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Molecular Mechanisms Underlying the Early Life Programming of the Liver

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Graduate Program in Physiology

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

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Molecular Mechanisms Underlying the Early Life Programming of the Liver

(Thesis format: Integrated Article)

by

Gurjeev Sohi

Graduate Program in Physiology & Pharmacology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Several clinical studies have demonstrated that intrauterine growth restriction (IUGR) offspring, faced with a nutritional mismatch postpartum, have an increased risk of developing the metabolic syndrome. The maternal protein restriction (MPR) rat model has been extensively studied to investigate the adverse effects of a nutritional mismatch in postnatal life of IUGR offspring. Previous studies have demonstrated that MPR leads to impaired function of the liver, an important metabolic organ for lipid and glucose homeostasis along with drug catabolism. However the underlying mechanisms which predispose these offspring to the metabolic syndrome remain elusive. In the following studies, low protein diet during pregnancy and lactation led to IUGR offspring with decreased liver to body weight ratios, followed by increased circulating and hepatic cholesterol levels in both sexes at day 21 and exclusively in the male offspring at day 130. These long-term increases in cholesterol levels mirrored decreases in cholesterol 7α-hydroxylase (Cyp7a1), a rate-limiting enzyme that catabolizes cholesterol to bile acids. This was due, in part, to diminished acetylation and enhanced methylation of histone H3 [K9,14], markers of chromatin silencing, at the promoter region of Cyp7a1. It was later demonstrated that these IUGR offspring exhibited increased molecular markers of hepatic endoplasmic reticulum (ER) stress and insulin resistance exclusively when they were faced with a nutritional mismatch in postnatal life. Finally, as cytochrome p450 (Cyp) dependent enzymes reside in the ER of the liver and are the primary enzymes involved in Phase 1 of drug metabolism, it was relevant to determine whether MPR lead to long-term alteration in their ability to metabolize drugs (e.g. statins for hypercholesterolemia). MPR offspring were shown to have an increase in enzymatic activity of the major drug metabolizing enzymes Cyp3a and Cyp2c. This was postulated to be due, in part, to transcriptional induction by constitutive androstane receptor. Collectively, these findings suggest that stable promoter-specific changes to post-translational histone modifications and elevated ER stress in the liver, are key molecular mechanisms whereby IUGR offspring receiving a nutritional mismatch in postnatal life develop high cholesterol and insulin resistance. Moreover, it can be postulated that these offspring require augmented doses of drugs in order to effectively alleviate these symptoms. This new knowledge would serve useful in the development of both prevention and management strategies to reduce the debilitating impact of metabolic diseases in low birth weight babies.
Keywords

Fetal Programming, Intrauterine Growth Restriction, Thrifty Phenotype, Low Protein Diet, Metabolic Syndrome, Cholesterol, Insulin Resistance, Liver, Epigenetics, Posttranslational Histone Modifications, Endoplasmic Reticulum Stress, Protein Translation, Growth, Drug Metabolism
To my mother, father and brother with so much love
Co-Authorship Statement

A portion of the “Introduction” was reproduced (adapted) from a Review Article:


GS and DBH researched pertinent material from the scientific literature and wrote the review article. AR helped with revisions on the review article. All authors approved the final version of the review article.

**Chapter 3:**


GS and DBH designed the experiments. KM and AR helped with animal care, blood and tissue collection. GS conducted the experiments. GS and DBH analyzed and interpreted the data. GS and DBH wrote the manuscript. All authors approved the final version of the manuscript.

**Chapter 4:**

Sohi G, Revesz A, Hardy DB (2013) Nutritional mismatch in postnatal life of low birth weight rat offspring leads to increased phosphorylation of hepatic eukaryotic initiation factor 2α in adulthood. *Metabolism.* (Accepted: METABOLISM-D-13-00099)

GS and DBH designed the experiments. AR helped with animal care and tissue collection. GS conducted the experiments. GS and DBH analyzed and interpreted the data. GS and DBH wrote the manuscript. All authors approved the final version of the manuscript.
Chapter 5:


GS, BLU and DBH designed the experiments. TJV helped with technical assistance on determining enzyme kinetics in liver microsomes via ultra performance liquid chromatography. GS and EJB conducted the experiments. GS and DBH analyzed and interpreted the data. GS and DBH wrote the manuscript.
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This thesis would not have been made possible if it was not for the support from Western University and the Government of Canada. Thank you for making an investment in graduate students and helping foster research of an international caliber.

Lastly, my education would not have been complete if it was not for the continued support of my mother and father. You have provided me with the foundation for success in whatever I desire to attempt. You are the pillars of strength in my life.
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<tr>
<td>11β-Hsd1</td>
<td>11β-hydroxysteroid dehydrogenase-1</td>
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<tr>
<td>Hmg-CoA reductase</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A reductase</td>
</tr>
<tr>
<td>Accα</td>
<td>Acetyl-coenzyme A carboxylase-α</td>
</tr>
<tr>
<td>Atf</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>Abca</td>
<td>Adenosine triphosphate binding cassette transporter</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>ArKO</td>
<td>Aromatase knockout</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>Cyp7a1</td>
<td>Cholesterol 7α-hydroxylase</td>
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<tr>
<td>Cetp</td>
<td>Cholesteryl ester transfer protein</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Car</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental origins of adult diseases</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>elf</td>
<td>Eukaryotic initiation factor</td>
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<tr>
<td>Fas</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FoxO1</td>
<td>Forkhead box protein O1</td>
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<td>Term</td>
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<tr>
<td>Grp78</td>
<td>Glucose regulated protein 78</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
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<tr>
<td>Gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>HDL</td>
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<td>Jmjd2</td>
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<td>Lxr</td>
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<td>Maternal protein restriction</td>
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<tr>
<td>Pltp</td>
<td>Phospholipid transfer protein</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PI</td>
<td>Placental insufficiency</td>
</tr>
<tr>
<td>Pxr</td>
<td>Pregnane X receptor</td>
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<tr>
<td>Rxr</td>
<td>Retinoid x receptor</td>
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<tr>
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<td>Stearoyl-CoA desaturase 1</td>
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1 INTRODUCTION

A portion of this Introduction has been reproduced (adapted) with permission from: Sohi G, Revesz A, Hardy DB (2011) Permanent implications of intrauterine growth restriction on cholesterol homeostasis. Semin Reprod Med 29(3): 246-56.
1.1 Cardiovascular Disease

While significant advances in medicine have been made over the past 50 years to improve overall health, cardiovascular disease (CVD) still remains the leading cause of premature death worldwide. Without dispute, the risk of developing cardiovascular disease increases in individuals diagnosed with the metabolic syndrome. For example, a 1998 prospective Québec cardiovascular study has demonstrated that adults with the metabolic syndrome have a ~18-fold risk of developing CVD compared to adults who do not exhibit this syndrome\(^1\). Moreover, the incidence of metabolic syndrome in Canada is reaching epidemic proportions, with one in three Canadian family practice patients between 40 and 60 years of age falling under this criteria\(^2\). The World Health Organization (WHO) has identified hypercholesterolemia and insulin resistance, in addition to obesity and hypertension as one of the major symptoms characterizing the metabolic syndrome\(^3, 4\). Specifically, WHO criteria for metabolic syndrome include insulin resistance as identified by type 2 diabetes or impaired fasting glucose and/or tolerance, alongside any two of the following: high blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic), high density lipoprotein (HDL) cholesterol <0.9 mmol/L in men or <1.0 mmol/L in women, plasma triglycerides ≥1.7 mmol/L, BMI >30 kg/m\(^2\) or urinary albumin excretion rate ≥20 μg/min\(^5\). While the criteria for metabolic syndrome only encompasses low levels of HDL plasma levels, hypercholesterolemia could be due to high levels of low density lipoprotein (LDL) or HDL levels depending on whether we are detecting it in the plasma levels of rodents (e.g. mice or rats) or humans. This is because humans predominantly carry cholesterol in the form of LDL while rodents predominantly carry cholesterol in the form of HDL\(^6\). Therefore, in this thesis “hypercholesterolemia” is with reference total circulating cholesterol levels.

1.2 Impact of Hypercholesterolemia and Impaired Glucose Tolerance on Cardiovascular Disease

Maintaining normal cholesterol levels and glucose homeostasis is critical for reducing the risk of developing CVDs. For example, a meta-analysis prospective study, using data from 58 clinical trials as well as nine cohort studies, indicates that in patients with
cardiovascular disease, a 1.8 mM reduction in low density lipoprotein (LDL) cholesterol by statins from pretreatment concentrations of 4.8 mM resulted in a 17% reduction in stroke and a 60% reduction in the risk of ischemic heart disease\(^7\). While statin therapies are considered safe and effective, incidences of statin induced skeletal muscle damage (\textit{i.e.} rhabdomyolysis) and hepatitis associated liver failure exist in some patients\(^5\). Similarly, individuals with impaired glucose tolerance have been estimated to have twice the risk for developing cardiovascular disease\(^8\). Therefore, targeted treatment of hyperglycemia, notably by metformin, is considered to be an effective strategy in preventing cardiovascular disease in diabetic patients\(^9\)\(^\text{-}10\). However, many individuals with impaired glucose intolerance and high cholesterol display no symptoms for many years, resulting in premature mortality due to a failure to receive timely diagnosis and treatment\(^11\)-\(^13\). Therefore, despite current therapeutic modalities which are aimed to reduce the incidence of the metabolic syndrome through lifestyle modifications (\textit{i.e.} changes in diet and physical activity) and/or pharmacological interventions, CVD still remains the leading cause of mortality in North America. As a consequence, this places an enormous human and economic burden on society, where 1 in every 2.9 deaths in the United States is due to CVD\(^14\)-\(^17\). In order to effectively alleviate the burden of this pandemic proactive development of strategies which focus on disease prevention prior to the manifestation of the adverse symptoms are warranted.

\subsection*{1.3 Developmental Origins of Adult Disease}

The ‘fetal origins’ or ‘Barker’ hypothesis suggests that adverse maternal events that lead to impaired fetal and neonatal growth results in offspring that are at a higher risk of developing chronic diseases in later life (Figure 1.1)\(^18\)-\(^20\). Epidemiological evidence strongly indicates that adverse events \textit{in utero} (\textit{i.e.} maternal hypoxia, undernutrition, infection, stress) can permanently alter physiological processes leading to the metabolic syndrome\(^21\),\(^22\). As a result, the last two decades have witnessed an increase in clinical and animal studies focused on understanding the role of the fetal environment in the long-term regulation or ‘programming’ of metabolic functions. Moreover, these studies have also sought to elucidate the molecular mechanisms involved in the etiology of these metabolic diseases in adult life. In understanding the role of the perinatal and/or postnatal
environment on long-term health, therapeutic intervention strategies can be targeted to prevent the onset of the metabolic syndrome.

Despite our best efforts, the rates of preterm and low birth weight pregnancies have increased over the previous two decades in Canada and the United States ([www.marchofdimes.com/peristats](http://www.marchofdimes.com/peristats))\(^\text{23}\). Given these trends, it is noteworthy that a majority of metabolic syndrome related diseases have been linked to adverse events in utero\(^\text{21, 24, 25}\). While low birth weight is the most common indicator used in clinical studies to correlate impaired fetal development and adverse outcomes in adulthood, the nature of the growth restriction (e.g. asymmetrical vs symmetrical IUGR) may alter this relationship. For instance, symmetric growth restriction refers to when the entire body is proportionally small. As a consequence, expert medical evaluation is required to distinguish whether the symmetric IUGR is due to pathological or normal development process\(^\text{26}\). This evaluation is important before a correlation between symmetric IUGR and adverse postnatal consequences can be investigated. On the contrary, placental insufficiency predominantly underlies most cases of asymmetric growth restriction, where the offspring has a normal head dimension but reduced abdominal circumference and limb size\(^\text{26}\). It is now well established that asymmetric IUGR has a strong correlation with increased risk of adverse outcomes in adult life. Lastly, aside from a careful consideration of the type of IUGR as a predictive indicator of these outcomes in adulthood, confounding factors also need to be accounted for. Most notably, these include influence of maternal socioeconomic status\(^\text{27}\), preexisting maternal CVD risk factors\(^\text{28}\), maternal smoking/high blood pressure\(^\text{29, 30}\) and maternal BMI\(^\text{31-33}\) on birth weight and adult diseases.

### 1.4 Thrifty Phenotype Hypothesis

The postnatal environment can also influence the correlation between IUGR and CVD. For instance adverse events during development induce fetal adaptations that allow for optimal survival in a similar predictive environment in postnatal life. However, the “thrifty phenotype” hypothesis suggests that these adaptations can become maladaptive if there is a ‘mismatch’ to the predictive environment\(^\text{34, 35}\). Clinically, low birth weight
offspring that underwent rapid postnatal catch-up growth due to mismatch in their postnatal environment (i.e. infant formula diets, overnutrition) displayed earlier indications of glucose intolerance, insulin resistance, dyslipidemia, obesity and higher death rates due to CVD (Figure 1.1). In addition, appropriate neonatal growth rates (i.e. due to breastfeeding) had a lower risk of hypercholesterolemia, type 2 diabetes, obesity and CVDs.

1.5 Clinical Perspective: Developmental Origins of Hypercholesterolemia and Impaired Glucose Tolerance

To date, numerous studies have demonstrated a strong inverse association between birth weight and metabolic risk factors associated with CVD (Figure 1.1). With respect to cholesterol, the first study to observe a link between development and subsequent dyslipidemia was conducted by Forsdahl in 1978. This study detected a significant positive correlation between poor living conditions in early childhood (based on the infant mortality rate) with higher cholesterol in men and women aged 35-49 years from within the same geographical area. In addition, a cohort of this study was also found to have an increased risk of coronary heart disease. Similarly, Amendt in 1976 observed adolescents born to diabetic mothers displayed early symptoms of diabetes including impaired glucose intolerance. Since these initial observations, the correlation of fetal growth to elevated cholesterol levels and glucose intolerance in adult life was popularized by the work of Barker and colleagues. Moreover, Barker also demonstrated that a positive correlation existed between birth weight and increased risk of death from ischemic heart disease. Many of the initial clinical data suggest strong support for low birth weight being linked to an atherogenic lipid profile and type 2 diabetes in adult life. However, some studies have indicated some inconsistencies with respect to this profile and ischemic heart disease, despite a strong correlation with type 2 diabetes. Some have argued that impaired fetal growth, as indicated by low birth weight, does not necessarily affect adult cholesterol levels enough to have any clinically significant effect in developing CVD. These authors further suggested that previous studies demonstrating strong inverse associations between birth weight and postnatal cholesterol levels were influenced by a publication bias resulting from inappropriate adjustment for...
current weight and other confounding factors\textsuperscript{31}. In addition, they argue that socioeconomic status has been demonstrated to have a greater impact on adult lipid levels when compared to birth weight\textsuperscript{61}.

In light of these arguments, it is still important not to underestimate the role of fetal development in the programming of adult cholesterol metabolism, type 2 diabetes and ischemic heart disease. First and foremost, a majority of studies use birth weight as an indicator of restricted fetal growth, although this may not be the best marker of fetal development. Secondly, while different \textit{in utero} conditions (\textit{i.e.} undernutrition, hypoxia, excess glucocorticoids) can result in low birth weight, it does not necessarily mean that all of these conditions impair the development of organs (\textit{i.e.} liver, small intestine) in a similar manner that is critical to cholesterol homeostasis. Interestingly, as the liver is a major site of cholesterol regulation, it has been demonstrated that abdominal circumference at birth, which provides an indirect measure of liver growth, has a stronger correlation with adult dyslipidemia than birth weight\textsuperscript{55}. The next generation of follow up studies need to better incorporate \textit{in utero} biomarkers for the earlier detection of abnormal liver development. Thirdly, care must be taken while drawing conclusions from meta analysis studies investigating links between birth weight and cholesterol levels later in life, as many studies used in the meta analysis examine cholesterol levels at different postnatal timepoints\textsuperscript{31,58}. This can influence the strength of the correlation because as one ages, adaptive responses to maintain cholesterol levels early in life may become maladaptive. Finally, these meta-analysis include studies which have variations with respect to gender, genetic makeup or postnatal environment, all of which may also further diminish this correlation\textsuperscript{62}. Since twins experience a relatively similar environment during fetal and postnatal development, clinical studies involving twins would be less prone to the effects of the previously discussed factors. In addition, monozygotic twins would be particularly useful, as their genetic variability would be minimal. However, there has been an issue concerning the use of twins in linking birth weight to metabolic disease given their low statistical size\textsuperscript{63,64}. Future clinical studies should address some of the highlighted shortcomings from earlier studies in order to better understand the implications of impaired development on long-term cholesterol regulation and ischemic heart disease. Given the time involved for these follow-up studies, animal models are
beneficial in aiding our understanding of the molecular mechanisms involved in the developmental origins of long-term cholesterol and glucose homeostasis.
**Figure 1.1**: Developmental origins of adult disease schematic. Several *in utero* insults leading to low birth weight offspring can increase the risk of metabolic syndrome in adulthood and subsequent risk of cardiovascular disease. This risk is further increased if the low birth weight offspring undergo rapid postnatal catch-up growth, leading to a thrifty phenotype.
1.6 Animal Models in the Developmental Origins of Hypercholesterolemia and Impaired Glucose Tolerance

Even before the formulation of the Barker’s hypothesis, animal models have successfully demonstrated that impaired fetal development compromises the ability of the offspring in regulating cholesterol or glucose in adult life. For example, premature weaning in rats was demonstrated to result in increased serum cholesterol levels and an inability to respond to a cholesterol challenge in the adult offspring\(^65, 66\). In addition, studies in guinea pigs have indicated that premature weaning leads to cholesterol dysregulation at postnatal day 10 and day 45\(^67\). This was attributed to decreases in cholesterol 7α-hydroxylase (Cyp7a1) activity, the rate limiting enzyme involved with the conversion of cholesterol to bile acids. Furthermore, sows with pharmacological induced gestational diabetes have been associated with offspring that exhibit impaired insulin response and glucose intolerance in neonatal life\(^68\). While these studies suggest that perturbations in fetal and neonatal life can have short-term consequences on cholesterol and glucose homeostasis, there are limited studies that address the effects of \textit{in utero} insults on long-term programming of glucose and cholesterol homeostasis. In addition, the long-term impact of factors that contribute to the “Thrifty Phenotype” in these offspring has not been elucidated. The most notable intrauterine insults associated with impaired fetal development are decreases in nutrients and oxygen delivery to the fetus. Therefore, it is important to delineate the contributions of both nutrition and hypoxia to long-term metabolic diseases.

1.6.1 Effects of Hypoxia on Long-term Cholesterol and Glucose Homeostasis

Using the rat as a model, maternal hypoxia during pregnancy has been demonstrated to lead to IUGR, impaired liver development, cardiac remodeling, diastolic dysfunction and increased sensitivity to ischemia in adulthood\(^69, 70\). While this model is an excellent model to study the fetal origins of CVD, the effect of maternal hypoxia on components of the metabolic syndrome preceding CVD (\textit{e.g.} hypercholesterolemia) are less understood.
Recent evidence suggests that maternal hypoxia derived offspring have impaired ability to regulate cholesterol leading to hypercholesterolemia when administered a high-fat dietary challenge at 10 months of age\textsuperscript{71}. Moreover, maternal hypoxia during pregnancy led to increased risk of developing CVD, as there were indications of early pathological appearances of atherosclerosis in adult offspring\textsuperscript{71}. Surgical ligation of the uterine artery (gestational day 19-21) in rats is a commonly used method to induce IUGR by reducing uteroplacental blood flow. This method has been previously demonstrated to decrease β-cell mass and impair glucose tolerance by 7 weeks of age\textsuperscript{72}. At 9 weeks of age the IUGR offspring display hyperlipidemia and become insulin resistant\textsuperscript{73}. Apart from reducing oxygen supply, decreasing blood flow by uterine ligation also reduces the transfer of amino acids, glucose and insulin. Therefore, it becomes difficult to delineate the direct effect of hypoxia on long-term disease risks in this experimental model of hypoxia. Placing rats in hypoxic chambers during gestation is usually the alternate approach undertaken to circumvent this issue\textsuperscript{69, 70}. Typically, rats are placed under an enclosed hypoxic chamber with an oxygen concentration of 10% during the last 6 days of gestation\textsuperscript{69, 70, 74}. Interestingly, prenatal hypoxia independent of undernutrition has been demonstrated to promote molecular markers of insulin resistance and increased cardiac susceptibility to ischemia injury\textsuperscript{69, 74}. However, recent work in our laboratory has demonstrated that this maternal hypoxia model leads to decreases in circulating glucose, but no change in cholesterol in 12 month offspring\textsuperscript{75}. Additional work is required to elucidate the molecular mechanisms behind this observation.

1.6.2 Maternal Iron Restriction in Rodents on Long-term Cholesterol and Glucose Homeostasis

Given that ~50% of pregnant women are iron deficient and that maternal anemia is linked to abnormalities in birth weight\textsuperscript{76}, animal models of iron restriction have been utilized to explore the link between impaired fetal development and long-term diseases. In rats, maternal iron restriction has been demonstrated to lead to decreases in fetal weight and plasma cholesterol levels, but an increase in fetal hepatic cholesterol\textsuperscript{77}. In addition, this corresponded with reduced expression of fetal hepatic expression of key cholesterol catabolism (\textit{i.e.} Cyp7a1) and triglyceride synthesis (\textit{i.e.} Srebp1-c) genes\textsuperscript{77}. Consequently,
there was a reduction in the hepatic expression of Srebp1-c downstream transcriptional target genes Fatty acid synthase (Fas) and Acetyl-coenzyme A carboxylase-α (Accα). Interestingly, maternal iron restriction did not alter plasma cholesterol levels and actually improved glucose tolerance in the offspring by three months. This suggests that iron restriction in utero has short-term effects on fetal liver function which may be corrected with restoration of maternal iron postpartum. This recovery in function is likely to be due to the highly plastic nature of the rat liver during the first three weeks of neonatal life. However, it still remains possible that maternal iron restriction may alter hepatic cholesterol and glucose regulation long-term (>3 months). In order to accurately extrapolate these findings to assess the impact of maternal iron deficiency on long-term liver function in humans, an iron deficient diet should continue to be administered for the first three weeks of postnatal life in these rat offspring, since in rodents liver differentiation develops late in gestation with a large portion of it occurring in postnatal life. In contrast, liver differentiation in humans already occurs by early to mid-gestation. Therefore, at birth the rat liver is ‘less’ mature and has greater plasticity than the human liver. This makes the rat liver more susceptible to further insult or recovery during early neonatal life.

### 1.6.3 Effects of Low Protein Diet on Long-term Cholesterol and Glucose Homeostasis

While iron restriction in pregnancy has been demonstrated to lead to IUGR and elevated fetal cholesterol levels, the maternal protein restriction (MPR) rat model is one of the most widely used animal models to investigate the correlation between IUGR and adult diseases. Since amino acids play a critical role in fetal growth, this MPR model is a relevant model to study the fetal origins of adult diseases. Furthermore, as placental insufficiency in humans can produce protein deficiency in the fetus, this MPR model shares features in common with IUGR, which represents 8% of newborns. Studies of Wistar rats have demonstrated that a MPR diet (8% protein vs 20% protein control) during gestation and lactation results in decreased birth weight (asymmetrical IUGR), and permanently reprograms the functions of various organs including the pancreas, muscle, adipose tissue and liver. In the liver, reduced maternal dietary protein is
associated with alterations in hepatic glucose output, decreased glucagon receptor and glucokinase expression, along with increases in hepatic lobular volume\textsuperscript{90}. As a result of MPR on the function of these organs, the offspring exhibit increased visceral fat\textsuperscript{88}, symptoms of type 2 diabetes\textsuperscript{93, 94}, and hypertension\textsuperscript{95, 96} in adulthood. Given that these symptoms characterize the metabolic syndrome\textsuperscript{97} which substantially increases the risk for CVD\textsuperscript{1}, this MPR model is very useful to study the fetal programming of CVD. However, the role of MPR on circulating cholesterol, another important clinical risk factor characterizing the metabolic syndrome\textsuperscript{97}, remains elusive.

