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Prenatal Programming of Hepatic Glucose and Cholesterol Regulation in Male Rat Offspring by Chronic Intermittent Hypoxia

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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PRENATAL PROGRAMMING OF HEPATIC GLUCOSE AND CHOLESTEROL REGULATION IN MALE RAT OFFSPRING BY CHRONIC INTERMITTENT HYPOXIA

(Thesis format: Integrated Article)

by

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ABSTRACT

Obstructive sleep apnea (OSA) is a chronic disorder involving repetitive interruptions in breathing during sleep. Sufferers of OSA are exposed to chronic intermittent hypoxia (CIH), characterized by cyclical reductions in oxygen availability. A number of studies have established a link between OSA and various cardiovascular and metabolic comorbidities in adulthood, including hypertension, obesity, and type II diabetes. While the consequences of OSA in adults have been well described, the cross-generational impact of this condition and potential effects on fetal development are not known. Epidemiological and animal studies have demonstrated that physiological insults during pregnancy lead to diminished growth of offspring and impairments in long-term metabolic health. The following studies were performed to investigate the alteration of metabolic health as a result of gestational exposure to CIH, the underlying physiological condition in OSA. Exposure to CIH during pregnancy resulted in asymmetrically growth restricted offspring with lower birthweights compared to normoxic offspring. In adulthood, male CIH offspring had higher body weights and higher visceral adipose tissue. Gestational CIH caused impairments in glucose homeostasis, as male CIH offspring were hyperglycemic, hyperinsulinemic, and showed impaired early-stage glucose tolerance in adulthood. Molecular analysis revealed that CIH offspring have diminished regulation of hepatic gluconeogenic pathways by the Liver X Receptor (LXR), leading to increased expression of hepatic markers associated with gluconeogenesis and increased glucocorticoid signalling. Adult male CIH offspring were also hypercholesterolemic with decreased expression of LXR target genes associated with cholesterol metabolism and excretion. Taken together, these results describe

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changes in hepatic function that result in impaired regulation of glucose and cholesterol homeostasis in adulthood. The findings of this thesis provide insight into the long-term consequences of insult during gestation, and highlight the importance of giving consideration to OSA in assessing maternal health and predicting fetal outcomes.

DEDICATION

This thesis is dedicated to my parents. Thank you for supporting me, for believing in me,

and for working incredibly hard for so many years.

CO-AUTHORSHIP STATEMENT

The contribution of co-authors is outlined below.

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LIST OF ABBREVIATIONS

11β-HSD1	11β-hydroxysteroid dehydrogenase type I
ABCA1	ATP-binding cassette transporter – subfamily A, member 1
ABCG8	ATP-binding cassette transporter – subfamily G, member 8
ApoE	Apolipoprotein E
BMI	Body mass index
ChREBP	Carbohydrate-responsive element binding protein
CIH	Chronic intermittent hypoxia
CYP7A1	Cholesterol 7α-hydroxylase
EDTA	Ethylenediaminetetraacetic acid
FFA	Free fatty acids
G6Pase	Glucose-6-phosphatase
GLUT	Glucose transporter
GR	Glucocorticoid receptor
HDL	High-density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
IGTT	Intraperitoneal glucose tolerance test

IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LXR	Liver X receptor
MAP	Mitogen activated protein
MLK	Mixed-lineage kinase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OSA	Obstructive sleep apnea
PEPCK	Phosphoenolpyruvate carboxykinase
TBS-T	Tris-buffered saline – Tween20

Chapter 1

Introduction

1.1 – SLEEP APNEA

Sleep apnea is a breathing disorder involving recurrent pauses in respiration during sleep. There are two primary forms of sleep apnea, central and obstructive. Obstructive sleep apnea is the most common form of sleep apnea, accounting for roughly 85 percent of all cases. Central sleep apnea is very rare, accounting for only 1 percent of all sleep apnea cases. The remaining 14 percent of individuals typically present with a mixed apnea condition that is a combination of both obstructive and central sleep apnea (Morgenthaler et al. 2006).

1.2 – CENTRAL SLEEP APNEA

Central sleep apnea is characterized by repetitive interruptions in breathing during sleep. Patients with central sleep apnea experience apneic episodes that limit the availability of oxygen to the body. However, central sleep apnea is typically accompanied by a lack of respiratory effort during cessation of air flow (Mellins et al. 1970, White 2005, Eckert et al. 2007). Individuals with central apnea often present with insomnia and mood disorders (White 1985) as well as excessive daytime sleepiness (Eckert et al. 2007). A number of manifestations have been reported, including narcotic-induced, congenital, and idiopathic central apneas (Eckert et al. 2007).

