05$) and DGAT2 (Figure 3-2G; $p < 0.05$) in the liver at six months of age, while there were no significant differences in hepatic ACCα, FAS, SCD, or FABP1 (Figure 3-2B–E). Adult female Δ9-THC-exposed offspring also demonstrated a significant increase in DGAT2 (Figure 3-2N; $p < 0.05$) relative to female controls, while all other enzymes involved in triglyceride synthesis were unchanged (Figure 3-2I–M). As p66Shc is linked to the accumulation of intracellular lipids (Berniakovich *et al.*, 2008), we further quantified the relative abundance of p66Shc protein in control and Δ9-THC-exposed offspring. Adult male offspring exposed to gestational Δ9-THC demonstrated increased hepatic p66Shc protein abundance at 6 months compared to control male offspring (Figure 3-2H; $p < 0.05$), while p66Shc was unchanged in adult

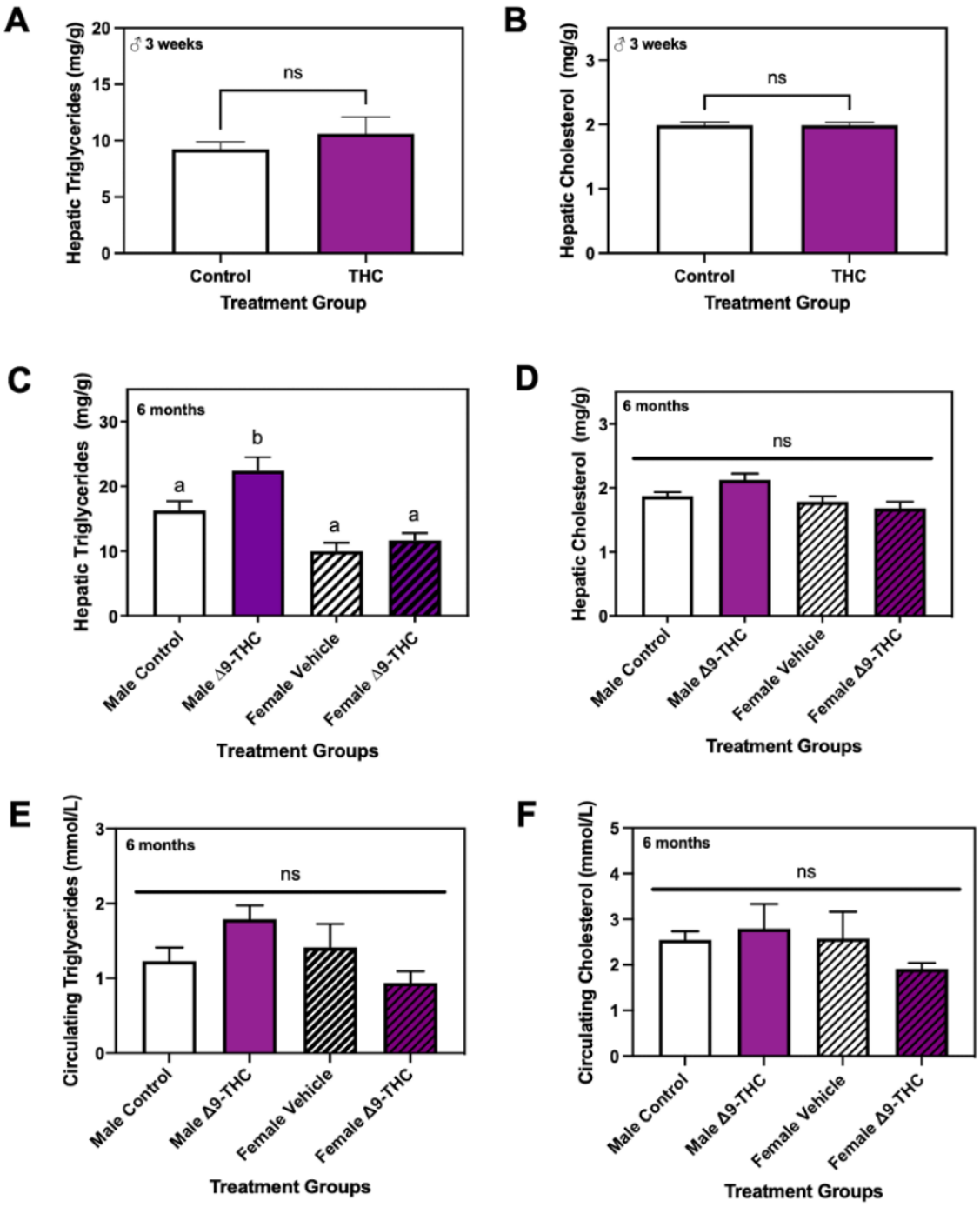
A
♂ 3 weeks
ns
Hepatic Triglycerides (mg/g)
20
15
10
5
0
Control
THC
Treatment Group
B
♂ 3 weeks
ns
Hepatic Cholesterol (mg/g)
3
2
1
0
Control
THC
Treatment Group
C
6 months
a
b
a
a
Hepatic Triglycerides (mg/g)
30
20
10
0
Male Control
Male Δ9-THC
Female Vehicle
Female Δ9-THC
Treatment Groups
D
6 months
ns
Hepatic Cholesterol (mg/g)
3
2
1
0
Male Control
Male Δ9-THC
Female Vehicle
Female Δ9-THC
Treatment Groups
E
6 months
ns
Circulating Triglycerides (mmol/L)
3
2
1
0
Male Control
Male Δ9-THC
Female Vehicle
Female Δ9-THC
Treatment Groups
F
6 months
ns
Circulating Cholesterol (mmol/L)
5
4
3
2
1
0
Male Control
Male Δ9-THC
Female Vehicle
Female Δ9-THC
Treatment Groups

Figure 3-1. The effects of gestational Δ9-tetrahydrocannabinol (Δ9-THC) on triglyceride and cholesterol levels in the liver and plasma of exposed offspring at three weeks and six months of age.

At six months of age, hepatic triglycerides (**A**) and cholesterol (**B**) were assessed in both male and female offspring (mg of lipid/g of tissue), along with circulating levels of triglycerides (**C**) and cholesterol (**D**; mmol/L). Hepatic triglycerides (**E**) and cholesterol (**F**) were also quantified in three-week old male offspring. Data are expressed as the mean ± SEM. The effects of Δ9-THC in three-week old male offspring were determined via Student's two-tailed unpaired *t*-test, while six-month old offspring results were analyzed using a two-way ANOVA followed by a Holm-Sidak-corrected multiple comparisons test. Groups labelled with different letters are significantly different from each other ($p < 0.05$), while groups labelled 'ns' were non-significant from each other.

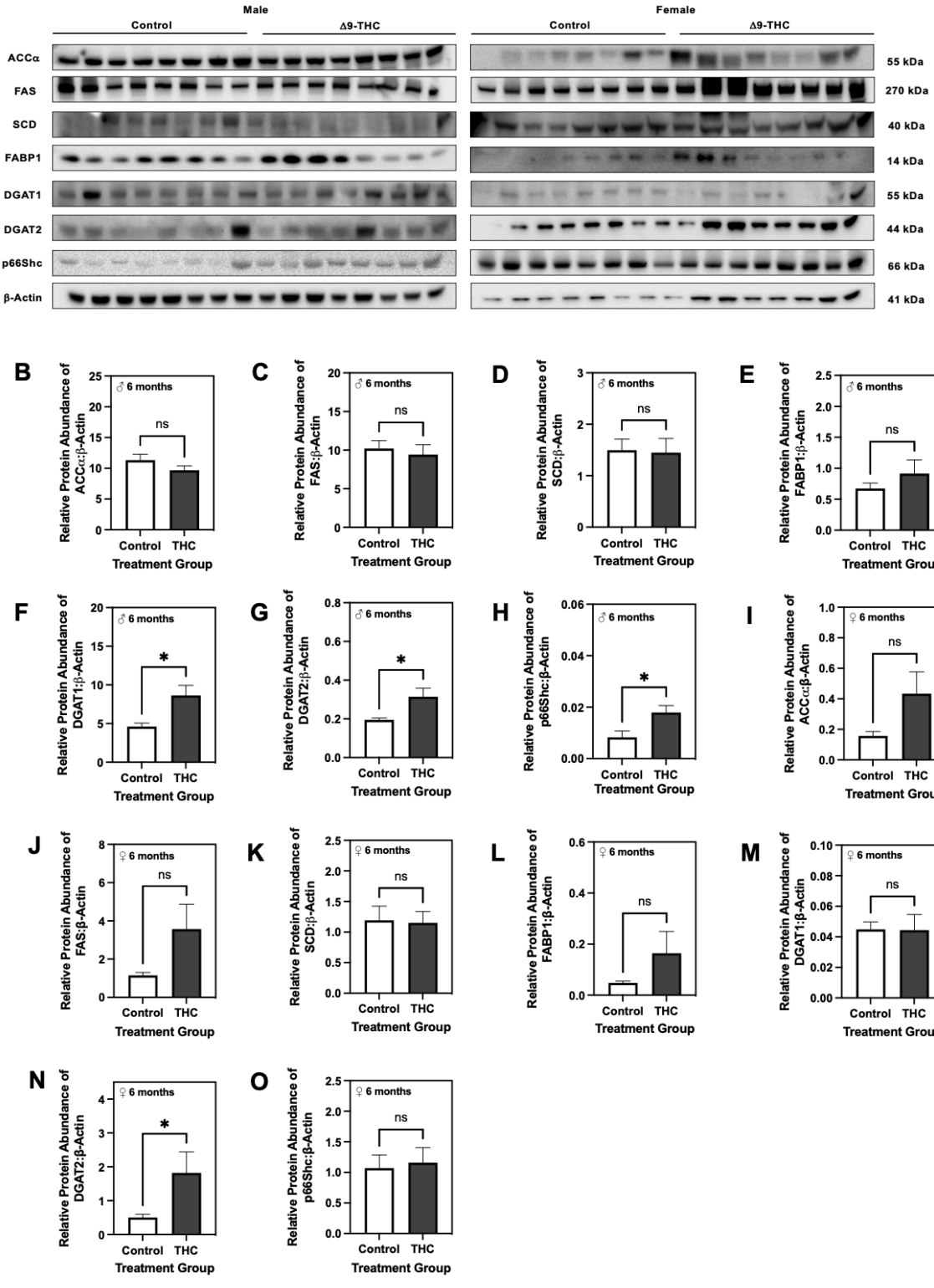
A
Male
Female
Control
Δ9-THC
ACCα
FAS
SCD
FABP1
DGAT1
DGAT2
p66Shc
β-Actin
55 kDa
270 kDa
40 kDa
14 kDa
55 kDa
44 kDa
66 kDa
41 kDa
B
C
D
E
F
G
H
I
J
K
L
M
N
O
♂ 6 months
♀ 6 months
ns
*
Relative Protein Abundance of ACCα:β-Actin
Relative Protein Abundance of FAS:β-Actin
Relative Protein Abundance of SCD:β-Actin
Relative Protein Abundance of FABP1:β-Actin
Relative Protein Abundance of DGAT1:β-Actin
Relative Protein Abundance of DGAT2:β-Actin
Relative Protein Abundance of p66Shc:β-Actin
Relative Protein Abundance of ACCα:β-Actin
Relative Protein Abundance of FAS:β-Actin
Relative Protein Abundance of SCD:β-Actin
Relative Protein Abundance of FABP1:β-Actin
Relative Protein Abundance of DGAT1:β-Actin
Relative Protein Abundance of DGAT2:β-Actin
Relative Protein Abundance of p66Shc:β-Actin
Control
THC
Treatment Group

Figure 3-2. Gestational exposure to 9-tetrahydrocannabinol (Δ9-THC) leads to upregulation of lipogenic enzymes in male offspring at six months of age.

(**A**) Representative western immunoblots illustrating hepatic expression of acetyl-coA carboxylase (ACCα), fatty acid synthase (FAS), stearoyl-coA desaturase (SCD), fatty acid binding protein 1 (FABP1), diacylglycerol acyltransferase (DGAT) 1, DGAT2, and p66Shc in male and female offspring at six months of age. Protein abundances of each enzyme for male offspring (**B**–**H**) and female offspring (**I**–**O**) were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. * Significant difference ($p < 0.05$).

female offspring (Figure 3-2O). Again, because changes were observed in hepatic lipid content exclusively in adult male offspring, we further examined the expression of these enzymes in males at three weeks of age. At three weeks, Δ9-THC-exposed male offspring had increased protein abundance of ACCα, SCD, FABP1, and DGAT2 (Figure 3-3B, D, E, and F; $P < 0.05$). However, protein levels of FAS, DGAT1, and p66Shc were unchanged in male offspring at three weeks of age (Figure 3-3B–D,F,H).

3.3.4 Gestational exposure to Δ9-THC does not affect protein levels of enzymes involved in aerobic metabolism at six months of age

Given that male offspring exhibit altered lipid metabolism and increased p66Shc, which are both implicated in mitochondrial dysfunction (Ipsen *et al.*, 2018; Lone *et al.*, 2018), we then assessed protein levels of enzymes involved in aerobic metabolism. At six months, the abundance of mitochondrial transcription factor A (TFAM), a protein that is essential in activating mitochondrial genome transcription, was unaltered in both male and female offspring exposed to gestational Δ9-THC (Figure 3-4B,F). Furthermore, male offspring exhibited no changes in the ratio of phosphorylated pyruvate dehydrogenase at serine residue 232 (p-PDH[Ser232]) to the total PDH, lactate dehydrogenase subunit A (LDHa), or citrate synthase (Figure 3-4C–E). Interestingly, six-month female offspring exposed to gestational Δ9-THC displayed a decrease in the ratio of p-PDH[Ser232] to total PDH (Figure 3-4G), while protein levels of LDHa and citrate synthase were unchanged (Figure 3-4H,I). However, at three weeks of age, male Δ9-THC offspring exhibited increased levels of TFAM (Figure 3-5B; $p < 0.01$) and LDHa (Figure 3-5D; $p < 0.01$), while the ratio of p-PDH[Ser232] to total PDH and levels of citrate synthase were unchanged (Figure 3-5C,E).

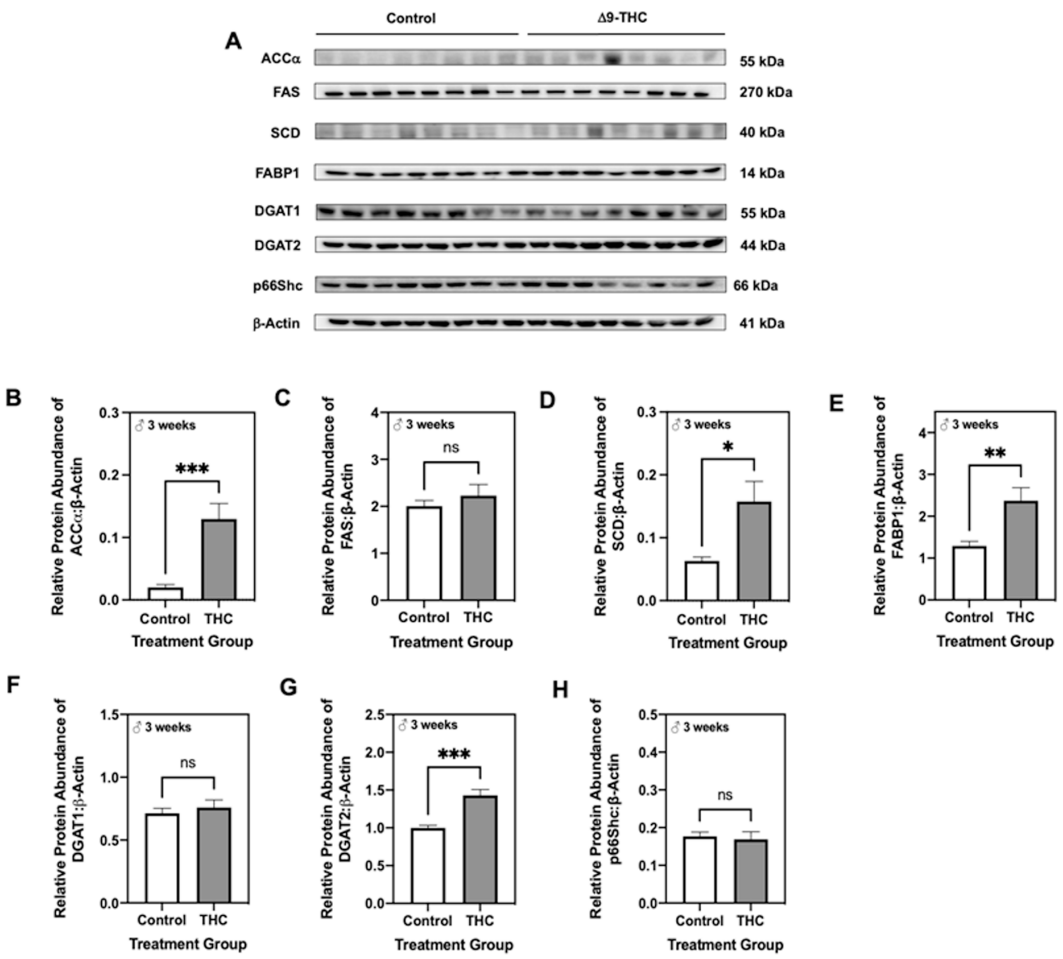

Figure 3-3. Three-week old male offspring exhibit increased lipogenic enzyme expression in the liver following gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC).

(**A**) Representative western immunoblots illustrating hepatic expression of acetyl-coA carboxylase (ACCα), fatty acid synthase (FAS), stearoyl-coA desaturase (SCD), fatty acid binding protein 1 (FABP1), diacylglycerol acyltransferase (DGAT) 1, DGAT2, and p66Shc in male offspring at three weeks of age. Protein abundances of (**B**) ACCα, (**C**) FAS, (**D**) SCD, (**E**) FABP1, (**F**) DGAT1, (**G**) DGAT2 and (**H**) p66Shc were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. * Significant difference ($p < 0.05$), ** significant difference ($p < 0.01$), *** significant difference ($p < 0.001$).

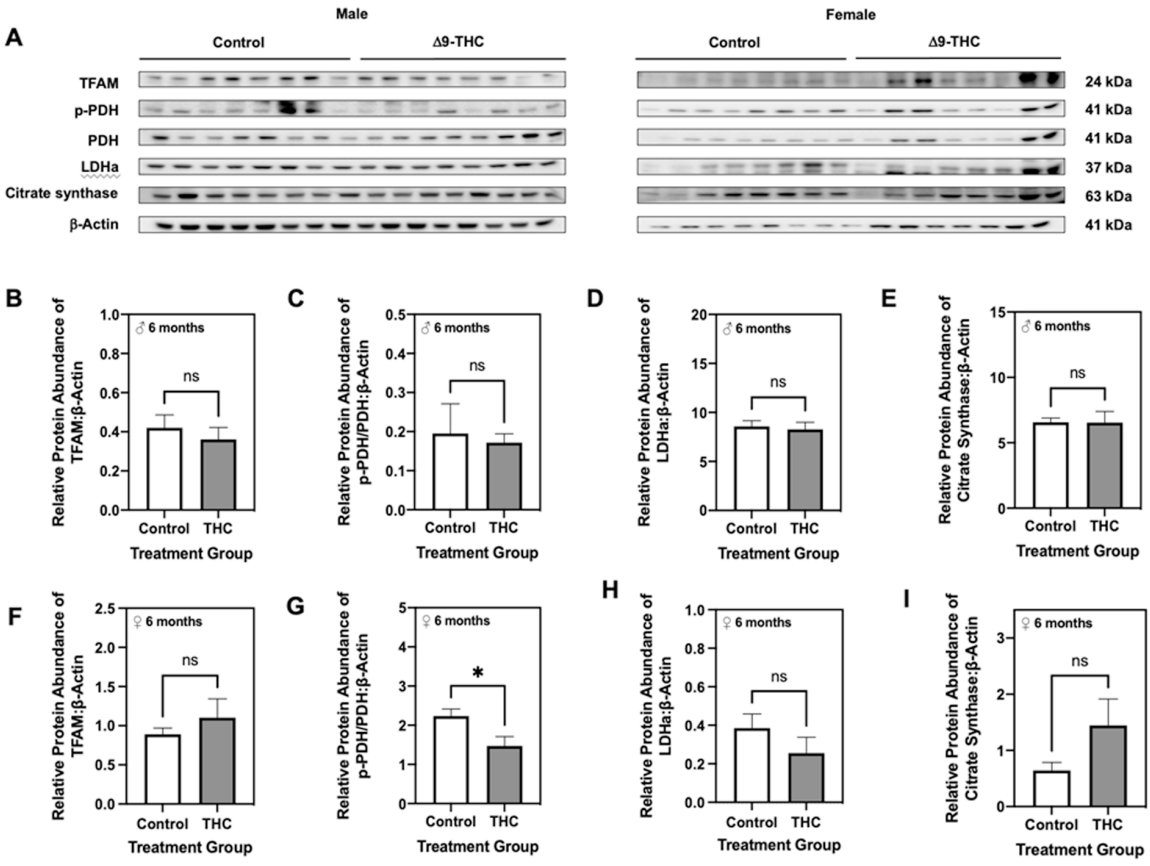

Figure 3-4. Gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) does not impair aerobic metabolism in male or female offspring at six months of age.

(**A**) Representative western immunoblots illustrating hepatic expression of mitochondrial transcription factor A (TFAM), phosphorylated pyruvate dehydrogenase (PDH) to total PDH, lactate dehydrogenase subunit A (LDHa), and citrate synthase in male and female offspring at six months of age. Protein abundances of each enzyme for male offspring (**B**–**E**) and female offspring (**F**–**I**) were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. * Significant difference ($p < 0.05$).

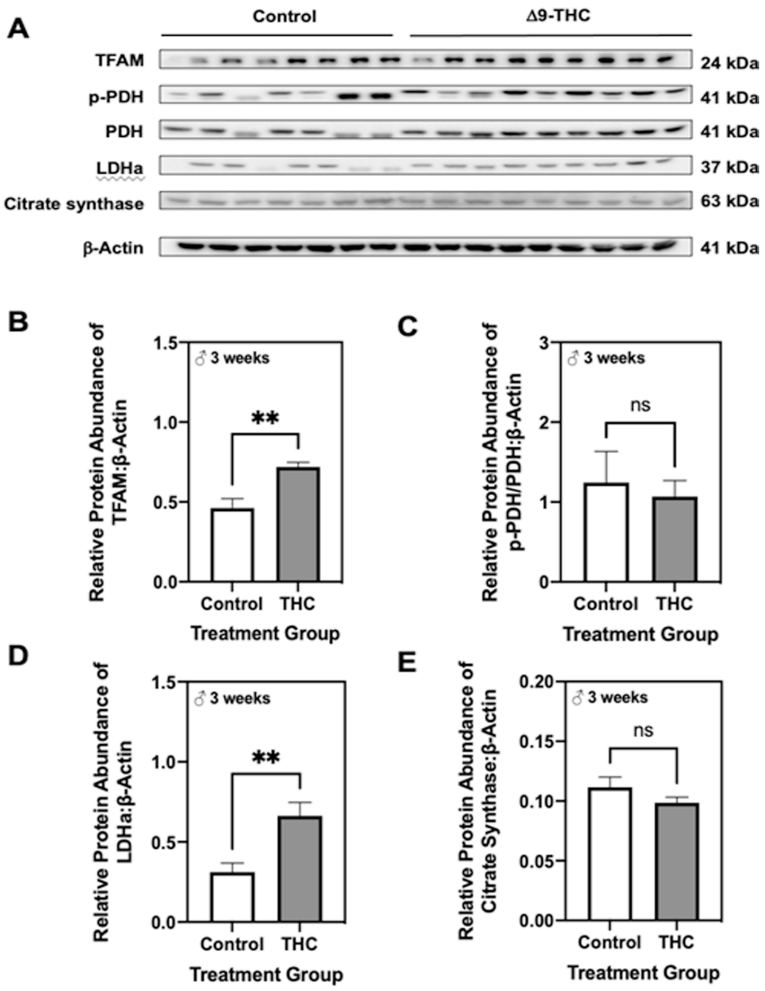

Figure 3-5. Three-week old male offspring exposed to Δ9-tetrahydrocannabinol (Δ9-THC) in utero have increased mitochondrial transcription factor A (TFAM) and lactate dehydrogenase subunit A (LDHa).