There is growing evidence that individual amino acids may be as important as the overall levels of macronutrients in determining size at birth and postnatal disease risk. For example, supplementing a MPR diet with glycine alone can prevent postnatal hypertension\textsuperscript{98}. Others have demonstrated that taurine supplementation to the MPR diet during pregnancy and lactation restores islet proliferation and pancreatic vascularization\textsuperscript{99, 100}. Future research on the role of micronutrients during development on the risk of postnatal diseases is anticipated with great interest. For a review on the role of macronutrients in the developmental origins of health and disease, please see a review article previously published by our laboratory\textsuperscript{101}.

Collectively, animal models of IUGR provide further support for the hypothesis that impaired development due to various maternal deficiencies can lead to impaired glucose and cholesterol homeostasis in adulthood\textsuperscript{77, 85, 102-104}. These deficiencies in humans and animals can include hypoxia\textsuperscript{71}, diminished protein\textsuperscript{105}, caloric restriction\textsuperscript{106}, excess glucocorticoids\textsuperscript{107, 108}, infections\textsuperscript{109} or deficiencies in the microenvironment including specific amino acids, vitamins and minerals\textsuperscript{78, 98, 110-112}. Although the correlation between impaired development and the risk of developing chronic disease in adult life is undoubtedly strong, the molecular mechanisms behind programming of pathophysiological processes are only beginning to be elucidated.
1.7 Molecular Mechanisms Underlying the Early Programming of Metabolic Syndrome

Regardless of the type of \textit{in utero} insult, animal models comprehensively demonstrate that the long-term regulation of cholesterol and glucose homeostasis is permanently altered in IUGR offspring. However, the molecular mechanisms underlying these events are only beginning to be uncovered.

1.7.1 Transcriptional Mechanisms Underlying the Early Programming of Metabolic Syndrome

Without question, understanding the role of transcription factors in this process will provide us with specific drug targets for the prenatal and postnatal prevention of these adult diseases. Nuclear receptors represent the largest family of transcription factors found in metazoans, binding to steroid hormones, fat-soluble vitamins, along with oxysterols and bile acids from the diet\textsuperscript{113}. Although the roles of many nuclear receptors are well defined, very little is known about their function in fetal programming. As mentioned previously, a subset of nuclear hormone receptors, namely the Liver X Receptors (LXR\textalpha{} and LXR\textbeta{}), are important mediators of cholesterol and glucose homeostasis in the adult by serving as important transcription factors\textsuperscript{113, 114}. Both receptors share 77\% homology and heterodimerize with the Retinoid X Receptor (RXR) to activate a wide variety of downstream target genes important for glucose synthesis and uptake, and cholesterol metabolism and efflux\textsuperscript{113, 114}. The LXR/RXR heterodimer can be activated by both RXR ligands (i.e. retinoids) and LXR ligands (i.e. oxysterols)\textsuperscript{113}. Upon ligand activation there is a recruitment of coregulators at the LXRE site of LXR target genes\textsuperscript{115}. The LXR\textalpha{} isoform is highly expressed in the liver but is also abundant in macrophages, the intestine, muscle, adipose and kidney, while LXR\textbeta{} is ubiquitously expressed\textsuperscript{113}. One of the main functions for LXRs is activating the expression of cholesterol 7a-hydroxylase (Cyp7a1), the rate-limiting enzyme for the conversion of cholesterol to bile acids. Studies where adult mice were fed a diet 100 times greater than their normal daily cholesterol intake found that they did not form atherosclerotic lesions\textsuperscript{116}. This was demonstrated to be due to an LXR\textalpha{}-mediated increase in the
expression of Cyp7a1\textsuperscript{116}. However, mice deficient in LXR\(\alpha\) lose the capacity to catabolize dietary cholesterol, via decreases in the expression of Cyp7a1, along with impairments in sterol and fatty acid synthesis\textsuperscript{117}. These studies implicate the important role of LXR\(\alpha\) in cholesterol homeostasis. The LXR\(\beta\) knockout mouse does not exhibit any alterations in bile acid metabolism\textsuperscript{118}, however mice deficient in both LXR\(\alpha\) and LXR\(\beta\) exhibit a more severe phenotype than LXR\(\alpha\) null mice alone with respect to cholesterol dysregulation\textsuperscript{119}. In addition to promoting cholesterol catabolism (\textit{i.e.} Cyp7a1), LXR\(\alpha\) and LXR\(\beta\) regulate transcription of a number of other target genes involved with cholesterol homeostasis, including efflux (ATP-binding cassette transporters \textit{ABCA1}, \textit{ABCG5} and \textit{ABCG8}) and high density lipoprotein (HDL)-mediated reverse transport (\textit{apoE}, \textit{PLTP}, \textit{CETP})\textsuperscript{115}. Mutations in several of these LXR target genes have major effects on circulating cholesterol concentrations and upon cardiovascular disease\textsuperscript{120}. For instance, humans lacking functional \textit{ABCA1} exhibit lower circulating HDL, increased arterial wall thickness, and a 6-fold increased risk for cardiovascular disease\textsuperscript{121}. In addition, humans or mice deficient in \textit{apoE} have elevated plasma cholesterol (LDL), triglycerides, and cardiovascular risk\textsuperscript{122, 123}. More recently, the role of LXR as an important regulator of glucose homeostasis has been established\textsuperscript{124}. Specifically, LXR has been found to repress critical genes involved with glucose production, including glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (Pepck) and 11\(\beta\)-hydroxysteroid dehydrogenase-1 (11\(\beta\)-HSD1), and increased expression of genes involved with glucose uptake, including glucose transporter 1 and 2\textsuperscript{125-127}. G6Pase and Pepck are genes involved with gluconeogenesis, while 11\(\beta\)-HSD1 increases glucose production by converting inactive corticosteroids to their active form (Figure 1.2).

Given that LXR\(\alpha\) plays a role in both cholesterol\textsuperscript{113} and glucose\textsuperscript{114} homeostasis, it is an attractive candidate for elucidating the molecular mechanisms underlying the etiology of the metabolic syndrome. To date, few studies have demonstrated links between nutrition, LXR\(s\) and liver function. Our laboratory has recently demonstrated that maternal hypoxia during pregnancy decreases the expression of hepatic G6Pase in adult offspring due to repressive changes at the DR-4 LXRE region (-258 to -169) of the rat \textit{G6Pase}
promoter\textsuperscript{75}. Both iron restriction and maternal protein restriction have been demonstrated to lead to decreased fetal LXR\textalpha\textsuperscript{77, 128}, but less is known about the long-term effects in postnatal life. Further work on the role of LXR\textalpha, RXR, and other lipid-sensing nuclear receptors are warranted to understand the transcriptional mechanisms involved in cholesterol dysregulation and impaired glucose tolerance.
Figure 1.2: Schematic Diagram of LXRα and some of its main downstream target genes involved with fatty acid synthesis, cholesterol homeostasis and glucose homeostasis. + = positively regulate, - = negatively regulate.
1.7.2 Epigenetic Mechanisms Underlying the Early Programming of Metabolic Syndrome

Epigenetics is an important mechanism in regulating the expression patterns of genes in a site and tissue specific manner in response to insults during development. The term ‘epigenetics’ was coined in 1942 by Conrad Waddington and was defined as ‘the branch of biology that studies the casual interactions between genes and their products, which bring the phenotype into being’\textsuperscript{129}. Most recently, Michael K. Skinner has defined epigenetics as ‘molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA sequence’\textsuperscript{130}. Epigenetic mechanisms essentially influence the long-term expression of a gene by altering the ability of the transcriptional machinery to interact with the chromatin environment. One way this can be accomplished is by direct DNA methylation via the addition of a methyl group to CpG sites on the DNA by members of the DNA methyltransferase family. Traditionally, an increase in methylation across CpG sites can impair initiation, elongation or termination of a gene. However, this may not always hold true as is the case with expression of Insulin-like growth factor-II (Igf-II), which is increased in IUGR offspring due to increased intragenic methylation\textsuperscript{131}. Animal models have previously demonstrated that a change in the environment as a result of an abundance or a limitation of nutrients during the period of development alters the expression of a variety of genes involved with DNA methylation, including DNA methyltransferases\textsuperscript{132-134}. However, very little is known about the impact of these alterations in correlation with promoter specific methylation status and long-term transcription of genes involved with cholesterol and glucose homeostasis in IUGR offspring\textsuperscript{135}. At the moment, clinical and animal studies are only beginning to demonstrate alterations in promoter specific methylation patterns of key genes associated with cholesterol dysregulation and type 2 diabetes in IUGR offspring, exclusively at an observational level\textsuperscript{128, 136, 137}.

The second major epigenetic mechanism involves influencing the chromatin environment via a number of post-translational modifications. Chromatin is mainly compromised of genomic DNA complexed with histone and non-histone proteins. Nucleosomes, the basic
building b/tarlocks of chromatin\textsuperscript{138}, are composed of 146 base pairs of DNA wrapped around two copies each of histones H2A, H2B, H3 and H4 which comprise the histone octamer\textsuperscript{12}. Histones are subject to a wide variety of post-translational modifications at over 60 amino acid residues\textsuperscript{139}, including methylation, acetylation, phosphorylation, ubiquitination and ADP-ribosylation\textsuperscript{139, 140}. The combinatorial nature of these covalent modifications reveals a “histone code”\textsuperscript{139, 140}. This code provides for a regulatory system that may dictate the transient, as well as long-term, transition between gene silencing or activation (Figure 1.3). Amongst all the different types of post-translational histone modifications, acetylation and methylation of lysine and phosphorylation of serine and threonine residues can be considered as well characterized. This is because their roles have been exhaustively examined through genome wide mapping studies which were geared towards detecting correlations between histone modification patterns and specific states of gene activity\textsuperscript{141}. The effects of other types of post-translational histone modifications on the overall chromatin structure are poorly understood and are mostly analyzed at the descriptive level\textsuperscript{141}. Of interest, acetylation and phosphorylation of histones have a high turnover rate, with a half life of only a few minutes\textsuperscript{142, 143}. In contrast, the turnover of histone lysine methylation is much slower and is similar to that of the entire histone (~1.3 days)\textsuperscript{144}. In addition, the greater the degree of methylation (i.e mono, di or tri-methylation), the greater the half-life and more stable the chromatin state\textsuperscript{144, 145}. Therefore, amongst all of the well characterized post-translational histone modifications, the tri-methylation of histone at lysine residues can be regarded as most suited towards maintaining long-term gene transcription\textsuperscript{140, 146-149}. Genes linked to cholesterol and glucose homeostasis can be upregulated or repressed depending on changes to the availability and demand of these two products in the body. Therefore, their promoter regions are expected to facultatively change from euchromatin to heterochromatin states, in order for transcription factors and co-regulators to appropriately access the region and exert their transcriptional effects. Therefore, amongst all the possible histone lysine residues which are known to be tri-methylated, focus should be aimed at those residues which have been previously observed to occur at both facultative heterochromatin as well as euchromatin regions of the genomic DNA\textsuperscript{150-158}. Furthermore, careful consideration should be given on determining which specific lysine
residue on the histones is tri-methylated. This is because depending on the type and location of the amino acid residue undergoing the posttranslational histone modification, one can observe either an increase or a decrease in gene transcription. For instance, an increase in the levels of tri-methylation at Histone H3 at lysine 4 and 36 is associated to be linked to active transcription. Conversely, if the increase in tri-methylation were to occur at Histone H3 lysine at 9 and 27, it would be associated with transcriptional repression.\(^{159}\)

It is important to realize that the different prenatal insults which lead to IUGR offspring seem to have both common and distinct adaptive responses initiated via epigenetic mechanisms. Therefore IUGR offspring derived from different insults may differ or be similar due to global, tissue, or site-directed epigenetic modifications. To date, there is very little known about the epigenetic alterations associated with expression of target genes in the liver throughout normal and abnormal fetal and postnatal development.\(^{91, 92, 160}\) Given that LXR\(\alpha\) has an important role in regulating transcription of genes linked to cholesterol homeostasis,\(^ {113}\) it becomes important to evaluate the underlying role of epigenetic mechanisms \(i.e.\) DNA methylation and posttranslational histone modifications) on regulating the long-term transcription of LXR\(\alpha\) and its target genes in an animal model of IUGR. However, there is limited knowledge about the posttranslational histone modifications which alter the activation of LXR target genes. One study in chick embryonic hepatocytes has demonstrated that LXR agonists increase the transcription of ACC\(\alpha\) via increased LXR binding to the LXRE within the 5’ upstream region of ACC\(\alpha\) along with enhanced acetylation of Histone H3.\(^ {161}\) In vitro studies by Yu \textit{et al.} have demonstrated that the LXR\(\alpha\) mediated transcriptional activation of the \textit{FAS} gene was associated with enriched Histone H3 and H4 acetylation at the LXRE within 30 minutes of ligand addition.\(^ {162}\) While these studies suggest that LXR target genes are subject to regulation by posttranslational histone modifications, little is known about the epigenetic alterations associated with expression of hepatic LXR target genes throughout normal or abnormal fetal and postnatal development. One study has reported that MPR during pregnancy results in the suppression of LXR\(\alpha\) expression in the fetal liver as a result of methylation of the upstream region of the \textit{LXR\(\alpha\)} promoter, but
effects in postnatal life were not investigated\textsuperscript{128}. As such, the influences of epigenetic mechanisms on LXR target genes on giving rise to the etiology of the metabolic syndrome also deserve consideration. Recently, our laboratory has demonstrated that maternal hypoxia during pregnancy leads to enhanced histone H3 methylation [lysine 9] surrounding the LXRE region (-258 to -169) of the rat \textit{G6Pase} promoter by 12 months of age\textsuperscript{75}, but the effects of under nutrition during perinatal life on long-term LXR activity at promoter of its target genes remain elusive.

Aside from post-translational histone modifications which may govern the long-term expression of genes, microRNAs (miRNAs) may also play a key role in the fetal programming of cholesterol and glucose homeostasis. miRNAs are short, noncoding RNA molecules of 20-25 nucleotides in length that regulate gene expression via messenger RNA (mRNA) degradation and/or translational repression\textsuperscript{163, 164}. By regulating the expression of target genes, miRNAs have been demonstrated to alter a variety of physiological processes including cell cycle regulation, differentiation, metabolism, and aging\textsuperscript{164}. miRNAs silence gene expression by binding to the 3′-Untranslated Region (3′-UTR) with partial sequence homology to induce cleavage or repression of productive translation\textsuperscript{165}. Given their ability to bind 3′-UTR with partial sequence homology, a single miRNA may have multiple targets in the genome\textsuperscript{165}. Conversely, given the nature of miRNA targeting, a single mRNA transcript can theoretically be targeted by several miRNAs\textsuperscript{165}. While miRNAs likely play an important role in the etiology of adult diseases and cancer, their role in fetal development and programming remain elusive. Recent microarray studies employing primers for a variety of miRNAs have demonstrated that maternal nutrient restriction can permanently alter the expression of miRNAs in the aortas of newborn and aging rat offspring\textsuperscript{163}. Moreover, circulating hypoxia regulated miRNAs have been demonstrated to be increased in pregnant women with fetal growth restriction\textsuperscript{166}. The role and identification of miRNAs altering the expression of genes involved in the fetal programming of cholesterol and glucose homeostasis remain to be identified, but are the subject of great interest.
Figure 1.3: Example of how posttranslational modifications to Histone H3 (Lysine 9) Leading to Chromatin Remodeling. A variety of histone demethylases can remove the methyl group and histone acetyl transferases (HATs) can add an acetyl group to the same histone H3 subunit at the Lysine 9 residue leading to an active chromatin environment. Conversely, a variety of histone deacetylases (HDACs) can remove the acetyl group and histone methyl transferases (HMTs) can add back the methyl group leading to an inactive chromatin environment.

HAT = Histone Acetylas
e
HMT = Histone Methyl Transferases
HDAC = Histone Deacetylases
1.7.3 Role of Sex Hormones in the Early Programming of Metabolic Syndrome

Sex steroid hormones remain an important mediator in long-term regulation of cholesterol and glucose in adults. It is well established that the onset and severity of numerous adult diseases differ between men and women. For example, men have higher 24-hour mean blood pressure, by approximately 6 to 10 mm Hg, compared to age matched premenopausal women, but this trend reverses after women reach menopause. In females, it is hypothesized that differences in sex hormones modulate regulatory systems leading to decreased hypertension and vascular dysfunction. Given the widespread origins of the metabolic syndrome, it is difficult to assess whether gender and, more specifically, sex hormones influence cholesterol catabolism and glucose homeostasis. Recent studies in animal models have suggested that perturbations to the maternal environment during pregnancy can lead to sex-specific, long-term consequences in postnatal life. For example, male offspring of rat dams exposed to 30% global nutrient restriction during pregnancy develop hypertension earlier than their female counterparts, whereas severe protein restriction (5% protein diet) during pregnancy results in programmed hypertension in both sexes. Moreover, an MPR diet during gestation and lactation results in pancreatic β cell dysfunction and visceral obesity exclusively in the adult male offspring at postnatal day 130. The sex specificity exists even at the epigenetic level in IUGR offspring. For example, uteroplacental insufficiency induced IUGR rats at postnatal day 21 have a global increase in the females and decrease in the males with respect to acetylation of Histone H3 (Lysine 9, 14) in the hippocampus and white matter. In addition, studies have now demonstrated that sex steroid hormones can influence epigenetic mechanisms, including post-translational histone modifications. Therefore, gender specific differences in the fetal programming of cholesterol and glucose homeostasis may be due in part to alterations in their “histone code”.
1.7.4 ‘Impaired Genesis Model’ in the Early Programming of Metabolic Syndrome

Another important mechanism whereby which early programming of adult diseases can occur is via the “impaired genesis model”. After a specific period of development in organs such as the brain, kidney and heart, there is a minimal level of replication and differentiation potential of the cells from these organs\textsuperscript{171-175}. This model suggests that any insults during these critical periods permanently alter the function of these organs by impacting the amount and quality of the long-lasting cells in these organs. For example, the heart of a newborn contains all the cardiomyocyte cells it requires for the rest of postnatal life. At term, 90% of these cardiomyocytes are binucleated and are terminally differentiated. As the heart displays limited plasticity after birth\textsuperscript{176}, any foreseeable insult during its development can cause permanent changes to its overall growth and function, which may later manifest into heart dysfunction in adult life. Placental insufficiency induced IUGR in sheep has been demonstrated to lead to a permanent increase in proportion of mononucleated cardiomyocytes\textsuperscript{177}. Similarly, several cases exist where IUGR display alterations in terminal composition and quantity of cells from other organs. For example, placental insufficiency during the second half of the pregnancy lead to IUGR guinea pigs, which have a decrease in the number of neurons in the hippocampus and cerebellum\textsuperscript{178}, a phenotype which could have a long-lasting impact on normal functioning of the hypothalamus-pituitary axis. Studies with both human and animal models have provided additional evidence supporting the impaired genesis model by demonstrating with use of different techniques that IUGR can lead to a lower number of nephrons, permanently programming kidney function\textsuperscript{171}. External stresses leading to IUGR can also impact pancreatic and muscle function by way of permanently reducing pancreatic \(\beta\) cell mass\textsuperscript{179} and increasing the proportion of type II b muscle fibers which are known to contain a lower number of mitochondria\textsuperscript{180}. The precise timing of the nutritional insult is also a critical factor in defining the overall impact of the impaired genesis model. This is primarily because the timing of the insult would have differing effects on the development of different organs and hence on disease risks. Moreover, the impact of an insult during the different windows of organ susceptibility can also differ
between species\textsuperscript{174}. Therefore, important consideration should be given when extrapolating findings from one species to another.

1.7.5 Endoplasmic Reticulum Stress in Underlying the Early Programming of Metabolic Syndrome

The endoplasmic reticulum helps maintain cellular function and survival, as it is essential for the synthesis, maturation and export of cellular proteins. A variety of insults such as hypoxia, nutrient deprivation, ischemia, viral or bacterial infection can result in excessive accumulation of misfolded or unfolded proteins in the ER lumen\textsuperscript{181}. The accumulation of these proteins triggers the activation of the ER stress pathway, which is also referred to as the unfolded protein response (UPR)\textsuperscript{181}. UPR aims to alleviate ER stress by decreasing global protein translation in order to reduce the load of the ER in processing these new proteins. A reduction in global protein translation is accomplished by an increase in phosphorylation of eukaryotic Initiation Factor (eIF)-2$\alpha$ at Serine (Ser) 51 residue, which leads to a rapid reduction in the initiation of mRNA translation\textsuperscript{182}. There is also an increase in ER chaperone protein levels \textit{i.e.} glucose regulated protein Grp78 and Grp94 to improve ER’s protein folding capacity\textsuperscript{183, 184}. Furthermore, ER stress induces the splicing of a highly conserved unconventional 26 bp intron of the X-box binding protein 1 (Xbp-1) mRNA, which encodes for a potent transcription factor involved with transcriptional upregulation of genes involved in coping with the ER stress\textsuperscript{183, 184}. Collectively, an increase in phosphorylation of eIF2$\alpha$ (Ser51), Grp94 and Grp78 protein levels, and splice variant XBP-1 mRNA level are considered hallmark indicators of elevated ER stress. Given the critical role of endoplasmic reticulum in overall cellular functions and survival, any stress to the ER would therefore aggravate the symptoms associated with the metabolic syndrome.

Over the last decade researchers have established a critical role of ER stress in giving rise to the risk factors, which characterize the metabolic syndrome in adults\textsuperscript{185, 186}. Predominantly, research has focused on identifying the various components in the ER stress pathophysiology that are linked to molecular mechanisms associated with insulin resistance and high cholesterol levels\textsuperscript{187-193}. An increase in insulin sensitivity associated
with weight loss demonstrated a substantial reduction in markers of UPR activation\textsuperscript{194}. P85α gene, a positive marker of insulin resistance, was demonstrated to bind to and cause the nuclear translocation of spliced XBP-1 in an ER stress dependent manner\textsuperscript{195}. Furthermore, Grp78 has been demonstrated to prevent an increase in phosphorylation of Akt1 at Ser 473 residue, a critical downstream event in the insulin signalling pathway\textsuperscript{196}. With respect to ER stress association with cholesterol dysregulation, translation attenuation in ER-stressed cells decreases Insig-1 levels\textsuperscript{193}. As a result, SREBP cleavage activating protein is released causing the translocation of SREBPs to the Golgi followed by proteolysis to generate the active SREBP transcription factors. These transcription factors are involved with activating target genes linked to lipid biosynthesis.

Interestingly, insults such as hypoxia, nutrient deprivation, ischemia, viral or bacterial infection that result in ER stress due to an accumulation of misfolded proteins, have also been implicated in placental insufficiency induced intrauterine growth restriction (PI-IUGR)\textsuperscript{182}. An elegant study by Yung et al. in 2008 clearly demonstrated a critical role of ER stress in placental dysfunction in human IUGR\textsuperscript{197}. As placental dysfunction primarily results in decreased nutrient and oxygen transport to the fetus\textsuperscript{5, 184-187}, one would anticipate ER stress to have an adverse effect on the development of IUGR fetal tissues. However, few studies have been conducted to investigate the evidence of UPR response in IUGR fetal tissues\textsuperscript{198}. Moreover, the role of ER stress in linking IUGR offspring to increased risk of developing the metabolic syndrome in adulthood remains poorly understood. A schematic summary of the major molecular mechanisms underlying the early life programming of the metabolic syndrome has been provided in Figure 1.4. This schematic allows for an appreciation of the interdependence which may exist between these mechanisms, a topic of future investigations.
Figure 1.4: Mechanisms underlying the early programming of metabolic syndrome. A variety of insults during pregnancy and weaning including hypoxia, dietary deficiencies, excess corticosteroids, infection and inflammation can impact important mechanisms which have been known to contribute to the pathophysiologies related to the metabolic syndrome. These mechanisms included epigenetic modifications, organelle stress, endocrine homeostasis and impaired organogenesis. Elucidating some of the interdependencies which may exist between these mechanisms, remain a topic of future investigations.
1.8 The Impact on Drug Metabolism by the Early Programming of Metabolic Syndrome

Several clinical studies have demonstrated a strong inverse association between birth weight and metabolic risk factors associated with CVD\textsuperscript{46-49, 54, 199, 200}. Animal studies provide further support for this association\textsuperscript{72, 90, 93, 104, 106, 201}. Therefore, low birth weight offspring would be more likely to be under medication (e.g. statins for hypercholesterolemia) for the management of symptoms associated with the metabolic syndrome. Moreover, use of these drugs would be anticipated to occur at an earlier time in their life. This is particularly relevant in cases where low birth weight offspring undergo accelerated growth in neonatal life and display these symptoms by adolescent age\textsuperscript{35, 36, 38-40, 202}. Yet, there is a lack of epidemiological evidence in humans to support the notion that there is indeed a higher usage of prescribed drugs in adult individuals that are born low birth weight.