The depressive effects of opiates on respiratory function have previously been described (Santiago, Edelman 1985, Shook, Watkins & Camporesi 1990). Recent

studies have demonstrated that prolonged use of opioid-based medications increased the disposition to central apnea in half of observed patients (Farney et al. 2003, Wang et al. 2005). The number of individuals with opioid-related sleep-disorder breathing is likely to increase due to the prevalence of opioid-based medications in the treatment of chronic pain (Luo, Pietrobon & Hey 2004, Eckert et al. 2007). Interestingly, repeated interruption of sleep has been shown to worsen feelings of physical pain, suggesting that opioid-induced central apneas may intensify the need and use of opioid-based medications (Eckert et al. 2007).

Congenital and idiopathic central apneas are rare conditions related to acquired and adapted abnormalities in respiratory control or chemoreceptor sensitivity (White 2005). Ventilation is in part driven by signals to respiratory control areas in the medulla from central chemoreceptors, which sense changes in the partial pressure of carbon dioxide (PCO_2)(Schlaefke 1981). Individuals with idiopathic and congenital central apneas tend to have chronically lower PCO_2 levels, preventing central chemoreceptors from activating respiratory centres in the brain stem (Xie et al. 1994, Xie et al. 1995, Xie et al. 1997). Furthermore, impairments in chemoreceptor sensitivity can result in persistently unstable ventilation (Khoo et al. 1982). Irregular breathing or a complete cessation of breathing can occur during sleep due to alterations in chemoresponse, as respiratory drive during sleep is reliant on communication between central chemical control mechanisms (Mellins et al. 1970, Tassinari et al. 1972).

1.3 – OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea (OSA) is a progressively worsening, chronic breathing disorder that occurs during sleep. Caused by a blockage of the upper-airway, OSA is characterized by a cyclical reduction in air flow to the lungs. Unlike central sleep apnea, OSA patients exhibit a continuous respiratory effort during interruption of breathing (Dempsey et al. 2010, Mannarino, Di Filippo & Pirro 2012). Two common events that occur in OSA patients are apneas and hypopneas. An apneic episode is a complete restriction of air flow, due to a total blockage of the upper-airway. A hypopneic episode is a greater than 50 percent reduction in air flow, caused by a significant narrowing of the upper-airway (Pack 2002). An apnea or hypopnea event during sleep reduces the volume of air reaching the lungs, limiting the ability of the organ to resupply oxygen and remove carbon dioxide from the blood.

Symptoms of OSA during sleep include chronic snoring, long pauses in breathing, frequent arousals, gasping for air upon arousal, and insomnia (Park, Ramar & Olson 2011). Symptoms of OSA while awake include daytime sleepiness (hypersomnolence), morning headaches, impaired cognitive function, and mood disorders (Park, Ramar & Olson 2011, Mannarino, Di Filippo & Pirro 2012). The condition is diagnosed following overnight assessment using polysomnography, a multi-parameter diagnostic tool that monitors neurological, musculoskeletal, cardiovascular, and respiratory changes during sleep (Church 2012). OSA is categorized according to the apnea-hypopnea index (AHI), which calculates the number of OSA-related episodes that occur per hour of sleep. By measuring the frequency of episodic occurrences, the

AHI provides a numerical guideline for assessing the severity of the condition within an individual. An AHI between five and fifteen episodes per hour is categorized as mild OSA, between fifteen and thirty as moderate OSA, and greater than thirty episodes per hour as severe OSA (Fleetham et al. 2006, Epstein et al. 2009).
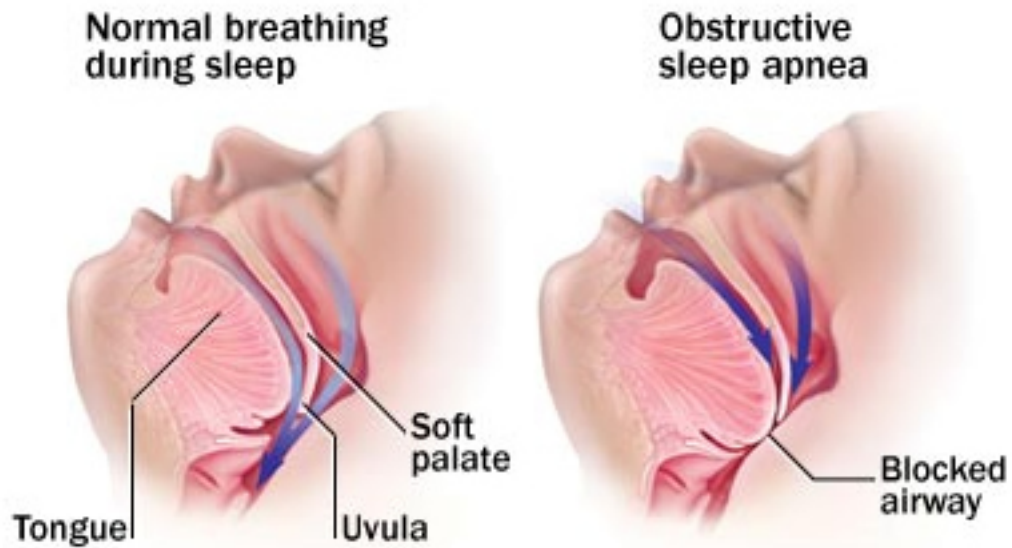
Figure 1.1: Illustrated depiction of obstructive sleep apnea