(**A**) Representative western immunoblots illustrating hepatic expression of mitochondrial transcription factor A (TFAM), phosphorylated pyruvate dehydrogenase (PDH) to total PDH, lactate dehydrogenase subunit A (LDHa), and citrate synthase in male and female offspring at six months of age. Protein abundances of (B) TFAM, (**C**) p-PDH[Ser232]/PDH, (**D**) LDHa and (**E**) citrate synthase were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. ** Significant difference ($p < 0.01$).

3.3.5 Gestational exposure to Δ9-THC leads to altered hepatic protein levels of superoxide dismutase 1 in adult male offspring

Given that the increased p66Shc is also associated with oxidative stress(Giorgio *et al.*, 2005), we further analyzed the abundance of antioxidant enzymes that are critical in combatting the damaging effects of ROS. Male offspring demonstrated decreased protein levels of SOD1 at six months of age (Figure 3-6C; $p < 0.05$), while catalase and SOD2 remained unchanged at this time point (Figure 3-6B,D). Lipid peroxidation was also unaffected at six months of age in male offspring exposed to Δ9-THC, as indicated by levels of 4-hydroxynonenol (4HNE; Figure 3-6E). At three weeks, male Δ9-THC-exposed offspring did not exhibit any changes in catalase, superoxide dismutase (SOD) 1 or SOD2 protein levels (Figure 3-6F–H). However, hepatic lipid peroxidation was increased as indicated by elevated levels of 4HNE (Figure 3-6I; $p < 0.05$).

3.3.6 Mitochondrial electron transport chain complexes are increased in male offspring at six months and three weeks of age following gestational exposure to Δ9-THC

The mitochondrial electron transport chain is known to have significant contribution to the production of ROS, particularly through the activity of complexes I and III (Brand, 2010). Given we observed changes in antioxidant and mitochondrial proteins in Δ9-THC offspring, we next quantified protein levels of each subunit of the electron transport chain. At six months, male offspring exposed to gestational Δ9-THC exhibited increased abundance of complexes I, III, and V (Figure 3-7B,D,F; $p < 0.05$), while levels of complexes II and IV were unchanged (Figure 3-7C,E). While adult female offspring demonstrated an increase in the protein abundance of complex I (Figure 3-7G; $p < 0.01$), the expression of all other subunits remained unchanged (Figure 3-7H–K). At three weeks, male Δ9-THC-exposed offspring also demonstrated increased levels of

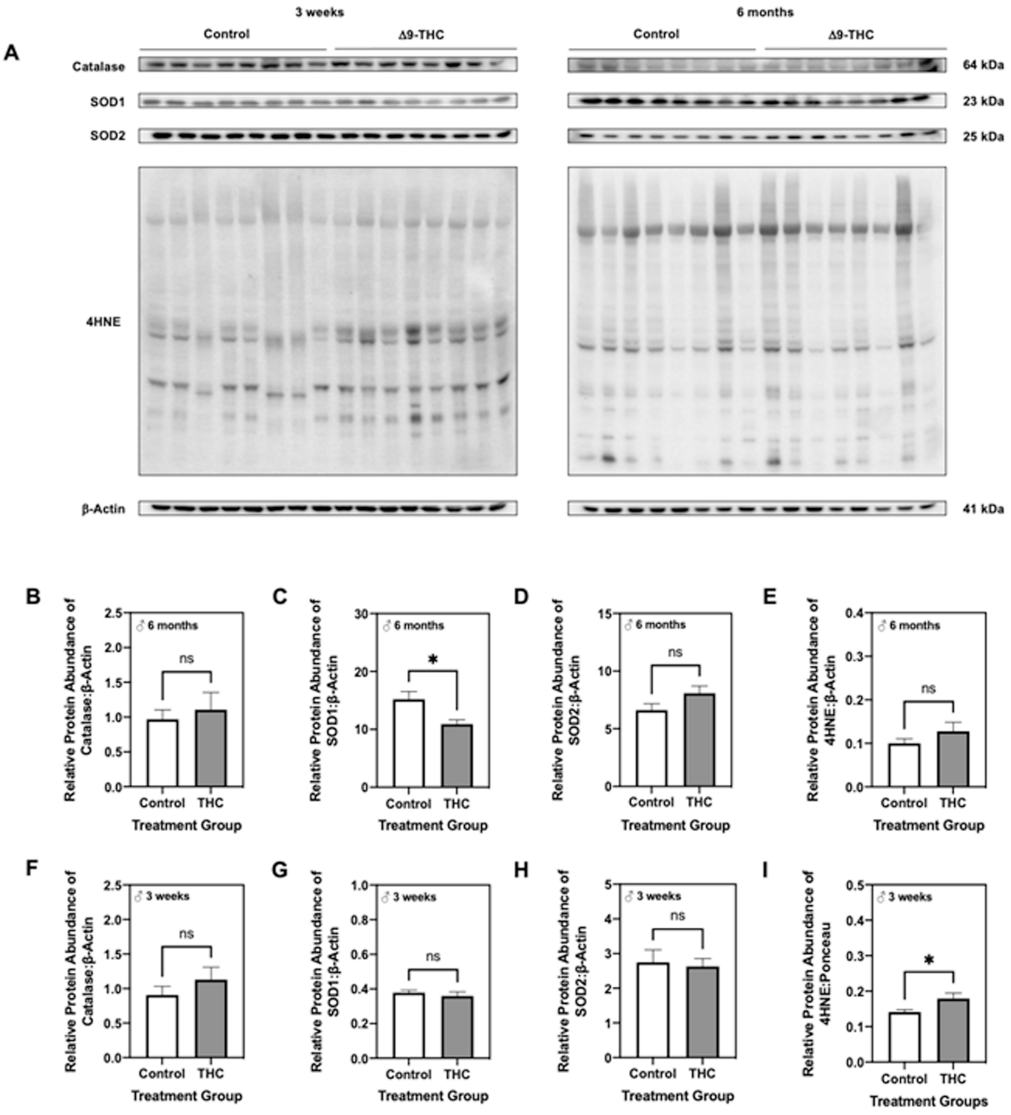

Figure 3-6. Gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) results in hepatic oxidative stress within male offspring at three weeks and six months of age.

(**A**) Representative western immunoblots illustrating hepatic expression of catalase, superoxide dismutase (SOD) 1, SOD2 and 4-hydroxynonenol (4HNE) in male offspring at three weeks and six months. Protein abundance of catalase (**B**,**F**), SOD1 (**C**,**G**), and SOD2 (**D**,**H**) were normalized to β-Actin ± SEM (n = 7–8/group), while 4HNE abundance (**E**,**I**) was expressed as means normalized to total protein abundance ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. * Significant difference ($p < 0.05$).

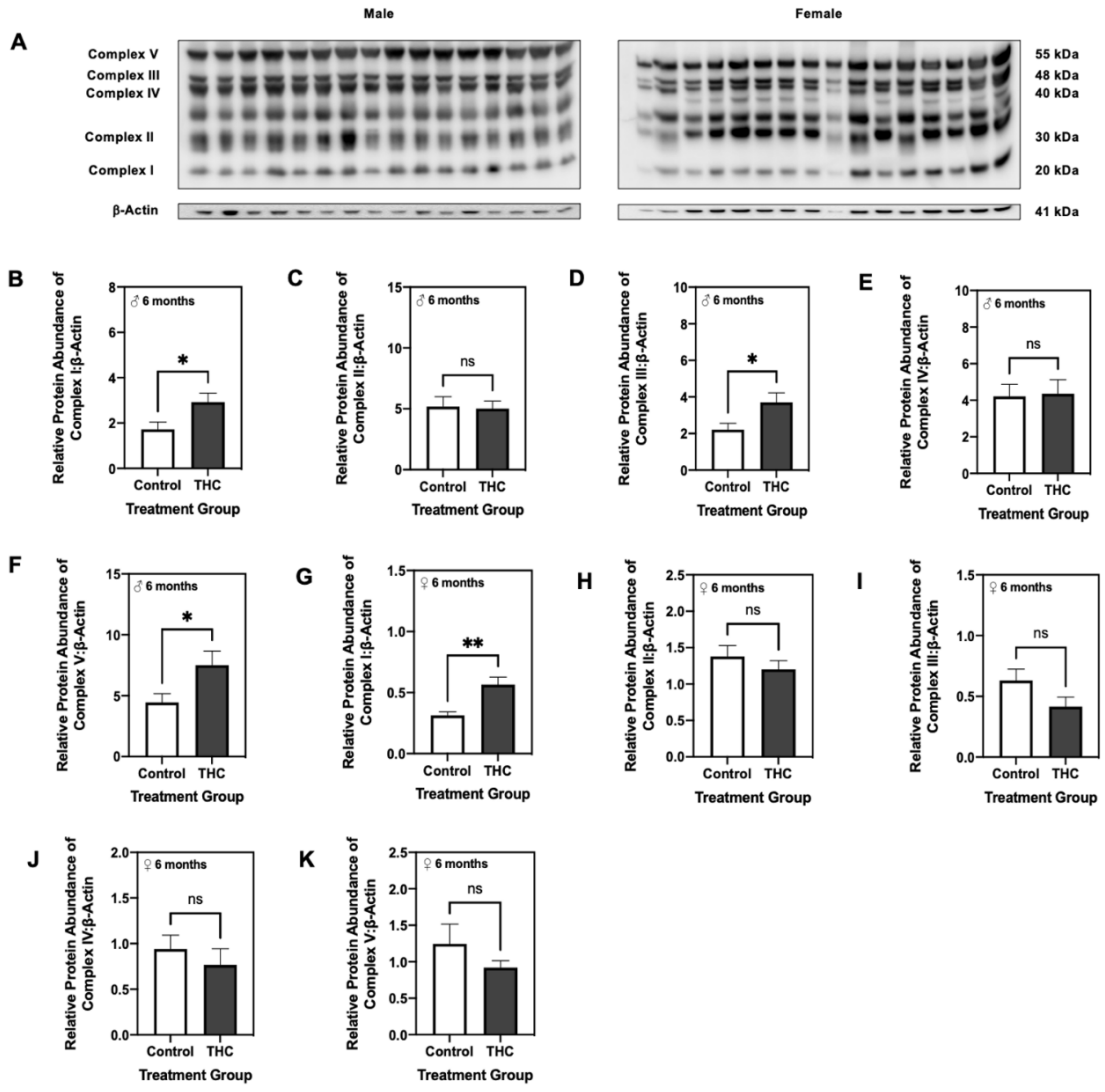

Figure 3-7. Gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) leads to increased complexes I, III and V of the electron transport chain in male offspring at six months of age.

(**A**) Representative western immunoblots illustrating hepatic expression of complexes I–V of the electron transport chain in male and female offspring at six months of age. Protein abundances of each enzyme for male offspring (**B**–**F**) and female offspring (**G**–**K**) were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. *Significant difference ($p < 0.05$), **significant difference ($p < 0.01$).

complexes I and III (Figure 3-8B,D; $p < 0.05$), while all other complexes were unchanged (Figure 3-8C,E,F).

3.3.7 Adult male offspring exposed to gestational ∆9-THC exhibit decreased hepatic transcript levels of miR-203a-3p and miR-29a/b/c

Various miRs are known to be dysregulated with metabolic disease, and we have previously shown that IUGR offspring with postnatal catch-up growth exhibit altered expression of miRs, specifically, miR-29a/b/c (Sohi *et al.*, 2015). To better understand the potential mechanisms behind the increase in p66Shc protein levels among adult male ∆9-THC-exposed livers, we first investigated whether hepatic transcript abundance of miR-203a-3p, which silences p66shc (Wang *et al.*, 2020), was altered in these same offspring. As expected, transcript abundance of miR-203a-3p was significantly decreased in the livers of adult male offspring with gestational exposure to ∆9-THC (Figure 3-9A; $p < 0.001$). Furthermore, livers from three-week old male ∆9-THC-exposed offspring displayed a trending increase in miR-203a-3p transcript abundance, but this change was not significant (Figure 3-9E). Similar to miR-203a-3p, hepatic transcript abundances of miR-29a/b/c was also decreased in six-month old male offspring exposed to gestational ∆9-THC (Figure 3-9B–D), while the expression of each isoform was unchanged at three weeks of age (Figure 3-9F–H).

3.4 Discussion

Impairments in the hepatic lipogenic pathway promote excessive production and storage of intracellular triglycerides, leading to the development of obesity and the metabolic syndrome (James *et al.*, 2004). In the current study, we demonstrate that gestational exposure to ∆9-THC leads to increased adipose to bodyweight ratio at six

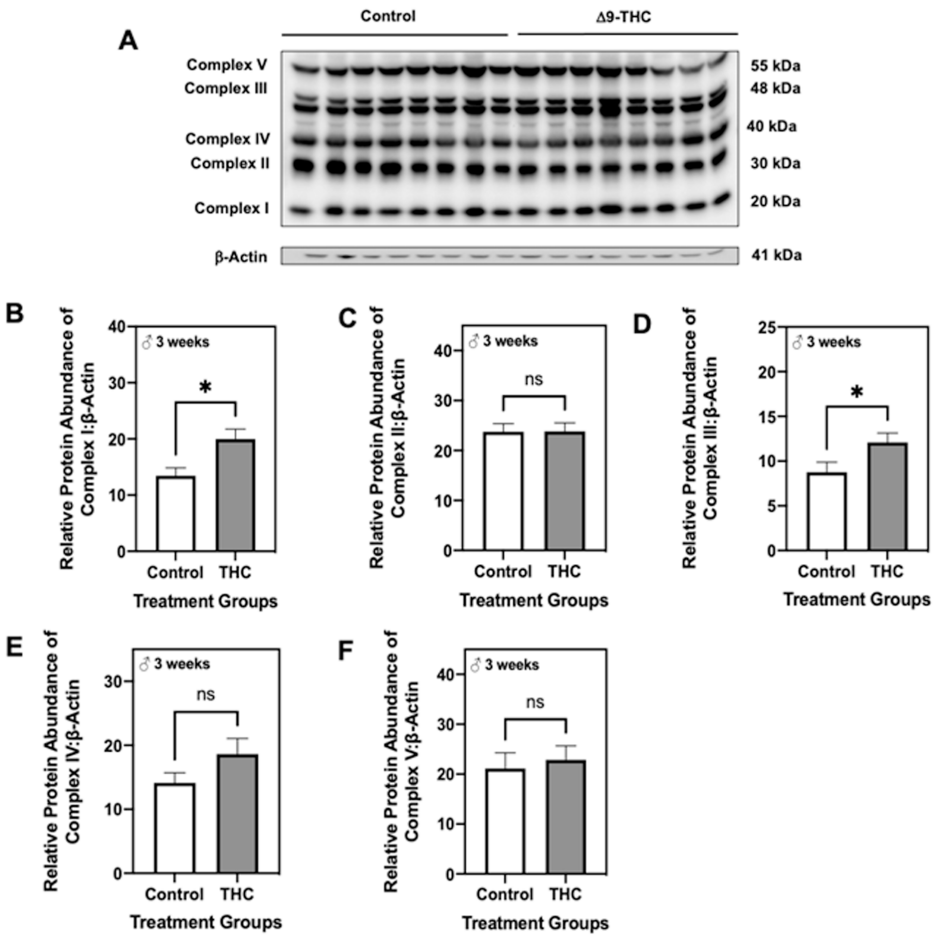

Figure 3-8. Gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) leads to increased complexes I and III of the electron transport chain in male offspring at three weeks of age.

(**A**) Representative western immunoblots illustrating hepatic expression of complexes I–V of the electron transport chain in male offspring at three weeks of age. Protein abundances of complex I (**B**), II (**C**), III (**D**), IV (**E**) and V (**F**) were normalized to β-Actin ± SEM (n = 7–8/group). All protein abundances were analyzed using a two-tailed unpaired Student's t-test. * Significant difference ($p < 0.05$).

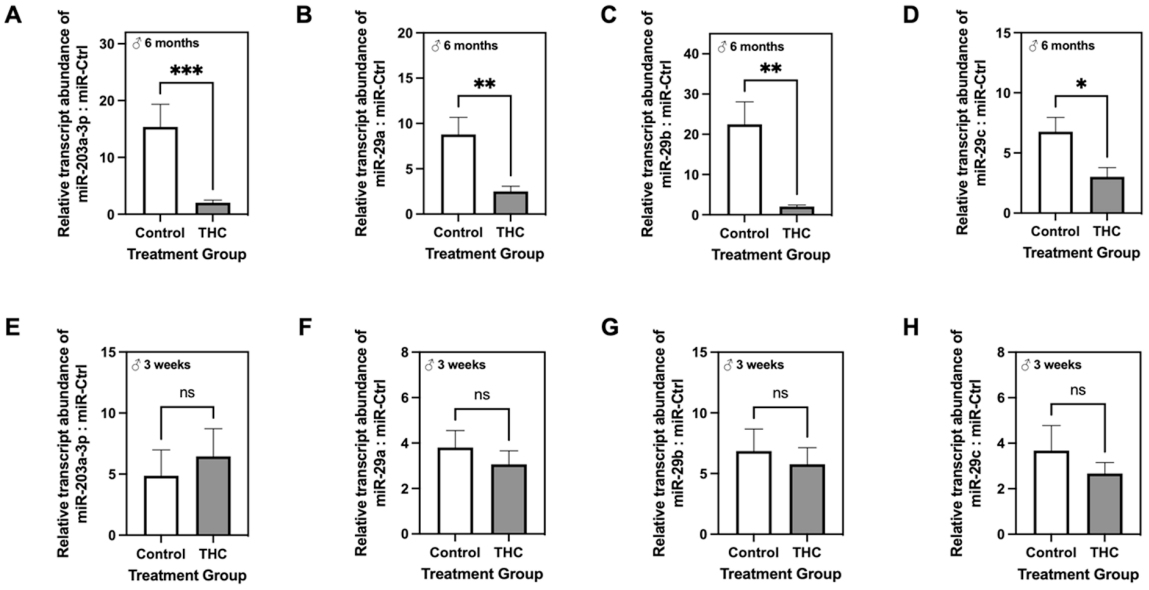

Figure 3-9. Quantitative RT-PCR analysis of miR-203a-3p and miR-29a/b/c in the livers of male offspring at six months (A–D) and three weeks (E–H) following gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC).

Relative amounts of all miRs were normalized to that of miRNA standard control. Data are expressed as means ± SEM (n = 7–8/group). Groups at each time point were compared using Student's two-tailed unpaired *t*-test. * Significant difference ($p < 0.05$), ** significant difference ($p < 0.01$), *** significant difference ($p < 0.001$).

months of age, along with elevated hepatic triglyceride levels in adult male offspring. The observed hyperlipidemia coincides with increased hepatic expression of lipogenic enzymes at three weeks and six months of age, culminating in accelerated de novo lipogenesis during adult life. Our results suggest that these changes in lipid metabolism occur in a sex-specific manner, as hepatic triglyceride levels were unchanged in adult female offspring exposed to gestational Δ9-THC. Livers taken from three-week old Δ9-THC-exposed male offspring also showed increased 4HNE abundance, suggesting that hepatic lipid peroxidation and oxidative stress first occurs in early life. Furthermore, oxidative stress and mitochondrial dysfunction appear to persist into adulthood when hepatic triglycerides are increased. Last, our study provides insight into the epigenetic mechanisms that may underly hepatic hyperlipidemia in growth-restricted offspring, as adult male offspring exposed to gestational Δ9-THC exhibited decreased expression of miR-203a-3p and miR-29a/b/c, all involved in mitochondrial homeostasis in the liver.

As mentioned previously, there is a relationship between birth weight and long-term metabolic health. We have reported that gestational exposure to Δ9-THC leads to symmetrical IUGR in rodent offspring, followed by whole body and hepatic catch-up growth by three weeks of age (Natale et al., 2020). The occurrence of catch-up growth, or rapid postnatal weight gain, is believed to further exacerbate the risk for metabolic diseases, such as obesity, as seen in birth cohorts studying growth-restricted infants from South Africa, Brazil, and the Unites States (Victora et al., 2003; Musa et al., 2015; Perng et al., 2016). Here we show that Δ9-THC-exposed offspring display increased visceral adipose to body weight ratio at six months of age, indicating the development of obesity in adult life. This is consistent with our previous studies of rodent offspring born from models of maternal nicotine exposure and maternal protein restriction, whereby visceral obesity was observed in adult offspring following postnatal catch-up growth (Gao et al., 2005; Guan et al., 2005). Clinically, obese individuals exhibit hypertriglyceridemia in the liver and plasma, often culminating in hepatic steatosis and later NAFLD (Riediger and Clara, 2011). In our model, gestational exposure to Δ9-THC led to an increase in hepatic triglycerides exclusively in male offspring at six months of age. Interestingly, there were

no differences in the levels of circulating triglycerides among male offspring, while circulating and hepatic cholesterol levels of both sexes were also unaffected. We have previously reported that female Δ9-THC exposed offspring exposed to gestational Δ9-THC do not exhibit differences in circulating estrogen or testosterone relative to control female offspring, so this protective effect cannot be attributed to changes in the circulating levels of androgenic hormones (Gillies et al., 2020). However, the differences in steroid receptor (i.e., androgen receptor/estrogen receptor) signaling in the liver might be involved (Park et al., 2011; Villa et al., 2012). Overall, there is great evidence to suggest that men are more susceptible than women to the development of hepatic steatosis and NAFLD (Browning et al., 2004; Hamaguchi et al., 2005; Suzuki et al., 2005; Tsuneto et al., 2010; Sung et al., 2012). Studies of healthy men and women demonstrate that men exhibit higher rates of de novo lipogenesis and decreased dietary fatty acid oxidation; therefore, the sexual dimorphism observed among Δ9-THC-exposed offspring may be attributed to changes in lipogenesis and lipolysis (Tran et al., 2010; Pramfalk et al., 2015).

Given the sex-specific differences in hepatic triglyceride content of Δ9-THC-exposed offspring, we next investigated the relative protein abundance of lipogenic enzymes among male and female adult offspring. At six months of age, male offspring exposed to gestational Δ9-THC exclusively displayed increased levels of both DGAT1 and DGAT2, along with an increase in the mitochondrial adaptor protein p66Shc. Deletion of DGAT and p66Shc are both independently associated with reduced intracellular accumulation of triglycerides (Berniakovich *et al.*, 2008; Leamy *et al.*, 2016). Therefore, the upregulation of both DGAT1/2 and p66Shc in the current study likely mediates the increase in hepatic triglycerides of male Δ9-THC exposed offspring. While adult female Δ9-THC exposed offspring demonstrated increases in DGAT2, this may not be sufficient in promoting increased de novo lipogenesis in the liver. Instead, a synergistic interaction of multiple enzymes is necessary to elicit changes in lipid metabolism. Since we observed lipogenic changes in male offspring exposed to gestational Δ9-THC in adulthood, we further assessed whether this occurred earlier (*e.g.,*

three weeks) coinciding with the completion of hepatic catch-up growth. Δ9-THC exposed male offspring exhibited increased abundance of ACCα, SCD, FABP1 and DGAT2 at three weeks of age, suggesting that catch-up growth may instigate de novo lipogenesis. Incidentally, recent studies have determined that FABP1 is an important regulator of Δ9-THC hepatic transport and biotransformation (Elmes *et al.*, 2015, 2019). Many hepatic pathophysiologies, including NAFLD, are associated with increased expression of FABP1, while its knockdown reduces hepatic triglyceride accumulation and lipid peroxidation (Mukai *et al.*, 2017). This is consistent with our current study, as three-week old male offspring displayed increased abundance of 4HNE (*i.e.,* a marker of lipid peroxidation) concomitant with elevated FABP1. Since FABP1 is involved in Δ9-THC metabolism, it is possible that these offspring have altered expression of FABP1 in response to exposure to Δ9-THC in utero. Mice with knockdown of hepatic FABP1 also exhibit decreased expression of DGAT1 and DGAT2 in the liver (Mukai *et al.*, 2017); therefore, early elevation of FABP1 in Δ9-THC-exposed male offspring could be involved in the upregulation of DGAT and de novo lipogenesis later in life.