Many clinical factors including age, sex, ethnicity, body mass index, diet (grapefruit and other fruit juices), drug interactions, lifestyle and genetics can all impact the pharmacokinetics of a drug in an individual\textsuperscript{203-208}. Surprisingly, little is known about whether low birth weight represents another significant clinical factor that can also alter the pharmacokinetics of a drug in adult life. This point deserves consideration because low birth weight offspring which experience conditions of limited supply of oxygen and nutrients during gestation, display a hallmark adaptive response where the brain is spared at the expense of peripheral organs, including the liver. The sparing occurs as a consequence of redistribution of blood flow from the peripheral organs (i.e. liver) in an attempt to maintain appropriate oxygen and nutrient supply to the brain\textsuperscript{209}. As a consequence, liver function is impaired in low birth weight infants\textsuperscript{210}. Furthermore, animal studies of IUGR have demonstrated long-term alterations in liver function\textsuperscript{89-92}. Since, the majority of prescribed drugs (~75\%) are metabolized in the liver\textsuperscript{211}, it is likely that low birth weight offspring with impaired liver function would alter the pharmacokinetics of drugs in adult life.
The cytochrome P450 (CYP) family of enzymes are responsible for metabolizing 75% of the drugs which are eliminated by the liver\(^\text{211}\). Reduced intravenous midazolam clearance in preterm infants provides indirect evidence suggesting alteration of hepatic CYP enzyme activity in low birth weight infants that are 2 to 15 days old\(^\text{212}\). To date, intravenous clearance of midazolam is considered the best probe to estimate hepatic CYP3A4 activity\(^\text{213}, 214\), an enzyme responsible for approximately 50% of all drugs metabolized by CYP family of enzymes\(^\text{211}\). However, there is little known about the impact of IUGR in humans on the long-term activity of these drug metabolizing CYP enzymes. Recently an animal study by Tajima et. al. in 2012 suggested that impaired development can have a long-term impact on drug metabolizing enzymes in the liver\(^\text{215}\). The study clearly demonstrated that when administering a high fat diet to pregnant mice there was a decrease in expression and activity of Cyp3a enzyme in the six weeks old offspring\(^\text{215}\). This coincided with the reduced clearance of trizolam, a substrate of Cyp3a\(^\text{215}\). Drug metabolizing enzymes, including Cyp3a and drug transporters, which are critical for the uptake and efflux of drugs, are known to be regulated by Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR). These two orphan nuclear receptors heterodimerize with the Retinoid X receptor (RXR), and subsequently bind to xenobiotic response elements at the promoter of drug metabolizing enzymes and transporter genes\(^\text{216}\). In addition to providing protection from xenobiotic toxicity, recent evidence demonstrates a physiologically important role of PXR and CAR in lipid and glucose metabolism\(^\text{217}\). Therefore, the hypercholesterolemia and impaired glucose tolerance observed in adult IUGR offspring may likely impact PXR and CAR’s ability to prevent toxicity from drugs in adult life. Conversely, CAR activation has been demonstrated to improve insulin sensitivity via glucose and lipid metabolic pathways\(^\text{218}\). However, PXR activation results in an increase in lipogenesis despite its ability to decrease gluconeogenesis\(^\text{217}, 219-221\). Not surprisingly, ligand activation of CAR during pregnancy has been demonstrated to prevent insulin resistance in offspring from high fat diet induced obese pregnant mice\(^\text{222}\). However, the effect of this on drug metabolizing enzymes in the liver was not examined. Interestingly, transient activation of CAR during neonatal life has been demonstrated to generate a stable epigenetic memory via promoter specific posttranslational histone modifications, which facilitate a long-term increase in
some of the Cyp drug metabolizing enzymes\textsuperscript{223}. Despite the recent evidence that early development impacts hepatic drug metabolizing enzymes long-term, there is still little known with respect to drug metabolism in adult life of IUGR offspring.

1.9 Summary

Numerous clinical and animal studies have provided evidence for an association between IUGR and an increased risk of developing cardiovascular disease. It is well established that impaired cholesterol and glucose homeostasis can significantly increase the risk of developing cardiovascular disease. Yet the mechanisms underlying the long-term impairment of cholesterol and glucose homeostasis in IUGR offspring are only beginning to be elucidated. Most animal models aim to investigate this by inducing IUGR by way of creating a hypoxic or a nutritionally deprived environment during gestation. The mechanisms implicated in the cholesterol and glucose homeostasis impairment in IUGR may include permanent alterations in organ development, hormone regulation, cellular stresses (\textit{i.e.} endoplasmic reticulum stress), and/or the global or promoter specific epigenetic environment. Lastly, considering that IUGR offspring would have a higher likelihood to be under medications (\textit{e.g.} statins for hypercholesterolemia), investigating whether alterations in these mechanisms influence their ability to metabolize drugs in adult life remains to be determined.
1.10 References


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2 HYPOTHESIS AND SPECIFIC AIMS

2.1 Focus of the Thesis

The incidence of metabolic syndrome in Canada is reaching epidemic proportions, with one in three Canadian family-practice patients between 40 and 60 years of age falling under this criterion\(^1\). Adults with the metabolic syndrome have a ~20-fold risk for developing cardiovascular disease (CVD)\(^1\), a leading cause of premature death worldwide. Most notably, impaired regulation of cholesterol and glucose can lead to a significant increased risk of developing CVD\(^2\)\(^3\). Several clinical studies have demonstrated that intrauterine growth restriction (IUGR) offspring, faced with a nutritional mismatch postpartum, have an increased risk of developing an atherogenic lipid profile and insulin resistance\(^4\)\(^-\)\(^10\). Maternal protein restriction (MPR) rat model has been extensively studied to investigate the adverse effects of a nutritional mismatch in postnatal life of IUGR offspring. The function of the liver, an important metabolic organ, has been previously reported to be impaired in the MPR derived offspring\(^11\)\(^-\)\(^14\). However, the underlying mechanisms, which predispose these offspring to cholesterol dysregulation and insulin resistance, remain elusive. The overall hypothesis of this thesis is that long-term alterations in the liver of IUGR offspring may mediate cholesterol dysregulation in adult life. The focus of the thesis is to identify alterations to key molecular mechanisms in the liver of MPR derived offspring that may lead to cholesterol dysregulation in adulthood. The thesis will also evaluate the ‘thrifty phenotype’ hypothesis with respect to whether these alterations become more harmful in conditions where the MPR derived IUGR offspring are faced with a nutritional mismatch in postnatal life. Moreover, the impact of this on the long-term ability to metabolize drugs in the liver will also be determined.
2.2 Specific Aim 1

To determine whether maternal protein restriction elevated cholesterol in adult rat offspring via repressive changes in histone modifications at the Cholesterol 7α-Hydroxylase promoter.

Adverse events in utero, such as intrauterine growth restriction (IUGR), can permanently alter epigenetic mechanisms leading to the metabolic syndrome, which encompasses a variety of symptoms including augmented cholesterol. The major site for cholesterol homeostasis occurs via the actions of hepatic cholesterol 7α-hydroxylase (Cyp7a1), which catabolizes cholesterol to bile acids. Post-translational histone modifications have been previously demonstrated to regulate Cyp7a1 transcription in adult mice15-17. In Chapter 3, we sought to determine whether posttranslational histone modifications influence the long-term expression of Cyp7a1 in IUGR.

Using a maternal protein restriction rat model we hypothesized that IUGR offspring may lead to elevated cholesterol in adult offspring via repressive changes in histone modification at the Cyp7a1 promoter. We describe in Chapter 3 that a low protein diet during pregnancy and lactation led to IUGR offspring with decreased liver to body weight ratios, followed by increased circulating and hepatic cholesterol levels in both sexes at day 21 and exclusively in the male offspring at day 130. The augmented cholesterol was associated with decreases in the expression of Cyp7a1. Chromatin immunoprecipitation (ChIP) revealed that this was concomitant with diminished acetylation and enhanced methylation of histone H3 [K9,14], markers of chromatin silencing, surrounding the promoter region of Cyp7a1. These epigenetic modifications originate in part due to dietary-induced decreases in fetal hepatic Jmjd2a expression, a histone H3 [K9] demethylase. Collectively, these findings suggest that the augmented cholesterol observed in low protein diet derived offspring is due to permanent repressive posttranslational histone modifications at the promoter of Cyp7a1. Moreover, this was the first study to demonstrate that maternal undernutrition leads to long-term cholesterol dysregulation in the offspring via epigenetic mechanisms.
2.3 Specific Aim 2

To determine whether nutritional mismatch in postnatal life of low birth weight offspring leads to elevated hepatic endoplasmic reticulum stress in adulthood.

Several clinical studies have demonstrated that low birth weight offspring, as a result of an adverse *in utero* environment, have an increased risk of developing the metabolic syndrome. Moreover, this risk is further exasperated when the low birth weight offspring is faced with a “nutritional mismatch” in the predictive postnatal environment. In rodent models, maternal protein restriction derived IUGR offspring when faced with a nutritional mismatch to normal protein in postnatal life have been previously demonstrated to result in impaired fetal growth, glucose intolerance and hypercholesterolemia (Chapter 3) in adulthood. Interestingly, there is strong evidence linking endoplasmic reticulum (ER) stress to both increased cholesterol and insulin resistance in adults. In Chapter 4, we sought to determine whether ER stress plays a role in predisposing low birth weight offspring to greater risk of metabolic syndrome.

Therefore, we hypothesized that MPR derived offspring faced with a nutritional mismatch are at a higher risk of developing metabolic syndrome in adult life due to presence of elevated hepatic ER stress. To address this hypothesis, pregnant Wistar rats were fed a control 20% protein diet (C) or a low 8% protein diet during pregnancy and postnatal life (LP1), during pregnancy and lactation (LP2), or exclusively during pregnancy (LP3). We describe in Chapter 4 that the LP2 and LP3 offspring which received a “nutritional mismatch” postpartum, displayed an elevation of established ER stress markers in the liver, including an increase in X box binding protein 1 mRNA splicing levels and elevated ER chaperones (Glucose regulated protein 78 and 94) at postnatal day 130. This was concomitant with attenuated protein synthesis, as indicated by increased phosphorylation of eukaryotic initiation factor 2α at Ser 51 residue. However, those offspring that were maintained on low protein diet postpartum (LP1) did not display elevated ER stress markers. These findings highlight a novel role of ER stress in predisposing low birth weight offspring, faced with a postnatal “nutritional mismatch”, to increased risk of developing the metabolic syndrome.
2.4 Specific Aim 3

To determine whether nutritional mismatch in postnatal life of low birth weight offspring leads to altered hepatic drug metabolism in adulthood.

Strong clinical evidence exists to support an association between low birth weight and increased risk of developing the metabolic syndrome. In rodent models, we have previously demonstrated that maternal protein restriction (MPR) results in impaired fetal growth and hypercholesterolemia in adulthood (Chapter 3). This was attributed to the long-term repression of hepatic cholesterol 7α-hydroxylase (Cyp7a1), a rate-limiting enzyme that catabolizes cholesterol to bile acids. Interestingly, decreased Cyp7a1 expression is associated with Endoplasmic Reticulum (ER) stress in a rat model of Hypothyroidism. Moreover, we have recently demonstrated ER stress markers to be elevated in the livers of adult MPR offspring (Chapter 4). As cytochrome p450 dependent (Cyp) enzymes reside in the ER of the liver and are the primary enzymes involved with Phase 1 of drug metabolism, in Chapter 5 we investigated whether their ability to metabolize substrates is altered in adult life of MPR derived low birth weight offspring.

Therefore, we hypothesized that MPR offspring may have an impaired ability to metabolize drugs in adulthood. To address this hypothesis, pregnant Wistar rats were either fed a control 20% protein diet (Control) or a low 8% protein diet throughout life (LP1) or exclusively during pregnancy and lactation (LP2). We describe in Chapter 5 that the Cyp3a, Cyp2b and Cyp2c enzymatic activity (Vmax/Km) increased at postnatal day 130, as evaluated by testosterone enzyme kinetics in liver microsomes via ultra performance liquid chromatography. The increase in activity was associated with an increase in mRNA levels of Cyp3a1, Cyp2c11, Cyp2b1 as determined through Q-RTPCR. This correlated well with an increase in expression of constitutive androstane receptor, which is known to regulate transcription of these Cyp enzymes. Collectively, these findings suggest that a low birth weight offspring may require greater amount of drugs which are metabolized by Cyp3a, Cyp2b and Cyp2c enzymes in order to exert their therapeutic effect. This is an extremely relevant finding as it is applicable to those individuals who are more likely to be prescribed medication (e.g. statins for
hypercholesterolemia) for the management of the symptoms associated with this syndrome.
2.5 References


MATERNAL PROTEIN RESTRICTION ELEVATES CHOLESTEROL IN ADULT RAT OFFSPRING DUE TO REPRESSIVE CHANGES IN HISTONE MODIFICATIONS AT THE CHOLESTEROL 7α-HYDROXYLASE PROMOTER

3.1 Introduction

High circulating cholesterol, in addition to hypertension, central obesity, and insulin resistance characterize the metabolic syndrome, and remains an important cardiovascular risk factor in adults\(^1,2\). The major site for the regulation of cholesterol homeostasis occurs in the liver, mainly through the conversion of cholesterol to bile acids, via the rate limiting enzyme cholesterol \(7\alpha\)-hydroxylase (Cyp7a1)\(^3\). The expression of Cyp7a1 is under the regulation of the orphan nuclear receptor, the Liver X Receptor (LXR)\(^4\). Male and female mice deficient in Cyp7a1 have elevated circulating cholesterol and decreased LDL receptors, collectively leading to a ‘proatherogenic’ phenotype\(^5\). While current therapies using the statin class of lipid-lowering drugs have been successful in treating patients with high cholesterol\(^6\), some patients experience adverse side effects such as rhabdomyolysis\(^7\). Thus, additional novel strategies are warranted to prevent the onset of hypercholesterolemia and reduce the long-term use of these drugs.

Clinical studies in humans have demonstrated that adverse events in utero that result in placental insufficiency-induced intrauterine growth restriction (PI-IUGR, representing 8% of newborns), can also alter physiological processes leading to the metabolic syndrome\(^8,9\). Moreover, 50% of metabolic syndrome-related diseases can be linked to adverse events in utero\(^10-12\). With respect to cholesterol, David Barker and colleagues have demonstrated that impaired fetal growth is linked to elevated LDL cholesterol and hypertension in adult humans\(^13,14\). Moreover, the association between birth weight and cholesterol was more apparent in males and independent of social class, current body weight, cigarette smoking, and alcohol consumption\(^13\). Given that hypercholesterolemia in mothers is associated with increased incidence of aortic fatty-streaks in fetal and adolescent life\(^15\), it is conceivable that postnatal cholesterol concentrations may be altered by the perinatal environment, though the underlying molecular mechanisms are unknown.

Various animal models have broadened our understanding of how early nutrition in pregnancy may influence liver development\(^16-19\). The majority of studies have demonstrated that maternal nutrient or protein restriction leads to decreased liver
growth\textsuperscript{16-19}. Other studies have demonstrated that a low protein diet during pregnancy leads to asymmetrical IUGR\textsuperscript{20}, associated with alterations in hepatic glucose output, decreased glucagon receptor and glucokinase expression, along with increases in insulin degradation and hepatic lobular volume\textsuperscript{16, 17}. However, very little is known about how maternal nutrition may alter cholesterol homeostasis in postnatal life. In one study, maternal dietary iron restriction led to a 21\% decrease in fetal growth and an increase in fetal liver cholesterol\textsuperscript{21}. This was further associated with decreased expression of Cyp7a1\textsuperscript{21}. More recently, a low protein diet throughout pregnancy in mice was demonstrated to lead to reduced LXR\(_\alpha\) expression at embryonic day 19.5\textsuperscript{22}, however the long-term effects of this \textit{in utero} nutritional insult on cholesterol homeostasis in postnatal life remain elusive.

Although transcriptional changes may mediate the expression of target genes involved in fetal programming, there is limited knowledge on the links between epigenetic mechanisms \textit{in utero} and the long-term expression of hepatic target genes\textsuperscript{18, 19, 23, 24}. Previous studies have demonstrated that diminished acetylation and/or an increased methylation of histone H3 [K9,14] has been associated with chromatin silencing and decreased RNA Polymerase II binding\textsuperscript{25, 26}. Elegant studies by Park \textit{et al.} have demonstrated that uterine-ligation induced IUGR rat offspring developed type 2 diabetes as a result of epigenetic silencing of pancreatic and duodenal homeobox 1 (Pdx1), a critical transcription factor regulating β cell differentiation\textsuperscript{27}. This included both deacetylation and hypermethylation of histone H3[K9]\textsuperscript{27}. Recently it was demonstrated that in a model of a low protein diet during pregnancy, LXR\(_\alpha\) expression was suppressed in the fetal liver as a result of DNA hypermethylation at the \textit{LXR\(_\alpha\)} promoter\textsuperscript{22}, however the long-term effects of this diet on LXR-target genes (\textit{e.g.} Cyp7a1) in postnatal life were not examined. In addition, the effects of a low protein diet on posttranslational histone modifications during liver development have never been explored.

In the present study, we tested the hypothesis that a low protein diet in pregnancy may impair cholesterol homeostasis long-term via repressive changes to histone modifications throughout development in a promoter-specific manner. We observed whether the timing
of the low protein diet throughout pregnancy and lactation led to increases in circulating cholesterol in both early (3 weeks) and late (18 weeks) postnatal life. To accomplish this, four dietary regimes (Control, C; Low Protein, LP1-LP3) were employed to assess the effects of a low protein diet throughout life (LP1), until the end of lactation (LP2), and exclusively during gestation (LP3). By examining all three low protein dietary regimes, it is possible to assess the impact of the duration of the low protein diet and the timing of protein restoration on liver development and circulating cholesterol. Given that Cyp7a1 plays a major role in regulating cholesterol catabolism in the liver, we also demonstrated if changes in circulating cholesterol were linked to alterations in Cyp7a1 expression. Finally, we investigated whether any changes to Cyp7a1 expression in fetal and postnatal life were due to differences in the acetylation and trimethylation of histone H3 [K9] surrounding the Cyp7a1 promoter.

3.2 Materials and Methods

3.2.1 Animals and Dietary Regimes

All procedures were performed in accordance with the guidelines set by the Canadian Council of Animal Care and upon approval of the Animal Care Committee of the University of Western Ontario. Female and male Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St-Constant, Quebec, Canada). These rats were housed in individual cages and maintained at room temperature on a 12:12 hour light-dark cycle. For three weeks, these rats were left to acclimatize to the animal care facility and their reproductive cycles were followed. At the onset of pro-oestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning.

At the onset of proestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning. Upon confirmation of impregnation (gestation day 1), the rats were fed either a control diet containing 20% protein (Bio-Serv, Frenchtown, NJ, USA, Product# F4576 – Rodent Diet, AIN-93G, Blue, ½” pellets) or a LP diet containing 8% protein (Bio-Serv, Frenchtown, NJ, USA,
Product#F4575 – Rodent Diet, AIN-93G, ½” pellets). The LP diet contained similar fat content and was made isocaloric (3.61 kcal/gram for control and 3.66 kcal/gram for LP diet) by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ, USA).

At birth, the litter size was reduced to 8 animals (4 females and 4 males), with weights closest to the litter mean. This ensured a standard litter size for all mothers. Four different dietary regimes were administered to these offspring (Table 3.2). Offspring derived from a maternal low protein diet were either administered the low protein diet throughout postnatal life (LP1), until the end of lactation (LP2) or until birth (LP3). Otherwise, they were given a control diet.

Food and water was provided ad libitum and food intake was recorded during pregnancy and in the offspring. At embryonic day 19 (e19), a subset of pregnant rats were sacrificed, and the weights of the fetus, placenta and liver were measured, and the fetal liver was excised and frozen. Another subset of rats was allowed to deliver spontaneously. The growth curves and food intake of these offspring were monitored by measuring their body weight and food consumption every third day. At both postnatal day 21 (d21), the selected time for weaning, and at postnatal day 130 (d130), the pups were also sacrificed for blood and medial lobe liver tissue analysis.

3.2.2 Plasma Lipid Measurements

Measurements of total cholesterol and triglycerides from plasma of all blood samples was automatically detected using the COBAS® analyzer (Roche, Mississauga, Ontario, Canada) at St. Joseph’s Health Care (London, Ontario, Canada). For the measurement of circulating cholesterol, cholesterol esters were cleaved by cholesterol esterase and subsequently converted to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. A colorimetric assay resulting from breakdown of hydrogen peroxide via the Trinder reaction allowed for the quantification of cholesterol levels.
3.2.3 Hepatic Cholesterol Measurements

In order to determine the total amount of cholesterol present in hepatic tissue, a (1:1) chloroform:methanol extraction was performed in order to extract and separate total cholesterol and phospholipids from aqueous matter. After samples were dried, the total amount of cholesterol was determined by the Amplex®Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA, USA) which produces resorufin as a cholesterol by-product. This was measured using a fluorescence spectrometer at 590 nm.

3.2.4 Real Time PCR Analysis

Total RNA from Wistar rat medial lobe liver tissue was extracted at e19, d21 and d130 by the one-step method of Chomczynski and Sacchi\(^28\) (TRIzol, Invitrogen, Carlsbad, CA, USA). RNA was treated with deoxyribonuclease to remove any contaminating DNA. 4 µg of the total RNA was reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sets directed against rat Jmjd2a, Jmjd2c, Jmjd2d, β-actin, and Hprt (ribosomal RNA) were generated via Primer Express software (PE Applied Biosystems, Boston, MA, USA) based on published sequences (Table 3.1). The relative abundance of each transcript was determined by real-time quantitative PCR as previously published\(^29\). For the quantitative analysis of mRNA expression, the Bio-Rad CFX384 Real Time System was employed using the DNA binding dye IQ™ SYBER green supermix (Bio-Rad, Mississauga, Ontario, Canada). The cycling conditions were 50 C for 2 min, 95 C for 10 min, followed by 45 cycles of 95 C for 15 sec, and 60 C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with ribosomal RNA (Hprt) or β-actin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope=-3.4) and equal priming efficiency for the different dilutions compared with their Ct values (data not shown). Given that all primer sets had equal priming efficiency, the ΔCt values (primer internal control) for each primer set were calibrated to the
experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared with calibrator was determined by the formula, $2^{\Delta\Delta Ct}$, in which $\Delta\Delta Ct$ is the calibrated Ct value.