The apnea/hypopnea episodes observed in individuals with obstructive sleep apnea are caused by a physical blockage of the airway during sleep These episodes limit the flow of air to the lungs, ultimately reducing the amount of oxygen that enters the blood.

1.3.1 – Physiological response to obstructive sleep apnea

Obstruction of the upper-airway during sleep limits the flow of air to the lungs during inspiration. The ability of the lungs to resupply oxygen to the circulation through alveolar gas exchange is diminished (Piiper et al. 1971), causing an inadequate amount of oxygen within the blood, termed hypoxia (Dean, Wilcox 1993). Thus, a recurring pattern of apneic and/or hypopneic episodes can cause a significant reduction in oxygen supply to the body. Repetitive closure of the airway exposes the body to a state of chronic intermittent hypoxia (CIH), which is characterized by frequent, short-term decreases in oxygen saturation of the blood. A decrease in oxygen transport through the circulation diminishes the supply to vital organs and tissues throughout the body (Tamisier et al. 2009).

During an episode of CIH, the body is exposed to a transient hypoxic state (Lavie, Polotsky 2009). The decline in blood-oxygen saturation causes activation of peripheral chemoreceptors (Fukuda et al. 1989, Fletcher 2001). Located in the aortic and carotid bodies, peripheral chemoreceptors are sensitive to changes in the circulating level of oxygen. The result of this activation is a surge in sympathetic nervous system activity proportional to the degree of hypoxia (Somers et al. 1995). Elevated sympathetic nervous system activity is associated with increases in blood pressure, force of contraction within the heart, and peripheral vasoconstriction (O'Donnell et al. 1996, Brooks et al. 1997, Nieto et al. 2000, Peppard et al. 2000). Previous models of CIH have demonstrated increased neuronal activation in brain stem areas that regulate sympathetic nervous system activity, including the nucleus of the solitary tract and noradrenergic cells within the ventrolateral medulla (Greenberg et al.

1999). Thus, each apneic/hypopneic episode contributing to the CIH state creates a temporary, but recurring, hypertensive state (Fletcher et al. 1991, Sunderram, Androulakis 2012).

In OSA patients, the hypoxic condition is corrected and breathing resumes following activation of brain centres controlling arousal. An arousal event involves a surge in autonomic activity that expels the sleeping individual from deeper stages of sleep and reopens the airway (Tomori et al. 2000). Activation of arousal centres in the forebrain is related to respiratory drive. As mentioned, individuals with OSA display a persistent respiratory effort during an apneic obstruction of the airway (Mannarino, Di Filippo & Pirro 2012). Arousal stimulus during sleep is proportional to respiratory effort and is controlled by mechanoreceptors in the upper-airway that signal to higher regions of the brain (Berry, Gleeson 1997, Exar, Collop 1999). This suggests that increased respiratory effort during an apneic episode may cause pressure changes in the airway that lead to arousal (Exar, Collop 1999).

1.3.2 – Physiological and anatomical determinants of obstructive sleep apnea risk

Apneic/hypopneic events can result from decreased muscular tone of the tongue and dilator muscles or asynchronous muscular activity in the upper-airway (Sullivan, Issa 1985, Park, Ramar & Olson 2011). As such, OSA-related events have a greater likelihood of occurring during rapid-eye-movement (REM) sleep, due to a decrease in airway muscle tone that is typically associated with this stage of sleep (Strollo, Rogers 1996). Individuals with greater fat deposition have a higher propensity for developing OSA. Obese patients may have a predisposition to airway disruption (Schwab et al.

1995), as MRI data show greater adiposity in the pharyngeal tissue, enlarged parapharyngeal fat pads, and larger neck circumference in individuals with OSA (Haponik et al. 1983, Horner et al. 1989). An increase of one standard deviation in any parameter of body size is associated with a three-fold increase in the risk of developing at least mild OSA (Young et al. 1993).