Oxidative stress and mitochondrial dysfunction are highly prevalent among metabolic diseases involving the liver. Elevated reactive oxygen species (ROS) and mitochondrial abnormalities are implicated in the pathogenesis of NAFLD. However, the mechanisms by which this occurs are not completely understood (Mantena *et al.*, 2008; Rolo *et al.*, 2012; Begriche *et al.*, 2013; Chen *et al.*, 2020). *In vitro* studies have established that ROS are detrimental to hepatic lipid metabolism and mitochondrial function, as treatment of HepG2 cells and primary hepatocytes with hydrogen peroxide promotes the accumulation of triglycerides and cholesterol (Seo *et al.*, 2019). Additionally, lipid-induced elevation of ROS has been shown to impair mitochondrial function in the human hepatoblastoma C3A cell line, along with increased gluconeogenesis and ketogenesis (Lockman *et al.*, 2012). Numerous animal models have also found that IUGR offspring exhibit indices of hepatic oxidative stress (*e.g.,* increased lipid peroxidation and ROS; altered expression and activity of antioxidant enzymes*)* and impaired mitochondrial function (*e.g.*, aberrant expression and activity of pyruvate dehydrogenase, citrate synthase, and complexes of the electron transport chain; disrupted

ATP synthesis) during neonatal and adult life (Wang *et al.*, 2008; Theys *et al.*, 2009; Moraes *et al.*, 2014; Devarajan *et al.*, 2019; Oke *et al.*, 2019). In particular, we have shown that hepatic p66Shc is elevated in adult male offspring subject to maternal protein restriction attributed to postnatal catch-up growth (Oke *et al.*, 2019). P66Shc is a key regulator of cellular redox state, lifespan, and mitochondrial metabolism, as it interacts with cytochrome C under conditions of cell stress to stimulate the production of ROS (Giorgio *et al.*, 2005). This mitochondrial-induced oxidative stress often leads to apoptosis, cellular senescence and compromised aerobic metabolism (Migliaccio *et al.*, 1999; Orsini *et al.*, 2004; Giorgio *et al.*, 2005; Trinei *et al.*, 2013), making p66Shc an attractive target in mitigating metabolic disease. Again, studies have also postulated that p66Shc is involved in the accumulation of intracellular lipids (Berniakovich *et al.*, 2008). Here, we found that adult male offspring exposed to gestational Δ9-THC have increased protein abundance of p66Shc in the liver, concomitant with decreased abundance of SOD1. While we did not directly measure hepatic ROS, these results suggest that oxidative stress occurs in the liver following exposure to gestational Δ9-THC and catch-up growth. To investigate this further, we assessed the levels of proteins involved in mitochondrial function and aerobic metabolism (*i.e.,* TFAM, p-PDH[Ser232], LDHa, citrate synthase, and complexes of the electron transport chain). While the expression of TFAM, p-PDH[Ser232], LDHa and citrate synthase was unchanged in adult male offspring, they did have increased amounts of complexes I, III and V. This is noteworthy as increases in complexes I and III are associated with elevated superoxide production via increased flow of electrons through the subunits of these enzymes (Brand, 2010). These data, in combination with our p66Shc and SOD1 findings, further support the idea that these offspring exhibit hepatic oxidative stress and altered oxidative phosphorylation. Complexes I and III also increased in three-week old male offspring, suggesting that oxidative stress occurs in early life following Δ9-THC exposure and catch-up growth.

To date, little is known about the epigenetic mechanisms underlying metabolic diseases in IUGR offspring. One such mechanism is miRs, which are small, non-coding RNA molecules that silence target genes through mRNA degradation or repressed protein translation. Numerous miRs have been demonstrated to have aberrant postnatal

expression in growth-restricted offspring, leading to cellular stress that precedes impaired function of metabolic organs (Sohi *et al.*, 2015; Firmin *et al.*, 2018; Zinkhan *et al.*, 2019; Saget *et al.*, 2020). Recent studies found that the translation of p66Shc protein is directly inhibited by miR-203a-3p, leading to the attenuation of liver injury and fibrosis in mice (Wang *et al.*, 2020). Here, we observed robust decreases in the expression of miR-203a-3p in the livers of male offspring exposed to gestational Δ9-THC, but only at six months of age. This is consistent with the observed increase in p66Shc protein levels at this time point, indicating that miR-203a-3p may be implicated in the development of oxidative stress and de novo lipogenesis in these offspring. We further quantified the expression of miR-29, as we have found this miR family to be altered in the livers of adult male protein-restricted offspring following catch-up growth (Sohi *et al.*, 2015). Similar to our findings of miR-203a-3p, adult male Δ9-THC-exposed offspring exhibited decreased hepatic expression of all three isoforms of miR-29 at six months of age. This is of great interest as miR-29 expression is downregulated in patients with advanced liver fibrosis and cirrhosis (Roderburg *et al.*, 2011). Conversely, when present at high levels, miR-29 can alleviate hepatocellular steatohepatitis, fibrosis and cirrhosis in mice (Roderburg *et al.*, 2011; Lin *et al.*, 2019; Yang *et al.*, 2019). Collectively, given that each of these miRs were down-regulated exclusively during adult life, it is conceivable that early catch-up growth of the liver culminates in long-term oxidative stress and impaired hepatic lipid metabolism through epigenetic regulation of gene expression. Future in vitro studies are warranted to investigate this relationship further to reveal additional metabolic targets that become dysregulated with altered expression of miR-203a-3p and miR-29a/b/c in the liver.

In summary, this study demonstrates for the first time that gestational exposure to Δ9-THC leads to hepatic hyperlipidemia in adult male offspring. We postulate that this occurs as a result of accelerated triglyceride synthesis (*i.e.,* de novo lipogenesis) in the liver, as multiple lipogenic enzymes are elevated at both three weeks and six months of age. Given that FABP1 is involved in the metabolism and transport of Δ9-THC, it is possible that gestational exposure to Δ9-THC in combination with catch-up growth leads to early elevation of FABP1 in the livers of male offspring. This may further contribute to

the observed increase in lipid peroxidation and lipogenic enzyme expression in male offspring at three weeks of age. Hepatic lipid overload is known to further increase oxidative stress and mitochondrial dysfunction. Therefore, this process may occur in a cyclical manner as indicated in Figure 3-10. Altered expression of miRs may also contribute to these molecular changes, as we have indicated that miR-203a-3p and miR-29a/b/c are downregulated in response to Δ9-THC exposure and catch-up growth. Additional long-term studies will be important in determining if this later culminates in hepatic pathologies such as NAFLD and cirrhosis of the liver. Given that Δ9-THC interacts with both the CB1 and CB2 receptors of the endocannabinoid system, it is also possible that differences in the expression of these receptors may exist upstream of the observed physiological and molecular effects. That said, the current study is somewhat limited in that we did not examine the expression of either CB1R or CB2R. While much remains unknown about the role of prenatal cannabinoids on offspring outcomes, it is clear that long-term metabolic health becomes compromised as a result. We have previously shown that exposure to Δ9-THC specifically has effect on glucose tolerance and cardiovascular function (Gillies *et al.*, 2020; Lee *et al.*, 2021), and our current study demonstrates that it further contributes to hyperlipidemia. Overall, these data provide great insight into the effects of gestational Δ9-THC exposure on the development and function of the liver, as well as the fundamental molecular mechanisms that underlie the metabolic dysfunction of growth-restricted offspring.

3.5 References

Barker DJ, Martyn CN, Osmond C, Hales CN and Fall CH (1993) Growth in utero and serum cholesterol concentrations in adult life. *BMJ (Clinical Research Ed.)* **307** 1524–1527.

Barra NG, Lisyansky M, Vanduzer TA, Raha S, Holloway AC and Hardy DB (2017) Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase

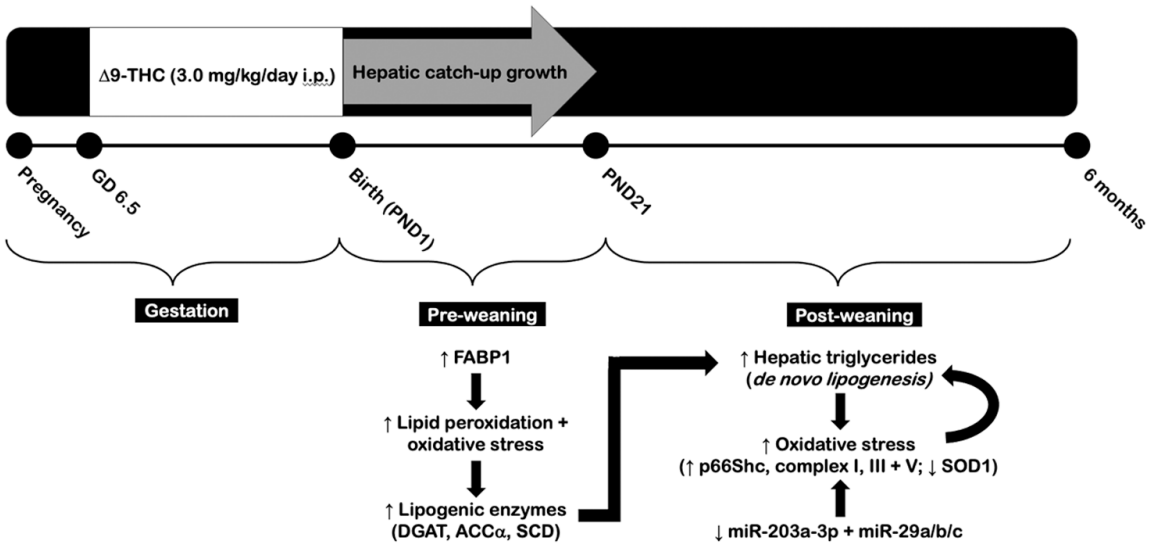

Figure 3-10. Proposed schematic illustrating the effects of gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC) on the male rat liver.

In summary, in utero exposure to Δ9-THC led to symmetrical intrauterine growth restriction in the affected offspring, followed by hepatic catch-up growth and increased visceral adiposity during adult life. Male offspring exposed to gestational Δ9-THC exhibited increased FABP1 and oxidative stress at three weeks of age, leading to increased de novo lipogenesis at six months. Adult male offspring also demonstrated oxidative stress and mitochondrial dysfunction, which may occur as a result of elevated hepatic triglyceride content. The presence of oxidative stress and mitochondrial dysfunction in combination with downregulated miR-203a-3p and miR-29a/b/c may further promote impaired lipid metabolism of the liver in a positive-feedback manner.

and impaired mitochondrial function in male rat offspring. *Journal of Applied Toxicology* **37** 1517–1526.

Beamish CA, Zhang L, Szlapinski SK, Strutt BJ and Hill DJ (2017) An increase in immature β-cells lacking Glut2 precedes the expansion of β-cell mass in the pregnant mouse. *PloS One* **12** e0182256.

Begriche K, Massart J, Robin MA, Bonnet F and Fromenty B (2013) Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. *Hepatology* **58** 1497–1507.

Benevenuto SG, Domenico MD, Martins MAG, Costa NS, de Souza ARL, Costa JL, Tavares MFM, Dolhnikoff M and Veras MM (2017) Recreational use of marijuana during pregnancy and negative gestational and fetal outcomes: An experimental study in mice. *Toxicology* **376** 94–101.

Berniakovich I, Trinei M, Stendardo M, Migliaccio E, Minucci S, Bernardi P, Pelicci PG and Giorgio M (2008) p66Shc-generated oxidative signal promotes fat accumulation. *Journal of Biological Chemistry* **283** 34283–34293.

Bouchard JF, Lépicier P and Lamontagne D (2003) Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart. *Life Sciences* **72** 1859–1870.

Brand MD (2010) The sites and topology of mitochondrial superoxide production. *Experimental Gerontology* **45** 466–472.

Brown RA, Dakkak H, Gilliland J and Seabrook JA (2019) Predictors of drug use during pregnancy: The relative effects of socioeconomic, demographic, and mental health risk factors. *Journal of Neonatal-Perinatal Medicine* **12** 179–187.

Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM and Hobbs HH (2004) Prevalence of hepatic steatosis in an urban

population in the United States: impact of ethnicity. *Hepatology (Baltimore, Md.)* **40** 1387–1395.

'Canadian Cannabis Survey 2019 - Summary - Canada.ca'.

Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ *et al.* (1998) Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **95** 13018–13023.

Chang X, Bian Y, He Q, Yao J, Zhu J, Wu J, Wang K and Duan T (2017) Suppression of STAT3 Signaling by Δ 9 -Tetrahydrocannabinol (THC) Induces Trophoblast Dysfunction. *Cellular Physiology and Biochemistry* **42** 537–550.

Chang JC, Tarr JA, Holland CL, De Genna NM, Richardson GA, Rodriguez KL, Sheeder J, Kraemer KL, Day NL, Rubio D *et al.* (2019) Beliefs and attitudes regarding prenatal marijuana use: Perspectives of pregnant women who report use. *Drug and Alcohol Dependence* **196** 14–20.

Chen Z, Tian R, She Z, Cai J and Li H (2020) Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radical Biology and Medicine* **152** 116–141.

Cheng K, Ji S, Jia P, Zhang H, Wang T, Song Z, Zhang L and Wang T (2020) Resveratrol Improves Hepatic Redox Status and Lipid Balance of Neonates with Intrauterine Growth Retardation in a Piglet Model. *BioMed Research International* **2020**.

Conner SN, Bedell V, Lipsey K, Macones GA, Cahill AG and Tuuli MG (2016) Maternal Marijuana Use and Adverse Neonatal Outcomes: A Systematic Review and Meta-analysis. *Obstetrics and Gynecology* **128** 713–723.

Deodati A, Argemí J, Germani D, Puglianiello A, Alisi A, De Stefanis C, Ferrero R, Nobili V, Aragón T and Cianfarani S (2018) The exposure to uteroplacental insufficiency is associated with activation of unfolded protein response in postnatal life. *PLOS ONE* **13** e0198490.

Devarajan A, Rajasekaran NS, Valburg C, Ganapathy E, Bindra S and Freije WA (2019) Maternal perinatal calorie restriction temporally regulates the hepatic autophagy and redox status in male rat. *Free Radical Biology and Medicine*.

Dinieri JA and Hurd YL (2012) Rat models of prenatal and adolescent cannabis exposure. *Methods in Molecular Biology (Clifton, N.J.)* **829** 231–242.

Elmes MW, Kaczocha M, Berger WT, Leung K, Ralph BP, Wang L, Sweeney JM, Miyauchi JT, Tsirka SE, Ojima I *et al.* (2015) Fatty Acid-binding Proteins (FABPs) Are Intracellular Carriers for Δ9-Tetrahydrocannabinol (THC) and Cannabidiol (CBD)*. *Journal of Biological Chemistry* **290** 8711–8721.

Elmes MW, Prentis LE, McGoldrick LL, Giuliano CJ, Sweeney JM, Joseph OM, Che J, Carbonetti GS, Studholme K, Deutsch DG *et al.* (2019) FABP1 controls hepatic transport and biotransformation of Δ 9 -THC. *Scientific Reports* **9** 7588.

ENGLISH DR, HULSE GK, MILNE E, HOLMAN CDJ and BOWER CI (1997) Maternal cannabis use and birth weight: a meta-analysis. *Addiction* **92** 1553–1560.

Eriksson J, Forsén T, Tuomilehto J, Osmond C and Barker D (2001) Size at birth, childhood growth and obesity in adult life. *International Journal of Obesity* **25** 735–740.

Faienza MF, Brunetti G, Ventura A, D'Aniello M, Pepe T, Giordano P, Monteduro M and Cavallo L (2013) Nonalcoholic Fatty Liver Disease in Prepubertal Children Born Small for Gestational Age: Influence of Rapid Weight Catch-Up Growth. *Hormone Research in Paediatrics* **79** 103–109.

Falcon M, Pichini S, Joya J, Pujadas M, Sanchez A, Vall O, García Algar O, Luna A, de la Torre R, Rotolo MC *et al.* (2012) Maternal hair testing for the assessment of fetal exposure to drug of abuse during early pregnancy: Comparison with testing in placental and fetal remains. *Forensic Science International* **218** 92–96.

Firmin S, Elmhiri G, Crepin D, Souidi M, Taouis M and Abdennebi-Najar L (2018) Formula derived Maillard reaction products in post-weaning intrauterine growth-restricted piglets induce developmental programming of hepatic oxidative stress independently of microRNA-21 and microRNA-155. *Journal of Developmental Origins of Health and Disease* **9** 566–572.

Fried PA (1976) Short and long-term effects of pre-natal Cannabis inhalation upon rat offspring. *Psychopharmacology* **50** 285–291.

Gao YJ, Holloway AC, Zeng ZH, Lim GE, Petrik JJ, Foster WG and Lee RM (2005) Prenatal exposure to nicotine causes postnatal obesity and altered perivascular adipose tissue function. *Obesity Research* **13** 687–692.

Gillies R, Lee K, Vanin S, Laviolette SR, Holloway AC, Arany E and Hardy DB (2020) Maternal exposure to Δ9-tetrahydrocannabinol impairs female offspring glucose homeostasis and endocrine pancreatic development in the rat. *Reproductive Toxicology (Elmsford, N.Y.)* **94** 84–91.

Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M *et al.* (2005) Electron transfer between cytochrome c and p66Shcgenerates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*.

Grundy D (2015) Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. *Experimental Physiology* **100** 755–758.

Guan H, Arany E, van Beek JP, Chamson-Reig A, Thyssen S, Hill DJ and Yang K (2005) Adipose tissue gene expression profiling reveals distinct molecular pathways that define visceral adiposity in offspring of maternal protein-restricted rats. *American Journal of Physiology.Endocrinology and Metabolism* **288** E663-73.

Gunn JKL, Rosales CB, Center KE, Nuñez A, Gibson SJ, Christ C and Ehiri JE (2016) Prenatal exposure to cannabis and maternal and child health outcomes: A systematic review and meta-analysis. *BMJ Open* **6** e009986.

Hamaguchi M, Kojima T, Takeda N, Nakagawa T, Taniguchi H, Fujii K, Omatsu T, Nakajima T, Sarui H, Shimazaki M *et al.* (2005) The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Annals of Internal Medicine* **143** 722–728.

Harbison RD and Mantilla-Plata B (1972) PRENATAL TOXICITY, MATERNAL DISTRIBUTION AND PLACENTAL TRANSFER OF TETRAHYDROCANNABINOL. *Journal of Pharmacology and Experimental Therapeutics* **180**.

Hayes EK, Lechowicz A, Petrik JJ, Storozhuk Y, Paez-Parent S, Dai Q, Samjoo IA, Mansell M, Gruslin A, Holloway AC *et al.* (2012) Adverse fetal and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental vasculature. *PloS One* **7** e33370–e33370.

Hurd YL, Wang X, Anderson V, Beck O, Minkoff H and Dow-Edwards D (2005) Marijuana impairs growth in mid-gestation fetuses. *Neurotoxicology and Teratology* **27** 221–229.

Ipsen DH, Lykkesfeldt J and Tveden-Nyborg P (2018) Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. *Cellular and Molecular Life Sciences: CMLS* **75** 3313–3327.

James PT, Rigby N and Leach R (2004) The obesity epidemic, metabolic syndrome and future prevention strategies. *European Journal of Cardiovascular Prevention & Rehabilitation* **11** 3–8.

Jensen-Urstad AP and Semenkovich CF (2012) Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger? *Biochimica et Biophysica Acta* **1821** 747–753.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLOS Biology* **8** e1000412.

Kim KH (1997) Regulation of mammalian acetyl-coenzyme A carboxylase. *Annual Review of Nutrition* **17** 77–99.

Klein C, Karanges E, Spiro A, Wong A, Spencer J, Huynh T, Gunasekaran N, Karl T, Long LE, Huang X-F *et al.* (2011) Cannabidiol potentiates Δ^9-tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. *Psychopharmacology* **218** 443–457.

Leamy AK, Hasenour CM, Egnatchik RA, Trenary IA, Yao C-H, Patti GJ, Shiota M and Young JD (2016) Knockdown of triglyceride synthesis does not enhance palmitate lipotoxicity or prevent oleate-mediated rescue in rat hepatocytes. *Biochimica Et Biophysica Acta* **1861** 1005–1014.

Lee K, Laviolette SR and Hardy DB (2021) Exposure to Δ9-tetrahydrocannabinol during rat pregnancy leads to impaired cardiac dysfunction in postnatal life. *Pediatric Research* 1–8.

Lin H-Y, Wang F-S, Yang Y-L and Huang Y-H (2019) MicroRNA-29a Suppresses CD36 to Ameliorate High Fat Diet-Induced Steatohepatitis and Liver Fibrosis in Mice. *Cells* **8**.

Lockman KA, Baren JP, Pemberton CJ, Baghdadi H, Burgess KE, Plevris-Papaioannou N, Lee P, Howie F, Beckett G, Pryde A *et al.* (2012) Oxidative stress rather than triglyceride accumulation is a determinant of mitochondrial dysfunction in in vitro models of hepatic cellular steatosis. *Liver International* **32** 1079–1092.

Lone A, Harris RA, Singh O, Betts DH and Cumming RC (2018) p66Shc activation promotes increased oxidative phosphorylation and renders CNS cells more vulnerable to amyloid beta toxicity. *Scientific Reports* **8** 17081–17081.

Luyckx VA, Compston CA, Simmen T and Mueller TF (2009) Accelerated senescence in kidneys of low-birth-weight rats after catch-up growth. *American Journal of Physiology-Renal Physiology* **297** F1697–F1705.

Ma N, Nicholson CJ, Wong M, Holloway AC and Hardy DB (2013) Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicology and Applied Pharmacology* **275** 1–11.

Maia J, Midão L, Cunha SC, Almada M, Fonseca BM, Braga J, Gonçalves D, Teixeira N and Correia-da-Silva G (2019) Effects of cannabis tetrahydrocannabinol on endocannabinoid homeostasis in human placenta. *Archives of Toxicology* **93** 649–658.

Malenczyk K, Keimpema E, Piscitelli F, Calvigioni D, Björklund P, Mackie K, Marzo V Di, Hökfelt TGM, Dobrzyn A and Harkany T (2015) Fetal endocannabinoids orchestrate the organization of pancreatic islet microarchitecture. *Proceedings of the National Academy of Sciences of the United States of America* **112** E6185–E6194.

Mantena SK, King AL, Andringa KK, Eccleston HB and Bailey SM (2008) Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol-

and obesity-induced fatty liver diseases. *Free Radical Biology and Medicine* **44** 1259–1272.

'Marijuana use during pregnancy and lactation' (2017). *Obstetrics and Gynecology* **130** e205–e209.

Mato S, Chevaleyre V, Robbe D, Pazos A, Castillo PE and Manzoni OJ (2004) A single in-vivo exposure to Δ9THC blocks endocannabinoid-mediated synaptic plasticity. *Nature Neuroscience* **7** 585–586.

Michalski CA, Hung RJ, Seeto RA, Dennis CL, Brooks JD, Henderson J, Levitan R, Lye SJ, Matthews SG and Knight JA (2020) Association between maternal cannabis use and birth outcomes: an observational study. *BMC Pregnancy and Childbirth* **20**.

Migliaccio E, Giogio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L and Pelicci PG (1999) The p66(shc) adaptor protein controls oxidative stress response and life span in mammals. *Nature*.

Miyazaki M and Ntambi JM (2003) Role of stearoyl-coenzyme A desaturase in lipid metabolism. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **68** 113–121.

Moraes C, Rebelato HJ, Amaral MEC, Resende TM, Silva EVC, Esquisatto MAM and Catisti R (2014) Effect of maternal protein restriction on liver metabolism in rat offspring. *The Journal of Physiological Sciences* **64** 347–355.

Mukai T, Egawa M, Takeuchi T, Yamashita H and Kusudo T (2017) Silencing of FABP1 ameliorates hepatic steatosis, inflammation, and oxidative stress in mice with nonalcoholic fatty liver disease. *FEBS Open Bio* **7** 1009–1016.

Musa MG, Kagura J, Pisa PT and Norris SA (2015) Relationship between early growth and CVD risk factors in adolescents. *Journal of Developmental Origins of Health and Disease* **7** 132–143.

Natale B V., Gustin KN, Lee K, Holloway AC, Laviolette SR, Natale DRC and Hardy DB (2020) Δ9-Tetrahydrocannabinol Exposure During Rat Pregnancy Leads To Symmetrical Fetal Growth Restriction and Labyrinth-Specific Vascular Defects in the Placenta. *Scientific Reports* **10** 1–15.

Nobili V, Marcellini M, Marchesini G, Vanni E, Manco M, Villani A and Bugianesi E (2007) Intrauterine Growth Retardation, Insulin Resistance, and Nonalcoholic Fatty Liver Disease in Children.