### 3.2.5 Chromatin Immunoprecipitation (ChIP)

ChIP was performed on snap frozen medial lobe liver tissue excised at e19, d21 and d130 from male and female offspring derived from the Control or LP2 dietary regime. ChIP was performed using a modification\(^3\) of previously published methods\(^4\). Briefly, a small piece of snap frozen liver was homogenized and incubated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA. Crosslinking was terminated by the addition of Glycine (0.125M, final concentration). The liver tissue was washed once with cold PBS and placed in 500 µl of SDS lysis buffer (Millipore, Etobicoke, Ontario, Canada) with protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). The lysates were sonicated on ice to produce sheared, soluble chromatin. The lysates were diluted ten times with the addition of ChIP dilution buffer (Millipore, Etobicoke, Ontario, Canada) and aliquoted to 400 µl amounts. Each of the aliquots was precleared with protein A/G Plus agarose beads (40 µl, Millipore, Etobicoke, Ontario, Canada) at 4°C for 30 minutes. The samples were microfuged at 12500 rpm to pellet the beads, and the supernatant containing the sheared chromatin was placed in new tubes. The aliquots were incubated with 4 µg of antibodies against RNA Polymerase II (cat #05-623B), acetylated histone H3 (lysine 9,14, cat #05-399), trimethylated histone H3 (lysine 9, cat # 07-442, all from Millipore, Etobicoke, Ontario, Canada), or Liver X Receptor (LXRα, cat# sc-13068x, Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight. Two aliquots were reserved as ‘controls’ – one incubated without antibody and the other with non-immune IgG (Millipore, Etobicoke, Ontario, Canada). Protein A/G Plus agarose beads (60 µl) were added to each tube, the mixtures incubated for 1 h at 4°C and the immune complexes collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in wash buffer I (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.25 M LiCl), and in 2 x TE buffer.
The beads were eluted with 250 μl elution buffer (1% SDS, 0.1mM NaHCO₃ + 20 μg salmon sperm DNA (Sigma-Aldrich, Oakville, Ontario, Canada) at room temperature. This was repeated once and eluates were combined. Crosslinking of the immunoprecipitated chromatin complexes and ‘input controls’ (10% of the total soluble chromatin) was reversed by heating the samples at 65°C for 4 h. Proteinase K (15 μg, (Invitrogen, Carlsbad, CA, USA) was added to each sample in buffer (50 mM Tris-HCl, pH 8.5, 1% SDS, 10 mM EDTA) and incubated for 1 h at 45°C. The DNA was purified by phenol-chloroform extraction and precipitated in EtOH overnight at 20°C. Samples and ‘input’ controls were diluted in 10-100 μl TE buffer just prior to PCR. Real-time PCR was employed using forward (5’-TGCTTTGGTCACACTCAAGTTCA-3’) and reverse (5’-GCAATTCCCCAAATCAAGGA-3’) primers that amplify a -164 bp to +65 bp region encompassing the TATA box and the Cyp7a1 initiation site, forward (5’-CGTAGCTCAGGCTCTGCGCCCTT-3’) and reverse (5’-CTGGCCTGCAC-AAGAAGATGCGGCTG-’) primers that amplify a -123 bp to +53 bp region encompassing the TATA box and the rat Gapdh initiation site, and forward (5’-GGCCGGGTAATGCTATTTTT-3’) and reverse (5’-CCGAAACAGTGGGTCTGACT-3’) primers that amplify a -156 bp to +25 bp region encompassing the rat Cyp7a1 LXRE site (-128 bp to -81 bp) and the TATA box (PE Applied Biosystems, Boston, MA, USA). Using serial dilutions of rat liver chromosomal DNA, these primers were employed because this region has equal efficiency in priming to their target sequences. Primers against distal regions of the Cyp7a1 promoter were employed to ensure this region has the greatest recruitment of RNA Polymerase II.

3.2.6 Preparation of Tissue Membrane Extracts and Immunoblot Analysis

Wistar rat liver tissue membrane extracts were prepared using modifications of previously published methods. Briefly, a small piece of snap frozen medial lobe liver tissue was homogenized in a 1.2 ml of buffer A solution (250mM Sucrose, 2mM MgCl₂, 20mM Tris-HCl at overall pH 7.5) with protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). The homogenate was centrifuged at 2000 g for 10 min at 4°C. The
supernatant was retained and buffer A was added. This was centrifuged at 120,000 g for 45 min at 4°C and the pellet was retained as the membrane fraction which was resuspended in 200 ul of buffer B (80 mM NaCl, 2mM CaCl$_2$, 1% Triton X-100, 50mM Tris-HCl at overall pH 8) with protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). Equal concentrations of membrane proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA), were fractionated in gradient polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred onto PVDF membrane (Millipore, Etobicoke, Ontario, Canada). Blots were probed using Cyp7a1 rabbit polyclonal antibody (1:500, cat #sc-25536, Santa Cruz Biotechnology, Santa Cruz, CA, USA), LXRα (Liver X Receptor (cat# sc-13068x, Santa Cruz Biotechnology, Santa Cruz, California) and monoclonal HRP conjugated β-Actin (1:50,000, cat#A3854, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 5 % milk-1xTris-buffered saline-Tween 20 buffer and with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:10,000, cat#711-035-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in 5 % milk-1x Tris-buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA).

### 3.2.7 Statistics

All results were expressed as the mean of arbitrary values ± the standard error of the mean (SEM). The significance of differences (p<0.05) between mean values were evaluated using the unpaired Student’s t-test for results from qRT-PCR, ChIP and Immunoblot analysis. Two and one-way analysis of variance (ANOVA) followed by a Bonferroni’s Multiple Comparison post hoc test, was used to evaluate significance of differences for results comparing the effect of all the dietary regimes.
**Table 3.1. Real Time PCR primers**

<table>
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<th>Gene</th>
<th>Primer (5’-3’)</th>
<th>Reference No.</th>
</tr>
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<tr>
<td>Jmjd2a</td>
<td>FWD GCG AGC AGG AGC TGG CAG AG</td>
<td>NM 001107966</td>
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<tr>
<td>Hmg-CoA Reductase</td>
<td>FOR TAC ATC CGT CTC CAG TCC AAA A</td>
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<tr>
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<tr>
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3.3 Results

3.3.1 A low protein diet during pregnancy and lactation leads to impaired body weight and liver growth

A low protein diet during pregnancy has been previously demonstrated to decrease birth weight\textsuperscript{16-19} and lead to increases in visceral fat, impaired glucose tolerance, and symptoms of type II diabetes in adulthood\textsuperscript{35-38} in the rat offspring. Although others have demonstrated that offspring derived from this low protein dietary regime were not hypertensive at 4 weeks of age\textsuperscript{39}, low protein females placed on a high carbohydrate diet after lactation exhibited elevated systolic blood pressure at 1 year of age\textsuperscript{40}. In this study, a low protein diet resulted in a significant decrease in fetal to placental weight ratio and a marked decrease in fetal liver to body weight ratio at embryonic day 19 (Table 3.2). In agreement with previous reports\textsuperscript{41, 42}, a low protein diet did not alter maternal food intake and there was no observed change in litter size or sex ratio (Figure 3.1A). To further examine the effect of the timing and duration of the low protein diet on postnatal growth and liver development, various dietary regimes (LP1, LP2, LP3) were implemented (Table 3.2). At day 21, there was a significant decrease in both body weight and liver to body weight ratio in the male and female offspring derived from the LP1/LP2 diet compared to control (Table 3.2). However, offspring of either sex derived from the LP3 dietary regime displayed no difference in body weight and liver to body weight ratios when compared to the offspring derived from the control diet. We next observed the effects of LP1-LP3 on liver and body growth at 130 days of age. We examined this age based on our previous study which demonstrated sex-specific differences in glucose homeostasis at this time point\textsuperscript{42}. While the body weights of LP1 or LP2 male and female offspring regimes remained significantly lower at day 130, their liver to body weight ratio was restored compared to the control animals (Table 3.2). Furthermore, no change in the food intake of the offspring was observed in the four dietary regimes (C, LP1-3; data not shown), suggesting that it was a difference in the protein content of the diet and not appetite that was a primary factor in inducing any of the observed developmental changes.
Table 3.2. The effect of LP dietary regimes LP1, LP2, LP3 on liver growth and body weight in rat offspring at embryonic day 19 (e19), postnatal day 21 (d21), and postnatal day 130 (d130)

Dietary regimes and the liver to body weight ratio of offspring. To assess body weight (g), the fetal to placental weight ratio was determined for offspring killed at e19, and the body weight was determined for offspring killed at d21 and d130. To assess liver growth, liver to body weight ratio was determined for all three developmental time points. All data was expressed as SEM. For e19, dietary effects were determined using Student’s unpaired t test whereas for d21 and d130, a one-way ANOVA followed by a Bonferroni’s Multiple Comparison post hoc test was performed. * = Significant difference (P < 0.05) vs. control (n = 10–14/group). N/A refers to when the LP dietary regimes were the same.
Figure 3.1: Effect of maternal low protein diet on A. Maternal Food Intake and B. Maternal Weight. Pregnant rats were given either a control diet (20% protein) or a low protein diet (8% protein) during gestation. Weight of food eaten in g/day/animal and maternal weight gain from gestation day 1 to gestation day 22 in grams were measured, respectively. Results were expressed as the mean ± SEM.* = significant difference (p<0.05). n=4-6/group, where each n represents a different mother.
3.3.2 A low protein diet during pregnancy and lactation leads to augmented circulating cholesterol in both sexes in early development, and exclusively in the male offspring in adulthood

To determine whether LP1-LP3 led to postnatal changes in cholesterol levels, total circulating cholesterol was measured in the offspring at day 21 and day 130. Total circulating cholesterol levels was observed to be significantly increased in the male and female offspring derived from the LP1/LP2 dietary regime at day 21 (Figure 3.2A) and exclusively in the male offspring derived from the LP2 dietary regime at day 130 (Figure 3.2B). In addition, hepatic total cholesterol concentrations were similarly increased in male LP2 offspring at day 130 (Figure 3.2C). In a small cohort of control animals given a low protein diet from birth to day 21 (reverse LP3), circulating cholesterol was not significantly different from the control or LP3 offspring (Figure 3.2A). The increase in hepatic cholesterol in LP2 males at day 130 was not attributed to alterations in Hmg-CoA reductase or the LDL receptor (Figure 3.3). Furthermore, when plasma triglycerides were measured at day 21 and day 130, there was no statistical difference in triglyceride concentrations between any dietary groups at both ages (data not shown).
Figure 3.2: The effect of different low protein dietary regimes on A. circulating cholesterol concentrations (mM) in rat offspring at postnatal d 21 (n=10-14/group), B. postnatal d 130 (n=10-14/group). C. The effect of LP2 on median lobe liver total cholesterol at postnatal d 130 (n=5-6/group). Results were expressed as the mean ± SEM. Dietary effects were determined using a two-way analysis of variance (ANOVA) followed by a Bonferroni’s Multiple Comparison post hoc test. ** = significant difference (p<0.01) & * = significant difference (p<0.05) vs male control, ## = significant difference (p<0.01) vs female control.
Figure 3.3: The effect of the LP2 dietary regime on hepatic 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (Hmg-CoA reductase) and low-density lipoprotein receptor (Ldl receptor) in A, C male and B, D female rat offspring at postnatal d 130, respectively. Relative hepatic Hmg-CoA reductase and Ldl receptor protein levels were determined using Western blot analysis. Total protein was isolated and Hmg-CoA reductase and Ldl receptor protein was detected on a western blot using the Hmg-CoA reductase and Ldl receptor primary antibody. The Hmg-CoA reductase and Ldl receptor protein levels were quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM.* = significant difference (p<0.05). n=5-6/group, where each n represents a single offspring derived from a different mother.
3.3.3 Decreases in Cyp7a1 protein levels coincide with the respective increases in total circulating cholesterol levels in offspring derived from the LP2 dietary regime at day 21 and day 130

To elucidate the underlying mechanisms for the augmented cholesterol in the LP2 offspring, immunoblot analysis was performed to determine the protein levels of hepatic Cyp7a1, the rate limiting enzyme involved in the conversion of cholesterol to bile acids, during fetal and postnatal development. At embryonic day 19, a low protein diet led to a significant decrease in hepatic Cyp7a1 protein levels (Figure 3.4A). At day 21, both the male and female offspring derived from the LP1/LP2 dietary regime also had significantly lower Cyp7a1 protein levels (Figure 3.4B,C), which was associated with the augmented circulating cholesterol observed in both sexes at this age (Figure 3.2A). Interestingly, when maternal proteins were restored to the low protein offspring during lactation (LP3), Cyp7a1 protein levels significantly increased compared to control (Figure 3.4D). Finally, when the expression of hepatic Cyp7a1 was examined in both sexes from the LP2 dietary regime at day 130, only the male LP2 offspring exhibited significant decreases in Cyp7a1 protein levels (Figure 3.4E,F). This gender-specific decrease in Cyp7a1 coincided with changes in circulating and hepatic cholesterol at this age (Figure 3.2B,C).
Figure 3.4. The effect of the LP2 dietary regime on hepatic Cyp7a1 protein levels in rat offspring at embryonic d 19 (A), male (B), and female (C) rat offspring at postnatal d 21 and male (E) and female (F) rat offspring at postnatal d 130. (D) The effect of LP3 dietary regime on hepatic Cyp7a1 protein levels in male rat offspring at postnatal d 21. Relative hepatic Cyp7a1 protein levels were determined using Western blot analysis. Total membrane protein was isolated and Cyp7a1 protein was detected on a Western blot using the Cyp7a1 primary antibody. The Cyp7a1 protein level was quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM. *, Significant difference (P < 0.05); n = 4–7/group, where each n represents a single offspring derived from a different mother.
3.3.4 The LP2 induced reduction in hepatic Cyp7a1 protein in the male offspring is due to a decreased recruitment of RNA Polymerase II at the Cyp7a1 initiation site throughout fetal and postnatal development

To explore the effects of LP2 on transcriptional mechanisms, chromatin immunoprecipitation (ChIP) was employed to examine the recruitment of RNA Polymerase II to the Cyp7a1 initiation site. Quantitative real-time PCR was used to amplify an approximate 100 bp genomic region surrounding the TATA box and the initiation site (Figure 3.5A,C,E,G). This region was demonstrated to have maximal enrichment of RNA Polymerase II compared to distal sites of the Cyp7a1 promoter (Figure 3.6). At embryonic day 19, it was observed that there was a significant decrease in the recruitment of RNA Polymerase II to the initiation site of hepatic Cyp7a1 in the low protein fetuses (Figure 3.5A). Moreover, the decreased binding of RNA Polymerase II to the Cyp7a1 promoter persisted in the LP2 derived male offspring at both day 21 and day 130 (Figure 3.5C,E). The decrease in recruitment of RNA polymerase II was specific to the Cyp7a1 promoter region given no significant alterations in its recruitment was observed at the promoter of Gapdh, a housekeeping gene (Figure 3.5B,D,F,H). ChIP performed using non immune IgG displayed negligible level of binding to either the Cyp7a1 and Gapdh promoter region. The decrease in the recruitment of RNA Polymerase to the promoter of Cyp7a1 in LP2 offspring corresponded with both the decrease in Cyp7a1 protein levels (Figure 3.4A,B,D) and augmented cholesterol (Figure 3.2A,B). Given that there were sex-specific differences in circulating cholesterol and Cyp7a1 expression between males and females at day 130, the recruitment of RNA Polymerase II to the Cyp7a1 promoter was examined at that age for both sexes. Unlike the males, ChIP revealed no corresponding differences in RNA Polymerase II binding to the promoter of Cyp7a1 in the females at this age (Figure 3.5G).
Figure 3.5. The effect of the LP2 dietary regime on the \textit{in vivo} RNA polymerase II (RNA Pol II) binding at the initiation site of hepatic \textit{Cyp7a1} (A, C, E, and G) and the initiation site of \textit{Gapdh} gene (B, D, F, and H) in rat offspring at embryonic d 19 (e19), male rat offspring at postnatal d 21, male and female rat offspring at postnatal d 130, respectively. ChIP was carried out on snap-frozen liver tissues by immunoprecipitation with antibody specific for RNA polymerase II. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real-time PCR via use of primers specific for the initiation sites on the promoter regions of hepatic \textit{Cyp7a1} and \textit{Gapdh}. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM. *, Significant difference ($P < 0.05$); $n = 4–6$/group, where each $n$ represents a single offspring derived from a different mother.
Figure 3.6. In vivo RNA Polymerase II recruitment at three sites upstream of the hepatic Cyp7a1 promoter by employing ChIP with use of primers encompassing -2000 bp, -500bp and the TATAA box regions of a control male rat offspring at postnatal day 21. ChIP was carried out on snap frozen liver tissues by immunoprecipitation with antibody specific for RNA Polymerase II. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real time PCR via use of primers specifically targeting the -2000 bp, -500 bp and the TATAA box regions on hepatic Cyp7a1. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.
3.3.5 Decreased recruitment of RNA Polymerase II in the LP2 dietary regime is associated with chromatin silencing of Cyp7a1 promoter region via posttranslational histone modifications

It is well established that decreased acetylation and/or an increased methylation of histone H3 [K9,14] is associated with chromatin silencing and decreased RNA Polymerase II binding. Therefore, to investigate whether posttranslational histone modifications influenced chromatin remodeling and RNA polymerase II recruitment to the Cyp7a1 promoter during LP2, we employed ChIP utilizing antibodies directed against histone H3 methylation [K9] and/or acetylation [K9,14]. At embryonic day 19, an increase in the acetylation of histone H3 (Figure 3.7A), but no change in trimethylation (Figure 3.7B) was observed surrounding the active site of Cyp7a1 promoter. This was despite the fact that the protein levels of Cyp7a1 were slightly reduced (Figure 3.4A). However, a significant decrease in the acetylation along with an increase in the methylation of histone H3 [K9], was observed at the hepatic Cyp7a1 promoter derived from LP2 male offspring at both day 21 (Figure 3.5C,D) and day 130 (Figure 3.5E,F). At both ages in LP2 males, these epigenetic changes correspond exactly with the observed decreased recruitment of RNA Polymerase II in the Cyp7a1 promoter (Figure 3.5C,E), diminished expression of Cyp7a1 (Figure 3.4B,D), and the observed increases in circulating and hepatic cholesterol (Figure 3.2A,B,C). In contrast, no alterations the acetylation [K9,14] or methylation [K9] of histone H3 were observed at the hepatic promoter of Gapdh at this age (Figure 3.8). Interestingly, in day 130 females, where no differences in circulating cholesterol or Cyp7a1 expression were observed (Figure 3.2B, 3.4E), there was also no corresponding changes in acetylation (Figure 3.7G) or methylation (Figure 3.7H) surrounding the active site of Cyp7a1.

Given that the methylation of histone H3 [K9] can be influenced by specific [K9] demethylases, we next examined whether changes in their expression facilitated the increases in the trimethylation of histone H3 [K9] associated in the LP2 males. We examined only the steady state mRNA levels of those [K9] demethylases (Jmjd2a,
Jmjd2b, Jmjd2c, Jmjd2d) that removed trimethyl groups from [K9] of histone H3\textsuperscript{26}. Real-time quantitative PCR revealed that significant decreases in the hepatic Jmjd2a mRNA were observed only at embryonic d19 in LP fetuses (Figure 3.9A). No significant changes in the hepatic mRNA levels of Jmjd2b and Jmjd2c were observed between diets at this age, although there was a trend for a decrease in their expression due to the LP diet (Figure 3.9B,C). The steady-state mRNA level of all of these [K9] demethylases was the same at d 21 (Figure 3.9D,E,F) and undetectable at d 130 in the control and LP2 dietary regimes (data not shown). The hepatic Jmjd2d mRNA was low to undetectable at all ages examined. This LP diet-induced decrease in the fetal hepatic expression of this [K9] demethylase may initiate the hypermethylation of histone H3 surrounding the Cyp7a1 promoter observed in LP2 offspring.
Figure 3.7: The effect of LP2 dietary regime on the *in vivo* level of acetylated histone H3 [K9,14] (A, C, E, and G) and trimethylated histone H3 [K9] (B, D, F, and H) at the hepatic *Cyp7a1* LXRE site in rat offspring at embryonic d 19 (e19), male rat offspring at postnatal d 21, male and female rat offspring at postnatal d 130, respectively. ChIP was carried out on snap-frozen liver tissues by immunoprecipitation with antibodies for acetylated histone H3 [K9,14] and trimethylated histone H3 [K9]. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real-time PCR via use of primers specific for the LXRE site on the promoter regions of hepatic *Cyp7a1*. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM. *, Significant difference (*P* < 0.05); *n* = 4–6/group, where each *n* represents an offspring derived from a different mother. RXR, Retinoid X receptor.
Figure 3.8: The effect of LP2 dietary regime on the in vivo level of **A.** Acetylated histone H3 [K9,14] and **B.** Tri-methylated histone H3 [K9] at the hepatic Gapdh initiation site in male rat offspring at postnatal day 130, respectively. ChIP was carried out on snap frozen liver tissues by immunoprecipitation with antibodies for acetylated histone H3 [K9,14] and trimethylated histone H3 [K9]. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real time PCR via use of primers specific for the initiation site on the promoter regions of hepatic Gapdh. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.* = significant difference (p<0.05). n=4-6/group, where each n represents an offspring derived from a different mother.
3.3.6 Protein restoration during lactation (LP3 dietary regime) enhances the recruitment of LXRα to the promoter of Cyp7a1 at postnatal day 21

Given the important role of LXRα in the transcriptional regulation of Cyp7a1, we next investigated whether hepatic LXRα levels were altered in the low protein offspring by postnatal day 21, the earliest time point of cholesterol dysregulation and impaired Cyp7a1 expression. Intriguingly, in both LP2 and LP3 offspring, increases in total hepatic LXRα protein levels (Figure 3.10A,B) were observed. We next employed ChIP to investigate the in vivo binding of LXRα to the promoter of Cyp7a1 containing a well characterized LXRE site. Despite an increase in total LXRα, LP2 offspring did not exhibit any alterations in the binding of LXRα to the promoter of Cyp7a1 at day 21 (Figure 3.10C). However, when the low protein offspring received a 20% protein diet during lactation (LP3), LXRα binding was enhanced (Figure 3.10D). This is in association with restored Cyp7a1 expression in this dietary cohort (Figure 3.4D).
Figure 3.9: Quantitative RT-PCR mRNA level analysis of Jmjd2a (A and D), Jmjd2b (B and E), and Jmjd2c (C and F) in the livers of rat offspring derived at embryonic d 19 (e19) and male rat offspring at postnatal d 21, respectively. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Jmjd2a, Jmjd2b, Jmjd2c, and Hprt. The relative levels of each mRNA transcript were normalized to that of the levels of each Hprt mRNA transcript. Results were expressed as the mean ± SEM. *, Significant difference (P < 0.05); n = 4–9/group at embryonic d 19, where each n represents an offspring derived from two different mothers and n = 5–6/group at postnatal d 21, where each n represents an offspring derived from a different mother.
Figure 3.10: The effect of LP1/LP2 (A and C) and LP3 (B and D) dietary regime on hepatic LXRα protein and binding levels to Cyp7a1 LXRE site in rat offspring at postnatal d 21. Relative hepatic LXRα protein levels were determined using Western blot analysis. Total protein was isolated and LXRα protein was detected on a Western blot using the LXRα primary antibody. The LXRα protein level was quantified using densitometry and normalized to that of β-actin protein levels. Relative hepatic LXRα binding levels to Cyp7a1 LXRE site were determined using ChIP analysis. ChIP was carried out on snap-frozen liver tissues by immunoprecipitation with antibody specific for LXRα. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real-time PCR via use of primers specific for the LXRE site on the promoter regions of hepatic Cyp7a1. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.* Significant difference (P < 0.05); n = 4–7/group, where each n represents a single offspring derived from a different mother. RXR, Retinoid X receptor.
3.4 Discussion

In this study we present evidence for the first time that a low protein diet during pregnancy and lactation led to hypercholesterolemia in both sexes at postnatal day 21, with augmented cholesterol persisting in the male offspring at day 130. This was found to coincide with transcriptional repression of Cyp7a1, governed in part due to changes in posttranslational histone modifications surrounding the Cyp7a1 promoter during postnatal life. Moreover, low protein diet-induced decreases in the steady-state levels of Jmjd2a mRNA in utero may mediate the increased trimethylation of histone H3 (lysine 9) associated with this promoter at postnatal day 21 and day 130. These identified epigenetic and transcriptional mechanisms help in further understanding the link between IUGR and long-term cholesterol dysregulation. Uncovering these molecular mechanisms is critical given 50% of metabolic syndrome-related diseases can be linked to adverse events in utero\textsuperscript{10-12}. In identifying some of the epigenetic mechanisms underlying the in utero origins of hypercholesterolemia, this study helps identify early life dietary and/or drug intervention strategies to lower cholesterol and reduce the incidence of the metabolic syndrome. Such strategies could reduce the long-term use of cholesterol lowering drugs in adulthood.