Individuals with tonsil-related irregularities or certain craniomandibular features may have greater risk of sleep-disordered breathing, due to anatomical characteristics that predispose the airway to narrowing or closing during sleep (Partinen et al. 1988). Furthermore, instances of familial prevalence that are not explained by metabolic health may be attributable to anatomical features. A family history of OSA may result from genetically-derived mandibular and craniofacial characteristics that place strain on the airway during sleep (Strollo, Rogers 1996). Additionally, this condition can be exacerbated by anatomical changes in the upper-airway that occur in individuals with OSA. Exposure to repetitive vibratory pressure (snoring) and elevated inspiratory pressure in a narrowed airway results in thickening of the soft palate due to edema (Woodson, Garancis & Toohill 1991).

1.3.3 – Populations affected by obstructive sleep apnea

Canadian public health surveys have estimated that approximately 900,000 adults have been diagnosed with OSA (Public Health Agency of Canada 2009). Symptoms associated with OSA include loud snoring, daytime sleepiness, having been observed to stop breathing during sleep, having a BMI greater than 30 kg/m^2, having

high blood pressure, being over the age of fifty, and being male (Nieto et al. 2000, Chung et al. 2008). Remarkably, more than one in four undiagnosed Canadian adults report at least three risk factors that are associated with a high risk of already having or developing OSA (PHAC 2009).

Population studies have demonstrated adult males are more than twice as likely to have OSA as adult women (Redline, Strohl 1998). The male sex has been designated an independent risk factor not only for increasing the likelihood of having OSA, but also for increasing the risk of developing moderate-to-severe OSA (more than fifteen episodes per hour) (Punjabi 2008). Evidence from the Wisconsin Sleep Cohort Study examining over 600 adult men and women showed that roughly one in four adult males and one in ten adult females had some form OSA (at least 5 episodes per hour). Furthermore, approximately ten percent of males and five percent of females in the study presented with moderate or severe OSA (more than 15 episodes per hour) (Young et al. 2009).

A study analyzing polysomnographic data from nearly 24,000 overnight patients concluded that the prevalence of OSA was three times greater in men and males had a higher mean AHI than females (Gabbay, Lavie 2012). While AHI increased linearly with age in both males and females, the rate of increase was greater in males. This suggests that, in untreated adults, OSA reaches moderate and severe stages more quickly in men (Gabbay, Lavie 2012). While the mean age of patients in the study was 51 years, men and women as young as 20 years of age were found to have OSA. Interestingly, women had less severe OSA compared to men in any comparable age group (Gabbay, Lavie 2012).

While the number of individuals experiencing apneic episodes is quite high, OSA is considered to be a dramatically underdiagnosed condition, as only four percent of men and two percent of women meet the symptomatic criteria for diagnosis (AHI greater than five concomitant with excessive daytime sleepiness)(Young et al. 2009). Sex-related differences in visceral fat deposition, a common indicator of OSA risk, may contribute to the prevalence disparity between males and female (Geer, Shen 2009). Referral for overnight testing using polysomnography is based on exhibiting symptoms to a physician. It has been suggested that OSA may be underdiagnosed in women due to the presentation of more subjective symptoms (Ralls, Grigg-Damberger 2012). Snoring, a factor commonly incorporated in the diagnosis, is less predictive of OSA in women than in men (Punjabi 2008). Women with OSA more often present complaints of insomnia and depression (Shepertycky, Banno & Kryger 2005, Valipour et al. 2007). Among OSA patients, complaints of insomnia are greater in women than in men (Subramanian et al. 2011b). Furthermore, women with OSA are found to report psychological comorbidities such as anxiety and depression at a much higher frequency than men with OSA (Uyar et al. 2011, Sampaio, Pereira & Winck 2012). Evidence from cohort studies suggest that a significantly larger proportion of the population is afflicted by sleep-disorder breathing than is clinically diagnosed (Young et al. 2009, Gabbay, Lavie 2012), perhaps warranting a revision of current diagnostic criteria.

Differences in the prevalence of OSA based on ethnicity are often explained by regional differences in rates of obesity (Ralls, Grigg-Damberger 2012). A recent study reported that the prevalence of OSA in Hispanic and white populations was nearly twice as high as that of East Asian populations (Yamagishi et al. 2010), however these rates

are likely attributable to the relative differences in BMI between groups. Interestingly, studies examining socioeconomic status have found that residing in disadvantaged neighbourhoods increased the risk of OSA in children more than three-fold after adjusting for ethnicity, premature birth, and obesity (Spilsbury et al. 2006, Goldstein et al. 2011). OSA is more prevalent in Canadian children living below the low-income threshold (poverty line) and single-parent households with low median incomes, after adjusting for age, ethnicity, and obesity (Brouillette et al. 2011). In addition, Canadian children afflicted by this condition were more likely to reside in disadvantaged neighbourhoods and suffer from more severe forms of OSA (Brouillette et al. 2011).