Oke SL, Sohi G and Hardy DB (2019) Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. *Reproduction (Cambridge, England)* **159** 27–39.

Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M *et al.* (2004) The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. *Journal of Biological Chemistry*.

Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K-U, Pak YK and Lee HK (2003) Fetal and Early Postnatal Protein Malnutrition Cause Long-Term Changes in Rat Liver and Muscle Mitochondria. *The Journal of Nutrition* **133** 3085–3090.

Park CJ, Zhao Z, Glidewell-Kenney C, Lazic M, Chambon P, Krust A, Weiss J, Clegg DJ, Dunaif A, Jameson JL *et al.* (2011) Genetic rescue of nonclassical ERα signaling normalizes energy balance in obese Erα-null mutant mice. *The Journal of Clinical Investigation* **121** 604–612.

Perng W, Hajj H, Belfort MB, Rifas-Shiman SL, Kramer MS, Gillman MW and Oken E (2016) Birth size, early life weight gain, and midchildhood cardiometabolic health. *Journal of Pediatrics* **173** 122-130.e1.

Pramfalk C, Pavlides M, Banerjee R, McNeil CA, Neubauer S, Karpe F and Hodson L (2015) Sex-Specific Differences in Hepatic Fat Oxidation and Synthesis May Explain the Higher Propensity for NAFLD in Men. *The Journal of Clinical Endocrinology and Metabolism* **100** 4425–4433.

Raez-Villanueva S, Debnath A, Hardy DB and Holloway AC (2022) Prenatal nicotine exposure leads to decreased histone H3 lysine 9 (H3K9) methylation and increased p66shc expression in the neonatal pancreas. *Journal of Developmental Origins of Health and Disease* **13** 156–160.

Ramírez-López MT, Arco R, Decara J, Vázquez M, Blanco RN, Alén F, Suárez J, De Heras RG and De Fonseca FR (2016) Exposure to a highly caloric palatable diet during the perinatal period affects the expression of the endogenous cannabinoid system in the brain, liver and adipose tissue of adult rat offspring. *PLoS ONE* **11**.

Ravelli GP, Stein ZA and Susser MW (1976) Obesity in young men after famine exposure in utero and early infancy. *The New England Journal of Medicine* **295** 349–353.

Riediger ND and Clara I (2011) Prevalence of metabolic syndrome in the Canadian adult population. *CMAJ : Canadian Medical Association Journal = Journal de l'Association Medicale Canadienne* **183** E1127-34.

Roderburg C, Urban G-W, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M *et al.* (2011) Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology (Baltimore, Md.)* **53** 209–218.

Rolo AP, Teodoro JS and Palmeira CM (2012) Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radical Biology and Medicine* **52** 59–69.

Saget S, Cong R, Decourtye L, Endale M-L, Martinerie L, Girardet C, Perret C, Clemessy M, Leneuve P, Dinard L *et al.* (2020) Changes in circulating miRNA19a-3p precede insulin resistance programmed by intra-uterine growth retardation in mice. *Molecular Metabolism* **42** 101083.

Schwope DM, Karschner EL, Gorelick DA and Huestis MA (2011) Identification of recent cannabis use: whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. *Clinical Chemistry* **57** 1406–1414.

Seo E, Kang H, Choi H, Choi W and Jun HS (2019) Reactive oxygen species-induced changes in glucose and lipid metabolism contribute to the accumulation of cholesterol in the liver during aging. *Aging Cell* **18**.

Silvestri C and Di Marzo V (2013) The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell Metabolism* **17** 475–490.

Smith SJ, Cases S, Jensen DR, Chen HC, Sande E, Tow B, Sanan DA, Raber J, Eckel RH and Farese Jr R V (2000) Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nature Genetics* **25** 87–90.

Sohi G, Marchand K, Revesz A, Arany E and Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. *Molecular Endocrinology (Baltimore, Md.)* **25** 785–798.

Sohi G, Revesz A, Ramkumar J and Hardy DB (2015) Higher Hepatic miR-29 Expression in Undernourished Male Rats During the Postnatal Period Targets the Long-Term Repression of IGF-1. *Endocrinology* **156** 3069–3076.

Sun X and Dey SK (2012) Endocannabinoid signaling in female reproduction. In *ACS Chemical Neuroscience*, pp 349–355. American Chemical Society.

Sung K-C, Kim B-S, Cho Y-K, Park D, Woo S, Kim S, Wild SH and Byrne CD (2012) Predicting incident fatty liver using simple cardio-metabolic risk factors at baseline. *BMC Gastroenterology* **12** 84.

Suomela E, Oikonen M, Pitkänen N, Ahola-Olli A, Virtanen J, Parkkola R, Jokinen E, Laitinen T, Hutri-Kähönen N, Kähönen M *et al.* (2016) Childhood predictors of adult fatty liver. The Cardiovascular Risk in Young Finns Study. *Journal of Hepatology* **65** 784–790.

Suzuki A, Angulo P, Lymp J, St Sauver J, Muto A, Okada T and Lindor K (2005) Chronological development of elevated aminotransferases in a nonalcoholic population. *Hepatology (Baltimore, Md.)* **41** 64–71.

Theys N, Clippe A, Bouckenooghe T, Reusens B and Remacle C (2009) Early Low Protein Diet Aggravates Unbalance between Antioxidant Enzymes Leading to Islet Dysfunction. *PLoS ONE* **4** e6110.

Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T *et al.* (2012) P53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. *Journal of Hepatology* **57** 837–843.

Tortoriello G, Morris CV, Alpar A, Fuzik J, Shirran SL, Calvigioni D, Keimpema E, Botting CH, Reinecke K, Herdegen T *et al.* (2014) Miswiring the brain: Δ9-tetrahydrocannabinol disrupts cortical development by inducing an SCG10/stathmin-2 degradation pathway. *The EMBO Journal* **33** 668–685.

Tran C, Jacot-Descombes D, Lecoultre V, Fielding BA, Carrel G, Lê K-A, Schneiter P, Bortolotti M, Frayn KN and Tappy L (2010) Sex differences in lipid and glucose kinetics after ingestion of an acute oral fructose load. *The British Journal of Nutrition* **104** 1139–1147.

Trinei M, Migliaccio E, Bernardi P, Paolucci F, Pelicci P and Giorgio M (2013) p66Shc, Mitochondria, and the Generation of Reactive Oxygen Species. In *Methods in Enzymology*, pp 99–110.

Tsuneto A, Hida A, Sera N, Imaizumi M, Ichimaru S, Nakashima E, Seto S, Maemura K and Akahoshi M (2010) Fatty liver incidence and predictive variables. *Hypertension Research* **33** 638–643.

Victora CG, Barros FC, Lima RC, Behague DP, Gon alves H, Horta BL, Gigante DP and Vaughan JP (2003) The Pelotas birth cohort study, Rio Grande do Sul, Brazil, 1982-2001. *Cadernos de Saúde Pública / Ministério Da Saúde, Fundação Oswaldo Cruz, Escola Nacional de Saúde Pública* **19** 1241–1256.

Villa A, Della Torre S, Stell A, Cook J, Brown M and Maggi A (2012) Tetradian oscillation of estrogen receptor α is necessary to prevent liver lipid deposition. *Proceedings of the National Academy of Sciences of the United States of America* **109** 11806–11811.

Wang J, Chen L, Li D, Yin Y, Wang X, Li P, Dangott LJ, Hu W and Wu G (2008) Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *Journal of Nutrition* **138** 60–66.

Wang Z, Zhao Y, Zhao H, Zhou J, Feng D, Tang F, Li Y, Lv L, Chen Z, Ma X *et al.* (2020) Inhibition of p66Shc Oxidative Signaling via CA-Induced Upregulation of miR-203a-3p Alleviates Liver Fibrosis Progression. *Molecular Therapy - Nucleic Acids* **21** 751–763.

Westfall RE, Janssen PA, Lucas P and Capler R (2006) Survey of medicinal cannabis use among childbearing women: patterns of its use in pregnancy and retroactive self-assessment of its efficacy against ‘morning sickness’. *Complementary Therapies in Clinical Practice* **12** 27–33.

Yamada M, Wolfe D, Han G, French SW, Ross MG and Desai M (2011) Early onset of fatty liver in growth-restricted rat fetuses and newborns. *Congenital Anomalies* **51** 167–173.

Yang Z, Zhao W, Zhang X, Mu R, Zhai Y, Kong L and Chen C (2008) Impact of famine during pregnancy and infancy on health in adulthood. In *Obesity Reviews*, pp 95–99. Obes Rev.

Yang Y-L, Kuo H-C, Wang F-S and Huang Y-H (2019) MicroRNA-29a Disrupts DNMT3b to Ameliorate Diet-Induced Non-Alcoholic Steatohepatitis in Mice. *International Journal of Molecular Sciences* **20**.

Zinkhan EK, Yu B and McKnight R (2019) Uteroplacental Insufficiency Impairs Cholesterol Elimination in Adult Female Growth-Restricted Rat Offspring Fed a High-Fat Diet. *Reproductive Sciences (Thousand Oaks, Calif.)* **26** 1173–1180.

Chapter IV

4 Oxidative stress and endoplasmic reticulum stress promote increased expression of hepatic miR-29 and fatty acid translocase in vitro

This chapter is a version of a manuscript under preparation entitled “Oxidative stress and endoplasmic reticulum stress promote increased expression of hepatic miR-29 and fatty acid translocase in vitro”.

4.1 Introduction

Intrauterine growth restriction (IUGR) is a pathological condition characterized by low birth weight and reduced organ growth. Several extrinsic risk factors are known to contribute to the etiology of IUGR, including maternal undernutrition, maternal drug exposure, maternal obesity, and placental insufficiency. Epidemiological studies have identified a relationship between birth weight and long-term metabolic health, and this is further exacerbated by postnatal catch-up growth (*i.e.,* rapid postnatal weight gain). This relationship is best explained by the Developmental Origins of Health and Disease (DOHaD) hypothesis, which proposes that adverse events *in utero* promote changes to fetal programming that are detrimental to offspring health. It is widely accepted that this occurs, in part, due to abnormal transcriptional regulation; however, little is known about the epigenetic mechanisms that contribute to this relationship. Epigenetics refers to molecular mechanisms that produce stable, heritable phenotypic changes without altering the underlying DNA sequence (Zhang *et al.*, 2020). Importantly, epigenetic mechanisms describe a higher level of transcriptional regulation that are separate from common transcription factors (Greally, 2018). These mechanisms include DNA methylation, post-translational histone modifications, and microRNAs. While each of these mechanisms have been implicated in IUGR, here we focus on the role of microRNAs in postnatal metabolism of the IUGR liver.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that act via silencing of target genes through mRNA degradation or inhibition of protein translation. High sequence complementarity between the 5' untranslated region (UTR) of miRNAs and the 3' UTR of target mRNAs results in mRNA degradation via enzymatic cleavage (Hammond, 2015). Alternatively, sequence mismatch between miRNAs and mRNA targets results in translational repression through the recruitment of proteins that block access of translational machinery (Hammond, 2015). Given the potential impact on gene expression, there has been some investigation into the role of miRNAs in the onset of metabolic disease of IUGR offspring. This includes our own work focused on the maternal protein restriction (MPR) rodent model of undernutrition, which identified that

hepatic miR-29a/b/c is upregulated in adult male offspring exclusively following postnatal catch-up growth (Sohi *et al.*, 2015). This was concomitant with decreased expression of hepatic insulin-like growth factor 1 (IGF-1), a hormone that is critical for fetal growth and development (Sohi *et al.*, 2015). These MPR offspring with catch-up growth further exhibit hepatic endoplasmic reticulum stress (Sohi *et al.*, 2013), along with mitochondrial dysfunction via aberrant markers of oxidative stress and aerobic metabolism in the liver (Oke *et al.*, 2019). Interestingly, we have recently demonstrated that adult male offspring with gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC, the psychoactive component of cannabis), display decreased hepatic transcript levels of miR-29 and miR-203a-3p at six months of age (Oke *et al.*, 2021). Again, this only occurred following catch-up growth, suggesting that rapid postnatal weight gain may influence miR expression. Importantly, male offspring from both the MPR model and the gestational Δ9-THC model exhibit hepatic hyperlipidemia in adult life (Sohi *et al.*, 2011; Oke *et al.*, 2021).These findings are of great interest, as the abnormal expression of miRNAs often precedes impaired function of metabolic organs and concomitant with cellular stress (Sohi *et al.*, 2015; Firmin *et al.*, 2018; Zinkhan *et al.*, 2019; Saget *et al.*, 2020). That said, further research is warranted to determine (1) the mechanisms that lead to altered expression of hepatic miR-29a/b/c, and (2) if miR-29a/b/c directly contributes to hepatic hyperlipidemia among IUGR offspring.

Although miR-29 and miR-203a-3p target different mRNA transcripts, there is evidence to suggest that both of these miRNAs are involved in the regulation of lipid biosynthesis and metabolism. Using the HepG2 cell line, miR-29a has been demonstrated to directly target the 3’ UTR of fatty acid translocase, also known as CD36 (Lin *et al.*, 2019). Upregulation of hepatic CD36 is associated with poor hepatic lipid metabolism and elevated body adiposity in rodents with non-alcoholic fatty liver disease (NAFLD; Wang *et al.*, 2019), while its knockdown *in vitro* and *in vivo* shows promise in alleviating hepatic lipid accumulation and increasing fatty acid oxidation (Wilson *et al.*, 2016; Li *et al.*, 2019). Considering that miR-29a suppresses the expression of CD36, these studies seem to be in favour of miR-29 as a protective agent against the metabolic consequences of various liver pathologies. Alternatively, miR-203a-3p has been demonstrated to

directly inhibit the translation of p66Shc protein in the human LX2 hepatic stellate cell line (Wang *et al.*, 2020). P66Shc is an adaptor protein that undergoes mitochondrial translocation under cell stress, and its activation further contributes to accelerated production of mitochondrial reactive oxygen species (ROS) and poor aerobic metabolism (Migliaccio *et al.*, 1999; Orsini *et al.*, 2004; Giorgio *et al.*, 2005). Additional studies suggest that p66Shc-induced ROS leads to the accumulation of intracellular lipids, giving rise to metabolic pathologies such as NAFLD and obesity (Berniakovich *et al.*, 2008; Tomita *et al.*, 2012).

Taking these observations into account, the role of hepatic miR-29 and miR-203a-3p in metabolic dysfunction of IUGR offspring seems promising. Given we have previously observed that IUGR offspring with catch-up growth exhibit ER stress, oxidative stress, and mitochondrial dysfunction (Sohi *et al.*, 2015; Oke *et al.*, 2019, 2021), the objective of this study was to determine if these forms of cellular stress directly lead to altered hepatic expression of miR-29 and miR-203a-3p in HepG2 cells. This cell line was selected because HepG2 cells are capable of producing and secreting proteins that are expressed in fetal hepatocytes, making them an appropriate *in vitro* model for the neonatal liver (Maruyama *et al.*, 2007). Furthermore, we intended to clarify the directionality in which expression of these miRNAs are changed in response to cell stress. To address this, we pharmacologically induced oxidative stress and ER stress separately in the HepG2 cell line. We hypothesized that both oxidative stress and ER stress would lead to altered expression of miR-29 and miR-203a-3p, independent of the pharmacological agent applied. We further anticipated that expression of hepatic p66Shc and CD36 would be altered in response to miR-29 and miR-203a-3p.

4.2 Materials and Methods

4.2.1 Animal Care and Dietary Regimen

All procedures were performed according to guidelines set by the Canadian Council of Animal Care (CCAC), with approval from the Animal Care Committee at The

University of Western Ontario. Male and female Wistar rats were purchased from Charles River (La Salle, St. Constant, QC), and allowed three weeks to acclimatize to environmental conditions of the animal care facility. Female rats were placed in cages with male rats upon proestrus, and pregnancy was confirmed via the presence of sperm in the vaginal smear (gestational day 1). Pregnant dams were fed either a control (20% casein) diet or a low (8% casein; LP) diet throughout gestation. Food was provided *ad libitum* throughout pregnancy, while diets were made isocaloric through the addition of sucrose to the LP diet (Sohi *et al.*, 2015). As previously reported, the addition of sucrose to the LP diet is considered a modest increase in carbohydrate content relative to the decrease in dietary protein (Oke *et al.*, 2019). Litter size was reduced to eight offspring per litter at birth by selecting pups with birth weights closest to the litter mean. Offspring born to control diet-fed dams were fed a control diet for the entire postnatal period. Alternatively, offspring born to protein-restricted dams were assigned to one of three dietary regimes (*i.e.,* low protein 1, low protein 2, or low protein 3; LP1, LP2, LP3). LP1 offspring received the LP for their entire lives, while LP2 offspring received the LP diet until three weeks of age before being switched to the control diet. LP3 offspring were fed the control diet for the entire postnatal period; that is, they were switched to the control diet immediately at birth. These three LP groups allow us to examine the effects of the low protein diet and catch-up growth separately, as hepatic differentiation is not complete until three weeks of age in the rat (Gruppuso and Sanders, 2016). Offspring were euthanized and necropsy was performed at four months of age (*i.e.,* postnatal day 130). This age was selected because it is the time at which IUGR offspring begin to exhibit metabolic dysfunction, as demonstrated by our own studies and others (Chamson-Reig *et al.*, 2009; Sohi *et al.*, 2011). All molecular analyses were performed using the right medial hepatic lobe, which was flash frozen in liquid nitrogen and stored long-term at -80 °C. Importantly, the offspring used here are the same as those used in Chapter II.

4.2.2 Cell Culture and Induction of Cellular Stress

The HepG2 human male hepatocellular carcinoma cell line was acquired from the American Type Culture Collection (ATCC). Cells were thawed and maintained at 37 °C

and 5% CO_2/95% atmospheric air. Cells were cultured in minimum essential medium (MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin solution (10 000 IU and 10 000 µg/mL, respectively; Fisher Scientific). All cells were passaged at least twice prior to plating for experimental treatment. Cells between passages 5 and 12 were seeded in six-well culture plates (Thermo Scientific) at a density of 2.0 x 10^5 cells/well and allowed to proliferate to approximately 80% confluency. To induce oxidative stress, cells were treated with either hydrogen peroxide solution (H_2O_2; 100, 200, or 400 µM; Millipore-Sigma) or rotenone solution (25, 50, or 100 µM; Millipore-Sigma), and allowed to proliferate for 6 and 24 hours in the presence of FBS. These concentrations and length of treatment were selected based on previous studies that reported successful induction of oxidative stress without affecting cell viability (Siddiqui *et al.*, 2013; Jiang *et al.*, 2014). Hydrogen peroxide is a reactive oxygen species (ROS) produced by numerous cellular organelles. Studies have found that hydrogen peroxide has an important role in cell signaling; however, high levels of hydrogen peroxide are cytotoxic as it can easily break down into hydroxyl radicals (Clarkson and Thompson, 2000; Jiang *et al.*, 2014). Because of this, hydrogen peroxide is commonly used to study the effects of oxidative stress *in vitro.* Alternatively, rotenone is a neurotoxic insecticide that inhibits complex I of the mitochondrial electron transport chain (ETC; Siddiqui *et al.*, 2013). By blocking the transfer of electrons from complex I to ubiquinone, rotenone increases the production of mitochondrial ROS (Radad *et al.*, 2006). Because of this, rotenone can be used to target mitochondria directly rather than inducing general oxidative stress.

To induce ER stress, cells were treated with either tunicamycin (1, 2, or 5 µg/mL; Millipore-Sigma) or thapsigargin (1, 2, or 5 µM; Millipore-Sigma) for 6 and 24 hours in the presence of FBS. Again, these concentrations and times were chosen based on previous studies of a similar nature (Naem *et al.*, 2013; Oke *et al.*, 2019). Tunicamycin induces ER stress by inhibiting enzymes that catalyze N-linked glycosylation during glycoprotein synthesis (Lei *et al.*, 2016). Our previous experiments found that treatment of HepG2s with 0.5 µg/mL of tunicamycin induces ER stress and increased p66Shc protein levels as early as one hour post-exposure (Oke *et al.*, 2019). Alternatively,

thapsigargin acts through inhibition of the sarcoplasmic/endoplasmic reticulum bound Ca^{2+}-ATPase (SERCA), thereby interfering with homeostasis of calcium levels within the ER (Naem *et al.*, 2013).

Cells treated with rotenone, tunicamycin, or thapsigargin were also treated with concentration-matched doses of dimethylsulfoxide (DMSO; Fisher Scientific) vehicular control. For each technical replicate, one well was left untreated. For each time point (*i.e.,* 6h and 24h), cells were collected for RNA isolation and quantitative real-time PCR (qRT-PCR) as described below.

4.2.3 RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was isolated from liver tissue and HepG2 cells using the one-step method of Chomczynski and Sacchi (TRIzol; Invitrogen). For miRNA targets, cDNA template was synthesized using Qiagen's miRCURY LNA RT kit, then diluted with nuclease-free water to 1:60 for use with qRT-PCR. For mRNA targets, cDNA template was synthesized using a High-Capacity cDNA RT kit (Applied Biosystems), then diluted with nuclease-free water to 1:40 for use with qRT-PCR. All cDNA template samples were stored long-term at -20 °C. For quantification of miR-29a/b/c and miR-203a-3p, primer assays were purchased from Qiagen (Table 4-1). Primer sets for mRNA targets of interest were designed using the National Center for Biotechnology Information and Ensembl genome browsers, followed by generation via Invitrogen Custom DNA Oligos (Table 4-2). Relative transcript abundances for all gene targets were determined via qRT-PCR as previously published (Barra *et al.*, 2017). Cq values obtained for miRNA targets were normalized to those of U6 snRNA control (Qiagen; Table 4-1), while Cq values for mRNA gene targets were normalized to geometric means of β-Actin and GAPDH (Table 4-2). Relative transcript abundance for all targets was calculated using the comparative ΔCt method.

Table 4-1. GeneGlobe Catalog Numbers for Qiagen miRCURY LNA miRNA primer assays used for quantitative real-time PCR.

MicroRNA	GeneGlobe (Catalog) Number
HSA-MIR-29A-3P	YP00204698
HSA-MIR-29B-3P	YP00204679
HSA-MIR-29C-3P	YP00204729
HSA-MIR-203A-3P	YP00205914
U6 SNRA	YP0020390

Table 4-2. Forward and reverse primer sequences for primers used in analysis of mRNA targets via quantitative real-time PCR.

Gene	Species	Primer Sequences (5'–3')	GenBank/ Reference
p66Shc	Human	**F:** AAGTACAATCCACTCCGG AATGA **R:** GGGCCCCAGGGATGAAG	NM_183001.5
	Rat	**F:** TACTTGGTTCGGGTGAGTGC **R:** GAGCAGGAAGTCCCGAC AAA	NM_053517.2
cd36	Human	**F:** GTTGAGAGCCTGTGCCT CAT **R:** TGGTTTCTACAAGCTCTGG TTCTTA	NM_001371075.1
	Rat	**F:** CAGTTTTGGATCTTTGAC GTGC **R:** TGCTGCTATTCTTTGCCAC TT	XM_039108092.1
GAPDH	Human	**F:** ATGGCATCAAGAAGGTG GTG **R:** CATACCAGGAAAATGAGC TTG	NM_001357943.2
	Rat	**F:** GGATACTGAGAGCAAGA GAGAGG **R:** TCCTGTTGTTATGGGGTC TGG	NM_017008.4
β-Actin	Human	**F:** CACCAACTGGGACGACAT **R:** ACAGCCTGGATAGCAACG	NM_001101.5
	Rat	**F:** CACAGCTGAGAGGGAAAT **R:** TCAGCAATGCCTGGGTAC	NM_031144

4.2.4 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9 software. Results were expressed as means of normalized values ± S.E.M. (standard error of the mean), and the threshold for statistical significance was set as $P < 0.05$. Cell culture experiments were performed and analyzed in technical replicates of 3 (*i.e.,* n=3/group), where each replicate represents an independent set of experiments using a different frozen cell stock or passage number. *Ex vivo* results for protein-restricted offspring were analyzed using a One Way ANOVA with Tukey's multiple comparisons test. *In vitro* results were analyzed using individual Student's unpaired t-tests; that is, each experimental group was compared to the untreated control group to determine if transcript abundance was significantly different. This statistical test was used rather than a one-way ANOVA because the purpose of these experiments was to determine if induction of oxidative stress or ER stress leads to altered miRNA expression, rather than to assess the effects of these drugs regarding time or concentration. Values obtained for cells treated with DMSO vehicular control were also compared to those of untreated cells to ensure that there were no adverse effects of DMSO.