As expected from previous studies by others\textsuperscript{(14-17)} and in our laboratory\textsuperscript{16-19}, a low protein diet resulted in reduced fetal to placental weight, neonatal growth, and liver to body weight ratio by postnatal day 21. Furthermore, this decrease in body weight persisted to adulthood (day 130) in offspring derived from a low protein diet during pregnancy and lactation, with (e.g. LP2) or without (e.g. LP1) a restoration in maternal protein content after lactation. This compliments previous studies suggesting that a low protein diet in early development has long-term implications on the overall growth of rats\textsuperscript{38}. The maternal protein restriction model of IUGR in rats is a relevant model to study the fetal origins of adult diseases given that amino acids play a critical role in fetal growth\textsuperscript{43}. Moreover, given placental insufficiency in humans can produce protein deficiency in the fetus\textsuperscript{44}, this model share features in common with human PI-IUGR. The impaired body weight is due to the low protein content in the diet given that maternal food intake was similar in all dietary regimes. Interestingly, the liver to body weight ratio
of offspring was not significantly different at postnatal day 130 in any dietary regime, suggesting that the impaired liver growth is ultimately recovered by adulthood, even though deficiencies in body weight may still be apparent.

As evident from their body weight at day 21, restoration of protein in the maternal diet at birth (e.g. LP3) resulted in complete catch-up growth in these offspring, with no changes in the liver to body weight ratio. Moreover, they also exhibited enhanced Cyp7a1 protein levels, contributing to the prevention of hypercholesterolemia. Given that during this period there is a high rate of replication, neogenesis, and apoptosis leading to extensive liver remodeling in the newborn, it is not surprising that a restoration of protein in the maternal diet during this developmental window rescued hepatic growth and function. Other studies have implicated the neonatal period as a period of plasticity in liver development. Stoffers et al. have demonstrated that in IUGR rats derived from uterine-ligated dams, neonatal administration of Exendin-4 (a GLP-1 analog), prevents the development of hepatic oxidative stress and insulin resistance. Further understanding the molecular mechanisms underlying this plasticity could lead to improved therapies designed to enhance hepatic growth and function long-term.

A low protein diet during pregnancy and lactation (e.g. LP1/LP2) was associated with hypercholesterolemia (~4.5 mM) at postnatal day 21 in both sexes. At this age, hepatic Cyp7a1 expression in these offspring was reduced two thirds compared to control. This is intriguing given that Cyp7a1 has been previously demonstrated to display maximal expression at postnatal day 21. A LP1/LP2-induced increase in circulating cholesterol by three weeks of age is of considerable interest with regards to the early development of cardiovascular disease. Interestingly, when a low protein diet was fed to nursing mothers fostering control diet offspring, circulating cholesterol levels was not significantly different, highlighting the importance of the in utero environment in the long-term regulation of cholesterol levels. In LP2 offspring at day 130, circulating and hepatic cholesterol was significantly higher (~30%) in the male LP2 offspring. The decrease in Cyp7a1 is likely the major contributor to the observed 30% increase in hepatic cholesterol given that there was no difference in the protein levels of Hmg-CoA
reductase (involved in cholesterol synthesis) and the LDL receptor (involved in cholesterol flux).

The augmented circulating and hepatic cholesterol observed in these LP2 offspring is similar to what is observed in Cyp7a1-deficient mice and in humans with a mutation in CYP7A1\textsuperscript{49, 50}. This elevation in baseline cholesterol in combination with other diet and lifestyle factors (\textit{i.e.} high fat diet) could make these animals more susceptible to hypertension and cardiovascular disease. For example, LP2 female offspring that were fed a control diet higher in carbohydrates after lactation exhibited elevated systolic blood pressure at 1 year of age\textsuperscript{40}. Although males were not examined in that study, it remains possible from our study that the augmented cholesterol from as early as three weeks could collectively contribute to a similar or worse phenotype. Moreover, the combination of impaired glucose tolerance\textsuperscript{38}, visceral obesity\textsuperscript{36}, and augmented cholesterol in these LP2 offspring would lead towards the development of metabolic syndrome.

It is interesting to note that while LP2 offspring had augmented cholesterol at day 130, neither LP1 or LP3 offspring exhibited this phenotype. This is likely due to the fact that a low protein environment during development results in adaptations that are better suited to a similar environment later on in life\textsuperscript{51}. Therefore, when a switch is made to a 20% protein diet after liver development\textsuperscript{52} (\textit{e.g.} LP2), the offspring become more susceptible to physiological disorders. It was surprising to find that the LP3 animals had normal circulating cholesterol in our study, given previous work which has demonstrated that rapid catch-up growth in humans is more tightly associated with components of the metabolic syndrome\textsuperscript{53}. The answer may be due to the differences in liver development and the duration of the low protein insult. Liver development in rodents and humans consists of embryonic cell specification, budding, and then differentiation\textsuperscript{52}. In rodents, liver differentiation develops later in gestation with a large portion of it occurring in postnatal life. In contrast, liver differentiation in humans already occurs in early to mid-gestation\textsuperscript{52}. Therefore, at birth the rat liver is ‘less’ mature and has greater plasticity than the human liver. This makes the rat liver more susceptible to further insult or recovery during that period of neonatal life. This plasticity in liver development is evident in the LP3 offspring whereby Cyp7a1 expression was restored at postnatal day 21 due to
increases in LXRα activation. This is in contrast to the LP2 offspring, which did not exhibit alterations in LXRα binding to the Cyp7a1 promoter. Collectively, this would suggest that it is the difference in the duration of the dietary low protein insult (LP2 = 43 days versus LP3 = 21 days) throughout the crucial stages of rat liver development that likely plays a greater role in long-term liver impairment than catch-up growth.

Epigenetic mechanisms have been previously demonstrated to underlie developmental programming of gene transcription. However, investigation of the epigenetic mechanisms linking an in utero insult to the development of disease in postnatal life is limited. Since a conserved CpG island is not found in the promoter region of the human and rodent Cyp7a1, it is more likely that posttranslational histone modifications may influence its long-term expression compared to DNA methylation. In the present study, significant decreases in the acetylation and increases in methylation of histone H3 [K9], promoting a repressive chromatin environment, was observed surrounding the hepatic Cyp7a1 promoter in LP2 male offspring at postnatal day 21. This persisted into adulthood (day 130), long after the switch to a control diet. The observed repressive chromatin environment correlated with decreased recruitment of RNA Polymerase II, diminished Cyp7a1 protein levels, and the observed increases in circulating cholesterol. This is not surprising as trichostatin A (TSA), a histone deacetylase inhibitor, has been previously demonstrated to increase Cyp7a1 expression and cholesterol metabolism when administered in mice. It is interesting to note that the significant increase in histone H3 trimethylation at postnatal days 21 was preceded by a decrease in the expression of the lysine 9 demethylase Jmjd2a during fetal life. This suggests that an early loss of demethylation in the fetal liver, due to a low protein diet, may initiate the enhanced trimethylation of histone H3 observed in LP2 offspring.

While an increase in the acetylation of histone H3 [K9,14] was observed surrounding the Cyp7a1 promoter at embryonic day 19, recruitment of RNA polymerase II to its promoter and Cyp7a1 protein levels were modestly reduced. Moreover, increases in the overall trimethylation of histone H3 (lysine 9) were not observed at this time in development. Therefore in fetal life, the decreased expression of Cyp7a1 is likely also due to transcriptional mechanisms. Recently, it was demonstrated that a low protein diet in mice
led to decreased expression of LXRα and certain downstream target genes at embryonic day 19.5 as a result of increased DNA methylation at LXRα promoter\textsuperscript{22}. Therefore, the decrease in recruitment of RNA polymerase II to the Cyp7a1 promoter in the fetuses derived from a low protein diet could possibly be due to decreased recruitment of LXRα at the Cyp7a1 LXRE site.

To date, very little is known about the links between malnutrition and epigenetic mechanisms during development, specifically with regards to the regulation of histone modifying enzymes. One study looking at fetal surfactant protein A (SP-A) expression demonstrated that lower oxygen tension \textit{in vitro} can promote alterations in these enzymes leading to a repressive chromatin environment\textsuperscript{55}. Given this low protein model leads to asymmetric IUGR\textsuperscript{20}, and that this brain-sparing effect is associated with fetal hypoxia\textsuperscript{56, 57}, this could conceivably mediate the observed decrease in JmjD2a expression. As a result, this would lead to the observed hypermethylation and hypoacetylation surrounding the promoter of Cyp7a1 during development.

Recent studies in animal models have suggested that perturbations to the maternal environment during pregnancy can lead to sex-specific, long-term consequences in postnatal life. For example, LP2 offspring exhibited insulin resistance\textsuperscript{42} and visceral obesity\textsuperscript{36} exclusively in the adult male offspring at postnatal day 130. In this study, circulating cholesterol increased while a concomitant decrease in Cyp7a1 protein levels and RNA Polymerase II recruitment was observed only in the adult male offspring at postnatal day 130. Furthermore, the male-specific silencing of Cyp7a1 expression observed at day 130 was governed, in part, due to male-specific repressive changes in posttranslational histone modifications surrounding the active site of the Cyp7a1 promoter. Given cholesterol and Cyp7a1 expression are misregulated in both sexes at day 21, but only in males at day 130, it is unlikely that imprinting is involved. Instead, it is plausible that gender-specific hormonal differences after lactation may mediate these programmed effects. It has been postulated that sex steroids (\textit{e.g} estrogen) may offer the female some protective effects against the development of these disease processes, including elevated blood pressure\textsuperscript{58}. Evidence from this comes from the aromatase knockout (ArKO) mouse, which cannot synthesize endogenous estrogens due to
disruption of the *Cyp19* gene\(^5^9\). *ArKO* females when challenged with a high cholesterol diet had higher circulating cholesterol and lower Cyp7a1 expression compared to wild-type females and males of either genotype\(^5^9\), where estrogen replacement reversed the hepatic steatosis\(^6^0\). However, the stimulatory effects of synthesizing estrogen may be only part of the reason for the sexual dimorphism observed in LP2 offspring. Given that LP2 males have suppressed levels of circulating testosterone compared to control males\(^4^2\), it is conceivable that the loss of this male sex steroid may also underlie the male-specific impairment of Cyp7a1 and cholesterol catabolism. Aside from sex steroids, another important difference in LP2 males and females in that study was circulating basal insulin concentrations. LP2 male offspring at postnatal day 130 have two-fold higher levels of circulating insulin\(^4^2\), which has been previously demonstrated to inhibit Cyp7a1 in both the rat hepatocytes and streptozotocin (STZ)-induced diabetic rats via decreases in the binding of the transcription factors FoxO1 and Smad3 to the promoter of *Cyp7a1*\(^6^1\). Moreover, studies have now established that these hormones can influence posttranslational histone modifications. For example, the estrogen receptor has been demonstrated to bind to coregulators (*i.e.* CBP/p300, SRC-1, SRC-2) that enhance promoter-specific histone H3 acetylation leading to an active chromatin state surrounding the promoter regions of estrogen responsive genes\(^6^2\)\(^-\)\(^6^4\). Therefore in LP2 females, it is conceivable that their ‘histone code’ was altered at the promoter of *Cyp7a1* via the actions of these hormones.

In conclusion, our results demonstrate that low protein diet-induced IUGR leading to augmented cholesterol in postnatal life results from permanent epigenetic silencing of the hepatic *Cyp7a1* promoter, via posttranslational histone modifications. Moreover, our study identifies the *in utero* environment as a novel risk factor for cholesterol dysregulation, and aids our understanding of why a prevalence of metabolic syndrome-related diseases can be linked to adverse events in fetal life. Given the plasticity of the liver in fetal and neonatal life, further studies will undoubtedly lead to early life dietary and/or drug intervention strategies to lower cholesterol and reduce the incidence of the metabolic syndrome in adulthood.
3.5 References


4 NUTRITIONAL MISMATCH IN POSTNATAL LIFE OF LOW BIRTH WEIGHT RAT OFFSPRING LEADS TO INCREASED PHOSPHORYLATION OF HEPATIC EUKARYOTIC INITIATION FACTOR 2α IN ADULTHOOD

3 The material in this chapter is based on a manuscript accepted (METABOLISM-D-13-00099): Sohi G, Revesz A, Hardy DB Nutritional Mismatch in Postnatal Life of Low Birth Weight Rat Offspring Leads to Increase Phosphorylation of Hepatic Eukaryotic Initiation Factor 2 α in Adulthood.(2013)
4.1 Introduction

Adverse events during development can be linked to an increased risk for developing obesity, cardiovascular disease (CVD) and diabetes. The “Predictive Adaptive Response” hypothesis suggests that these adverse events would induce fetal adaptations that result in a “survivor phenotype” which is advantageous in a similar predictive future reproductive environment\(^1\). However, if there is a ‘mismatch’ to this predictive environment, these adaptations would lead to a “thrifty phenotype” which is maladaptive\(^2, 3\). Clinically, preterm infants that exhibited catch-up growth due to higher nutrient exposure have increased markers of the metabolic syndrome by adolescence\(^4\)\(^\text{-}^8\). Similarly, in experimental rodent models leading to low birth weight, accelerated postnatal growth rates due to overfeeding is associated with hypertension\(^9\), obesity\(^10\), hypercholesterolemia\(^11\), insulin resistance\(^10\) and reduced longevity\(^12\). While the link between impaired fetal growth and the risk for developing chronic disease in adulthood is undoubtedly strong, the underlying mechanisms are still being elucidated. Some of these mechanisms include epigenetics, alterations in transcription factor (\(i.e.\) nuclear receptors) activity, mitochondrial dysfunction and impaired organogenesis\(^13, 14\).

Another mechanism underlying the development of the metabolic syndrome in low birth weight offspring may be due to impairment of protein synthesis. Protein synthesis is considered critical for normal growth and has been associated with risk factors that contribute to onset of metabolic diseases. For example, obese male subjects complicated with type 2 diabetes have lower rates of whole-body protein synthesis\(^15\). Moreover, guinea pigs with hypercholesterolemia exhibit decreased protein synthesis in the heart, brain, kidney and liver\(^16\). In metazoans, protein translation is known to be negatively regulated at the initiation stage by increases in the phosphorylation of eukaryotic initiation factor (eIF)-2\(\alpha\) at Serine (Ser) 51 residue\(^17, 18\). Interestingly, \(in vitro\) studies provide robust evidence for an inverse correlation between phosphorylation of eIF2\(\alpha\) (Ser51) and phosphorylation of Akt1 (Ser473), a marker of insulin sensitivity\(^19\)\(^\text{-}^22\). To address this relationship in the context of insulin signaling, a study by Achard and Labutt using HepG2 human hepatoma cells demonstrated that palmitate treatment increased phospho-eIF2\(\alpha\) (Ser51) within 1 hour and decreased phospho-Akt1 (Ser473) in response
to insulin simulation 18 hours later. Yet, the link between phosphorylation of eIF2α to phosphorylation of Akt1 (Serine 473) has not been fully examined in a physiological context \textit{(i.e. maternal nutrition)} \textit{in vivo}. Moreover, little is known about how a nutritional mismatch between fetal and neonatal life would influence these two established markers of protein translation and insulin resistance.

The maternal protein restriction (MPR) rat model has been extensively studied to support the “predictive adaptive response” hypothesis. For instance, low birth weight offspring derived from MPR have a reduced lifespan when recuperated under a normal protein diet at birth or after weaning. On the other hand, in the absence of protein restoration, rat offspring tend to live longer. A reduction in longevity can be attributed to an earlier onset of metabolic risk factors in these intrauterine growth restricted (IUGR) offspring, including insulin resistance. Our laboratory and others have previously observed impaired liver function to be associated with elevated hepatic cholesterol, glucose and oxidative stress in MPR offspring, including insulin resistance. Given that impaired liver function is a key contributor to the etiology of the metabolic syndrome, we chose to examine whether these MPR offspring receiving nutritional mismatch exhibit increases in hepatic phosphorylation of eIF2α (Ser51) in postnatal life. In addition, we sought to determine whether this corresponded with decreases in phosphorylation of Akt1 (Ser473) \textit{in vivo}. Moreover, given that the phosphorylation of eIF2α (Ser51) is also increased in response to endoplasmic reticulum (ER) stress, which is associated with the metabolic syndrome, we aimed to measure markers of hepatic ER stress (Glucose regulated protein 78/94 and spliced Xbp1) in these MPR offspring. To address these questions, three separate dietary regimes were employed in pregnant rats. These include a normal (20%) protein diet administered throughout life (control), a low (8%) protein diet throughout life (LP1), until weaning (LP2), or exclusively prenatally (LP3).

4.2 Materials and Methods

4.2.1 Animals and Dietary Regimes

All procedures were performed in accordance with the guidelines set by the Canadian
Council of Animal Care and upon approval of the Animal Care Committee of the University of Western Ontario. Female and male Wistar rats at breeding age (250 g; 8-10 weeks old) were purchased from Charles River (La Salle, St. Constant, Quebec, Canada). These rats were housed in individual cages and maintained at room temperature on a 12-h light, 12-h dark cycle. For 3 weeks, these rats were left to acclimatize to the animal care facility, and their reproductive cycles were followed. At the onset of proestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning. Upon confirmation of impregnation (gestation day 1), the rats were fed either a control diet containing 20% protein (Bio-Serv, Frenchtown, NJ, USA, Product# F4576 – Rodent Diet, AIN-93G, Blue, ½” pellets) or a LP diet containing 8% protein (Bio-Serv, Frenchtown, NJ, USA, Product#F4575 – Rodent Diet, AIN-93G, ½” pellets). The LP diet contained similar fat content and was made isocaloric (3.61 kcal/gram for control and 3.66 kcal/gram for LP diet) by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ, USA). At birth, the litter size was reduced to 8 animals (4 females and 4 males), with weights closest to the litter mean. This ensured a standard litter size for all mothers and no overnutrition. Three different dietary regimes were administered to these offspring (Figure 4.1A). Offspring derived from a maternal LP diet were either administered the LP diet throughout postnatal life (LP1), until the end of weaning (LP2), or exclusively prenatally (LP3). Food and water was provided ad libitum, and food intake was recorded during pregnancy and in the offspring. For consistency, only male offspring were used for the study because early-life programming is known to occur in a sexually dimorphic manner, which was not the focus of this study. At embryonic day 19 (e19), 16 pregnant rats (8 control and 8 low protein diet pregnancies) were sacrificed and the fetal livers were excised and snap frozen for immunoblot analysis. Another subset of 24 rats (8 control and 16 low protein diet pregnancies) were allowed to deliver spontaneously. The food intake of these offspring were monitored by measuring their food consumption every third day, and have been previously published. At postnatal day 130 (d130), 8 pups, each representing a single offspring from a different mother, were sacrificed per dietary regime (control, LP1, LP2 and LP3) and the medial lobe liver tissue was excised and frozen for quantitative RT-PCR and immunoblot analysis.
4.2.2 Postnatal Growth Rate Analysis

From parturition to postnatal day 130, offspring body weights were recorded daily. The growth curves for the four dietary regimes were obtained by plotting average body weight (grams) ± the SEM against time (days). The growth curves displayed a best fit to a sigmoidal curve with variable slope. Therefore, the data sets were analyzed under non-linear regression using GraphPad Prism 5™. The steepness of the curve was determined by the slope factor value, which in this study reflected growth rate of the offspring.

4.2.3 Real Time PCR Analysis

Total RNA from Wistar rat medial lobe liver tissue was extracted at e19 and postnatal d130 by the one-step method of Chomczynski and Sacchi (Trizol, Invitrogen, Carlsbad, CA)226. RNA was treated with deoxyribonuclease to remove any contaminating DNA. Of the total RNA, 4μg were reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). Primer sets directed against total rat Xbp-1 forward (5’-CTCAGAGGCAGTGCCAAGG-3’) and reverse (5’-CTCTGGGGAGACATTAG-3’) and β-actin forward (5’-ACGAGGCCCAGAGCCAAGA-3’) and reverse (5’-TTGGTTACAATGCCGTGTTC-3’) were generated via Primer Express software (PE Applied Biosystems, Boston, MA, USA). The relative abundance of each transcript was determined by real-time quantitative PCR as previously published25. For the quantitative analysis of mRNA expression, the Bio-Rad CFX384 Real Time System was employed using the DNA binding dye IQ SYBER green supermix (Bio-Rad, Mississauga, Ontario, Canada). The cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with β-actin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope=-3.4) and equal priming efficiency for the different dilutions compared with their Ct values (data not shown). Given that all
primer sets had equal priming efficiency, the ΔCt values (primer internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value). The relative abundance of each primer set compared with calibrator was determined by the formula, $2^{\Delta \Delta Ct}$, in which $\Delta \Delta Ct$ is the calibrated Ct value.

### 4.2.4 Quantitative Real Time PCR Analysis of Xbp-1 spliced mRNA Levels

This assay was performed using modifications of previously published methods\(^\text{25, 35}\). Briefly, 4μg of RNA reverse transcribed to cDNA was added to a tube of 3.5 mM MgCl\(_2\), 0.12 mM each of dNTP (A, T, G, C), 1 x PCR reaction buffer (-MgCl\(_2\)) (Invitrogen), 0.15 μM rat Xbp-1 primer forward (5’-GCTTGTGATTGAGAACCAGG-3’) and reverse (5’AGGCTTGGGTATACATGG-3’), 0.05 units/μL Taq DNA Polymerase, Native (Invitrogen). The mixture was incubate at 50°C for 2min, 95°C for 10 min, followed by 2 cycles of 95°C for 15 sec, and 60°C for 1 min. 2.25 units//μL Pst1 restriction enzyme (Invitrogen) was added to the reaction mixture for 2 hours to digest the double stranded cDNA of unspliced Xbp-1. The remaining mixture containing the spliced Xbp1 cDNA was used towards determining Xbp1-spliced mRNA levels via quantitative real-time PCR analysis.

### 4.2.5 Preparation of Tissue Total Protein Extracts and Immunoblot Analysis

Frozen Wistar rat liver tissue were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% C\(_{24}\)H\(_{39}\)NaO\(_4\), supplemented with 1mM NaV, 50mM NaF and 25mM C\(_3\)H\(_7\)O\(_6\)PNa\(_2\).XH\(_2\)O), and protease inhibitor cocktail (Roche). The homogenate was sonicated using five pulses of 1 second each with amplitude of 30 % (Misonix Sonicator 4000, Newtown, CT, USA). Following sonication, the homegenate was centrifuged at 300 g for 15 min at 4°C. The supernatant was retained and centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was retained as the total
cellular protein fraction. Equal concentrations of proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA), were fractionated in gradient polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride membrane (Millipore). Blots were probed using p-eIF2α (S51) rabbit monoclonal antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA), eIF2α rabbit polyclonal antibody (1:1000, Cell Signaling), p-Akt1 (S373) rabbit polyclonal antibody (1:1000, Abcam Inc., Cambridge, MA), Akt-1 rabbit polyclonal antibody (1:1250, Abcam), Kdel ER marker mouse monoclonal antibody (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and monoclonal horseradish peroxidase-conjugated β-actin (1:50000, Sigma-Aldrich Oakville, Ontario, Canada) diluted in 5% milk-1×Tris-buffered saline-Tween 20 buffer. Horseradish peroxidase-conjugated donkey antirabbit IgG (1:10000, Jackson ImmunoResearch Laboratories, West Grove, PA) or horseradish peroxidase-conjugated donkey antimouse IgG (1:5000, Jackson ImmunoResearch Laboratories) diluted in 5% milk 1×Tris-buffered saline-Tween 20 buffer were used as the secondary antibodies, respectively. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA).

4.2.6 Statistics

All results were expressed as the mean of arbitrary values ± the SEM. The significance of differences (P < 0.05) between mean values was evaluated using the unpaired Student’s t test for results from growth profile data, quantitative RT-PCR, and immunoblot analysis.