1.3.4 – Cardiovascular comorbidities of obstructive sleep apnea

1.3.4.1 - Hypertension

The impact of OSA on cardiovascular function is of considerable significance, as morbidity and mortality associated with cardiovascular disease presents a substantial burden to the public health system. Interestingly, OSA has been identified as a treatable cause of secondary hypertension (Chobanian et al. 2003). Recent population studies have demonstrated that increasing severity of OSA is accompanied by worsening hypertension, a relationship that is maintained when adjusted for BMI (Young et al. 1997). Compared with control subjects, OSA patients have increased risk of developing hypertension with increasing AHI. Individuals with moderate to severe OSA have a nearly three-fold increase in the risk of becoming hypertensive (Peppard et al. 2000). Animal studies have previously demonstrated that intermittent hypoxia models mimicking OSA lead to the development of hypertension (Dematteis et al. 2009, Belaidi

et al. 2009). Human studies have also described a link between OSA and resistant hypertension (Ruttanaumpawan et al. 2009). Recent evidence has emerged demonstrating that treatment of OSA can have a significant impact on alleviating hypertension (Becker et al. 2003, Andren, Sjoquist & Tegelberg 2009, Dernaika, Kinasewitz & Tawk 2009, Aihara et al. 2010, Barbe et al. 2010, Lozano et al. 2010).

1.3.4.2 – Heart failure

A recent cohort study found that over 80 percent of patients with congestive heart failure suffered from sleep apnea (Paulino et al. 2009). The consequences of repetitive fluctuation of intrathoracic pressure have previously been described, including left-ventricle hypertrophy and cardiac dysfunction (Buda, MacKenzie & Wigle 1981, Buda, Schroeder & Guilleminault 1981). Likewise, recent findings have demonstrated that OSA-induced exposure of the cardiovascular system to recurring hypoxia, increases in systemic and pulmonary pressures, and large shifts in intrathoracic pressure impairs contractility of the heart (Kasai, Bradley 2011). Longitudinal studies have found that adult men with severe OSA were nearly 60 percent more likely to develop heart failure than men without OSA (Gottlieb et al. 2010). Several studies have demonstrated that treatment of OSA with medical devices or pharmaceutical intervention can improve left ventricle function (Dohi et al. 2008, Johnson et al. 2008, Khayat et al. 2009) and increase survival rates (Kasai et al. 2008, Javaheri et al. 2011).

1.3.4.3 – Arrhythmia

Patients with OSA have a substantially higher risk of bradyarrhythmias (Monahan et al. 2009), including sinoatrial arrest and complete heart block (Daccarett et al. 2008). Furthermore, individuals with OSA have nearly a four-fold higher risk of complex arrhythmias, such as atrial fibrillation (Mehra et al. 2006) due to structural changes in the atria and pulmonary vein caused by recurring fluctuations in intrathoracic pressure (Lim et al. 2009). While there is little evidence to insinuate that treatment of OSA can ameliorate cardiac arrhythmias, it has been suggested that alleviating the condition may improve outcomes of arrhythmia treatments (Gopalakrishnan, Tak 2011). OSA has been linked with higher failure rates for atrial fibrillation treatments, including pulmonary vein isolation and ablation (Hoyer et al. 2010, Matiello et al. 2010).

1.3.4.4 – Pulmonary Hypertension

Rodent models utilizing intermittent hypoxia to mimic OSA have demonstrated that CIH exposure causes pulmonary hypertension and remodelling of the pulmonary artery and right ventricle (Gopalakrishnan, Tak 2011). Pulmonary hypertension (categorized as a mean pulmonary arterial pressure greater than 20 mmHg) may be caused by mechanisms similar to those contributing to systemic hypertension in OSA patients. Pulmonary arterial pressure is believed to increase during the arousal phase of an apneic episode, similar to systemic arterial pressure, due to a surge in sympathetic nervous system activity (Smith et al. 1998). It is estimated that nearly 40 percent of individuals with OSA may be afflicted with pulmonary hypertension (Sajkov et al. 1999). Previous studies have demonstrated that treatment of OSA can alleviate pulmonary

hypertension and improve the response of pulmonary vasculature to hypoxia (Sajkov et al. 2002).