4.3 Results

4.3.1 Adult male protein-restricted offspring exhibit increased hepatic transcript levels of miR-203a-3p following postnatal catch-up growth

Given we observed a significant decrease in hepatic miR-203a-3p and miR-29a/b/c transcript levels among adult male offspring with catch-up growth following gestational exposure to Δ9-THC (Oke *et al.*, 2021), we followed up with analysis of miR-203a-3p expression in the livers of adult male protein-restricted offspring with (*i.e.* LP2/LP3 offspring) and without (*e.g.* LP1 offspring) postnatal catch-up growth. Our laboratory has

previously demonstrated that these MPR offspring with catch-up growth exhibit significantly increased hepatic transcript abundance of miR-29a/b/c (Sohi *et al.*, 2015). Moreover, we have demonstrated that these same offspring exhibit elevated markers of oxidative stress and aberrant aerobic metabolism in (Oke *et al.*, 2019). Interestingly, we found that miR-203a-3p was increased exclusively in the livers of LP3 offspring (Figure 4-1; $P < 0.05$). While this group of offspring is protein-restricted throughout gestation, they also undergo the greatest degree of catch-up growth given that they are switched to a control diet at birth. Of note, miR-203a-3p transcript levels were not significantly altered in LP1 or LP2 offspring relative to that of the control group (Figure 4-1).

4.3.2 MiR-203a-3p transcript is undetectable in the HepG2 cell line

Following the collection of each treatment group, we began by measuring transcript levels of miR-203a-3p in HepG2 cells. We first performed qRT-PCR experiments to measure this miRNA in the untreated control group and hydrogen peroxide-treated groups for each technical replicate; however, we found that the resultant Cq values were very high (*i.e.,* > 39). This indicated that miR-203a-3p was poorly expressed, and it suggests that the HepG2 cell line is not an ideal *in vitro* model in studying this miRNA. We further attempted to measure miR-203a-3p in all samples treated with tunicamycin to ensure that ER stress did not induce its expression, and we again found that it was undetectable.

4.3.3 Short-term (6-hour) treatment with pharmacological inducers of oxidative stress and ER stress has no effect on miR-29 transcript abundance

To determine if miR-29 expression is upregulated following short-term oxidative stress or ER stress, we performed qRT-PCR in HepG2 cells following 6h treatment with hydrogen peroxide, rotenone, tunicamycin, or thapsigargin. Because miR-29 has three isoforms, we measured the relative transcript abundance for each of miR-29a, -29b, and –

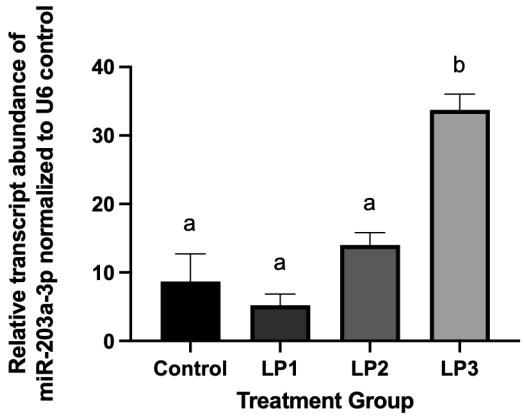

Figure 4-1. Maternal protein restriction (MPR) followed by postnatal catch-up growth leads to upregulation of miR-203a-3p in the livers of male offspring at four months of age.

Quantitative RT-PCR analysis of miR-203a-3p was performed following necropsy of livers from control and MPR Wistar rat offspring. Relative amounts of miR-203a-3p were expressed as means normalized to U6 miRNA control ± SEM (n=8/group). Transcript abundances for each individual group were analyzed using One Way ANOVA and Tukey's multiple comparisons test. Groups marked with different letters are significantly different ($P<0.05$).

29c. Our analyses showed that each of the three isoforms remained unchanged following 6h treatment with any drug (data not shown), suggesting that exposure to these drugs was too acute to influence miR-29 expression. While we did not directly measure markers of oxidative or ER stress, the drug concentrations used in this study have previously been shown to successfully induce oxidative stress or ER stress in HepG2 cells without having drastic effects on cell viability (Naem *et al.*, 2013; Siddiqui *et al.*, 2013; Jiang *et al.*, 2014; Oke *et al.*, 2019). That said, many studies indicated a treatment length of 24h; therefore, it is possible that a 6h exposure was too acute to cause oxidative or ER stress.

4.3.4 24-hour treatment of HepG2 cells with hydrogen peroxide does not influence miR-29 expression

Given we did not observe significant changes in miR-29 expression following 6h treatment, we next performed qRT-PCR for samples exposed to each drug for 24h. We began by assessing levels of miR-29a/b/c in cells treated with hydrogen peroxide. As mentioned previously, hydrogen peroxide treatment has been used to induce oxidative stress in HepG2 cells, while its effects are ameliorated by co-treatment with N-Acetyl-Serotonin (NAS; Jiang *et al.*, 2014). Interestingly, treatment of primary rat vascular smooth muscle cells with 200 μM hydrogen peroxide has been shown to result in decreased miR-29b transcript (Lin *et al.*, 2009). Nonetheless, the effects of hydrogen peroxide on miR-29 expression in HepG2s have not been investigated. In the current study, we found that all three isoforms of miR-29 were unchanged following 24h treatment with all three doses of hydrogen peroxide (Figure 4-2).

4.3.5 MiR-29 is elevated in HepG2 cells following 24-hour exposure to rotenone, an inhibitor of complex I

To ensure that the effects observed on miR-29 expression are not drug-specific, we next measured levels of miR-29 in cells treated with rotenone for 24h. Again, rotenone-induced oxidative stress is of mitochondrial origin via targeting of complex I in the ETC. Here we discovered that 24h treatment of HepG2s with 25 μM and 50 μM

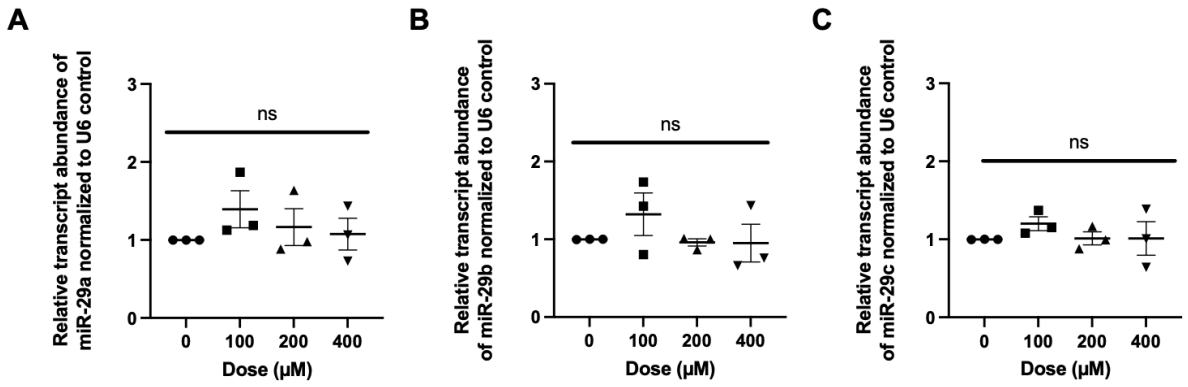

Figure 4-2. Induction of oxidative stress with hydrogen peroxide does not influence transcript abundance of miR-29a/b/c in the HepG2 cell line following 24h treatment.

Cells were plated in a six-well plate at 2 x 10^5 cells/well and let grown to 80% confluency. Cells were then treated for 24h with either 100 μM, 200 μM, or 400 μM of hydrogen peroxide. RNA was collected from each group, followed by qRT-PCR analysis with the complementary cDNA template. Fold change difference of (A) miR-29a, (B) miR-29b, and (C) miR-29c were expressed as means from three biological replicates normalized to U6 miRNA control ± SEM. Transcript abundances for each individual point were analyzed using a two-tailed unpaired Student's *t*-test and compared to the untreated control group. n.s.= non-significant ($P < 0.05$)

rotenone led to increased miR-29a and miR-29c transcript (Figure 4-3A, C; $P < 0.05$), and treatment with 25 μM, 50 μM, and 100 μM rotenone led to increased miR-29b (Figure 4-3A-C; $P < 0.05$). Since we did not see changes in miR-29 expression following exposure to hydrogen peroxide, these results suggest that miR-29 may be specifically influenced by mitochondrial-induced oxidative stress rather than cytosolic oxidative stress.

4.3.6 24-hour treatment of HepG2 Cells with thapsigargin, but not tunicamycin, leads to increased miR-29 transcript abundance

Because we have previously found that protein-restricted offspring display hepatic ER stress coinciding with increased expression of miR-29 (Sohi *et al.*, 2013), we next quantified levels of miR-29 transcript in HepG2 cells treated with tunicamycin or thapsigargin for 24h. Our results here showed that the expression of each miR-29 isoform was unchanged following treatment with each dose of tunicamycin (Figure 4-4). Conversely, treatment with thapsigargin resulted in increased levels of miR-29b and miR-29c; these isoforms exhibited a trending increase after treatment with 1 μM and 5 μM (Figure 4-5B,C), while treatment with 2 μM resulted in a significant increase (Figure 4-5B,C; $P < 0.05$). Abundance of miR-29a transcript was unaffected by treatment with thapsigargin (Figure 4-5A).

4.3.7 HepG2 cells treated with rotenone and thapsigargin have increased transcript abundance of fatty acid translocase (CD36)

To follow up with our analyses of miR-29 expression corresponding to its proposed targets, we next assessed transcript levels of p66Shc for each of the cellular treatment groups. We did not observe changes in p66Shc expression following treatment with rotenone, tunicamycin or thapsigargin (Figures 4-6C, E, G). This is in alignment with our previous studies where we showed that p66Shc protein, but not transcript levels,

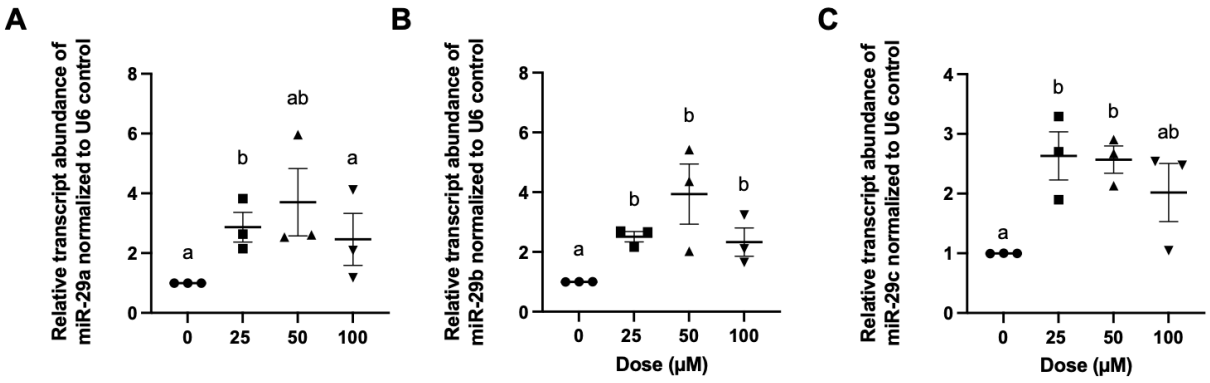

Figure 4-3. Induction of oxidative stress with complex I inhibitor rotenone results in increased transcript abundance of miR-29a/b/c in the HepG2 cell line following 24h treatment.

Cells were plated in a six-well plate at 2 x 10^5 cells/well and let grown to 80% confluency. Cells were then treated for 24h with either 25 μM, 50 μM, or 100 μM of rotenone. RNA was collected from each group, followed by qRT-PCR analysis with the complementary cDNA template. Fold change difference of (A) miR-29a, (B) miR-29b, and (C) miR-29c were expressed as means from three biological replicates normalized to U6 miRNA control ± SEM. Transcript abundances for each individual point were analyzed using a two-tailed unpaired Student's *t*-test and compared to the untreated control group. Groups marked with different letters are significantly different ($P<0.05$).

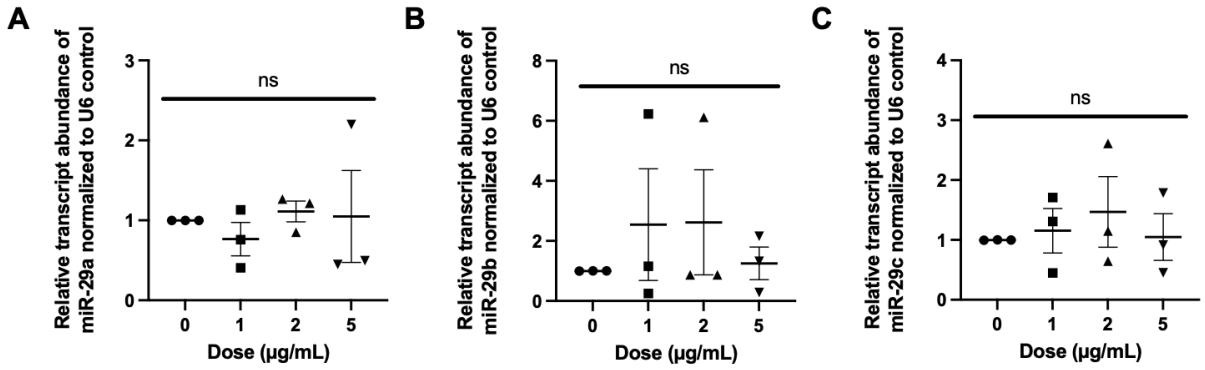

Figure 4-4. Induction of endoplasmic reticulum (ER) stress with tunicamycin has no effect on transcript abundance of miR-29a/b/c in the HepG2 cell line following 24h treatment.

Cells were plated in a six-well plate at 2 x 10^5 cells/well and let grown to 80% confluency. Cells were then treated for 24h with either 1, 2, or 5 µg/mL of tunicamycin. RNA was collected from each group, followed by qRT-PCR analysis with the complementary cDNA template. Fold change difference of (A) miR-29a, (B) miR-29b, and (C) miR-29c were expressed as means from three biological replicates normalized to U6 miRNA control ± SEM. Transcript abundances for each individual point were analyzed using a two-tailed unpaired Student's *t*-test and compared to the untreated control group. n.s.= non-significant ($P < 0.05$)

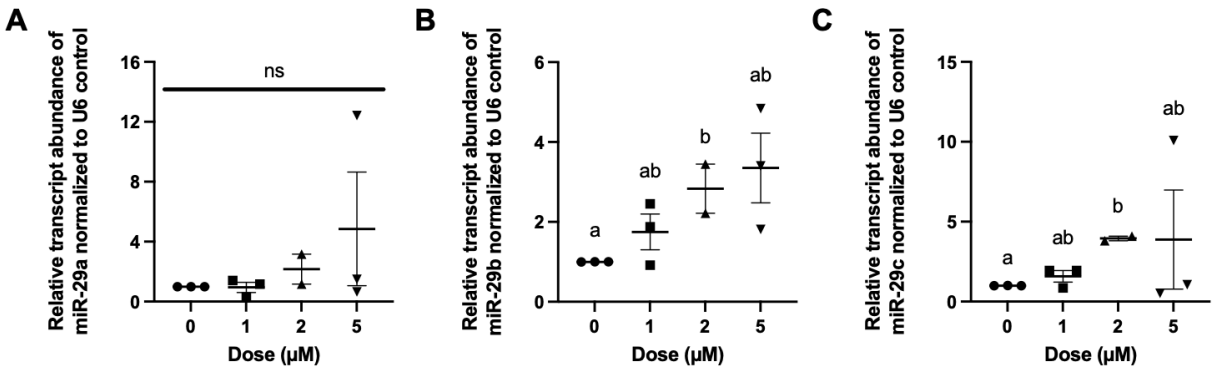

Figure 4-5. Induction of endoplasmic reticulum (ER) stress with thapsigargin leads to increased miR-29 transcript abundance in the HepG2 cell line following 24h treatment.

Cells were plated in a six-well plate at 2 x 10^5 cells/well and let grown to 80% confluency. Cells were then treated for 24h with either 1, 2, or 5 μM of thapsigargin. RNA was collected from each group, followed by qRT-PCR analysis with the complementary cDNA template. Fold change difference of (A) miR-29a, (B) miR-29b, and (C) miR-29c were expressed as means from three biological replicates normalized to U6 miRNA control ± SEM. Transcript abundances for each individual point were analyzed using a two-tailed unpaired Student's *t*-test and compared to the untreated control group. Groups marked with different letters are significantly different ($P<0.05$).

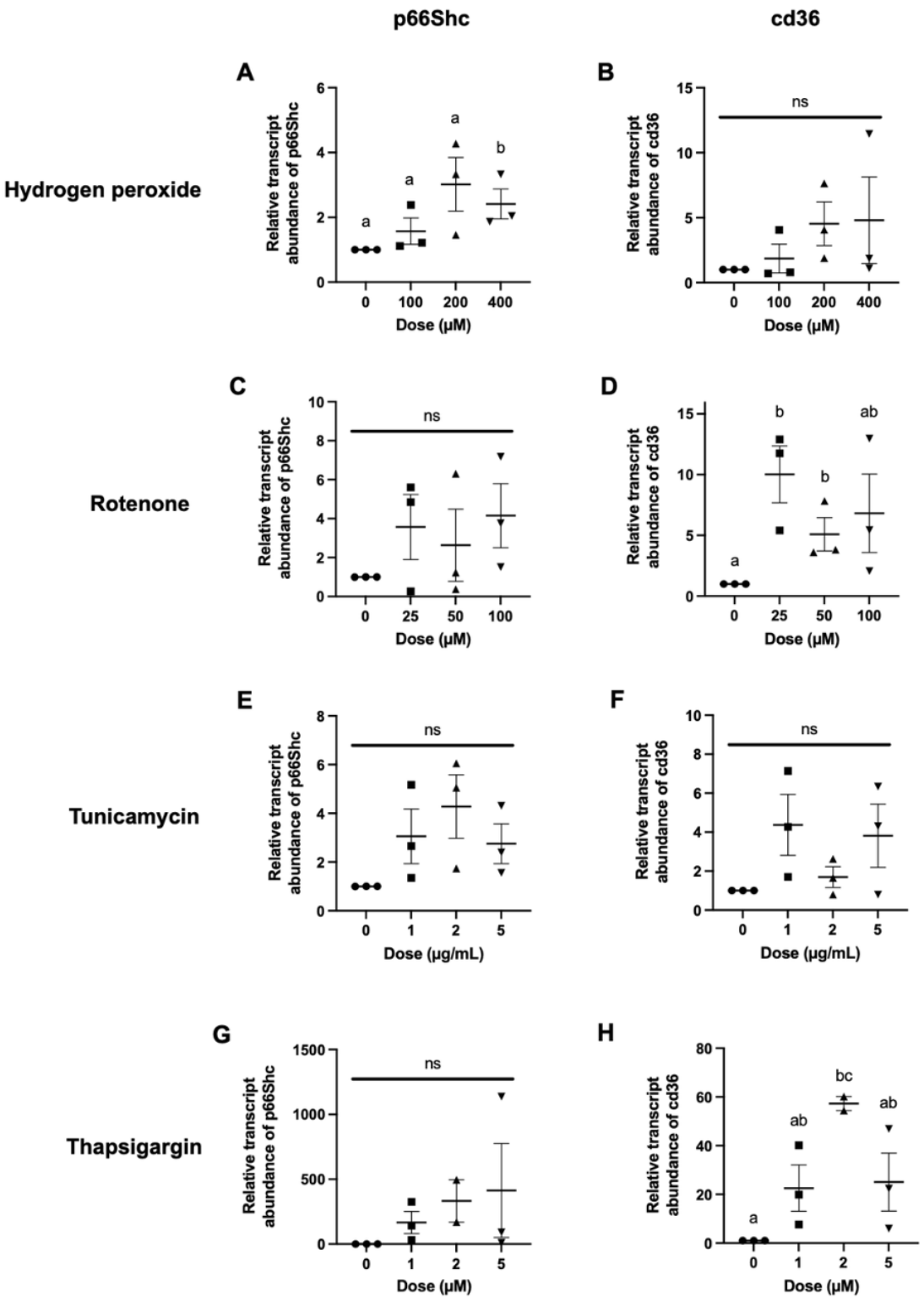
p66Shc
cd36
A
B
Hydrogen peroxide
Relative transcript abundance of p66Shc
Relative transcript abundance of cd36
Dose (μM)
a
a
a
b
ns
C
D
Rotenone
Dose (μM)
ns
a
b
b
ab
E
F
Tunicamycin
Dose (μg/mL)
ns
G
H
Thapsigargin
Dose (μM)
ns
a
ab
bc
ab

Figure 4-6. 24-hour exposure to rotenone or thapsigargin leads to increased expression of fatty acid translocase (CD36) in the HepG2 cell line.

Cells were plated in a six-well plate at 2 x 10^5 cells/well and let grown to 80% confluency. Cells were then treated for 24h with hydrogen peroxide (100, 200, 400 μM), rotenone (25, 50, 100 μM), tunicamycin (1, 2, 5 μg/mL), or thapsigargin (1, 2, 5 μM). RNA was collected from each group, followed by qRT-PCR analysis with the complementary cDNA template. Fold change difference of p66Shc (A, C, E, G) and CD36 (B, D, F, H) were expressed as means from three biological replicates normalized to the geometric means of GAPDH and β-Actin ± SEM.

are altered with tunicamycin treatment. These results suggest that p66Shc may be regulated at the level of protein translation rather than gene transcription (Oke *et al.*, 2019). At the same time, 24h treatment with 400 μM hydrogen peroxide led to increased p66Shc mRNA abundance (Figure 4-6A; $P < 0.05$). Exposure to 100 μM and 200 μM hydrogen peroxide for 24h did not significantly alter p66Shc levels (Figure 4-5A). Given that miR-29a is known to target fatty acid translocase (CD36), we also measured levels of CD36 transcript in each group of cells. Not surprisingly, treatment with hydrogen peroxide and tunicamycin did not induce changes in expression of *cd36* after 24h (Figures 4-6B, F). Conversely, cells treated with 25 μM and 50 μM rotenone exhibited increased transcript abundance of CD36 (Figures 4-6B; $P < 0.05$). This was surprising as we saw increased levels of miR-29 with these concentrations; however, it also suggests that hepatic lipid metabolism was altered through means other than miR-29. Treatment with 2 μM thapsigargin also produced increased levels of CD36 transcript (Figure 4-6H; $P < 0.0001$), which is interesting as this group also expressed increased miR-29b/c.

4.3.7 Adult male offspring subject to maternal protein restriction have decreased transcript abundance of fatty acid translocase (CD36) following postnatal catch-Up growth

Finally, we came full circle by measuring levels of CD36 in our *in vivo* liver samples taken from the MPR rodent model. We have previously reported that these offspring do not exhibit changes in p66Shc transcript at four months of age; however, LP2 offspring display increased p66Shc protein at this age (Oke *et al.*, 2019). As expected, there were no significant differences between any of the LP groups and the control group regarding p66Shc transcript levels (Figure 4-7A). Interestingly, LP3 offspring did exhibit increased p66Shc mRNA relative to LP1 and LP2 offspring (Figure 4-7A; $P < 0.05$). Given that these offspring also have increased miR-29 expression in the liver, we next quantified mRNA levels of CD36. Our analyses found that each LP group

showed decreased levels of CD36 mRNA relative to the control group (Figure 4-7B; $P < 0.05$), while there were no significant differences in CD36 levels between the LP groups.

4.4 Discussion

Here we demonstrate that induction of oxidative stress and ER stress via rotenone and thapsigargin, respectively, each lead to increased expression of hepatic miR-29. Induction of general oxidative stress via hydrogen peroxide does not influence miR-29 expression but does contribute to increased transcript levels of p66Shc at high concentrations. Conversely, inhibition of complex I with rotenone leads to increased miR-29 alongside increased CD36 mRNA. While induction of ER stress with tunicamycin does not affect miR-29 expression or that of its downstream targets, treatment with thapsigargin increases miR-29 in a dose-dependent manner with a simultaneous increase in CD36 transcript levels. Our previous studies demonstrate that miR-29 is upregulated in the livers of adult male protein-restricted offspring following catch-up growth (Sohi *et al.*, 2015). Here we show that these offspring also exhibit decreased mRNA expression of its constitutive target, CD36, regardless of whether catch-up growth had occurred. While we have further found that miR-203a-3p is altered in livers of IUGR offspring (Oke *et al.*, 2021), the current study identifies that this miRNA is poorly expressed in hepatocytes. Collectively, our results suggest that mitochondrial-induced oxidative stress and ER-stress may be important in regulating miR-29 expression as an underlying mechanism of hepatic lipid transport, particularly in hepatocytes of growth-restricted offspring.