4.3 Results

4.3.1 Overall Growth Rates are Greater for MPR Offspring Derived from a Low Protein Diet Until Birth (LP3) or Weaning (LP2)

Offspring derived from all four dietary regimes (C, LP1-3) had a growth curve pattern that matched a sigmoidal curve with variable slope and displayed an R² value of 0.99 (Figure 4.1C). Maternal low protein diet derived low birth weight offspring which were
recuperated under a normal protein diet until birth (LP3) or weaning (LP2) had a higher overall growth rate when compared to those maintained on a low protein diet throughout life (LP1) (Figure 4.1B). The overall growth rates were inferred by the slope factor values (Figure 4.1C). When compared to the Control group, the growth rates of LP2 offspring were significantly higher (p<0.05) and LP1 were significantly lower (p<0.01, Figure 4.1C). Interestingly, the overall growth rates of LP3 were identical to that of the Control group, despite the fact that LP3 offspring were born low birth weight (Figure 4.1B, C).
Figure 4.1: The effect of maternal protein restricted (MPR) dietary regimes on growth profile in wistar rats. The growth profile was examined for A. the four separate dietary regimes which include a normal (20%) protein diet administered throughout life (Control) and a low (8%) protein diet administered throughout life (LP1), until weaning (LP2) or exclusively prenatally (LP3). B. the body weight (grams) were plotted against days in postnatal life and C. the slope factor value were determined under a non-linear regression (non fit curve) by setting line of best fit to a sigmoidal curve with a variable slope in GraphPad Prism 5™. Results were expressed as the mean ± SEM. *, Significant difference (P <0.05) compared to the Control group; n=14-22/group, where each n represents a single offspring derived from a different mother.
4.3.2 Offspring derived from a low protein diet until birth (LP3) or weaning (LP2) exhibit increased phosphorylation of hepatic eIF2α (Ser51) by postnatal day 130.

In this study, we observed that hepatic protein translation was inhibited exclusively in the livers of adult maternal low protein diet derived IUGR offspring who recuperated under a normal protein diet in postnatal life. These included offspring derived from a LP2 and LP3 dietary regime, where significant increases in phosphorylation of eIF2α (Ser51) were observed in the livers at postnatal day 130 compared to Control (Figure 4.2B, C). Interestingly, in IUGR offspring subjected to protein restriction throughout pregnancy and postnatal life (LP1), a significant decrease in phosphorylation of eIF2α (Ser51) was observed in the livers at postnatal day 130 compared to Control (Figure 4.2A). In addition, the phosphorylation levels of eIF2α (Ser51) inversely correlated with phosphorylation levels of Akt1 (Ser473) with respect to LP1 and LP2 offspring (Figure 4.2A, B). This correlation was absent at postnatal day 130 in the LP3 offspring (Figure 4.2C).
Figure 4.2: The effect of MPR dietary regimes on hepatic phosphorylated eIF2α (Ser51) protein levels at d130. The effect of A. LP1, B. LP2 and C. LP3 dietary regimes on phosphorylated protein kinase B (Akt1) at Serine 473, Akt1, eukaryotic initiation factor 2α (eIF2α) at Serine 51 and eIF2α protein levels in the livers of male offspring at postnatal day 130. Relative p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α protein levels were determined using Western blot analysis. Total protein was isolated and p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α protein were detected on a Western blot using p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α primary antibody. Their protein levels were quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM. *, Significant difference (P <0.05); n=5–7/group, where each n represents a single offspring derived from a different mother.
4.3.3 Phosphorylation of eIF2α (Ser51) protein levels were unaltered by maternal protein restriction in the fetal livers at embryonic day 19

Since an increase in phosphorylation of eIF2α (Ser51) was observed by adulthood in MPR offspring that received a Control protein diet postpartum (LP3) or post-weaning (LP2), we further pursued whether hepatic protein translation attenuation was exclusive to adult life or was persistent from fetal life. At embryonic day 19, there were no changes phosphorylated eIF2α (Ser51) protein levels (Figure 4.3). Moreover, hepatic phospho-Akt1 (Ser473) protein levels were also normal compared to the Control (Figure 4.3).
Figure 4.3: The effect of MPR on phosphorylated eIF2α (Ser51) protein levels in fetal livers at embryonic d19. The effect of maternal low protein diet on phosphorylated protein kinase B (Akt1) at Serine 473, Akt1, eukaryotic initiation factor 2 α (eIF2α) at Serine 51 and eIF2α protein levels in the fetal livers at embryonic day 19. Relative p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α protein levels were determined using Western blot analysis. Total protein was isolated and p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α protein were detected on a Western blot using p-Akt1 (S473), Akt1, p-eIF2α (S51) and eIF2α primary antibody. Their protein levels were quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM. *, Significant difference (P < 0.05); n=7/group, where each n represents a single offspring derived from a different mother.
4.3.4 Elevated ER chaperones in the livers of adult offspring derived from a LP diet exclusively during pregnancy (LP3) and during pregnancy and lactation (LP2)

Another marker indicating that the perinatal environment may lead to ER stress in adulthood is through an increase in ER chaperone proteins. Adult offspring derived from a LP diet exclusively during pregnancy (LP3) and during pregnancy and lactation (LP2) resulted in elevated hepatic ER chaperones by postnatal day 130 (Figure 4.4B, C). Specifically, there was a significant increase in ER chaperones Grp78 in the LP2 derived offspring, and Grp94 in the LP3 derived offspring in adulthood compared to Control (Figure 4.4B, C). Interestingly, LP1 derived offspring displayed no changes to ER chaperone Grp94 and 78 protein levels (Figure 4.4A).
Figure 4.4: The effect of MPR dietary regimes on hepatic ER chaperones protein levels at d130. The effect of A. LP1, B. LP2 and C. LP3 dietary regimes on Glucose regulatory protein 94 and 78 (Grp94 & Grp78) protein levels in the livers of male offspring at postnatal day 130. Relative Grp94 and Grp78 protein levels were determined using Western blot analysis. Total protein was isolated and Grp94 and Grp78 protein were detected on a Western blot using KDEL primary antibody, which recognizes the carboxyl terminus KDEL sequence of Grp94 and Grp78. Their protein levels were quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM. *, Significant difference (P <0.05); n=5–7/group, where each n represents a single offspring derived from a different mother.
4.3.5 Increases in Xbp-1 spliced variant mRNA levels coincide with the elevated ER chaperone protein levels and enhanced phosphorylation of eIF2α (Ser51) in the livers of adult offspring derived from LP2 and LP3 dietary regimes

The spliced variant Xbp-1 is a more potent form of Xbp-1 transcription factor which activates a wide variety of genes involved with ER stress activation pathway, including ER chaperone proteins\textsuperscript{36,37}. In this study, we observed an increase in spliced variant Xbp-1 mRNA levels in the livers of LP2 and LP3 derived offspring in adulthood compared to Control (Figure 4.5B, C). The increase in spliced Xbp-1 mRNA levels partially correlated with the increase in protein levels of spliced Xbp-1 downstream transcriptional targets, the ER chaperone proteins (Figure 4.4B, C). Moreover, spliced Xbp-1 mRNA levels in the livers of LP1 derived offspring were unchanged, which correlates with no change to ER chaperones Grp94 and 78 in these offspring (Figure 4.4A).
Figure 4.5: The effect of MPR dietary regimes on hepatic Xbp-1 spliced variant mRNA levels at d130. The effect of A. LP1, B. LP2 and C. LP3 dietary regimes on X-box binding protein (Xbp-1) spliced variant mRNA levels in the livers of male offspring at postnatal day 130 via quantitative RT-PCR analysis. RNA was extracted and reverse transcribed for quantitative RT-PCR using primer specific for Xbp-1. Reverse transcribed RNA was first amplified by Taq DNA Polymerase for two PCR cycles and then treated with PstI restriction enzyme to allow for splicing of all unspliced Xbp-1 transcripts. Quantitative RT-PCR was then performed to amplify exclusively the Xbp-1 spliced transcript. The relative levels of each Xbp-1 spliced mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. *, Significant difference (P <0.05); n=7/group, where each n represents a single offspring derived from a different mother.
4.4 Discussion

In this study, we demonstrated that there is an increase in phospho-eIF2α (Ser51) in the adult livers of IUGR rat offspring exclusively when they undergo accelerated growth rates as a consequence of a nutritional protein mismatch. Moreover, when there is no mismatch between fetal and postnatal nutrition, the IUGR offspring experience slower growth rates and do not exhibit hepatic protein translation attenuation in adult life. This further supports the central tenet of the ‘thrifty phenotype’ hypothesis that long-term adverse metabolic effects are most apparent when there is a mismatch between early nutritional deprivation and later nutritional affluence\textsuperscript{2, 3, 38}. Collectively, this study helps to identify appropriate dietary intervention strategies for reducing the incidence of the metabolic syndrome in IUGR offspring by highlighting the beneficial effects of matching the prenatal and postnatal environment to maintain protein translation in the liver. It is conceivable that such strategies could reduce the long-term usage of drugs prescribed to manage symptoms of the metabolic syndrome.

MPR derived IUGR offspring that recuperated under a normal protein diet post-weaning (LP2) or postpartum (LP3) displayed accelerated growth rates compared to MPR offspring maintained on a low protein diet throughout fetal and postnatal life (LP1). These accelerated growth rates (i.e. those exhibited by LP2 and LP3 offspring) mirrored decreases in hepatic protein translation as reflected through increases in phospho-eIF2α (Ser51) levels. Conversely, slower growth rates due to an absence of a nutritional mismatch (e.g. LP1) resulted in a decrease in phospho-eIF2α (Ser51) in adulthood. Since no change was observed in the levels of hepatic phospho-eIF2α (Ser51) at embryonic day 19, accelerated growth rates is likely the more significant contributing factor to protein translation attenuation in adult life, as opposed to the direct actions of the low protein diet itself during pregnancy. However, to provide further support for the “predictive adaptive response” hypothesis, it would be of interest to investigate the effects of low protein diet exclusively administered during lactation or post-lactation. Our previous studies have suggested that a low protein diet exclusively in fetal life or during pregnancy and lactation did not alter circulating cholesterol\textsuperscript{25}, indicating that a longer fetal and neonatal low protein insult is necessary for impaired hepatic function long-term. Interestingly, four
serine/threonine protein kinases have been identified to phosphorylate eIF2α. These include general control nonrepressed 2 kinase (GCN2), heme-regulated inhibitor kinase (HRI), double stranded RNA-activated kinase (PKR) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) which are activated by amino acid depletion, hypoxia, viral infection and accumulation of misfolded proteins, respectively\textsuperscript{17, 18}. However, this study did not investigate whether these kinases were involved in increasing levels of phospho-eIF2α (Ser51) observed in LP2 and LP3 offspring. Moreover, other stages of protein synthesis regulation by important amino acid sensing modulators such as through the mammalian target of rapamycin kinase (mTOR) were also not examined but deserve consideration\textsuperscript{19}.

Intriguingly, the LP2 and LP3 offspring display an accelerated growth rate despite the observed increase in hepatic phospho-eIF2α (Ser51), an indicator of reduced protein translation. The changes in growth occurred in spite of the fact that food intake was not different between dietary groups\textsuperscript{25}. Rather, this might be explained by changes in adiposity since visceral fat mass and the fat to body weight ratio have been reported to be twice as high in these LP2 offspring relative to the control\textsuperscript{26, 27}. Therefore, it is plausible that an increase in visceral obesity may predominantly underlie the increases in accelerated growth (as assessed by bodyweight) in the LP2 and LP3 offspring, even though the overall protein synthesis may be reduced in the liver. Interestingly, a decrease in whole body protein synthesis has been previously reported in male type 2 diabetic patients with obesity\textsuperscript{15}. It is also noteworthy that even though the low birth weight LP2 offspring have a higher slope factor, reflecting higher overall growth rate, they do not fully catch-up in size to the normal birth weight control offspring as they reach their growth plateau sooner. With respect to the impact of phospho-eIF2α and on liver growth, it is noteworthy the liver:body weight ratio was reduced 50\% in LP offspring at embryonic day 19 (when phospho-eIF2α was unchanged), they were caught up by day 130\textsuperscript{25}. This suggests that the observed increases in phospho-eIF2α in LP2 and LP3 offspring at postnatal day 130 may occur after the liver has undergone differentiation and growth.

An inability to induce protein translation is likely due to decreased insulin sensitivity in
the liver. In this study we observed an increase in phospho-Akt1 (Ser473) to be associated with a decrease in phospho-eIF2α (Ser51) in LP1 offspring. Conversely, a decrease in phospho-Akt1 (Ser473) was observed to be associated with an increase in phospho-eIF2α (Ser51) in the LP2 offspring. This finding confirms previous in vitro studies which have observed a bi-directional association between phosphorylated eIF2α (Ser51) and phosphorylated Akt1 (Ser473) by administering pharmacological inhibitors of Akt1 and eIF2α. Despite elevated phospho-eIF2α (Ser51) levels, the LP3 offspring did not display corresponding decreases in phospho-Akt1 (Ser473). This may be due to the fact that some of the adverse effects of a low protein diet are rescued by protein restoration during the first three weeks of neonatal life, a period of plasticity in the developing rat liver. It can be postulated that this correlation may eventually hold true in the LP3 offspring later on in life provided phospho-eIF2α (Ser51) remain elevated past day 130. Collectively, these results support the notion that elevated phospho-eIF2α (Ser51) could be contributing to a reduction in hepatic insulin sensitivity in LP2 and LP3 derived adult offspring. Interestingly, LP2 offspring have been previously demonstrated to exhibit increases in HOMA-IR, glucose intolerance and decreases in hepatic glucokinase activity. Alternatively, it remains to be determined whether a decrease in phospho-eIF2α (Ser51) and slower growth rates in LP1 IUGR offspring is beneficial to increasing hepatic glucokinase activity, insulin sensitivity, and glucose tolerance. It is noteworthy that these LP1 offspring do live longer.

Interestingly, protein translation attenuation via increases in phospho-eIF2α (Ser51) is also one of the hallmark responses of elevated ER stress. ER stress is a result of excessive accumulation of misfolded or unfolded proteins in the ER lumen due to a variety of environmental insults. The accumulation of these proteins triggers the activation of the ER stress pathway, which is also referred to as the unfolded protein response (UPR). UPR aims to alleviate ER stress by decreasing global protein translation in order to reduce the load of the ER in processing these new proteins. In addition to a reduction of global protein translation, there is also an increase in ER chaperone protein levels (i.e. glucose regulated protein Grp78 and Grp94) to improve ER’s protein folding capacity. Furthermore, ER stress induces the splicing of a highly conserved unconventional
bp intron of the X-box binding protein 1 (Xbp-1) mRNA, which encodes for a potent transcription factor involved with transcriptional upregulation of genes involved with coping with the ER stress\textsuperscript{36, 37}. Collectively, an increase in phosphorylation of eIF2α (Serine 51), Grp94 and Grp78 protein levels, and splice variant XBP-1 mRNA level are considered hallmark indicators of elevated ER stress. Given the critical role of endoplasmic reticulum in overall cellular functions and survival, any stress to the ER would therefore aggravate the symptoms associated with the metabolic syndrome. We were interested in measuring these markers of UPR given ER stress has been demonstrated to lead to aspects of the metabolic syndrome (insulin resistance, obesity and hypercholesterolemia)\textsuperscript{31-33, 41} which are also exhibited in MPR offspring\textsuperscript{25-27}. In addition to increases in phospho-eIF2α (Ser51) in the adult livers of LP2 and LP3 offspring, we also observed increases in Xbp-1 splice variant mRNA levels. Since splice variant Xbp-1 transcription factor is known to upregulate the transcription of ER chaperones\textsuperscript{36, 37}, we expected corresponding increases in ER chaperones Grp78 and Grp94 protein levels. However, in both LP2 and LP3 only one of the two ER chaperone protein levels were significantly elevated. It is still noteworthy that the ER chaperone Grp78, a downstream target gene of spliced Xbp-1, has been demonstrated to prevent an increase in phospho-Akt1 (Ser473)\textsuperscript{42}. Therefore the increase in hepatic spliced Xbp1 mRNA and Grp78 protein levels in the LP2 offspring may also help explain for our observed decreases in hepatic phospho-Akt1 (Ser473) and the reported insulin resistance in these offspring\textsuperscript{27}.

In summary, we provide evidence that in addition to epigenetic and transcriptional mechanisms underlying the ‘developmental origins of adult disease’, protein translation attenuation may mediate long-term insulin resistance and other aspects of the metabolic syndrome. Confirming the main tenets of the ‘thrifty hypothesis’, our results also demonstrated that only in the situation whereby MPR offspring were faced with a protein dietary mismatch from fetal to postnatal life did they display reduced hepatic protein translation in adulthood. Moreover, these offspring were more susceptible to developing insulin resistance and other markers of the metabolic syndrome as they displayed indicators of elevated ER stress. The management of early life diet to reduce accelerated
postnatal growth and improve hepatic protein synthesis in IUGR offspring may prove to be an important intervention strategy to decrease the incidence of the metabolic syndrome in adulthood.
4.5 References


5 NUTRITIONAL MISMATCH IN POSTNATAL LIFE OF LOW BIRTH WEIGHT RAT OFFSPRING LEADS TO ELEVATED HEPATIC DRUG METABOLISM IN ADULTHOOD

* The material in this chapter is based on a manuscript in preparation: Sohi G, Barry EJ, Velenosi TJ, Urquhart BL, and Hardy DB Nutritional Mismatch in Postnatal Life of Low Birth Weight Rat Offspring Leads to Elevated Hepatic Drug Metabolism in Adulthood. (2013)
5.1 Introduction

Clinical studies have reported a strong correlation between low birth weight and metabolic risk factors associated with cardiovascular disease (CVD)\(^1\textsuperscript{-5}\). Therefore, the likelihood of prescribing medication for the management of these metabolic symptoms (\textit{e.g.} statins for hypercholesterolemia) can be considered to be greater in these offspring. This may be particularly relevant in cases of low birth weight offspring, which undergo nutrition-induced accelerated growth in neonatal life and display an earlier onset of these symptoms\(^6\textsuperscript{-11}\). The underlying reason behind this phenomenon can be explained by the “Predictive Adaptive Response” hypothesis, which suggests that adverse events during development induce adaptations suited for survival in a similar predictive environment but can become maladaptive if a ‘mismatch’ to the predictive environment occurs, leading to a “thrifty phenotype”\(^12\textsuperscript{-14}\). Clinically, this hypothesis has been supported by evidence where accelerated growth due to higher nutrient exposure in preterm infants results in an increase in markers of the metabolic syndrome by adolescence\(^15\textsuperscript{-19}\). However, very little is known about drug disposition of low birth offspring in adult life. Moreover, the role of nutrition mismatch induced accelerated growth has not been examined.

Several clinical and animal studies have observed liver dysfunction in postnatal life of low birth weight infants\(^20\textsuperscript{-24}\). Given that approximately 75\% of prescribed drugs are metabolized in the liver\(^25\), liver dysfunction may alter the pharmacokinetic parameters of several drugs in postnatal life of these infants. Amongst the prescribed drugs eliminated by the liver, three fourths of these are metabolized by the cytochrome P450 (CYP) family of enzymes\(^25\). In humans, reduced intravenous midazolam clearance, a measure of hepatic CYP3A4 activity, has been observed in preterm infants aged between 2 to 15 days\(^26\textsuperscript{-28}\). Interestingly, the CYP3A4 isozyme is responsible for the metabolism of approximately half of all marketed drugs\(^29\). Yet, it remains to be investigated whether pharmacokinetics of drugs in the liver are altered in adult life of low birth weight offspring.

Animal models make it feasible to investigate alterations in pharmacokinetics of a drug due to changes in hepatic CYP enzyme activity. The long-term programming of Cyp3a
activity was recently demonstrated by Tajima et al. in mice offspring born of mothers receiving a high fat diet during pregnancy. Specifically, they correlated a decrease in hepatic Cyp3a activity in six week old offspring with reduced triazolam substrate clearance. Although an important finding, it remains to be established whether similar long-term programming of drug metabolizing CYP enzyme activities occur when in utero insults result in low birth weight offspring. In rodent experimental models low birth weight offspring that undergo accelerated growth rates due to a postnatal nutrition mismatch have been previously associated with hypertension, obesity, hypercholesterolemia, insulin resistance and reduced longevity. Therefore, it would be of interest to also determine the adverse consequences of altering the postnatal nutrition environment of low birth weight offspring on long-term programming of hepatic drug metabolizing CYP enzymes.

Since worldwide maternal under-nutrition is a leading source of impaired perinatal development, and given amino acids play a critical role in fetal growth, the maternal protein restriction (MPR) dietary regime is a relevant animal model to study the developmental origins of adult diseases (DOHaD). In addition, MPR shares features common with placental insufficiency induced intrauterine growth restriction (PI-IUGR), which occurs in 8% of pregnancies and produces protein deficiency in the developing fetus. We and others have previously demonstrated that maternal protein restriction (MPR, 8% protein) during pregnancy and lactation results in low birth weight offspring (asymmetrical IUGR), which display impaired liver function in adulthood. In addition, we have observed that these offspring display high circulating and hepatic cholesterol levels exclusively when they are faced with a nutritional mismatch of a normal (20%) protein diet post-weaning. This was attributed to the long-term repression of a hepatic cytochrome P450 enzyme, cholesterol 7α-hydroxylase (Cyp7a1), which is rate-limiting for the catabolism of cholesterol to bile acids. It is noteworthy that decreased Cyp7a1 expression is also associated with Endoplasmic Reticulum (ER) stress in a rat model of hypothyroidism. This is of great interest considering that we have recently demonstrated that these MPR offspring have elevated ER stress markers in their livers at four months of age. In contrast, MPR derived offspring that were maintained on a low protein diet throughout life did not display hypercholesterolemia or hepatic ER
stress. As Cyp enzymes (i.e. Cyp3a1, Cyp3a2, Cyp2c11 and Cyp2b1) reside in the ER of the liver and are the primary enzymes involved with Phase 1 of drug metabolism\textsuperscript{43}, we tested the hypothesis that MPR derived low birth offspring may have an impaired activity of drug-metabolizing Cyp enzymes in adulthood when faced with a nutritional mismatch in postnatal life. To address this, the effects of a low protein diet throughout life (LP1) or until the end of lactation (LP2) were compared relative to a normal protein diet throughout life of the offspring (C).

5.2 Materials and Methods

5.2.1 Animals and Dietary Regimes

All procedures were performed in accordance with the guidelines set by the Canadian Council of Animal Care and upon approval of the Animal Care Committee of the University of Western Ontario. Female and male Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St.Constant, Quebec, Canada). These rats were housed in individual cages and maintained at room temperature on a 12-h light, 12-h dark cycle. For 3 weeks, these rats were left to acclimatize to the animal care facility and their reproductive cycles were followed. At the onset of proestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning. Upon confirmation of impregnation (gestation day 1), the rats were fed either a control diet containing 20% protein (Bio-Serv, Frenchtown, NJ, USA, Product# F4576 – Rodent Diet, AIN-93G, Blue, ½” pellets) or a LP diet containing 8% protein (Bio-Serv, Frenchtown, NJ, USA, Product#F4575 – Rodent Diet, AIN-93G, ½” pellets). The LP diet contained similar fat content and was made isocaloric by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ, USA). At birth, the litter size was reduced to eight animals (four females and four males), with weights closest to the litter mean. This ensured a standard litter size for all mothers. Three different dietary regimes were administered to these offspring. Offspring derived from a maternal LP diet were either administered the LP diet throughout postnatal life (LP1) or until the end of weaning (LP2). Food and water was provided ad libitum, and food intake was recorded during pregnancy and in the offspring. At embryonic day 19 (e19), a subset of pregnant rats were sacrificed and the
fetal livers were excised and snap frozen for quantitative RT-PCR. Another subset of rats was allowed to deliver spontaneously. The food intake of these offspring were monitored by measuring their food consumption every third day, and have been previously published\textsuperscript{41}. At postnatal day 130 (d130), the pups were also sacrificed and the medial lobe liver tissue was excised and frozen for quantitative RT-PCR and for testosterone enzyme kinetics in liver microsomes via ultra performance liquid chromatography. We did not examine the female offspring to prevent confounding factors related to their estrous cycle and their hormonal profile. Moreover, the maternal low protein model has been demonstrated to exhibit early life programming effects in a sexually dimorphic manner, which was not the focus of this study\textsuperscript{41, 44, 45}.  