1.3.5 – Metabolic comorbidities of obstructive sleep apnea

1.3.5.1 – Obesity

Obesity is an important pathogenic risk factor for OSA in adults and children (Redline et al. 1999, Young, Peppard & Taheri 2005, Tauman, Gozal 2006, Kaditis et al. 2008, Li et al. 2010). Resulting from a chronic imbalance between energy consumption and energy expenditure, obesity typically manifests as an accumulation of visceral adipose tissue and increase in overall body weight (Williams, Fruhbeck 2009). Western populations categorize obesity as having a body mass index greater than 30 kg/m^2 (World Health Organization, 2000). Obesity has a major influence on health status, but is readily treatable and thus a preventable cause of morbidity and mortality (Lam, Mak & Ip 2012). Prospective studies have demonstrated that a 5 kg/m^2 increase in body mass index is associated with a 40 percent increased risk of mortality from cardiovascular disease, 90 percent increased risk for diabetic and hepatic complications, and 20 percent increased risk for respiratory diseases. Furthermore, a body mass index between 30 and 35 is associated with a reduction in median survival by 2 to 4 years, while a body mass index between 40 and 45 is associated with a reduction of 8 to 10 years (Lam, Mak & Ip 2012).

Increases in body weight can affect the risk of OSA onset, as well as the severity of pre-existing apneas (Phillips et al. 1999, Newman et al. 2005). A significant proportion of adult OSA patients present with high visceral adiposity (Grunstein et al.

1993), which is commonly associated with accumulation of adipose tissue in the upper-airway and a larger overall neck circumference (Welch et al. 2002). Given the emergence of obesity as a global epidemic, it is likely that the consequences of this metabolic condition will precipitate as an increase in the prevalence of OSA. However it must be noted that obesity and OSA share a complex and multi-factorial relationship.

OSA is commonly associated with a greater accumulation of visceral adipose tissue (Phillips et al. 2000). In addition, OSA patients typically have greater difficulty losing weight compared to individuals of similar body mass without OSA (Phillips et al. 1999). Obesity is a commonly targeted factor for resolving OSA, as a decrease in body weight will additionally benefit the treatment of other potential comorbidities, including cardiovascular disease (McClendon et al. 2010). A prospective study examining candidates for bariatric surgery found that nearly half of individuals had OSA, with a mean AHI over 50 (Haines et al. 2007). Remarkably, post-surgery results indicated an 18 kg/m^2 decline in body mass index and a 36-point decline in AHI (Haines et al. 2007). A study investigating gastric banding surgery in severely obese men (average body mass index of 53 kg/m^2) with moderate to severe OSA reported a post-surgery average weight loss of 45 kg and 49-point decrease in AHI (Dixon, Schachter & O'Brien 2005). Similarly, pharmacological treatments of obesity have also shown to improve the severity of OSA. Weight loss trials using sibutramine, a serotonin/noradrenaline reuptake inhibitor marketed as an anti-obesity drug, demonstrated an average body weight decrease of 8 kg and 16-point decline in AHI score over a period of 24-weeks (Yee et al. 2007). Similar results were also reported following a 6-month trial with sibutramine in non-diabetic men with moderate or severe OSA (Phillips et al. 2009).

1.3.5.2 – Glucose dyshomeostasis and insulin resistance

The relationship between severity of OSA and glucose homeostasis has previously been investigated (Drager et al. 2009, Levy, Bonsignore & Eckel 2009, Lui, Ip 2010). Data from cohort studies suggest that OSA is associated with higher fasting blood glucose levels (Punjabi et al. 2004). Numerous cross-sectional studies have demonstrated a strong association between OSA and impaired glucose tolerance (Meslier et al. 2003, Tassone et al. 2003, Theorell-Haglow et al. 2008, Seicean et al. 2008), as well as between OSA and type II diabetes (Renko et al. 2005, West, Nicoll & Stradling 2006, Einhorn et al. 2007). Interestingly, studies examining insomnia have found that poor sleep quality is associated with increased incidence of diabetes (Nilsson et al. 2004, Meisinger et al. 2005) and higher fasting insulin levels (Suarez 2008), after adjusting for confounding variables. With repetitive arousal and insomnia being major components of sleep behaviour in OSA patients (Park, Ramar & Olson 2011), it is conceivable that OSA may contribute to the development of type II diabetes and impaired insulin regulation through its contribution to sleep fragmentation.