Studies have demonstrated that growth of the fetal liver is compromised at the benefit of other organs; therefore, the liver stands to gain the most weight during postnatal life (Desai and Hales, 1997). This is concerning because postnatal catch-up growth is further detrimental to offspring metabolism, particularly in regard to lipid handling. Using multiple models of IUGR, we have shown that hepatic lipid biosynthesis and metabolism is greatly impacted following catch-up growth of the liver (Sohi *et al.*,

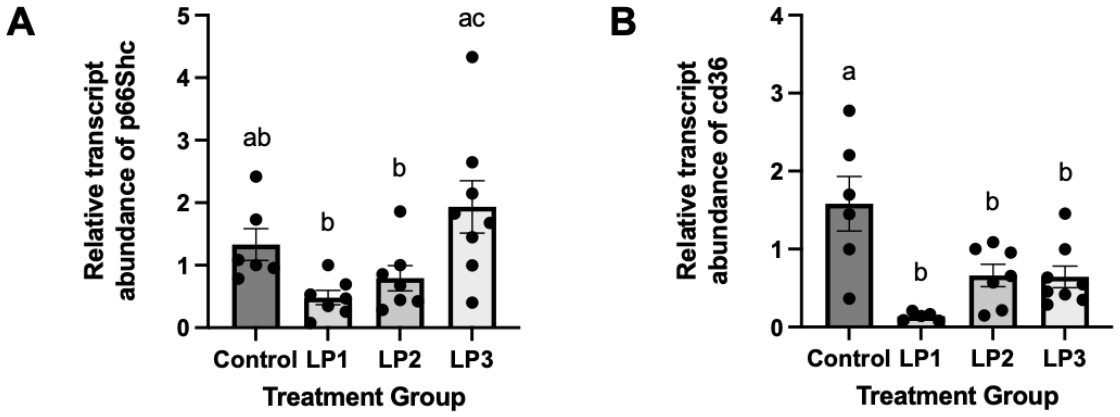

Figure 4-7. Maternal protein restriction leads to decreased transcript abundance of fatty acid translocase (CD36) in the livers of male offspring at four months of age.

Pregnant dams were subjected to an 8% protein diet during gestation, and pups were randomly assigned to one of three postnatal dietary regimens at birth: 8% protein diet throughout life (LP1), 8% protein diet until three weeks of age + 20% (control) diet after three weeks (LP2), or 20% protein diet throughout life (LP3). Following necropsy, RNA was isolated from livers of each group and qRT-PCR analysis was performed with the complementary cDNA. Fold change difference of (A) p66Shc and (B) CD36 were expressed as means normalized to the geometric means of GAPDH and β-Actin ± SEM (n=8/group). Transcript abundances for each individual group were analyzed using One Way ANOVA and Tukey's multiple comparisons test. Groups marked with different letters are significantly different ($P<0.05$).

2011; Ma *et al.*, 2013; Oke *et al.*, 2021). Our studies have further implicated oxidative stress, mitochondrial dysfunction, and ER stress in this relationship (Barra *et al.*, 2017; Oke *et al.*, 2019, 2021), each of which occur with various metabolic pathologies. While these forms of cell stress have been associated with altered levels of miR-29 and miR-203a-3p in the liver, it remains unknown whether they directly influence hepatic miRNA expression as a contributor to abnormal hepatic function. Here we show that oxidative stress and ER stress each give rise to elevated miR-29 expression in HepG2 cells, but only following treatment with rotenone or thapsigargin. Notably, the mechanism of action for these drugs is associated with mitochondrial function. While rotenone interferes directly with mitochondrial metabolism by inhibiting complex I of the ETC, the effects of thapsigargin are more indirect through interference with calcium signaling. Mitochondrial calcium influx occurs at mitochondrial-associated ER membranes (MAMs), which are sites along the ER that permit physical attachment to mitochondria (Hayashi *et al.*, 2009). Exposure to thapsigargin results in slow, sustained influx of calcium from the ER to mitochondria, leading to increased mitochondrial membrane potential and apoptosis (Deniaud *et al.*, 2008). Both rotenone and thapsigargin have been demonstrated to accelerate mitochondrial ROS production in HepG2 cells, leading to mitochondrial-induced apoptosis (Siddiqui *et al.*, 2013; Wang *et al.*, 2016). Given that we observed changes in miR-29 expression with exposure to these drugs, but not hydrogen peroxide or tunicamycin, it seems plausible that oxidative stress and mitochondrial dysfunction act as a signal in triggering changes to hepatic miR-29 expression. This is in alignment with our animal studies, as oxidative stress, mitochondrial dysfunction, and altered miR-29 expression occur together following catch-up growth (Sohi *et al.*, 2015; Oke *et al.*, 2019). That said, we have not directly measured ROS or performed functional assays of mitochondrial function in the current study. Completion of these experiments would be necessary to validate that oxidative stress and mitochondrial dysfunction are in fact occurring, which could be done easily through use of the Seahorse XF^{e}24 Extracellular Flux Analyzer (Agilent).

In recent years, miR-29 has been investigated as a possible non-invasive biomarker for the progression of various liver diseases. Not surprisingly, numerous

studies have identified miR-29 as being pleiotropic in nature. Many target genes of miR-29, including CD36, are involved in pathways affecting hepatic function (*e.g.,* nutrient transport and metabolism, oxidative stress, inflammation, and programmed cell death; Wilson *et al.*, 2016; Li *et al.*, 2019; Lin *et al.*, 2019; Wang *et al.*, 2019). Each of these pathways are known to promote non-alcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC), liver fibrosis, and cirrhosis (Lin *et al.*, 2020). However, there is no clear consensus on the directionality of miR-29 expression which impact hepatic function. We have previously found that male MPR offspring exhibit increased hepatic miR-29 expression following catch-up growth (Sohi *et al.*, 2015), while male offspring exposed to gestational Δ9-THC display decreased hepatic miR-29 (Oke *et al.*, 2021). Patients with advanced liver fibrosis and cirrhosis have been shown to exhibit decreased expression of miR-29 (Roderburg *et al.*, 2011), while high miR-29 expression is also believed to have a protective effect against NASH, fibrosis and cirrhosis in mice (Roderburg *et al.*, 2011; Lin *et al.*, 2019; Yang *et al.*, 2019). Notably, these studies suggest that miR-29 may mitigate oxidative stress in a murine model of NASH (Yang *et al.*, 2019). This could explain our *in vitro* data, as miR-29 may be upregulated to combat the negative effects of rotenone and thapsigargin. Since we saw changes in miR-29 expression at 24h but not 6h, it is plausible that initiation of this process does not occur immediately. This may also explain why CD36 transcript levels are elevated alongside miR-29 at 24h rather than being suppressed, while additional culture time may be necessary in allowing for mRNA degradation to occur. Instead, protein translation of CD36 may also be affected by miR-29, as miRNAs can act through mRNA degradation or translational repression. Ultimately, the contrasting expression patterns of miR-29 observed in our own studies and those of others may occur due to timing and severity of insult.

In addition to measuring levels of miR-29, we investigated the effects of oxidative stress and ER stress on miR-203a-3p expression. As stated, translation of the adaptor protein p66Shc is repressed by miR-203a-3p (Wang *et al.*, 2020). Elevated p66Shc is associated with oxidative stress, poor aerobic metabolism, and the accumulation of intracellular lipids (Migliaccio *et al.*, 1999; Orsini *et al.*, 2004; Giorgio *et al.*, 2005;

Berniakovich *et al.*, 2008; Tomita *et al.*, 2012); therefore, it's not surprising that p66Shc is elevated in livers affected by non-alcoholic steatohepatitis (NASH), NAFLD, and fibrosis (Tomita *et al.*, 2012; Zhao *et al.*, 2019). P66Shc knockdown in rodents with these diseases helps to alleviate oxidative damage and mitochondrial function, thereby preventing disease progression (Haga *et al.*, 2010; Zhao *et al.*, 2019). Additionally, targeting of p66Shc by miR-203a-3p specifically has been shown to attenuate liver injury in rodent models (Wang *et al.*, 2020). Here we saw a significant increase in hepatic miR-203a-3p abundance among MPR offspring that had been switched to a control diet at birth. Our previous studies have shown that these same offspring have decreased p66Shc protein abundance (Oke *et al.*, 2019), which may occur due to increased miR-203a-3p. While we had intended to examine miR-203a-3p expression alongside miR-29 in our *in vitro* model, we discovered that miR-203a-3p is poorly expressed in the HepG2 cell line. These data are in agreement with a previous study that found miR-203a-3p to be poorly expressed in HepG2 cells due to hypermethylation within its promoter region (Diao *et al.*, 2014). With this in mind, it seems that miR-203a-3p is expressed exclusively in hepatic stellate cells (HSCs). The liver is an incredibly heterogeneous organ made up of multiple cell types; however, hepatocytes and HSCs are the major two cell types that are involved in the progression of NASH and NAFLD. While hepatocytes are responsible for regulating hepatic metabolism, HSCs are mesenchymal cells that maintain extracellular matrix (ECM) in the liver (Si-Tayeb *et al.*, 2010). Chronic activation of HSCs leads to increased production of ECM proteins such as collagen, thereby leading to fibrosis of the liver (Si-Tayeb *et al.*, 2010). Our analysis of hepatic miR-203a-3p in MPR offspring utilized whole-liver samples; therefore, it's likely that we were able to detect this miRNA in the HSCs of each sample. Isolation of primary hepatocytes and HSCs from MPR offspring would be necessary to confirm this finding, as we could compare the expression of miR-203a-3p in each cell type. Overall, future *in vitro* studies using cell lines should avoid using HepG2 cells to study miR-203a-3p in any capacity.

In summary, this study demonstrates that expression of miR-29 in the liver may be regulated by oxidative stress and ER stress. We are the first to demonstrate that this relationship may be mediated by mitochondria, as miR-29 upregulation occurred exclusively following treatment with drugs known to influence mitochondrial function. This is in agreement with our studies of protein-restricted offspring, which have shown that catch-up

growth leads to increased miR-29 alongside oxidative stress, ER stress, and mitochondrial dysfunction in the adult liver. As mentioned, one limitation of our study is that we did not directly investigate levels or localization of ROS *in vitro* or in hepatic tissue *ex vivo*. Additional studies are necessary to determine if ROS production is heightened in cells with elevated miR-29 abundance, along with supplementary measures of mitochondrial function. Moreover, it would be interesting to assess whether increased abundance of miR-29 leads to accumulation of intracellular lipids. This would further support the involvement of miR-29 as an underlying mechanism of NASH and NAFLD. Given that we saw increased expression of CD36 at 24h *in vitro*, long-term studies could also determine if CD36 levels become repressed by miR-29 as seen in adult protein-restricted offspring. Overall, these data are important not only in understanding the progression of metabolic disease in IUGR offspring, but also the general mechanisms underlying health and disease across all populations.

4.6 References

Barra NG, Lisyansky M, Vanduzer TA, Raha S, Holloway AC and Hardy DB (2017) Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase and impaired mitochondrial function in male rat offspring. *Journal of Applied Toxicology* **37** 1517–1526.

Berniakovich I, Trinei M, Stendardo M, Migliaccio E, Minucci S, Bernardi P, Pelicci PG and Giorgio M (2008) p66Shc-generated oxidative signal promotes fat accumulation. *Journal of Biological Chemistry* **283** 34283–34293.

Chamson-Reig A, Thyssen SM, Hill DJ and Arany E (2009) Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. *Experimental Biology and Medicine (Maywood, N.J.)* **234** 1425–1436.

Clarkson PM and Thompson HS (2000) Antioxidants: what role do they play in physical activity and health? *The American Journal of Clinical Nutrition* **72** 637S-646S.

Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C and Brenner C (2008) Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene* **27** 285–299.

Desai M and Hales CN (1997) Role of fetal and infant growth in programming metabolism in later life. *Biological Reviews of the Cambridge Philosophical Society* **72** 329–348.

Diao Y, Guo X, Jiang L, Wang G, Zhang C, Wan J, Jin Y and Wu Z (2014) miR-203, a Tumor Suppressor Frequently Down-regulated by Promoter Hypermethylation in Rhabdomyosarcoma *. *Journal of Biological Chemistry* **289** 529–539.

Firmin S, Elmhiri G, Crepin D, Souidi M, Taouis M and Abdennebi-Najar L (2018) Formula derived Maillard reaction products in post-weaning intrauterine growth-restricted piglets induce developmental programming of hepatic oxidative stress independently of microRNA-21 and microRNA-155. *Journal of Developmental Origins of Health and Disease* **9** 566–572.

Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M *et al.* (2005) Electron transfer between cytochrome c and p66Shcgenerates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*.

Greally JM (2018) A user's guide to the ambiguous word 'epigenetics'. *Nature Reviews Molecular Cell Biology* **19** 207–208.

Gruppuso PA and Sanders JA (2016) Regulation of liver development: implications for liver biology across the lifespan. *Journal of Molecular Endocrinology* **56** R115–R125.

Haga S, Morita N, Irani K, Fujiyoshi M, Ogino T, Ozawa T and Ozaki M (2010) p66Shc has a pivotal function in impaired liver regeneration in aged mice by a redox-dependent mechanism. *Laboratory Investigation* **90** 1718–1726.

Hammond SM (2015) An overview of microRNAs. *Advanced Drug Delivery Reviews* **87** 3–14.

Hayashi T, Rizzuto R, Hajnoczky G and Su T-P (2009) MAM: more than just a housekeeper. *Trends in Cell Biology* **19** 81–88.

Jiang J, Yu S, Jiang Z, Liang C, Yu W, Li J, Du X, Wang H, Gao X and Wang X (2014) N-Acetyl-Serotonin Protects HepG2 Cells from Oxidative Stress Injury Induced by Hydrogen Peroxide. *Oxidative Medicine and Cellular Longevity* **2014** e310504.

Lei L, Zhu Y, Gao W, Du X, Zhang M, Peng Z, Fu S, Li X, Zhe W, Li X *et al.* (2016) Alpha-lipoic acid attenuates endoplasmic reticulum stress-induced insulin resistance by improving mitochondrial function in HepG2 cells. *Cellular Signalling* **28** 1441–1450.

Li Y, Yang P, Zhao L, Chen Y, Zhang X, Zeng S, Wei L, Varghese Z, Moorhead JF, Chen Y *et al.* (2019) CD36 plays a negative role in the regulation of lipophagy in hepatocytes through an AMPK-dependent pathway. *Journal of Lipid Research* **60** 844–855.

Lin Y, Liu X, Cheng Y, Yang J, Huo Y and Zhang C (2009) Involvement of MicroRNAs in Hydrogen Peroxide-mediated Gene Regulation and Cellular Injury Response in Vascular Smooth Muscle Cells. *Journal of Biological Chemistry* **284** 7903–7913.

Lin H-Y, Wang F-S, Yang Y-L and Huang Y-H (2019) MicroRNA-29a Suppresses CD36 to Ameliorate High Fat Diet-Induced Steatohepatitis and Liver Fibrosis in Mice. *Cells* **8**.

Lin H-Y, Yang Y-L, Wang P-W, Wang F-S and Huang Y-H (2020) The Emerging Role of MicroRNAs in NAFLD: Highlight of MicroRNA-29a in Modulating Oxidative Stress, Inflammation, and Beyond. *Cells* **9** 1041.

Ma N, Nicholson CJ, Wong M, Holloway AC and Hardy DB (2013) Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicology and Applied Pharmacology* **275** 1–11.

Maruyama M, Matsunaga T, Harada E and Ohmori S (2007) Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biological & Pharmaceutical Bulletin* **30** 2091–2097.

Migliaccio E, Giogio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L and Pelicci PG (1999) The p66(shc) adaptor protein controls oxidative stress response and life span in mammals. *Nature*.

Naem E, Haas MJ, Wong NCW and Mooradian AD (2013) Endoplasmic reticulum stress in HepG2 cells inhibits apolipoprotein A-I secretion. *Life Sciences* **92** 72–80.

Oke SL, Sohi G and Hardy DB (2019) Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. *Reproduction (Cambridge, England)* **159** 27–39.

Oke SL, Lee K, Papp R, Laviolette SR and Hardy DB (2021) In Utero Exposure to Δ9-Tetrahydrocannabinol Leads to Postnatal Catch-Up Growth and Dysmetabolism in the Adult Rat Liver. *International Journal of Molecular Sciences* **22** 7502.

Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M *et al.* (2004) The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. *Journal of Biological Chemistry*.

Radad K, Rausch W-D and Gille G (2006) Rotenone induces cell death in primary dopaminergic culture by increasing ROS production and inhibiting mitochondrial respiration. *Neurochemistry International* **49** 379–386.

Roderburg C, Urban G-W, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M *et al.* (2011) Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology (Baltimore, Md.)* **53** 209–218.

Saget S, Cong R, Decourtye L, Endale M-L, Martinerie L, Girardet C, Perret C, Clemessy M, Leneuve P, Dinard L *et al.* (2020) Changes in circulating miRNA19a-3p precede insulin resistance programmed by intra-uterine growth retardation in mice. *Molecular Metabolism* **42** 101083.

Siddiqui MA, Ahmad J, Farshori NN, Saquib Q, Jahan S, Kashyap MP, Ahamed M, Musarrat J and Al-Khedhairy AA (2013) Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. *Molecular and Cellular Biochemistry* **384** 59–69.

Si-Tayeb K, Lemaigre FP and Duncan SA (2010) Organogenesis and Development of the Liver. *Developmental Cell* **18** 175–189.

Sohi G, Marchand K, Revesz A, Arany E and Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. *Molecular Endocrinology (Baltimore, Md.)* **25** 785–798.

Sohi G, Revesz A and Hardy DB (2013) Nutritional mismatch in postnatal life of low birth weight rat offspring leads to increased phosphorylation of hepatic eukaryotic initiation factor 2 alpha in adulthood. *Metabolism: Clinical and Experimental*.

Sohi G, Revesz A, Ramkumar J and Hardy DB (2015) Higher Hepatic miR-29 Expression in Undernourished Male Rats During the Postnatal Period Targets the Long-Term Repression of IGF-1. *Endocrinology* **156** 3069–3076.

Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T *et al.* (2012) P53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. *Journal of Hepatology* **57** 837–843.

Wang C, Li T, Tang S, Zhao D, Zhang C, Zhang S, Deng S, Zhou Y and Xiao X (2016) Thapsigargin induces apoptosis when autophagy is inhibited in HepG2 cells and both processes are regulated by ROS-dependent pathway. *Environmental Toxicology and Pharmacology* **41** 167–179.

Wang Y, Koch M, di Giuseppe R, Evans K, Borggrefe J, Nöthlings U, Handberg A, Jensen MK and Lieb W (2019) Associations of Plasma CD36 and Body Fat Distribution. *The Journal of Clinical Endocrinology & Metabolism* **104** 4016–4023.

Wang Z, Zhao Y, Zhao H, Zhou J, Feng D, Tang F, Li Y, Lv L, Chen Z, Ma X *et al.* (2020) Inhibition of p66Shc Oxidative Signaling via CA-Induced Upregulation of miR-203a-3p Alleviates Liver Fibrosis Progression. *Molecular Therapy - Nucleic Acids* **21** 751–763.

Wilson CG, Tran JL, Erion DM, Vera NB, Febbraio M and Weiss EJ (2016) Hepatocyte-Specific Disruption of CD36 Attenuates Fatty Liver and Improves Insulin Sensitivity in HFD-Fed Mice. *Endocrinology* **157** 570–585.

Yang Y-L, Kuo H-C, Wang F-S and Huang Y-H (2019) MicroRNA-29a Disrupts DNMT3b to Ameliorate Diet-Induced Non-Alcoholic Steatohepatitis in Mice. *International Journal of Molecular Sciences* **20**.

Zhang L, Lu Q and Chang C (2020) Epigenetics in Health and Disease. *Advances in Experimental Medicine and Biology* **1253** 3–55.

Zhao Y, Wang Z, Feng D, Zhao H, Lin M, Hu Y, Zhang N, Lv L, Gao Z, Zhai X *et al.* (2019) p66Shc Contributes to Liver Fibrosis through the Regulation of Mitochondrial Reactive Oxygen Species. *Theranostics* **9** 1510–1522.

Zinkhan EK, Yu B and McKnight R (2019) Uteroplacental Insufficiency Impairs Cholesterol Elimination in Adult Female Growth-Restricted Rat Offspring Fed a High-Fat Diet. *Reproductive Sciences (Thousand Oaks, Calif.)* **26** 1173–1180.

Chapter V

5 Discussion and Conclusions

5.1 Discussion and Significance of Research

The Developmental Origins of Health and Disease (DOHaD) hypothesis outlines a relationship between birth weight and long-term metabolic health. Epidemiological and animal studies have demonstrated that the fetal liver is particularly compromised due to intrauterine growth restriction (IUGR), while hepatic postnatal catch-up growth exacerbates the hyperlipidemia observed in adult life. While these studies have highlighted the consequences of postnatal catch-up growth, the underlying molecular mechanisms remain poorly understood. This includes mitochondrial dysfunction, which has been shown to occur among IUGR offspring in postnatal life (Park *et al.*, 2003; Moraes *et al.*, 2014; Barra *et al.*, 2017; Woodman *et al.*, 2018). Mitochondria are energy-producing organelles that are essential in regulating metabolism, but cellular stress (*e.g.,* oxidative stress and ER stress) can interfere with mitochondrial function and produce a variety of metabolic pathologies. Epigenetic mechanisms such as microRNAs have also been implicated in IUGR; therefore, it is plausible that epigenetic regulation of gene expression may influence any mitochondrial dysfunction observed.

The overall hypothesis of my thesis is that independent of gestational insult, postnatal catch-up growth leads to hepatic mitochondrial dysfunction in IUGR offspring, culminating in hyperlipidemia. The main findings that support this hypothesis are:

1) Postnatal catch-up growth disrupts hepatic lipid biosynthesis and mitochondrial function across multiple models of IUGR, particularly in a sex-specific manner.
2) Postnatal catch-up growth has an indirect effect on the regulation of miR-29 expression due to impairment of mitochondrial function.

Overall, my work provides insight into the molecular consequences of catch-up growth in the IUGR liver. These studies advance our understanding of mitochondrial dysfunction in response to catch-up growth, along with the basic molecular mechanisms by which hepatic miR-29 expression is regulated. We have demonstrated that IUGR in combination with catch-up growth is detrimental to hepatic lipid handling, and that mitochondria are particularly susceptible to cellular stress following the completion of hepatic differentiation. Taken together, these results could lead to the development of therapeutic strategies in clinical practice during both the prenatal and postnatal period.

5.1.1 Postnatal catch-up growth disrupts hepatic biosynthesis and mitochondrial function in a sex-specific manner

Epidemiological studies indicate that low birth weight offspring with catch-up growth are at high risk for hepatic pathologies in adulthood, including non-alcoholic fatty liver disease (NAFLD) and diabetes (Ravelli *et al.*, 1998; Nobili *et al.*, 2007; Suomela *et al.*, 2016). In Chapters II and III, I investigated the contribution of catch-up growth upon hepatic mitochondrial function as an underlying cause of hyperlipidemia in IUGR offspring. To do this, we investigated two different rodent models of IUGR: (1) the maternal protein restriction (MPR) model of undernutrition, and (2) gestational exposure to Δ9-tetrahydrocannabinol (Δ9-THC).

5.1.1.1 Chapter II: Outcomes

In Chapter II, I performed molecular techniques (*i.e.,* western immunoblotting and qRT-PCR) to investigate markers of mitochondrial function in the livers of male offspring born from the MPR model. In this model, pregnant dams are randomly assigned to a normal (20%) protein diet, or a low (8%; LP) protein diet. Offspring born to LP dams are further assigned to one of three dietary regimens: (1) LP for all life (LP1), (2) LP until three weeks of age + normal protein for the remainder of life (LP2), or (3) normal protein for all life (LP3). This model beneficial in that it provides an in-depth look at how the liver responds to catch-up growth both before and after it is fully

developed. While the human liver is fully developed at term (*i.e.,* gestational week 37), the rodent liver does not complete hepatic differentiation until three weeks of age (Gruppuso and Sanders, 2016). By switching protein-restricted offspring to a normal protein diet at different developmental time points, we can directly influence the timing of catch-up growth in rodents such that it reflects the human scenario. For example, administration of a low protein diet until three weeks of age in rodents is physiologically relevant to human development, as this marks the end of liver development in rodents.