5.2.2 Real Time PCR Analysis  

Total RNA from Wistar rat medial lobe liver tissue was extracted at e19 and d130 by the one-step method of Chomczynski and Sacchi\textsuperscript{46} (TRIzol, Invitrogen, Carlsbad, CA, USA). RNA was treated with deoxyribonuclease to remove any contaminating DNA. 4 µg of the total RNA was reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sets directed against rat Cyp2b1, Cyp3a1, Cyp3a2, Cyp2c11, Car, Pxr, and β-actin were generated via Primer Express software (PE Applied Biosystems, Boston, MA, USA) based on published sequences (Table 5.1). The relative abundance of each transcript was determined by real-time quantitative PCR as previously published\textsuperscript{47}. For the quantitative analysis of mRNA expression, the Bio-Rad CFX384 Real Time System was employed using the DNA binding dye IQ\textsuperscript{TM} SYBR green supermix (Bio-Rad, Mississauga, Ontario, Canada). The cycling conditions were 50 C for 2 min, 95 C for 10 min, followed by 45 cycles of 95 C for 15 sec and 60 C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with β-actin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope=-3.4) and equal priming efficiency for the different dilutions compared with their Ct values (data not
shown). Given that all primer sets had equal priming efficiency, the $\Delta$Ct values (primer internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared with calibrator was determined by the formula, $2^{\Delta\Delta\text{Ct}}$, in which $\Delta\Delta$Ct is the calibrated Ct value.

5.2.3 Hepatic Microsome Isolation

Wistar rat liver microsomes were isolated by differential centrifugation using methods described previously by Velenosi et al. Briefly, 0.9% NaCl solution was used to rinse liver tissue. The rinsed tissue was homogenized in 1.15% KCl solution containing 1 mM EDTA and was centrifuged at 9000g for 20 min at 4°C. The subsequent supernatant was centrifuged at 105,000g for 60 min at 4°C. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4 and protein concentration was determined by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA). Microsomal protein extract was stored at -80°C for further analysis.

5.2.4 Hepatic Metabolism of Testosterone by Cyp3a, Cyp2b and Cyp2c Enzymes

Metabolic activity of Cyp3a and Cyp2c in hepatic microsomes was determined using methods previously described by Velenosi et al. (2012). Testosterone was selected as a probe for Cyp3a, Cyp2b and Cyp2c enzyme activity based on previously documented selective metabolism by specific rat P450 isozymes. 50 mM potassium phosphate buffer and 2 mM MgCl2 (pH 7.4) with 1 mg/ml hepatic microsomal protein equating to a final volume of 250μl was used for timed enzymatic reactions. Linear rate of production of metabolites was determined by varying time, protein and relevant substrate concentrations prior to conducting enzymatic reactions. Formation of testosterone metabolites (2α-OH testosterone, 6β-OH testosterone and 16α-OH testosterone) was determined to be linear at 10 minutes. The reactions were initiated by addition of 1mM NADPH to microsomal samples containing varying concentrations of testosterone. The reaction was terminated by addition of 50 μl of ice-cold acetonitrile followed by 15-minute incubation on ice and centrifugation to pellet precipitated protein.
5.2.5 Testosterone Metabolite Analysis by Ultraperformance Liquid Chromatography with Photodiode Array (UPLC-PDA) Detection

Testosterone metabolite analysis was performed by solid-phase extraction followed by UPLC-PDA using methods previously described\(^{48}\). The solid-phase extraction cartridge (C18, Strata-X Polymeric Reverse Phase 33 μm; Phenomenex, Torrance, CA) was conditioned according to manufacturer’s specification. Carbamazepine was used as an internal standard for testosterone quantification. The analytes and internal standard were passed across the packing of the cartridge by gravity. The cartridges were then washed with 1 ml of Milli-Q water followed by 1 ml of 50:50 methanol/water. 1 ml of methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid was used to elute the analytes into clean glass test tubes. The eluent was dried, reconstituted in mobile phase and injected on a Phenomenex Kinetex C18 column (1.7 μm particle size, 50 x 2.1 mm; Torrence, CA) for testosterone and analyte separation. The column was maintained at 40°C in a Waters ACQUITY UPLC H-Class System. Mobile phase flow, gradient used and detection wavelengths were the same as previously published\(^{48}\).

5.2.6 Statistics

All results were expressed as the mean of arbitrary values ± the standard error of the mean (SEM). The significance of differences (p<0.05) between mean values were evaluated using the unpaired Student’s \( t \)-test for ChIP and testosterone enzyme kinetics in liver microsomes via ultra performance liquid chromatography. One-way analysis of variance (ANOVA) followed by a Bonferroni’s Multiple Comparison post hoc test, was used to evaluate significance of differences for results comparing the effect of all the dietary regimes for qRT-PCR analysis.
### Table 5.1. Real Time PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'-3')</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp3a1</td>
<td>FWD ACC AGT GGA AGA CTC AAG GAG ATG T</td>
<td>XM 003751127</td>
</tr>
<tr>
<td></td>
<td>REV TCA CAG GGA CAG GTT TGC CTT TCT</td>
<td>XM 003751127</td>
</tr>
<tr>
<td>Cy3a2</td>
<td>FWD GCT CTT GAT GCA TGG TTA AAG ATT TG</td>
<td>BC 089765</td>
</tr>
<tr>
<td></td>
<td>REV ATC CAC AGA CCT TGC CAA CTC CTT</td>
<td>BC 089765</td>
</tr>
<tr>
<td>Cy2c11</td>
<td>FWD CCC TGA GGA CTT TTG GGA TGG GC</td>
<td>BC 088146</td>
</tr>
<tr>
<td></td>
<td>REV GGG GCA CCT TTG CTC TTC CTC</td>
<td>BC 088146</td>
</tr>
<tr>
<td>Cy2b1</td>
<td>FWD GCT CAA GTA CCC CCA TGT CG</td>
<td>XM 003750353</td>
</tr>
<tr>
<td></td>
<td>REV ATC AGT GTA TGG CAT TTT ACT GCG G</td>
<td>XM 003750353</td>
</tr>
<tr>
<td>Car</td>
<td>FWD CCT TTT CCG TTC CCT GAC CA</td>
<td>AB 204900</td>
</tr>
<tr>
<td></td>
<td>REV AGG CAG AAC GTA GTG TTG AGT</td>
<td>AB 204900</td>
</tr>
<tr>
<td>Pxr</td>
<td>FWD TGC ACA CAG GTT CCT GTT CCT GA</td>
<td>AF 151377</td>
</tr>
<tr>
<td></td>
<td>REV GGG GTG CGT GTC CTG GAT GC</td>
<td>AF 151377</td>
</tr>
<tr>
<td>β-actin</td>
<td>FWD ACG AGG CCC AGA GCA AGA</td>
<td>NM 031144</td>
</tr>
<tr>
<td></td>
<td>REV TTG GTT ACA ATG CCG TGT TCA</td>
<td>NM 031144</td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 A switch to a normal protein diet in postnatal life of MPR derived low birth weight offspring leads to increases in hepatic Cyp3a1, Cyp2c11 and Cyp2b1 mRNA expression at postnatal day 130

In this study, we wanted to investigate the long-term effects of MPR derived low birth weight offspring on expression and function of major drug metabolizing cytochrome P450 (CYP) enzymes in the liver. Q-RT-PCR analysis was conducted to examine the steady-state mRNA expression of hepatic Cyp3a1, Cyp3a2, Cyp2c11 and Cyp2b1 at postnatal day 130. A 1.79, 1.45 and 1.94 fold increase in Cyp3a1, Cyp2c11, Cyp2b1 respectively was observed in the livers of MPR offspring who were placed on a normal protein diet post-weaning (LP2) compared to the Control (Figure 5.1A, C, D). Interestingly, in MPR offspring subjected to protein restriction throughout pregnancy and postnatal life (LP1), there was no difference in hepatic Cyp3a1, Cyp3a2, Cyp2c11 and Cyp2b1 mRNA expression (Figure 1A, B, C, D). Upon comparing the two MPR dietary regimes, the LP2 offspring displayed a 1.93, 1.27 and 3.04 fold elevation in hepatic Cyp3a1, Cyp2c11 and Cyp2b1 mRNA expression, respectively, relative to LP1 (Figure 1A, C, D). There was also a noticeable trend towards an increase in Cyp3a2 mRNA expression in LP2 offspring compared to Control and LP1 offspring (P<0.06) (Figure 5.11B).
Figure 5.1: Quantitative RT-PCR mRNA level analysis of A. Cyp3a1, B. Cyp3a2, C. Cyp2c11, D. Cyp2b1 in the livers of rat offspring derived at postnatal d 130. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Cyp3a1, Cyp3a2, Cyp2c11, and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. * Control vs LP2 Significant difference ($P < 0.05$), # LP1 vs LP2 Significant difference ($P < 0.05$); $n = 7–8$/group, where each $n$ represents an offspring derived from a different mother.
5.3.2 Elevated Cyp3a1, Cyp2c11 and Cyp2b1 mRNA levels correlated with increases in their drug metabolizing activity

To evaluate whether postnatal nutritional mismatch in MPR derived low birth weight offspring also impacted long-term function of hepatic Cyp3a, Cyp2c and Cyp2b enzyme activities, testosterone metabolism assay was performed using rat liver microsomes. This assay is an excellent indicator of Cyp3a, Cyp2c and Cyp2b enzyme activity, as testosterone metabolites $2\alpha$OH-testosterone, $16\alpha$OH-testosterone and $6\beta$OH-testosterone have been previously observed to be generated by Cyp2c, Cyp2b and Cyp3a enzymes, respectively. Full enzyme kinetics of these metabolites was determined for this study. $V_{\text{max}}/K_{m}$, a measure of intrinsic clearance for $2\alpha$OH-testosterone, $16\alpha$OH-testosterone and $6\beta$OH-testosterone, was significantly elevated by 2.0, 1.9 and 3.25 fold respectively in LP2 offspring at postnatal day 130 (Figure 5.2 A, B, C). The increases in intrinsic clearance of testosterone metabolites corresponded well with increases in Cyp2c11, Cyp2b1 and Cyp3a1 mRNA expression, respectively (Figure 5.1D, C, A). The Michaelis-Menten kinetic parameters for $2\alpha$OH-testosterone, $16\alpha$OH-testosterone and $6\beta$OH-testosterone are presented in Table 5.2.
Figure 5.2: Michaelis-Menten plots of A. 2αOH-testosterone, B. 16αOH-testosterone and C. 6βOH-testosterone after incubation of rat liver microsomes with 1mM NADPH and various concentrations of testosterone. Liver microsomes were extracted and timed enzyme reaction was performed for testosterone metabolite analysis via solid-phase extraction followed by UPLC-PDA. Each data point on the curves were expressed as the mean ± SEM.; n = 5–6/group, where each n represents an offspring derived from a different mother.
Table 5.2. Michaelis-Menten kinetic values for P450 probe substrates in Control and LP2 offspring rat liver microsomes at postnatal d 130

<table>
<thead>
<tr>
<th></th>
<th>2α -OH Testosterone</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ (µM)</td>
<td>Vₘₐₓ (pmol/min/mg protein)</td>
<td>Vₘₐₓ/Kₘ (µl/min/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>86 ± 19</td>
<td>500 ± 34</td>
<td>7.12 ± 1.17</td>
</tr>
<tr>
<td>LP2</td>
<td>50 ± 9</td>
<td>615 ± 38*</td>
<td>14.22 ± 2.48*</td>
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<tr>
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<th>6β -OH Testosterone</th>
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<td></td>
<td>Kₘ (µM)</td>
<td>Vₘₐₓ (pmol/min/mg protein)</td>
<td>Vₘₐₓ/Kₘ (µl/min/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>172 ± 18</td>
<td>182 ± 27</td>
<td>0.97 ± 0.16</td>
</tr>
<tr>
<td>LP2</td>
<td>123 ± 19</td>
<td>214 ± 21</td>
<td>1.85 ± 0.22**</td>
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<table>
<thead>
<tr>
<th></th>
<th>16α -OH Testosterone</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ (µM)</td>
<td>Vₘₐₓ (pmol/min/mg protein)</td>
<td>Vₘₐₓ/Kₘ (µl/min/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>80 ± 12</td>
<td>665 ± 58</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>LP2</td>
<td>43 ± 8*</td>
<td>873 ± 83</td>
<td>26 ± 6*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

*P < 0.05 compared with control.

**P < 0.001 compared with control.
5.3.3 Elevated Cyp3a1, Cyp2c11 and Cyp2b1 expression and function coincided with increases in constitutive androstane receptor (Car)

We next evaluated whether the elevated activity and steady-state mRNA levels of Cyp3a1, Cyp2c11, Cyp2b1 coincided with upregulation of the orphan nuclear receptors Car and Pxr. These nuclear receptors serve as master transcription factors which regulate the transcription of several xenobiotic detoxification enzymes. As depicted in Figure 5.3, administering a normal protein diet post-weaning to maternal low protein diet derived low birth weight offspring (LP2) resulted in significantly increased expression of hepatic Car mRNA levels compared to control in adulthood. This increase was prevented in IUGR offspring maintained on a low protein diet throughout fetal and postnatal life (LP1).
Figure 5.3: Quantitative RT-PCR mRNA level analysis of A. Pxr and B. Car in the livers of rat offspring derived at postnatal d 130. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Pxr, Car and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. *, Significant difference (P < 0.05); n = 7–8/group, where each n represents an offspring derived from a different mother.
5.3.4 Major hepatic drug metabolizing Cyp enzymes were unaltered by MPR at postnatal day 21.

Since an increase in Cyp3a, Cyp2b and Cyp2c11 expression and activity was observed by adulthood in MPR offspring that received a control protein diet post-weaning (LP2), we further pursued whether this increase was exclusively due to a switch in diet post-weaning or was persistent before the normal protein was restored at postnatal day 21. Interestingly, MPR offspring displayed no significant difference in Cyp3a1, Cyp3a2, Cyp2b1 and Cyp2c11 mRNA levels compared to control at postnatal day 21 (Figure 5.4).
Figure 5.4: Quantitative RT-PCR mRNA level analysis of A. Cyp3a1, B. Cyp3a2, C. Cyp2c11, D. Cyp2b1 in the livers of rat offspring derived at postnatal d 21. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Cyp3a1, Cyp3a2, Cyp2c11, and β-actin. The relative levels of each mRNA transcript were normalized to that of the levels of each β-actin mRNA transcript. Results were expressed as the mean ± SEM. * Control vs LP2 Significant difference ($P < 0.05$), # LP1 vs LP2 Significant difference ($P < 0.05$); n = 7–8/group, where each n represents an offspring derived from a different mother.
5.4 Discussion

In this study we present the novel finding that MPR derived low birth weight rat offspring have elevated Cyp3a, Cyp2b and Cyp2c activity in adulthood, exclusively when faced with a nutritional mismatch in postnatal life. This was found to coincide with increases in their steady-state mRNA levels, which may be due, in part, to transcriptional induction by constitutive androstane receptor (Car). Interestingly, when low birth weight offspring were maintained on a low protein diet post-lactation, they exhibited no differences in expression of these Cyp enzymes. Collectively, this study suggests that IUGR offspring receiving an inappropriate dietary intervention strategy may require an adjustment to their drug dosages to effectively ameliorate the symptoms of the metabolic syndrome. It is conceivable that adopting personalized drug treatment strategies in postnatal life of low birth weight offspring may prove to be an effective therapeutic approach.

Contrary to our initial hypothesis, administering a normal protein diet post-weaning to maternal low protein diet derived low birth weight offspring (LP2) resulted in elevated expression of drug metabolizing enzymes Cyp3a1 and Cyp2c11 in adulthood. The LP2 offspring displayed corresponding increases in intrinsic enzymatic activity of these Cyp enzymes. Given that testosterone is also a substrate for these Cyp enzymes, it is noteworthy that circulating testosterone has been previously reported to be decreased in these LP2 offspring\(^{44}\). Our data now provide evidence for the mechanism underlying this observation. Conversely, no differences in the steady-state mRNA expression of these Cyp enzymes were observed when the IUGR offspring were maintained on a low protein diet (LP1). Moreover, no changes in expression of these Cyp enzymes were observed at postnatal day 21, which represents the end of lactation, a time point where normal protein was restored for LP2 offspring. This suggests that the postnatal nutritional mismatch is likely the more significant contributing factor to elevated Cyp3a1 and Cyp2c11 expression in adult life, as opposed to the direct actions of the low protein diet itself during pregnancy and lactation. Since, increases in Cyp3a and Cyp2c activity correlated with Cyp3a1 and Cyp2c11 steady-state mRNA expression in LP2 offspring, we
postulated transcriptional mechanisms to underlie the increases in activity. Specifically, we attempted to investigate the role of xenobiotic sensing nuclear receptors Pxr and Car which represent major transcription factors involved with the transcriptional induction of drug-metabolizing Cyp enzymes in humans. In this study we observed an exclusive elevation of Car mRNA levels in LP2 offspring. Interestingly, IUGR offspring maintained on a low protein diet throughout fetal and postnatal life (LP1) displayed no change in the xenobiotic sensing nuclear receptors Pxr and Car. Since, Car binding sites have not been well characterized towards explaining the transcriptional induction of Cyp3a1, Cyp3a2 and Cyp2c11 genes in rats, we evaluated whether increases in Car correlated with the well characterized increases in Cyp2b1 expression. As anticipated, there was a noticeable increase in both hepatic Cyp2b1 mRNA and activity levels in the LP2 adult offspring. Therefore, we propose that the increases in hepatic Car expression also underlie the elevated expression and activity of Cyp3a1 and Cyp2c11 in these offspring.

In support of the main tenet of the “predictive adaptive response” hypothesis, MPR derived low birth weight rat offspring when faced with a nutritional mismatch, have been previously reported to display high cholesterol, visceral obesity, hypertension, type 2 diabetes and reduced longevity. Conversely, in the absence of protein restoration, rat IUGR offspring have been observed to live longer. Given that the liver plays a key role in metabolism, any alterations in its function can lead to the development of the metabolic syndrome and reduced lifespan. We recently observed elevated hepatic ER stress exclusively in MPR derived IUGR offspring, which received a nutritional mismatch in postnatal life. Interestingly, an elegant study by Pascual et. al. in 2008 demonstrated that tunicamycin-induced ER stress in HepG2 cells induced the expression of CYP2B6, the human ortholog of Cyp2b1. The molecular mechanism behind this induction was attributed to ER stress activation of liver enriched activating transcription factor 5 (ATF5), which shares close sequence homology to the more ubiquitously expressed ATF4. ATF4 activation is known to occur via its selective translation during a period of global protein translation attenuation due to elevated phosphorylation of eukaryotic initiation factor 2α. Therefore, it is possible that activation of ATF5 due to the
previously observed hepatic increases in phosphorylation of eukaryotic initiation factor 2α\(^56\), may also cause the induction in Cyp2b1 expression observed in the study. Interestingly, ATF5 has been observed to interact with C/EBPα family of transcription factors, as well as synergistically potentiate the actions of nuclear receptor CAR, which are both established regulators of the CYP2B6 gene\(^57\). With the use of Algen PROMO™ software, we have identified multiple putative C/EBPα binding sites at the promoter of all the Cyp enzymes examined in this study. Therefore, it is conceivable that under conditions of high stress (\textit{i.e.} ER stress), as exhibited in LP2 low birth weight offspring, elevation of these xenobiotic metabolizing enzymes may serve as an adaptive response to improve its detoxification capacity.

To date there have been no clinical studies conducted to evaluate the effects of low birth weight on the pharmacokinetics of drugs in adulthood. This is particularly relevant to drugs that would likely be used to manage the symptoms of the metabolic syndrome that are observed in these offspring. For instance, despite strong clinical and animal evidence linking IUGR to hepatic dysfunction and elevated cholesterol levels in adult life, there is little known about whether statin pharmacokinetic or pharmacodynamic parameters are altered. Out of all the statins that target the liver, simvastatin, lovastatin and atorvastatin are metabolized primarily through CYP3A4, and fluvastatin is metabolized through CYP2C9\(^57\). Since, the rat orthologs of CYP3A4 (\textit{i.e.} Cyp3a1 and Cyp3a2) and CYP2C11 (\textit{i.e.} Cyp2c11) activity was elevated in adulthood, it is likely that low birth weight offspring would metabolize statins in the liver at a faster rate. Our data suggest that low birth weight offspring may require augmented doses of statins to maintain efficacy towards reducing circulating cholesterol levels, a hypothesis which still remains to be tested. Moreover, in order to completely understand the impact of low birth weight on alterations in pharmacokinetics of drugs, several additional factors need to be evaluated, mainly changes in plasma binding protein levels and drug transporter function. It is also important to determine whether different insults leading to low birth weight would similarly impact drug pharmacokinetic measures in adult life.
In summary, this study highlights that low birth weight offspring faced with a nutritional mismatch in postnatal life have elevated activity of major hepatic phase I drug metabolizing enzymes in adulthood. Moreover, maintaining these low birth weight offspring on a low protein diet prevented the increases in expression of these enzymes. It is plausible that these offspring sense the nutritional mismatch as an unanticipated insult and consequently respond by increasing their detoxification capacity via the activation of nuclear receptor Car. In light of this study, careful consideration is recommended for optimizing drug dosing to ameliorate symptoms of metabolic syndrome in IUGR offspring that display nutritional mismatch induced accelerated growth.
5.5 References


6 DISCUSSION AND CONCLUSIONS
6.1 Summary and Discussion

6.1.1 Chapter Three

The aim of Chapter Three was to elucidate the molecular mechanisms which may lead to long-term cholesterol dysregulation of MPR derived low birth weight offspring. Given placental insufficiency in humans can produce protein deficiency in the fetus, this maternal protein restriction model shares features in common with IUGR, which represents 8% of newborns\(^{1-3}\). The hypothesis was that IUGR offspring may lead to elevated cholesterol levels in adult offspring via repressive changes in histone modifications at the \textit{Cyp7a1} promoter. As shown in Chapter Three, long-term hepatic \textit{Cyp7a1} transcriptional repression in IUGR offspring derived from a low protein diet during pregnancy and lactation underlies the increases in circulating and hepatic cholesterol levels in both sexes at postnatal day 21 and exclusively in the male offspring at postnatal day 130. This long-term repression in transcription was concomitant with diminished acetylation and enhanced methylation of histone H3 [K9,14], markers of chromatin silencing, surrounding the promoter region of \textit{Cyp7a1}. These epigenetic modifications originated in part due to diet-induced decreases in fetal hepatic Jmjd2a expression, a histone H3 [K9] demethylase. This chapter provides the first evidence highlighting the important role of epigenetic mechanisms (\textit{i.e.} post-translational histone modifications) in predisposing under nutrition derived low birth weight offspring to increased risk of hypercholesterolemia. In addition, these findings overturn the dogma that fetal programming effects are permanent since restoration of proteins in the maternal diet during the neonatal period re-established the expression of \textit{Cyp7a1} to prevent hypercholesterolemia. This restoration was associated with an increase in LXR\(\alpha\) expression.

6.1.2 Chapter Four

The aim of Chapter Four was to determine whether nutritional mismatch in postnatal life of low birth weight offspring would lead to elevated hepatic endoplasmic reticulum (ER) stress in adulthood. The hypothesis was that MPR derived offspring faced with a
nutritional mismatch are at a higher risk of developing metabolic syndrome in adult life due to presence of elevated hepatic ER stress. As shown in Chapter Four, exclusively when MPR derived low birth weight offspring received a “nutritional mismatch” postpartum, did they display elevated hepatic ER stress markers, including an increase in X box binding protein 1 mRNA splicing levels and elevated ER chaperones (Glucose regulated protein 78 and 94) at postnatal day 130. This was concomitant with attenuated protein synthesis, as indicated by increased phosphorylation of eukaryotic initiation factor 2 alpha at Serine 51 residue. Interestingly, maintaining the low birth weight offspring on a low protein diet throughout life prevented hepatic ER stress. These findings suggest that in addition to epigenetic and transcriptional mechanisms underlying the ‘developmental origins of adult disease’, chronic activation of the ER stress pathway may also mediate long-term insulin resistance and hypercholesterolemia.