Several observational studies have reported an association between OSA and insulin resistance (Strohl et al. 1994, Ip et al. 2000, Makino et al. 2006, Tkacova et al. 2008). Furthermore, the degree of insulin resistance increases in proportion with severity of OSA, independent of confounding variables including age, ethnicity, sex, and body mass index (Ip et al. 2002, Punjabi et al. 2002, Bonsignore, Eckel 2009). Evidence supporting the treatment of OSA as a remedy for type II diabetes and insulin resistance has thus far been inconsistent. While several studies have demonstrated that treatment

of OSA with medical devices improves insulin sensitivity (Harsch et al. 2004, Lindberg et al. 2006) and type II diabetes (Babu et al. 2005, Hassaballa et al. 2005), a number of studies have failed to find any appreciable changes in metabolic health (Smurra et al. 2001, Trenell et al. 2007, Vgontzas et al. 2008).

1.3.5.3 – Liver disease

Non-alcoholic fatty liver disease (NAFLD) is a persistent liver disease that is estimated to affect as many as two thirds of individuals suffering from obesity (Younossi et al. 2008). Common symptoms of this liver disease include inflammation of the liver, elevated rates of hepatic lipid production/retention, and liver fibrosis (Ahmed, Byrne 2010, Musso et al. 2011). NAFLD is known to contribute to the onset of cardiovascular disease and diabetes (Ghouri, Preiss & Sattar 2010, Musso, Cassader & Gambino 2011). An association has also been described for NAFLD with hyperlipidemia (Ahmed, Byrne 2010). In addition, persistent hypoxic insults have been shown to precipitate NAFLD (Minoguchi et al. 2006).

A study examining 106 individuals with OSA found that 66 percent of patients also suffered from NAFLD (Turkay et al. 2012). Furthermore, AHI score, decreased blood-oxygen saturation, and duration of hypoxia during an apneic event were all good predictors of the severity of NAFLD (Turkay et al. 2012). Similarly, a negative correlation has been described between blood-oxygen saturation and levels of hepatic type III procollagen, a marker of liver fibrosis (Tatsumi, Saibara 2005). Rodent models utilizing CIH to investigate the potential role of sleep apnea have suggested that OSA alters hepatic susceptibility and exacerbates the consequences of metabolic insults,

including genetic-induced obesity (Li et al. 2005a, Li et al. 2005b), high-fat diet (Savransky et al. 2007a), and high-cholesterol diet (Drager et al. 2012). OSA has been identified as a factor that not only precipitates NAFLD (Savransky et al. 2007a, Savransky et al. 2007b, Takayama et al. 2009), but also as a factor that hastens the transition from NAFLD to non-alcoholic steatohepatitis (NASH), an end-stage form of liver disease characterized by excessive accumulation of lipids within hepatocytes and cirrhosis of the liver (Ahmed, Abu & Byrne 2010).

1.3.6 – Current treatments for obstructive sleep apnea

1.3.6.1 – Positive airway pressure therapy

The most common treatment method for OSA is the use of a continuous positive airway pressure (CPAP) device (Gay et al. 2006, Giles et al. 2006). The CPAP machine utilizes air pressure to sustain an open airway and prevent collapse during sleep (Montesi et al. 2012). Numerous studies have demonstrated that treatment of OSA using CPAP causes a significant reduction in AHI score (Engleman et al. 1999, Montserrat et al. 2001, Stuck, Leitzbach & Maurer 2012, Ha, Hirai & Tsoi 2013, Salord et al. 2013). In addition, there is evidence to suggest that CPAP ameliorates many of the physiological and behavioural consequences of OSA, including reductions in daytime somnolence (Xu et al. 2012), OSA-induced hypertension (Montesi et al. 2012), and the risk of motor vehicle accidents (Tregear et al. 2010). While there may be substantial benefit to treatment of OSA with CPAP devices, there are concerns regarding patient-compliance. Use of CPAP machines has been associated with side effects resulting in irritation and discomfort (Pepin et al. 1996, Baltzan, Elkholi &

Wolkove 2009). This has translated into a low compliance rates among patients (Shapiro, Shapiro 2010), with some studies reporting less than 50 percent compliance (Engleman, Martin & Douglas 1994, Kushida et al. 2006, Veasey et al. 2006).

A recently described variation of CPAP therapy is the auto-titrating positive airway pressure device (APAP) (Hailey et al. 2005, Morgenthaler et al. 2008, Ip et al. 2012, Xu et al. 2012). While CPAP machines exert a basal pressure level high enough to eliminate any apneic or hypopneic event from occurring, APAP devices continuously modulate the pressure being applied based on changes in airway resistance (Ip et al. 2012). This regulation of airway pressure may be advantageous, given that pressure levels in the airway can change based on body position and sleep stage (Xu et al. 2012). With resistance being constantly monitored, the pressure applied by APAP devices can be increased when airflow is impeded and decreased during exhalation, which may be of importance as some patients are irritated by or have difficulty breathing out against pressures applied by CPAP devices (Randerath et al. 1999, Xu et al. 2012). Utilization of APAP is an effective treatment option for OSA patients that may not have a higher tolerance for CPAP devices (Parish, Miller & Hentz 2008), as APAP compliance is known to be higher than that for CPAP devices (Smith, Lasserson 2009).