Our laboratory has previously demonstrated that male LP2 offspring display hypercholesterolemia at four months of age (Sohi *et al.*, 2011). In Chapter II, I discovered that these offspring further exhibit hepatic oxidative stress and poor aerobic metabolism. At four months of age, livers from LP2 males have elevated markers of oxidative stress (increased p66Shc, 4-hydroxynonenol, and superoxide dismutase; decreased catalase) and aberrant makers of aerobic metabolism (increased p66Shc, phosphorylated pyruvate dehydrogenase [Ser232], lactate dehydrogenase subunit alpha; decreased complex II, citrate synthase, and mitochondrial transcription factor A). Again, these changes occur exclusively in adult life among male offspring that experienced catch-up growth following the perinatal period (*i.e.,* the completion of hepatic differentiation). The impairments in mitochondrial function may also underlie some of the hepatic endocrine deficits (*e.g.,* decreased IGF-1, decreased pAkt [Ser473]) and aberrant drug metabolism (*e.g.,* increased Cyp3A and Cyp2c11 activity) exhibited exclusively by LP2 offspring (Sohi *et al.*, 2011, 2013, 2014, 2015). Conversely, LP1 and LP3 offspring do not display oxidative stress or mitochondrial dysfunction at three weeks of age or during adult life. This is consistent with our previous studies and those of others, which suggest that LP1 and LP3 offspring are metabolically healthy (Hales *et al.*, 1996; Sohi *et al.*, 2011). My data is further supportive of the DOHaD hypothesis, which states that a 'mismatch' in nutrient availability gives rise to alterations in fetal programming such that long-term metabolic function is negatively impacted (Hales and Barker, 2001). Because LP1 offspring remain on a LP diet during postnatal life, they do not experience this mismatch and are spared the adverse molecular changes. Although LP3 offspring do undergo a switch in protein content, it is possible that dietary intervention prior to the point of

terminal differentiation can rescue the liver from the detrimental effects of IUGR with catch-up growth. Taken together, these data suggest that the IUGR liver is particularly vulnerable to catch-up growth after it is fully developed.

5.1.1.2 Chapter II: Clinical significance

From a clinical perspective, the results observed in the MPR model are highly informative as they reinforce that care should be taken to avoid postnatal catch-up growth. Again, many human cohorts have demonstrated that postnatal catch-up growth in low birth weight offspring leads to dyslipidemia, as in a study by Singhal *et al.* on preterm infants who were formula-fed after birth (Singhal *et al.*, 2004). As adolescents, individuals who were fed nutrient-dense formula exhibited high ratios of low-density lipoprotein (LDL) to high-density lipoprotein (HDL), indicative of increased risk for cardiovascular disease and stroke (Singhal *et al.*, 2004). They further displayed high serum concentrations of C-reactive protein (CRP), a measure of inflammation that is associated with atherosclerosis (Singhal *et al.*, 2004). Interestingly, preterm infants who were fed banked breastmilk had lower levels of LDL to HDL and CRP relative to those who were formula-fed (Singhal *et al.*, 2004). While these results do highlight the importance of breastfeeding, they more importantly elucidate the significance of catch-up growth on long-term metabolism. Here I show that maintenance on a LP diet during the entire postnatal period (LP1) does not lead to mitochondrial dysfunction in MPR offspring, while our previous work has found these offspring to have normal hepatic function as adults (Sohi *et al.*, 2011; Oke *et al.*, 2019). Each of these studies agree with the main principle of DOHaD, as the mismatch in nutrient availability between prenatal and postnatal life leads to hepatic hyperlipidemia. This information could influence how healthcare professionals counsel patients both during and after pregnancy, as they should emphasize the importance of a balanced prenatal and postnatal diet in optimizing health throughout the lifespan. Moreover, the identification of mitochondrial targets could lead to therapeutic interventions during postnatal life as a means of mitigating the effects of catch-up growth.

5.1.1.3 Chapter III: Outcomes

In Chapter III, I used an alternative model of IUGR to validate my findings from the MPR model. That is, I sought to confirm that IUGR in combination with postnatal catch-up growth has a negative effect on hepatic lipid metabolism and mitochondrial function, irrespective of the type of gestational insult. In our model of gestational Δ9-THC exposure, offspring exhibit symmetrical IUGR followed by whole body and hepatic catch-up growth by three weeks of age (Natale *et al.*, 2020). Here I demonstrate that male Δ9-THC-exposed offspring exhibit hypertriglyceridemia in the liver at six months of age, while hepatic triglyceride levels were unchanged in female offspring and three-week old male offspring. By three weeks, male offspring have an increased abundance of enzymes involved in lipid transport and biosynthesis (*e.g.,* fatty acid binding protein 1, acetyl-CoA carboxylase alpha, stearoyl-CoA desaturase, and diacylglycerol acyltransferase). This occurred alongside increased hepatic 4-hydroxynonenol, p66Shc, complex I, and complex III at three weeks, suggesting that oxidative stress and impaired aerobic metabolism had again occurred with catch-up growth in this model of IUGR. Since hepatic triglyceride levels were not yet affected at this time point, I propose that this early incidence of mitochondrial dysregulation instigates the increased hepatic *de novo* lipogenesis at six months. Adult male Δ9-THC-exposed offspring continued to exhibit oxidative stress and mitochondrial dysfunction (increased p66Shc, complex I, III and V; decreased SOD1) at six months, which could be both a contributing factor and a consequence of hepatic hyperlipidemia. To determine if catch-up growth is indeed the primary causative factor, it would be interesting to repeat these analyses in Δ9-THC-exposed offspring at birth prior to catch-up growth. This would provide us with a greater overall understanding of the direct effects of specific gestational insults independent of catch-up growth.

5.1.1.4 Chapter III: Clinical significance

In addition to validating the detriments of catch-up growth, the information gained here from our gestational Δ9-THC rodent model illustrates the hepatic consequences of

maternal cannabis use during pregnancy. Despite the legalization of cannabis in Canada and select American states, very little is known about the safety of maternal cannabis use with respect to its effects on fetal development and postnatal metabolism. Not surprisingly, our laboratory's research has identified that gestational exposure to Δ9-THC compromises fetal growth (Natale *et al.*, 2020), while my postnatal studies have expanded upon its consequences on offspring liver function and obesity in adult life (Oke *et al.*, 2021). Because dyslipidemia and obesity are risk factors for type II diabetes and cardiovascular disease, my data may explain the glucose intolerance and impaired cardiac function that is also observed in Δ9-THC-exposed offspring (Gillies *et al.*, 2020; Lee *et al.*, 2021). These data may also provide some context to the human scenario, as a recent study based out of Colorado established that fetal exposure to cannabis leads to a 2.6% increase in adiposity (*i.e.,* fat mass) and elevated fasting glucose and insulin levels during childhood (mean age=4.7 years; Moore *et al.*, 2022). Collectively, data from this model could again influence how healthcare professionals counsel their patients during pregnancy, and it may encourage patients to deter cannabis use during pregnancy in protection of the fetus.

5.1.1.5 The sex-specific effects of postnatal catch-up growth

Overall, these data indicate that male offspring with catch-up growth are more susceptible to hepatic hyperlipidemia and mitochondrial dysfunction than female offspring. In both the MPR and Δ9-THC models, adult male offspring experience hepatic hyperlipidemia while females are unaffected. I further demonstrated that oxidative stress and mitochondrial dysfunction occur exclusively in male offspring from both models (Table 5-1), suggesting that these forms of cell stress may contribute to the male-specific progression of hyperlipidemia (Sohi *et al.*, 2011; Oke *et al.*, 2019). This is in alignment with human studies of healthy men and women, whereby men have higher rates of *de novo* lipogenesis and decreased fatty acid oxidation (Tran *et al.*, 2010; Pramfalk *et al.*, 2015). Of note, my studies determined that hepatic p66Shc protein levels were increased in male offspring with catch-up growth from both models, suggesting that this protein may have an important role in establishing the sex-specific mitochondrial dysfunction of

Table 5-1. Summary of protein markers of oxidative stress and mitochondrial dysfunction analyzed in offspring born from rodent models of Maternal Protein Restriction and Gestational Δ9-THC.

Protein Marker	Maternal Protein Restriction	Gestational Δ9-THC
P66Shc	↑	↑
Pin1	↑	n.s.
4-HNE	↑	n.s.
SOD1	↑	↓
SOD2	↑	n.s.
Catalase	↓	n.s.
p-PDH	↑	n.s.
LDHa	↑	n.s.
Citrate synthase	↓	n.s.
Electron transport chain	↓ complex II	↑ complexes I, III, V
TFAM	↓	n.s.

Protein markers labelled with ↑ were significant increased, while those with ↓ were significantly decreased ($P < 0.05$). n.s.= non-significant.

IUGR offspring. It has also been proposed that differences in androgenic hormone levels (*i.e.,* estrogen and testosterone) are protective against the development of liver pathologies in females compared to males (Szafran and Smielak-Korombel, 1998; Ozaki *et al.*, 2001). Studies have demonstrated that estrogen actually potentiates mitochondrial ROS production (Felty *et al.*, 2005; Tian *et al.*, 2016), but this has been disputed by studies of C57Bl/6J mice showing that males and females exhibit similar production of mitochondrial ROS and lifespan despite females having higher estrogen levels (Sanz *et al.*, 2007). It has also been shown that estrogen levels are not altered in male or female MPR offspring in adult life, while similar data were observed in Δ9-THC-exposed adult females (Chamson-Reig *et al.*, 2009; Gillies *et al.*, 2020). Alternatively, activation of estrogen receptors has been shown to maintain lipid and glucose metabolism in male mice (Qiu *et al.*, 2017). Given that estrogen receptors have been identified in mitochondria (Yager and Chen, 2007), it is possible that activation of these receptors by estrogen leads to altered transcription of mtDNA. Many mitochondrial proteins involved in aerobic metabolism are mitochondrial-encoded; therefore, their expression may be affected by estrogen receptor activation and subsequently influence lipid handling. Given that male MPR offspring have been shown to exhibit decreased serum testosterone levels at four months of age due to enhanced metabolic activity of hepatic Cyp3A and Cyp2c11 (Chamson-Reig *et al.*, 2009; Sohi *et al.*, 2014), it's plausible that testosterone signaling may have a role in promoting mitochondrial dysfunction instead of estrogen.

5.1.2 Postnatal catch-up growth likely has an indirect effect of the regulation of miR-29 expression through interference with mitochondrial function

In Chapters II and III, oxidative stress and mitochondrial dysfunction were investigated as major drivers of hepatic hyperlipidemia in IUGR offspring with catch-up growth. Importantly, our laboratory has shown that MPR offspring exclusively with catch-up growth also exhibit endoplasmic reticulum (ER) stress (*i.e.*, higher peIF2α, Grp94) in adult life (Sohi *et al.*, 2013). MPR offspring with catch-up growth also have

aberrant expression of hepatic miRNAs, including miR-29a/b/c (Sohi *et al.*, 2015). However, it remains unknown whether this occurs due to oxidative stress, ER stress, or mitochondrial dysfunction. In Chapters III and IV, I characterized the expression of miR-29 and miR-203a-3p in male IUGR offspring from the MPR and Δ9-THC models. Suppression of miR-29 and miR-203a-3p have been shown to contribute to dyslipidemia through elevation of their targets, including fatty acid translocase (CD36) and p66Shc, respectively (Roderburg *et al.*, 2011; Tomita *et al.*, 2012; Yang *et al.*, 2019; Zhao *et al.*, 2019; Wang *et al.*, 2020). As outlined in Chapters I and IV, CD36 permits transmembrane NEFA transport; therefore, increased levels of CD36 have been associated with hepatic lipid accumulation and poor lipid metabolism (Lin *et al.*, 2019). Transgenic mice with constitutive expression of hepatic miR-29 do exhibit decreased levels of CD36, but this is counteracted by feeding of a high fat diet and suggests that CD36 expression is more responsive to nutrient availability rather than miR-29 expression (Lin *et al.*, 2019). Similarly, elevated p66Shc leads to increased lipid accumulation and oxidative stress (Berniakovich *et al.*, 2008; Wang *et al.*, 2020). While we have already shown that hepatic miR-29 is increased in MPR offspring with catch-up growth (Sohi *et al.*, 2015), analyses of miR-203a-3p abundance showed that only LP3 offspring have increased miR-203a-3p at four months (Chapter IV**)**. Conversely, hepatic expression of both miR-29 and miR-203a-3p was decreased in Δ9-THC-exposed males (Chapter III). Although there are discrepancies in miR expression between these two models, this may be attributed to heterogeneity of the liver. MiR-29 and miR-203a-3p have vastly different expression profiles across liver cell types (*e.g.,* hepatocytes versus HSCs; Roderburg *et al.*, 2011; Kogure *et al.*, 2012; Diao *et al.*, 2014), so it is possible that different cell populations are affected by catch-up growth and cell stress in these two models of IUGR.

In Chapter IV, I studied the direct effects of oxidative stress and ER stress on miR-29 and miR-203a-3p *in vitro.* We have previously observed changes in hepatic miRNAs among adult male MPR offspring with catch-up growth (Sohi *et al.*, 2015); however, it is unclear from this *in vivo* data whether oxidative stress or ER stress have impact on miRNA expression. *In vitro* studies were therefore necessary to determine if

miR-29 and miR-203a-3p are affected by these forms of cell stress, and to investigate which type of cell stress has greater influence on miRNA expression. To do this, I used pharmacological inducers of oxidative stress (hydrogen peroxide and rotenone) and ER stress (tunicamycin and thapsigargin) in HepG2 cells, followed by quantification of miRNAs and their target mRNAs via qRT-PCR. The human male HepG2 cell line is an appropriate *in vitro* model for studying the neonatal liver, as these cells produce and secrete proteins that are exclusive to fetal hepatocytes (Maruyama *et al.*, 2007). While 6h exposure to each drug did not elicit changes in miRNA expression, miR-29 was increased in response to treatment with rotenone and thapsigargin for 24h. Surprisingly, cells treated with rotenone and thapsigargin for 24h further exhibited heightened transcript abundance of CD36, indicating that lipid transport may be increased and lead to intracellular lipid accumulation. Given that CD36 is a target of miR-29, we were surprised to see that rotenone and thapsigargin increased CD36; however, it is conceivable that oxidative stress may instigate other transcriptional and/or epigenetic changes enhancing CD36 transcription. My analyses of miR-203a-3p expression in HepG2 cells revealed that this miRNA is poorly expressed in hepatocytes; therefore, alternative cell lines should be used to examine miR-203a-3p in the liver. Previous studies of hepatic miR-203a-3p have focused on its expression in HSCs of the diseased liver and the human LX-2 HSC line, suggesting that miR-203a-3p may have a role in regulating extracellular matrix production rather than metabolism (Wang *et al.*, 2020).

My *in vitro* experiments further demonstrated that miR-29 and CD36 are increased in response to treatment with rotenone and thapsigargin, but not hydrogen peroxide or tunicamycin. Importantly, rotenone and thapsigargin are known to influence mitochondrial function, albeit through different mechanisms. Rotenone is an inhibitor of complex I of the electron transport chain (Siddiqui *et al.*, 2013), while thapsigargin acts through interference with calcium signaling (Deniaud *et al.*, 2008; Naem *et al.*, 2013). Both drugs have been shown to increase mitochondrial production of ROS in HepG2 cells, ultimately resulting in apoptosis (Siddiqui *et al.*, 2013; Wang *et al.*, 2016). It is for this reason that the evidence for miR-29 being directly increased by ER stress is inconclusive, as it is not known whether thapsigargin acted through oxidative stress or

ER stress. Nonetheless, since miR-29 expression was increased exclusively following treatment with these two drugs, it seems plausible that miR-29 expression is primarily regulated by mitochondrial dysfunction (Figure 5-1). Taken together, these data provide further evidence for the role for mitochondrial dysfunction in determining the fate of hepatic lipids.

5.2 Limitations

While this thesis has greatly advanced our understanding of DOHaD, it also contains several limitations that should be addressed. Throughout the entirety of this thesis, my main objective was to characterize the role of postnatal catch-up growth across various models of IUGR. In the MPR model, the numerous timepoints of dietary intervention (*i.e.,* LP1 vs. LP2 vs. LP3) allow for analyses of mitochondrial markers in offspring both with and without catch-up growth. However, the studies performed as part of Chapter II only included male offspring. This was intentional to avoid any confounding effects of the female estrus cycle, and because female MPR offspring do not exhibit hepatic hyperlipidemia in adult life (Sohi *et al.*, 2011; Oke *et al.*, 2019). That said, livers from female offspring should be further analyzed to conclude that these mitochondrial deficits are truly sex specific. Additionally, in Chapter III, I performed experiments using livers of Δ9-THC-exposed offspring at three weeks and six months of age only. Given that catch-up growth is complete by three weeks in these offspring, my studies have quantified markers of mitochondrial dysfunction and hepatic lipid metabolism exclusively after catch-up growth. To confirm that catch-up growth is indeed responsible for the mitochondrial dysfunction and hepatic hyperlipidemia observed in this model, I would need to perform additional experiments in newborn offspring at postnatal day one, prior to the initiation of catch-up growth. Should these protein markers and hepatic triglyceride levels be unchanged at birth, I could conclude that catch-up growth is at fault for the mitochondrial and metabolic deficits observed following gestational exposure to Δ9-THC.

In Chapters II and III, I performed molecular techniques on *ex vivo*, whole liver samples from our rodent models of IUGR. I found that adult male offspring from both models exhibit oxidative stress and mitochondrial dysfunction following catch-up growth, and I used the HepG2 cell line to assess the direct effects of cell stress on epigenetic mechanisms. While these data are informative and supportive of the DOHaD hypothesis, it is difficult to discern the effects of catch-up growth on individual cell types in the liver. As mentioned previously, the liver is a heterogeneous organ with cell types of varying functions. Each hepatic cell type has a unique genetic (and epigenetic) profile that regulates various developmental pathways, as seen in my *in vitro* studies of miR-29 and miR-203a-3p in the HepG2 cell line. Therefore, it is likely that each cell type in the IUGR liver is affected differently by catch-up growth. To address this, multiple cell lines representing the various types of liver cells are necessary to distinguish the effects of cell stress on hepatic miRNA expression. For example, the LX2 human immortalized cell line could be used as a model for HSCs, as done in studies by Wang *et al.* (Wang *et al.*, 2020). Additionally, while we have established that miR-29 expression is influenced by mitochondrial dysfunction, it remains unanswered if aberrant miRNA expression can also induce mitochondrial dysfunction. This classic 'chicken and egg' scenario could be addressed through transfection of miR-29 mimics and inhibitors, followed by analyses of the same mRNA and protein markers examined in Chapters II and III.

5.3 Future Directions

This thesis investigated the role of postnatal catch-up growth on mitochondrial function in growth-restricted offspring. We implemented two rodent models of IUGR to interpret the molecular mechanisms by which mitochondrial dysfunction leads to hepatic hyperlipidemia in adult life. Supplementary *in vitro* studies allowed us to further elucidate these mechanisms, but future studies are warranted to better understand the effects of catch-up growth on mitochondrial function in the liver.

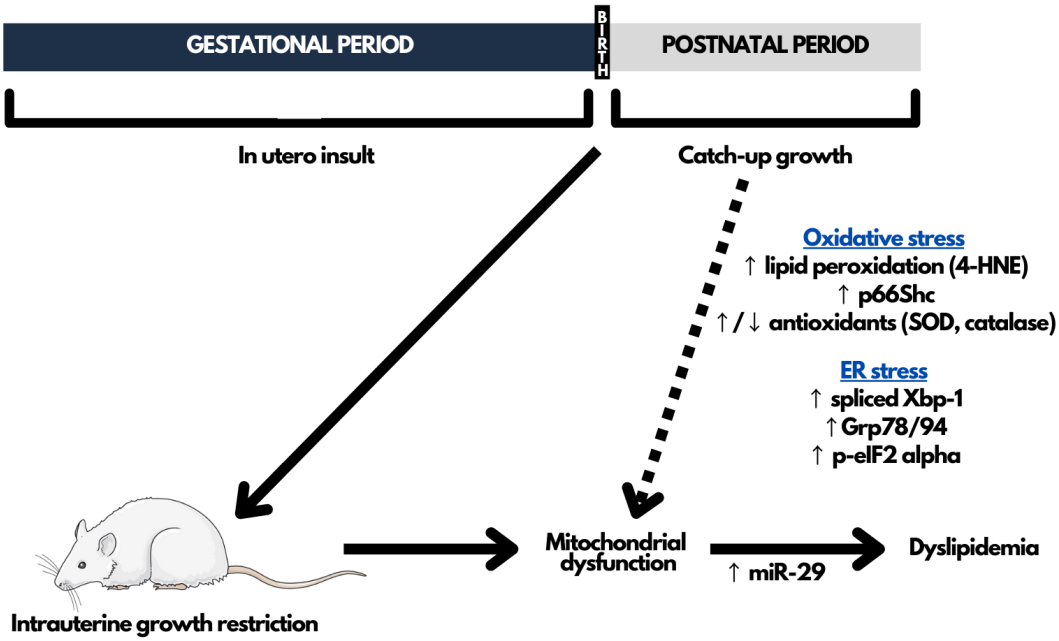

Figure 5-1. Mitochondrial dysfunction leads to increased miR-29 as an underlying mechanism of hepatic hyperlipidemia in adult growth-restricted offspring with postnatal catch-up growth.

Offspring affected by intrauterine growth restriction (IUGR) exhibit low birth weight and reduced organ to body weight ratios, followed by rapid postnatal weight gain (*i.e.,* catch-up growth) after birth. This catch-up growth leads to oxidative stress and ER stress during postnatal life, thereby promoting mitochondrial dysfunction in the liver. As a result, hepatic miR-29 becomes increased in IUGR offspring with catch-up growth, which may serve as an underlying mechanism of hyperlipidemia observed in adult life. Together, it appears that mitochondrial dysfunction is a critical source of metabolic dysfunction in growth-restricted offspring exclusively following postnatal catch-up growth.

First, there are many avenues by which we could follow up with our studies of gestational exposure to Δ9-THC. In Chapter III, I investigated mitochondrial dysfunction and hepatic hyperlipidemia in offspring at three weeks and six months of age. At six months, male Δ9-THC-exposed offspring exhibited increased hepatic triglyceride levels and elevated adipose to body weight ratio (Oke *et al.*, 2021). As these animals age, it is possible that they develop NAFLD and cardiovascular disease. By returning to this model and allowing male offspring to age beyond six months, we could assess the progression of NAFLD and cardiovascular disease through histopathological and biochemical analyses. These would include immunohistochemical analyses of liver, skeletal muscle, and cardiac tissues with Masson trichrome stain (for connective tissue deposition) and Oil Red O (for intracellular lipid accumulation). Measures of cardiac function, including echocardiogram assessment, could also be performed in adult offspring as done previously by our lab for offspring at three weeks of age (Lee *et al.*, 2021). Gestational Δ9-THC exposure and catch-up growth led to increased thickness of the left ventricle anterior wall, along with decreased cardiac output at this time point. Again, since dyslipidemia is a risk factor for cardiovascular disease, it's likely that these impairments in cardiac function are worsened with age due to poor lipid metabolism.

Given that my interests lie in the role of mitochondria in hepatic lipid metabolism, specific isolation of primary hepatocytes from IUGR offspring in either model would allow us to perform *in vitro* functional assays measuring oxidative stress and aerobic metabolism. These experiments would be performed using cells isolated from newborn, three-week old, and adult offspring to investigate the effects of catch-up growth on cellular metabolism. The Seahorse XF^{e}24 Extracellular Flux Analyzer (Agilent) in our laboratory could be used to measure oxygen consumption rate (OCR) and extracellular acidification (ECAR) rate in IUGR primary hepatocytes, both before and after catch-up growth. These *in vitro* assays can be used to measure basal levels of OCR and ECAR in response to fatty acid substrates and inhibitors of mitochondrial enzymes. In addition to these metabolic studies, RNA-sequencing (RNA-seq) could be further utilized to address issues arising from liver heterogeneity and sex-specificity. In doing so, these studies

would provide a better understand of how mitochondrial dysfunction in hepatocytes contributes to the progression of hyperlipidemia.