6.1.3 Chapter Five

Given that low birth weight children that undergo accelerated growth display an earlier onset of metabolic disease symptoms and are more likely to be prescribed medication to manage their symptoms (e.g. statins for hypercholesterolemia), the aim of Chapter Five was to investigate whether hepatic drug metabolism is altered in adult life of low birth weight offspring. In addition, it was of interest to determine whether this association is positively correlated in low birth offspring that display accelerated growth when faced with a nutritional mismatch postpartum. The hypotheses was that MPR derived low birth weight offspring may have an impaired ability to metabolize drugs in adulthood. As shown in Chapter Five, a low protein diet during pregnancy and lactation leads to increases in Cyp3a, Cyp2b and Cyp2c enzymatic activity ($V_{max}/K_m$) at postnatal day 130. This was attributed primarily due to increases in mRNA levels of Cyp3a1, Cyp2b1 and Cyp2c11. These elevated mRNA levels originate in part due to induction in expression of constitute androstane receptor, which is known to activate gene transcription of these Cyp enzymes. Interestingly, low birth weight offspring that were maintained on low protein diet postpartum did not display any alterations in Cyp enzyme expression. Collectively, these findings suggest that a low birth weight offspring when faced with a nutritional mismatch postpartum may require greater doses of drugs, which are metabolized by
Cyp3a, Cyp2b and Cyp2c enzymes, in order to exert their therapeutic effects. This is an important finding as it is applicable to those individuals who are more likely to be prescribed medication (e.g. statins for hypercholesterolemia) for the management of the symptoms associated with the metabolic syndrome.

6.2 Future Directions

Chapter Three and Four of the thesis provide a mechanistic insight which improves our understanding of why low birth weight babies are predisposed to increased risk of metabolic syndrome. In identifying some of the underlying mechanisms, this thesis helps identify early life dietary and/or drug intervention strategies which can be potentially used to lower cholesterol and reduce the incidence of the metabolic syndrome. Such strategies could reduce the long-term use of cholesterol lowering drugs in adulthood. Moreover, considering that adults that were born low birth weight have a higher likelihood of prescribing these drugs, chapter Five emphasizes the importance of personalized pharmacological therapy in these individuals. However, upon collectively analyzing the findings from the thesis, it is critical to realize that there are a number of important questions that still need to be addressed. The following sections of the thesis aim to highlight some of these important questions.

6.2.1 Molecular Mechanisms Underlying Sex-Specific Effects observed in Adult Life of Low Birth Weight Offspring

These studies have demonstrated that elevated circulating cholesterol levels in LP2 offspring are associated with impaired Cyp7a1 expression in both sexes at three weeks (pre-weaning) and exclusively in the males at four months of age. This sex-specific effect is consistent with findings from other animal model studies. For example, LP2 offspring exhibit insulin resistance and visceral obesity exclusively in the adult male offspring at postnatal day 130. While the mechanisms underlying these sex-specific programming effects have not been elucidated, it has been hypothesized that sex steroids (i.e. estrogen) protect the female from the development of these disease processes, including elevated blood pressure. Evidence for this comes from the Aromatase Knockout (ArKO) mouse, which cannot synthesize endogenous estrogens due to
disruption of the $\text{Cyp19}$ gene\textsuperscript{8}. ArKO females challenged with a high cholesterol diet have higher circulating cholesterol levels and lower Cyp7a1 expression compared to wild-type females and males of either genotype\textsuperscript{8}. Moreover, estrogen replacement reversed the hepatic steatosis in male ArKO mice\textsuperscript{9}. Interestingly, this reversal is only possible at lower doses of estrogen which are comparable to physiological levels in the female rats as two previous studies have demonstrated a biphasic response of CYP7A1 to estrogen levels in rats\textsuperscript{10, 11}. Lower doses of 17β Estradiol (an endogenous agonist) resulted in an induction of CYP7A1 expression, while at higher doses there was a repression of CYP7A1 expression. The molecular mechanism mediating this biphasic dose response has not been fully elucidated yet. The effects of estrogen may only be part of the reason for the sexual dimorphism, as a loss of circulating testosterone has been previously observed in MPR males\textsuperscript{5}. It is plausible that the loss of this androgen also underlies male-specific impairment of Cyp7a1 and cholesterol catabolism. Elevated activity of testosterone metabolizing Cyp3a, Cyp2b and Cyp2c enzymes observed in adult life of MPR derived male offspring (Chapter 5) now provide evidence for the mechanism underlying this observation. However, to pinpoint the role of these Cyp enzymes in male specific decreases in circulating testosterone, their expression in MPR females would need to be evaluated. In order to fully elucidate whether estrogen or testosterone are protective towards preventing high cholesterol observed in MPR derived adult male offspring, one can design in vivo experiments which involve castration of MPR derived male offspring followed by a timely supplementation of estrogen or testosterone. In conjunction, supplementing estrogen or testosterone in ovariectomized MPR derived female offspring would also be considered to conclusively determine the role of each hormone in the MPR derived male-specific impairment of cholesterol homeostasis. It is also important to account for the role of other hormones in this impairment. For instance, MPR male offspring at 130 days of age have two-fold higher levels of circulating insulin\textsuperscript{5}, which has been known to inhibit Cyp7a1 transcription in both rat hepatocytes and streptozotocin (STZ)-induced diabetic rats via decreases in the binding of transcription factors Fox01 and Smad3 to the promoter of Cyp7a1\textsuperscript{12}. Lastly, studies have now demonstrated that sex steroid hormones can influence epigenetic mechanisms, including post-translational histone modifications\textsuperscript{13}. Therefore, future
studies should evaluate whether alterations in the “histone code” due to endocrine disruptions mediate these sex-specific effects. Conversely, the impact of the “histone code” should also be examined in mediating these endocrine disruptions in low birth weight offspring.

6.2.2 Role of Histone Modifying Enzymes in Initiating and Maintaining Transcription of Genes Regulating Cholesterol Homeostasis in Low Birth Weight Offspring

An imbalance of histone modifying enzymes can result in aberrant histone modification levels in several disease processes. Consequently, these enzymes have gained a lot of attention as potential therapeutic targets over the past decade\textsuperscript{14}. Yet, no animal study has attempted to examine changes to the global expression profiles of all known histone modifying enzymes in placental insufficiency (PI) induced-IUGR. Two studies have descriptively investigated changes to in vivo binding of a few histone modifying enzymes in order to understand the post-translational histone modifications occurring at the promoter region of pdx-1 in pancreatic \( \beta \)-cells and glut-4 in skeletal muscles, which underlie the development of type 2 diabetes in IUGR rat offspring\textsuperscript{15,16}. However, the role of histone modifying enzymes in linking PI-IUGR to other risk factors of the metabolic syndrome remains unexplored. The two main natural consequences of placental insufficiency are low oxygen and nutrient delivery to the fetus, which can have an effect on the expression and activity of histone modifying enzymes. For example, hypoxia has been demonstrated to alter the expression and activity of a variety of histone modifying enzymes\textsuperscript{17-21}. Furthermore, hypoxia has been demonstrated to increase di-methylation of Histone H3 K9 mediated by G9a methyltransferase\textsuperscript{22}, an enzyme known to be involved with bile acid mediated Cyp7a1 repression\textsuperscript{23}. As MPR has been well documented to result in placental insufficiency with low oxygen and nutrient deliver to the IUGR fetus\textsuperscript{24-28}, a change in expression and activity of a wide variety of histone modifying enzymes during development would be expected in this model. Furthermore, given that MPR resulted in long-term repression of Cyp7a1, there is a need to examine the changes in expression of histone modifying enzymes which result in stable repressive post-translational histone modifications. Thus far, the steady-state mRNA levels of those [K9] demethylases (Jmjd2a, Jmjd2b, Jmjd2c, Jmjd2d) that remove three methyl groups from
[K9] of Histone H3 has been examined\(^9\). Q-RT-PCR revealed that a decrease in the hepatic Jmjd2a mRNA was observed at e19 (data not shown). Furthermore, expression of histone modifying enzymes needs to be correlated with their \textit{in vivo} binding changes at the promoter of Cyp7a1 with the use of ChIP. This would provide insight into their role in establishing and maintaining the chromatin environment at Cyp7a1 promoter region and in regulating long-term Cyp7a1 transcription. This role can be further strengthened by performing \textit{in vitro} studies by determining whether Cyp7a1 expression is altered due to siRNA knockdown of the candidate histone modifying enzyme. The long-term goal should be to further characterize the function of histone modifying enzymes in altering long-term transcription of a wide variety of other cholesterol regulatory genes involved with catabolism (Cyp8b1)\(^{30}\), efflux (ATP binding Cassette Transporters A1, G5 and G8)\(^{31}\) and synthesis (Srebp-2)\(^{31}\) or uptake (LDL Receptor)\(^{31}\). This would lead to the identification of key histone modifying enzymes that are more specific in prevention of hypercholesterolemia. Additionally, it will be the first time research would be geared towards investigating how a particular histone modifying enzyme plays a role in influencing epigenetic mechanisms governing cholesterol regulation in general. A similar approach can be implemented to investigate the role of histone modifying enzymes on genes involved with glucose homeostasis. Moreover, the mRNA, protein and ChIP library established from these experiments can be used for collaborative projects involved with the investigation of the role of histone modifying enzymes in long-term transcriptional regulation of hepatic genes associated with other disease processes.

6.2.3 Preventing the Developmental Origins of Hypercholesterolemia and Glucose Intolerance

Hepatic differentiation in mammals begins at an early stage \textit{in utero}, during which liver has a major hematopoietic function until birth\(^{32}\). In rodents, the liver bud is formed containing bipotential progenitor cells that differentiate into either hepatocytes or ductal cells at mid-gestation\(^{32}\). In the last three days of gestation, a high rate of fetal hepatocyte proliferation results in the tripling of total liver weight\(^{33}\). This is followed by a transition of fetal to adult rat hepatocytes in the first week of postnatal life\(^{34}\). Given that there is a high rate of neogenesis, replication and apoptosis during this neonatal period in rats\(^{33}\), it
represents a critical window for therapy designed to improving hepatic function and proliferation. It has been previously demonstrated that the neonatal administration of Exendin-4 (a GLP-1 analogy) in IUGR rats derived from uterine-ligated dams rescued β-cell function and prevented the development of diabetes\textsuperscript{35-37}. This was attributed to be due to the restoration of expression of a key transcription factor Pdx-1, via epigenetic mechanism induced alterations at its promoter region. In addition, Exendin-4 treatment during this neonatal period has been demonstrated to prevent the development of hepatic oxidative stress and insulin resistance\textsuperscript{35}. These studies remarkably illustrate that neonatal intervention can influence both pancreatic and liver development long-term. Future studies should be aimed at investigating how we can exploit this plasticity in liver development to correct the abnormalities that result from an adverse in utero environment.

Attempts were made to correct the hypercholesterolemia in MPR derived IUGR offspring by administering an LXR agonist (GW3695) from postnatal day 5 to 15 (Figure 6.1). The LXR agonist GW3695 was chosen for these studies in MPR offspring for several reasons: (1) restoration of proteins during neonatal life (LP3) stimulated LXR expression and restored cholesterol homeostasis, (2) hepatic LXR expression is impaired in MPR offspring at day 130\textsuperscript{38}, (3) diminished hepatic LXR in MPR offspring also mediates their impaired glucose tolerance\textsuperscript{36} and (4) GW3695 has been previously used in neonatal rat studies \textit{in vivo} to effectively boost LXR activity\textsuperscript{39, 40}. Interestingly, a week later (postnatal day 21), these offspring had decreased circulating LDL:HDL cholesterol ratios which was concomitant with elevated LXRα and Cyp7a1 expression\textsuperscript{41}. Furthermore, this was associated with a permissive chromatin environment as assessed by increases in the acetylation of histone H3 [lysine 9] surrounding the LXRE of the \textit{Cyp7a1} promoter\textsuperscript{41}. Investigations to determine whether these changes are maintained in adulthood as a consequence of this neonatal LXR are currently underway. Future studies should also focus on determining whether this intervention will reverse the glucose impairment observed in these offspring in adult life.

In Chapter Four it was demonstrated that a mismatched diet leads to increased ER stress in IUGR rats. However, little is known about whether early intervention with diet or
drugs could alleviate ER stress and symptoms of the metabolic syndrome long-term. In adults, a number of studies have demonstrated significant clinical implications of therapies to improve metabolic control by alleviating ER stress. In humans, weight loss has been associated with a decrease in ER stress markers in the adipose and liver tissues. Furthermore, chemical and molecular chaperones have received a lot of attention as agents improving metabolic control and insulin sensitivity in animal models, as well as in obese humans. For instance, the chemical chaperone sodium 4-phenylbutyrate (PBA), has been demonstrated to reduce ER stress induced by either hepatocellular injury or chronic oleate exposure in mice. In humans, oral administration of PBA has been observed to alleviate insulin resistance and β cell dysfunction. Although PBA is not safe to use during pregnancy, it is routinely used in children for the treatment of urea cycle disorders. Oral Tauroursodeoxycholic Acid (TUDCA) is another example of chemical chaperone that has been demonstrated to improve insulin sensitivity of the liver and skeletal muscle in animals and humans. Interestingly, unconjugated Ursodeoxycholic acid (UDCA) is clinically approved for the treatment of intrahepatic cholestasis during pregnancy. In addition, UDCA has been demonstrated to reduce apoptosis in human placental explants treated with increasing levels of bile acids. However, the use of TUDCA for the purpose of preventing placental insufficiency by increasing its ER folding capacity has never been investigated. In addition, the use of PBA or TUDCA during neonatal life has not been considered in preventing the onset of the metabolic syndrome in IUGR offspring. It is conceivable that short-term intervention, which exploits liver plasticity during development, by administration of chemical ER chaperones would help prevent the observed increases in cholesterol levels and glucose intolerance in the adulthood. Studies with similarity to the LXR agonist neonatal intervention approach are currently underway to determine if oral administration of TUDCA (1g/Kg body weight) will prevent ER stress and rescue
the high cholesterol levels and glucose impairment of MPR derived IUGR offspring in adult life (Figure 6.1).
Figure 6.1: Neonatal Drug Intervention Studies. Cholesterol and glucose homeostatic parameters will be examined in four separate dietary regimes which include a normal (20%) protein diet administered a vehicle (Vehicle + CONTROL) or a drug (Drug + CONTROL) and a low (8%) protein diet during pregnancy and weaning alongside administration of a Vehicle (Vehicle + LP2) or a drug (Drug + LP2) from postnatal day 5 to 15. Vehicle and drugs were administered intra-peritoneal (IP). Vehicle: DMSO (50mg/kg IP). Drug: LXRα Agonist GW3695 (50mg/kg IP) or ER chaperone TUDCA (1g/kg administered orally).
6.2.4 Investigating Alterations in Drug Metabolism in Adulthood of IUGR Offspring

Based on the findings presented in this thesis, IUGR offspring are more likely to be prescribed drugs for management of metabolic risk factors. However, epidemiological studies have not been conducted to determine if this is indeed true. In Chapter Five, a MPR rat model was used to demonstrate that IUGR offspring have elevated activity of important hepatic drug metabolizing Cyp enzymes in adulthood. These findings suggested that IUGR offspring would require augmented doses of drugs in adult life in order to exert their therapeutic effect when compared to normal birth weight offspring. Further experimentation is required in order to validate this point. A quick indicator of whether pharmacokinetics of a drug is altered in animals can be derived from the zoxazolamine paralysis assay, where a decrease in duration of paralysis is related to faster clearance of zoxazolamine\textsuperscript{57}. For a more thorough and conclusive analysis, pharmacokinetic parameters of drugs of interest need to be correlated with their respective drug disposition molecular pathways in adult life of IUGR offspring. These pathways mainly include Phase I and Phase II drug metabolizing enzymes, plasma binding proteins, uptake proteins and transporters in several tissues. Moreover, it remains to be determined whether different insults leading to IUGR would similarly impact drug pharmacokinetic measures in adult life.

6.3 Conclusions

The impact of adverse events during development on an individual’s long-term wellbeing is undoubtedly strong. In most cases these adverse events can be prevented from occurring by intervention. However, there are several scenarios where adverse events remain unnoticed or have already impacted developmental processes by the time they are recognized. Therefore, development of biomarkers, which provide earlier identification of impaired development would be invaluable in these situations. However, until this can be achieved, it is imperative to increase our understanding of the underlying mechanisms which program offspring impacted by these adverse events to an increased risk of adult diseases. Therefore, the focus of this thesis was to investigate these mechanisms in order
to enhance our understanding of why low birth weight offspring have increased risk of developing the metabolic syndrome in adult life (Figure 6.2). In the first section of the thesis, elevated cholesterol levels in low birth weight offspring were shown to be linked to long-term repressive changes in histone modifications at the promoter of Cholesterol 7α-Hydroxylase, a rate-limiting enzyme involved with cholesterol catabolism. The second section of the thesis provided novel evidence to suggest that in addition to epigenetic and transcriptional mechanisms underlying the ‘developmental origins of adult disease’, activation of hepatic ER stress may mediate long-term insulin resistance and hypercholesterolemia observed in these offspring (Figure 6.2). Moreover, these two sections provide support to the “Predictive Adaptive Response” hypothesis, as the impairment in hepatic cholesterol catabolism and ER stress was present exclusively in the IUGR rat offspring which received a nutritional mismatch and displayed accelerated growth rates in postnatal life. By understanding the role of these molecular mechanisms in mediating these developmental abnormalities, better strategies for preventing the onset of adult diseases can be developed. The third section of this thesis focuses on investigating mechanisms which will enable the development of strategies for risk management as opposed to risk prevention (Figure 6.2). Given that IUGR offspring are more likely to be prescribed medication for management of the metabolic syndrome, the impact of IUGR on Phase I of drug metabolizing enzymes in adulthood was investigated in Chapter Five. The findings demonstrated that IUGR offspring when faced with a nutritional mismatch had elevated Cy3a, Cyp2b and Cyp2b enzyme activity, suggesting the need for augmented doses of drugs metabolized by these enzymes in order to manage the symptoms of the metabolic syndrome in adult life. Overall, it is anticipated that the findings presented in this thesis will lead to a better understanding of the molecular mechanisms underlying the early programming of the liver.
Figure 6.2: Schematic summary of the Thesis
6.4 References


7 APPENDIX
Appendix 1

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7.2 Appendix 2

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- Best Oral Presentation Award, Development Origins of Health & Disease Symposium, Ann Arbor, MI, USA (2008)

**PUBLICATIONS LIST**

**REFERRED PAPERS (IN PRESS)**


2. Sohi G, Marchand K, Revesz A, Arany E, and Hardy DB. Maternal Protein Restriction Elevates Cholesterol in Adult Rat Offspring Due to Repressive Changes in Histone Modifications at the Cholesterol 7α-Hydroxylase Promoter, *Molecular Endocrinology*. 2011 May; 25(5);785-98.


**REFERRED PAPERS (IN PREPARATION)**

5. Sohi G, Barry EJ, Velenosi TJ, Urquhart BL, Hardy DB (2013) Maternal Protein Restriction Leads to Elevated Hepatic Drug Metabolism in Low Birth Weight Adult Rat Offspring. *In preparation*


SELECTED PUBLISHED ABSTRACTS

Total Number of Presentations: First Presenter: 31 Co-Author: 30


5. Sohi G, Revesz A, Arany E, and Hardy DB. Elevated hepatic Endoplasmic Reticulum in Adult Rat Offspring Derived from Maternal Protein Restriction. Canadian Student Health Research Forum, Winnipeg, ON, June 11-12, 2012 (Top 5% PhD students of Schulich School of Medicine and Dentistry representing Western University).


17. **Sohi G**, Weese K, Arany E, and **DB Hardy**. Maternal Protein Restriction (MPR) in Rats Leads to Increased Cholesterol in the Male Offspring and Decreased Expression of Hepatic Liver X Receptor (LXR)-Target Genes, *Developmental Origins of Health and Disease Symposium*, University of Michigan, Ann Arbor, MI, October 17th, 2008 (*Best Abstract Award*).
RELATED WORK EXPERIENCE

Teaching Assistant, Western University, London, ON (2008 - 2013)

- Physiology 1021y – Human Physiology (2010-2013)
- Physiology 3130y – Physiology Laboratory (2008-2010)

Research Assistant, Schulsich School of Medicine & Dentistry, London, ON (2008)

- Helped establish and manage a 1800 sq. ft. laboratory space
- Allocated decisions for $350,000 funded by the Canadian Foundation of Innovation directed at the purchasing of “state of the art” laboratory equipment
- Managed and organized “Product Presentations” by competing biotechnology companies
- Evaluated logistic requirements of future laboratory usage and established training sessions for graduate students

MANAGEMENT, LEADERSHIP & VOLUNTEER EXPERIENCE

Clinical Trial Assistant, Center for Clinical Investigation & Therapeutics, London, ON (2013 - Present)

- Assisted Dr. Brad Urquhart on clinical study aimed at investigating kidney-liver cross talk mechanisms in kidney disease patients by evaluating oral dose of fexofenadine and intravenous dose of midazolam

Director of Research Media, Kukuzoo Studios, Toronto, ON (2012 - 2013)

- Establishing contacts with leading scientists and institutes for the use of animation videos to promote public awareness of scientific discoveries and initiatives


- Consulted with the Society of Saint Vincent de Paul on developing creative ways to increase thrift store sales by 20% in order to effectively support low income families

Graduate Recruiter, Schulsich School of Medicine & Dentistry, London, ON (2010 - 2013)

- Served as a representative at graduate recruitment fairs at the national and international level

Instructor, Let’s Talk Science, London, ON (2010 - Present)

- Organized hands-on scientific experiments aimed at inspiring youth in the fields of science, engineering, and technology, and motivating them to become innovators of the next generation

Director, LIVERight Student Association, Western University, London, ON (2009 - 2013)

- Lead a team of undergraduate students to raise awareness of liver diseases and helped fundraise $5000 for the Canadian Liver Foundation
- Initiated a research study to evaluate the awareness of Hepatitis A and B among university students across Canada

**Fundraiser, Big Brothers and Big Sisters of London and Area,** London, ON (2012)
- Raised $500 for the event Bowl for Kids Sake by organizing a bowling team.

**Volunteer, The Canadian Medical Hall of Fame,** London, ON (2010 - 2011)
- Help organize student tours of our research laboratory at the University of Western Ontario
- Engaged students by designing basic experimental protocols for them to conduct

**Head Coach, Mississauga Minor Basketball Association,** Mississauga, ON (2007-2008)
- Supervised and served as a role model for a Midget (Under 15) Boys Basketball Team.

**Respite Assistant, Community Living,** Mississauga, ON (2005 - 2007)
- Taught the intellectually disabled important education and living skills
- Helped them integrate with society by organizing community events

**SUPERVISORY EXPERIENCE**

Supervised four honors 4th year thesis project students, Andrew Revesz, Thin Vo, Noelle Ma, Michael Wong. My responsibilities included facilitating the development of suitable research projects and training students in various techniques pertinent to their projects. Three of them are now on their way to completing their masters and each of them have 1-2 publications in press. I have also supervised undergraduate student Eric J Barry (4th undergraduate at McMaster University) and Megan M Smith (recently completed her MSc at University of Toronto). Moreover, I have provided hands on training to perform Chromatin Immunoprecipitation and/or Western Blot Analysis to Christina Raykha (PhD Candidate), Waseem Iqbal (PhD Candidate) and Thomas Velenosi (PhD Candidate).

**KNOWLEDGE TRANSLATION ACTIVITIES**

**Director of Research Media, Kukuzoo Studios,** Toronto, ON (2012 - 2013)

Knowledge Translation Activity: Community Engagement

**Target Stakeholder:** General Public

Outcome: Collaborated with Dr. Adrian Owen, a world-renowned neuroscientist, on developing an engaging animation explainer video which highlights his groundbreaking research discovery that involved communication with vegetative state patients to the general public.
URL Link: http://www.youtube.com/watch?v=l_MbvfKtgi4&list=UU9oplgdixPpMBAK0Shs5LSQ&index=1

Evidence of Uptake: The youtube video has resulted in new collaboration with other scientists from the Brain and Mind Institute, Rotman Institute and Philosophy, St. Michael's Hospital and University of British Colombia. This video is the first point of introduction for anyone interested in learning more about Dr. Adrian Owen's research (www.owenlab.org).


Presenter, Strong Bones, Strong Minds and Strong Muscles, Western University, ON (2012 - 2013)

Knowledge Translation Activity: Community Engagement

Target Stakeholder: General Public

Outcome: Engaged the elderly population at a retirement home to appreciate the practical significance of my PhD research

Evidence of Uptake: Received positive feedback at the end of the presentation