Mitochondrial dysfunction is a multifaceted cellular phenotype with a wide range of molecular consequences. Here I have only scratched the surface in how mitochondria are affected by catch-up growth, and there are many directions in which we could advance these studies. There is evidence to show that mitochondrial dynamics (*i.e.,* fission and fusion) is highly sensitive to cell stress and nutrient status, particularly through signaling via the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways. Nutrient deprivation has been demonstrated to cause mitochondrial elongation and a preference for fatty acid oxidation over glucose metabolism (Yu and Pekkurnaz, 2018). Conversely, nutrient overload leads to mitochondrial fragmentation and inefficient bioenergetics (Yu and Pekkurnaz, 2018). During my studies of MPR offspring, I found that transcript abundance for markers of both mitochondrial fusion (Opa1 and Mfn1) and fission (Drp1) are upregulated in livers of adult males with catch-up growth (Figure B-1, Appendix B). Given the connection between mitochondrial dynamics and metabolism, it would be valuable to pursue these preliminary data further by directly assessing mitochondrial morphology. This would again require isolation of primary hepatocytes before and after catch-up growth, followed by electron microscopy to clarify whether mitochondrial fission or fusion occurs. Furthermore, it would be interesting to determine the effects of catch-up growth on mitophagy, a specialized form of autophagy that removes damaged or dysfunction mitochondria from the cell. Much like mitochondrial dynamics, mitophagy is regulated by nutrient sensing pathways via AMPK and mTOR signaling. Interestingly, there are reports indicating that successful mitophagy also relies on mitochondrial fission. To investigate autophagy, either electron microscopy or fluorescence microscopy could monitor the formation of autophagosomes. Given that offspring with catch-up growth prioritize anabolic pathways over energy catabolism, and they exhibit mitochondrial dysfunction, I predict that catch-up growth leads to both mitochondrial fission and mitophagy in the IUGR liver.

Finally, it would be interesting to explore the therapeutic potential of oxidative stress and ER stress inhibitors in either the MPR or Δ9-THC model. With a 'proof-of-principle' approach, these models would be repeated exactly as before; however, inhibitors of oxidative stress or ER stress would be administered either gestationally or postnatally. Administration of these inhibitors only during pregnancy or postnatal life would determine if postnatal intervention in the offspring is sufficient (*e.g.,* exclusively during lactation), or if perinatal intervention is necessary. Indeed, there are many pharmaceutical and dietary interventions that have already been investigated as mitigators of oxidative stress or ER stress. For oxidative stress, various antioxidant compounds are of interest because of their ability to inhibit lipid peroxidation and scavenge free radicals. For example, vitamins C and E, resveratrol, melatonin, and pyrroloquinoline quinone (PQQ), are antioxidants that can be ingested through the diet. To date, there is some existing research on dietary supplementation of these antioxidants during pregnancy, but their success in preventing IUGR is inconclusive (Chen *et al.*, 2006; Poston *et al.*, 2006; Ornoy *et al.*, 2009; Bourque *et al.*, 2012; Lemley *et al.*, 2012; Wood, 2021). That said, postnatal administration of these compounds in IUGR offspring could still help to prevent the oxidative stress caused by catch-up growth and stop the progression of metabolic disease later in life. Since oxidative stress and ER stress often occur together, it would be interesting to assess the effects of these oxidative stress inhibitors on ER stress as well. Alternatively, administration of tauroursodeoxycholic acid (TUDCA) could be used to directly target ER stress. TUDCA is a naturally occurring bile acid produced by intestinal bacteria, and it has been shown to alleviate ER stress by improving the protein folding capabilities of the ER (Omura *et al.*, 2013). In addition, it is a known anti-apoptotic agent as it can stabilize intrinsic mitochondrial pathways that lead to oxidative stress and apoptosis (Vang *et al.*, 2014). It is for these reasons that TUDCA has been previously studied as potential treatment for many neurodegenerative and metabolic diseases, making it an attractive candidate for postnatal treatment in IUGR offspring.

5.4 Summary

Intrauterine growth restriction (IUGR) is a pathological condition characterized by low birth weight and compromised organ development. Epidemiological studies of gestational famine suggest a relationship between birth weight and long-term metabolic dysfunction, while the occurrence of postnatal catch-up growth can exacerbate this detrimental relationship. In this thesis, I present the novel discovery that catch-up growth promotes oxidative stress and mitochondrial dysfunction in the growth-restricted liver. This occurs across multiple rodent models of IUGR, suggesting that the combination of IUGR and catch-up growth has greater impact on mitochondrial function than the type of gestational insult. These trends occur exclusively among male IUGR offspring, leading to hepatic hyperlipidemia during adult life. Epigenetic mechanisms appear to have a role in this process, as hepatic expression of miR-29 is altered following catch-up growth. *In vitro* experiments further suggest that alterations in miR-29 expression may be mitochondrial-driven. Collectively, my data suggest that hepatic catch-up growth has great impact on mitochondrial function and lipid handling in growth-restricted offspring, and that this occurs in a sex-specific manner. Again, this could contribute to the development of postnatal therapies among IUGR offspring, thereby leading to decreased incidence of the metabolic syndrome.

5.4 References

Barra NG, Lisyansky M, Vanduzer TA, Raha S, Holloway AC and Hardy DB (2017) Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase and impaired mitochondrial function in male rat offspring. *Journal of Applied Toxicology* **37** 1517–1526.

Berniakovich I, Trinei M, Stendardo M, Migliaccio E, Minucci S, Bernardi P, Pelicci PG and Giorgio M (2008) p66Shc-generated oxidative signal promotes fat accumulation. *Journal of Biological Chemistry* **283** 34283–34293.

Bourque SL, Dolinsky VW, Dyck JR and Davidge ST (2012) Maternal resveratrol treatment during pregnancy improves adverse fetal outcomes in a rat model of severe hypoxia. *Placenta* **33** 449–452.

Chamson-Reig A, Thyssen SM, Hill DJ and Arany E (2009) Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. *Experimental Biology and Medicine (Maywood, N.J.)* **234** 1425–1436.

Chen YH, Xu DX, Zhao L, Wang H, Wang JP and Wei W (2006) Ascorbic acid protects against lipopolysaccharide-induced intra-uterine fetal death and intra-uterine growth retardation in mice. *Toxicology* **217** 39–45.

Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C and Brenner C (2008) Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene* **27** 285–299.

Diao Y, Guo X, Jiang L, Wang G, Zhang C, Wan J, Jin Y and Wu Z (2014) miR-203, a Tumor Suppressor Frequently Down-regulated by Promoter Hypermethylation in Rhabdomyosarcoma *. *Journal of Biological Chemistry* **289** 529–539.

Felty Q, Xiong W-C, Sun D, Sarkar S, Singh KP, Parkash J and Roy D (2005) Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. *Biochemistry* **44** 6900–6909.

Gillies R, Lee K, Vanin S, Laviolette SR, Holloway AC, Arany E and Hardy DB (2020) Maternal exposure to Δ9-tetrahydrocannabinol impairs female offspring glucose homeostasis and endocrine pancreatic development in the rat. *Reproductive Toxicology (Elmsford, N.Y.)* **94** 84–91.

Gruppuso PA and Sanders JA (2016) Regulation of liver development: implications for liver biology across the lifespan. *Journal of Molecular Endocrinology* **56** R115–R125.

Hales CN and Barker DJ (2001) The thrifty phenotype hypothesis. *British Medical Bulletin* **60** 5–20.

Hales CN, Desai M, Ozanne SE and Crowther NJ (1996) Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. *Biochemical Society Transactions* **24** 341–350.

Kogure T, Costinean S, Yan I, Braconi C, Croce C and Patel T (2012) Hepatic miR-29ab1 expression modulates chronic hepatic injury. *Journal of Cellular and Molecular Medicine* **16** 2647–2654.

Lee K, Laviolette SR and Hardy DB (2021) Exposure to Δ9-tetrahydrocannabinol during rat pregnancy leads to impaired cardiac dysfunction in postnatal life. *Pediatric Research* 1–8.

Lemley CO, Meyer AM, Camacho LE, Neville TL, Newman DJ, Caton JS and Vonnahme KA (2012) Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology* **302** R454-67.

Lin H-Y, Wang F-S, Yang Y-L and Huang Y-H (2019) MicroRNA-29a Suppresses CD36 to Ameliorate High Fat Diet-Induced Steatohepatitis and Liver Fibrosis in Mice. *Cells* **8**.

Maruyama M, Matsunaga T, Harada E and Ohmori S (2007) Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biological & Pharmaceutical Bulletin* **30** 2091–2097.

Moore BF, Sauder KA, Shapiro ALB, Crume T, Kinney GL and Dabelea D (2022) Fetal Exposure to Cannabis and Childhood Metabolic Outcomes: The Healthy Start Study. *The Journal of Clinical Endocrinology & Metabolism* dgac101.

Moraes C, Rebelato HJ, Amaral MEC, Resende TM, Silva EVC, Esquisatto MAM and Catisti R (2014) Effect of maternal protein restriction on liver metabolism in rat offspring. *The Journal of Physiological Sciences* **64** 347–355.

Naem E, Haas MJ, Wong NCW and Mooradian AD (2013) Endoplasmic reticulum stress in HepG2 cells inhibits apolipoprotein A-I secretion. *Life Sciences* **92** 72–80.

Natale BV, Gustin KN, Lee K, Holloway AC, Laviolette SR, Natale DRC and Hardy DB (2020) Δ9-Tetrahydrocannabinol Exposure During Rat Pregnancy Leads To Symmetrical Fetal Growth Restriction and Labyrinth-Specific Vascular Defects in the Placenta. *Scientific Reports* **10** 1–15.

Nobili V, Marcellini M, Marchesini G, Vanni E, Manco M, Villani A and Bugianesi E (2007) Intrauterine Growth Retardation, Insulin Resistance, and Nonalcoholic Fatty Liver Disease in Children.

Oke SL, Sohi G and Hardy DB (2019) Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. *Reproduction (Cambridge, England)* **159** 27–39.

Oke SL, Lee K, Papp R, Laviolette SR and Hardy DB (2021) In Utero Exposure to Δ9-Tetrahydrocannabinol Leads to Postnatal Catch-Up Growth and Dysmetabolism in the Adult Rat Liver. *International Journal of Molecular Sciences* **22** 7502.

Omura T, Asari M, Yamamoto J, Oka K, Hoshina C, Maseda C, Awaya T, Tasaki Y, Shiono H, Yonezawa A *et al.* (2013) Sodium tauroursodeoxycholate prevents paraquat-induced cell death by suppressing endoplasmic reticulum stress

responses in human lung epithelial A549 cells. *Biochemical and Biophysical Research Communications* **432** 689–694.

Ornoy A, Tsadok MA, Yaffe P and Zangen SW (2009) The Cohen diabetic rat as a model for fetal growth restriction: vitamins C and E reduce fetal oxidative stress but do not restore normal growth. *Reproductive Toxicology (Elmsford, N.Y.)* **28** 521–529.

Ozaki T, Nishina H, Hanson MA and Poston L (2001) Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *The Journal of Physiology* **530** 141–152.

Park KS, Kim SK, Kim MS, Cho EY, Lee JH, Lee K-U, Pak YK and Lee HK (2003) Fetal and Early Postnatal Protein Malnutrition Cause Long-Term Changes in Rat Liver and Muscle Mitochondria. *The Journal of Nutrition* **133** 3085–3090.

Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH and Consortium V in P (VIP) T (2006) Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet* **367** 1145–1154.

Pramfalk C, Pavlides M, Banerjee R, McNeil CA, Neubauer S, Karpe F and Hodson L (2015) Sex-Specific Differences in Hepatic Fat Oxidation and Synthesis May Explain the Higher Propensity for NAFLD in Men. *The Journal of Clinical Endocrinology and Metabolism* **100** 4425–4433.

Qiu S, Vazquez JT, Boulger E, Liu H, Xue P, Hussain MA and Wolfe A (2017) Hepatic estrogen receptor α is critical for regulation of gluconeogenesis and lipid metabolism in males. *Scientific Reports* **7** 1661.

Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN and Bleker OP (1998) Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351** 173–177.

Roderburg C, Urban G-W, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M *et al.* (2011) Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology (Baltimore, Md.)* **53** 209–218.

Sanz A, Hiona A, Kujoth GC, Seo AY, Hofer T, Kouwenhoven E, Kalani R, Prolla TA, Barja G and Leeuwenburgh C (2007) Evaluation of sex differences on mitochondrial bioenergetics and apoptosis in mice. *Experimental Gerontology* **42** 173–182.

Siddiqui MA, Ahmad J, Farshori NN, Saquib Q, Jahan S, Kashyap MP, Ahamed M, Musarrat J and Al-Khedhairy AA (2013) Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. *Molecular and Cellular Biochemistry* **384** 59–69.

Singhal A, Cole TJ, Fewtrell M and Lucas A (2004) Breastmilk feeding and lipoprotein profile in adolescents born preterm: follow-up of a prospective randomised study. *Lancet* **363** 1571–1578.

Sohi G, Marchand K, Revesz A, Arany E and Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. *Molecular Endocrinology (Baltimore, Md.)* **25** 785–798.

Sohi G, Revesz A and Hardy DB (2013) Nutritional mismatch in postnatal life of low birth weight rat offspring leads to increased phosphorylation of hepatic eukaryotic initiation factor 2 alpha in adulthood. *Metabolism: Clinical and Experimental*.

Sohi G, Barry EJ, Velenosi TJ, Urquhart BL and Hardy DB (2014) Protein Restoration in Low-Birth-Weight Rat Offspring Derived from Maternal Low-Protein Diet Leads to Elevated Hepatic CYP3A and CYP2C11 Activity in Adulthood. *Drug Metabolism and Disposition: The Biological Fate of Chemicals* **42** 221–228.

Sohi G, Revesz A, Ramkumar J and Hardy DB (2015) Higher Hepatic miR-29 Expression in Undernourished Male Rats During the Postnatal Period Targets the Long-Term Repression of IGF-1. *Endocrinology* **156** 3069–3076.

Suomela E, Oikonen M, Pitkänen N, Ahola-Olli A, Virtanen J, Parkkola R, Jokinen E, Laitinen T, Hutri-Kähönen N, Kähönen M *et al.* (2016) Childhood predictors of adult fatty liver. The Cardiovascular Risk in Young Finns Study. *Journal of Hepatology* **65** 784–790.

Szafran H and Smielak-Korombel W (1998) [The role of estrogens in hormonal regulation of lipid metabolism in women]. *Przeglad Lekarski* **55** 266–270.

Tian H, Gao Z, Wang G, Li H and Zheng J (2016) Estrogen potentiates reactive oxygen species (ROS) tolerance to initiate carcinogenesis and promote cancer malignant transformation. *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine* **37** 141–150.

Tomita K, Teratani T, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, Nishiyama K, Mataki N, Irie R, Minamino T *et al.* (2012) P53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. *Journal of Hepatology* **57** 837–843.

Tran C, Jacot-Descombes D, Lecoultre V, Fielding BA, Carrel G, Lê K-A, Schneiter P, Bortolotti M, Frayn KN and Tappy L (2010) Sex differences in lipid and glucose kinetics after ingestion of an acute oral fructose load. *The British Journal of Nutrition* **104** 1139–1147.

Vang S, Longley K, Steer CJ and Low WC (2014) The Unexpected Uses of Urso- and Tauroursodeoxycholic Acid in the Treatment of Non-liver Diseases. *Global Advances in Health and Medicine* **3** 58–69.

Wang C, Li T, Tang S, Zhao D, Zhang C, Zhang S, Deng S, Zhou Y and Xiao X (2016) Thapsigargin induces apoptosis when autophagy is inhibited in HepG2

cells and both processes are regulated by ROS-dependent pathway. *Environmental Toxicology and Pharmacology* **41** 167–179.

Wang Z, Zhao Y, Zhao H, Zhou J, Feng D, Tang F, Li Y, Lv L, Chen Z, Ma X *et al.* (2020) Inhibition of p66Shc Oxidative Signaling via CA-Induced Upregulation of miR-203a-3p Alleviates Liver Fibrosis Progression. *Molecular Therapy - Nucleic Acids* **21** 751–763.

Wood A (2021) Characterizing the Effects of Pyrroloquinoline Quinone (PQQ) Supplementation on Skeletal Muscle Mitochondrial Function and Myogenesis During Oxidative Stress and IUGR. *Electronic Thesis and Dissertation Repository*.

Woodman AG, Mah R, Keddie D, Noble RMN, Panahi S, Gragasin FS, Lemieux H and Bourque SL (2018) Prenatal iron deficiency causes sex-dependent mitochondrial dysfunction and oxidative stress in fetal rat kidneys and liver. *The FASEB Journal* **32** 3254–3263.

Yager JD and Chen JQ (2007) Mitochondrial estrogen receptors – new insights into specific functions. *Trends in Endocrinology & Metabolism* **18** 89–91.

Yang Y-L, Kuo H-C, Wang F-S and Huang Y-H (2019) MicroRNA-29a Disrupts DNMT3b to Ameliorate Diet-Induced Non-Alcoholic Steatohepatitis in Mice. *International Journal of Molecular Sciences* **20**.

Yu SB and Pekkurnaz G (2018) Mechanisms Orchestrating Mitochondrial Dynamics for Energy Homeostasis. *Journal of Molecular Biology* **430** 3922–3941.

Zhao Y, Wang Z, Feng D, Zhao H, Lin M, Hu Y, Zhang N, Lv L, Gao Z, Zhai X *et al.* (2019) p66Shc Contributes to Liver Fibrosis through the Regulation of Mitochondrial Reactive Oxygen Species. *Theranostics* **9** 1510–1522.

Appendix A: Copyright Agreement

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Apr 06, 2022

This Agreement between The University of Western Ontario -- Shelby Oke ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	5283040634172
License date	Apr 06, 2022
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Springer eBook
Licensed Content Title	Effects of Protein Deficiency on Perinatal and Postnatal Health Outcomes
Licensed Content Author	Shelby L. Oke, Daniel B. Hardy
Licensed Content Date	Jan 1, 2019
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	Text extract
Number of text extracts	1

Will you be translating?	no
Circulation/distribution	500 - 999
Author of this Springer Nature content	yes
Title	The molecular mechanisms of hepatic mitochondrial dysfunction in growth-restricted offspring
Institution name	The University of Western Ontario
Expected presentation date	Jun 2022
Portions	"Epigenetic Mechanisms Linking Protein Restriction and Adverse Metabolic Outcomes"; pages 1428-1430
Requestor Location	The University of Western Ontario 1151 Richmond Street Dental Sciences Building 2019 London, ON N6A 3K7 Canada Attn: The University of Western Ontario
Total	0.00 CAD

Appendix B: Preliminary Data for Future Studies

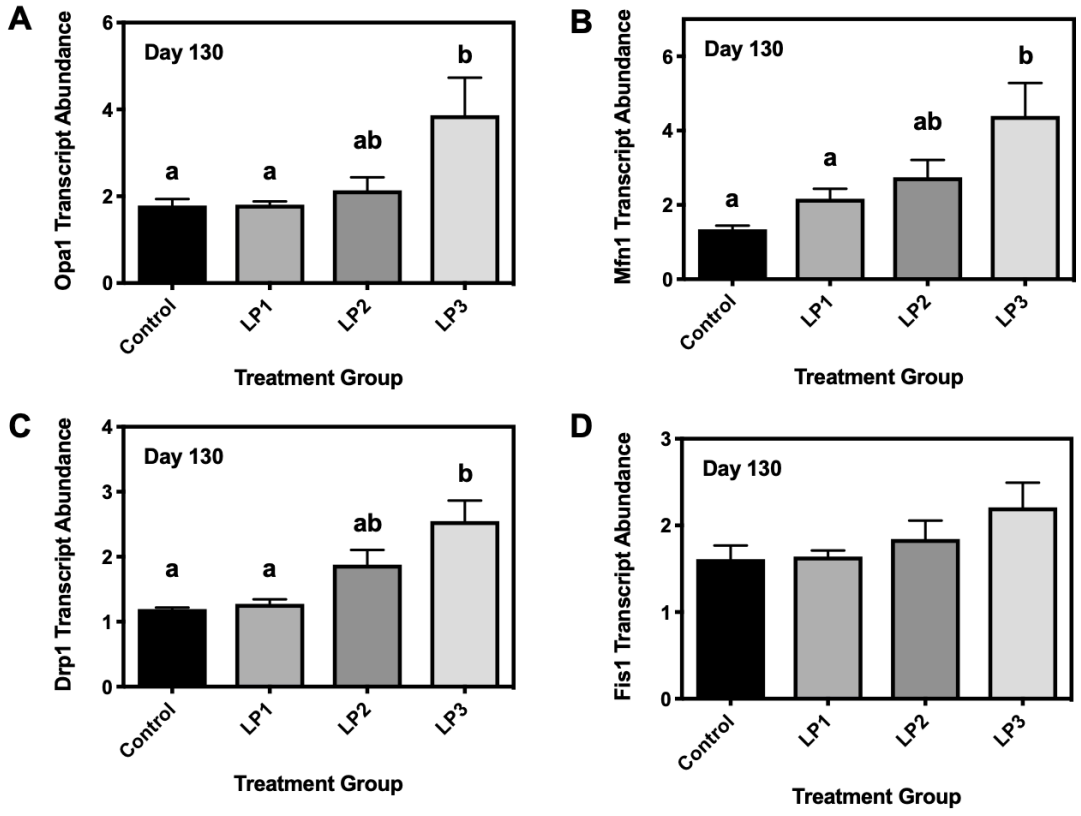

Figure B-1. Maternal protein restriction leads to upregulated markers of mitochondrial fusion and fission in livers of offspring with postnatal catch-up growth.

Pregnant dams were subjected to an 8% protein diet during gestation, and pups were randomly assigned to one of three postnatal dietary regimens at birth: 8% protein diet throughout life (LP1), 8% protein diet until three weeks of age + 20% (control) diet after three weeks (LP2), or 20% protein diet throughout life (LP3). Following necropsy, RNA was isolated from livers of each group and qRT-PCR analysis was performed with the complementary cDNA. Fold change difference of (A) opa1, (B) mfn1, (C) drp1, and (D) fis1 were expressed as means from normalized to the geometric means of GAPDH and β-Actin ± SEM (n=8/group). Transcript abundances for each individual point were analyzed using a two-tailed unpaired Student's *t*-test and compared to the untreated control group. Groups marked with different letters are significantly different ($P < 0.05$).

Curriculum Vitae

Name:	Shelby L. Oke
Post-secondary Education and Degrees:	The University of Western Ontario London, Ontario, Canada 2012–2016 B.Sc.
	The University of Western Ontario London, Ontario, Canada 2016–2022 Ph.D.
Honours and Awards:	Archie and Alma Etherington Family Scholarship 2012–2013
	Children's Health Research Institute Epigenetics Trainee Fellowship 2016–2019
	Obstetrics and Gynaecology Graduate Scholarship 2017–2018
	George W. Stavraky Teaching Award 2018
	Children's Health Research Institute Trainee Fellowship 2019–2020
	Gordon J. Mogenson Award for Research Excellence in Physiology 2020
	Natural Sciences and Engineering Research Council Doctoral Canada Graduate Scholarship 2020–2023

Related Work Experience	Work-Study Student The University of Western Ontario 2015–2016
	Teaching Assistant The University of Western Ontario 2016–2021

Publications:

Oke, Shelby L. *et al.* (2021). In utero exposure to Δ9-tetrahydrocannabinol leads to postnatal catch-up growth and dysmetabolism in the adult rat liver. International Journal of Molecular Sciences, 22 (14), 2502.

Oke, Shelby L. and Hardy, Daniel B. (2021). The role of cellular stress in intrauterine growth restriction and postnatal dysmetabolism. International Journal of Molecular Sciences, 22 (13), 6986.

Oke, Shelby L. *et al.* (2020). Perinatal protein restriction with postnatal catch-up growth leads to elevated p66Shc and mitochondrial dysfunction in the adult rat liver. Reproduction, 159, 27–39.

Oke, Shelby L. and Hardy, Daniel B. (2017). Effects of protein deficiency on perinatal and postnatal health outcomes. The Handbook of Famine, Starvation, and Nutrient Deprivation, 1–23.