Figure 1.2: Illustrated depiction of mask worn during continuous positive airway pressure (CPAP) treatment

CPAP is the most common method of treatment for obstructive sleep apnea. The CPAP machine utilizes a constant air pressure stream to maintain an open airway during sleep and prevent the occurrence of apneic or hypopneic episodes

1.3.6.2 – Oral appliances

Oral appliances are an alternative to CPAP treatment commonly used for patients with mild or moderate OSA. These devices, worn during sleep, structurally alter the upper airway to reduce the likelihood of collapse. Two oral appliances often utilized for OSA treatment are tongue-retaining devices and mandibular advancement devices (Sutherland, Cistulli 2011, Marklund, Verbraecken & Randerath 2012). Mandibular advancement devices, the most commonly used and most effective oral appliance (Hoekema, Stegenga & De Bont 2004), cause the mandible to protrude and be maintained in a forward position (Iftikhar et al. 2013). Mandibular advancement devices have been shown to reduce snoring (Mehta et al. 2001) and daytime somnolence (Gotsopoulos et al. 2002, Naismith et al. 2005, Petri et al. 2008). A number of randomized control trials have determined that, while not as efficacious as CPAP treatment, mandibular advancement device usage results in pronounced improvement in AHI score (mean reduction of 55 percent by mandibular devices versus mean reduction of 80 percent by CPAP treatment) (Johnston et al. 2002, Lawton, Battagel & Kotecha 2005, Lam et al. 2007, Hoekema et al. 2008).

1.3.6.3 – Surgical treatments

Surgical procedures for the treatment of OSA focus on skeletal or soft-tissue modification of pharyngeal or nasal airway structures. The most common surgical method employed is uvulopalatopharyngoplasty, which involves enlargement of the airway by removal of the uvula, posterior palate, and potentially the tonsils (Aurora et al. 2010). Surgery is often recommended for patients with poor oral appliance or CPAP

compliance, or for individuals that experience pain or discomfort from these treatments. However, meta-analysis of literature related to surgical procedures for the treatment of OSA, including uvulopalatopharyngoplasty, are typically ineffective as a sole therapy method, as patients typically retain moderate to high AHI scores post-surgery (Aurora et al. 2010). Alternatively, it has been suggest that soft-tissue surgeries may be advantageous as an adjunct treatment to more conventional methods, including oral appliance usage and positive airway pressure devices (Jacobson, Schendel 2012).
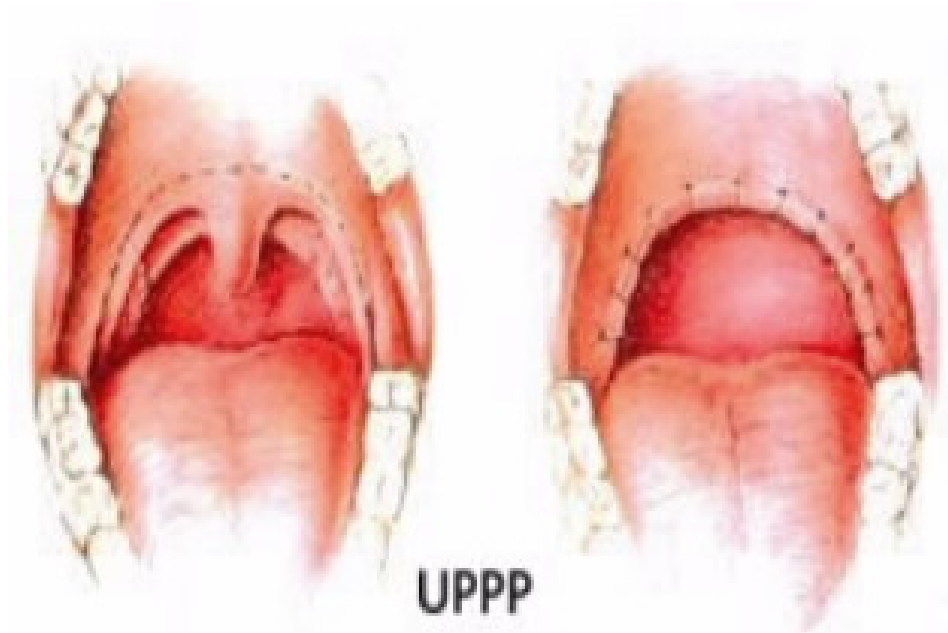
Figure 1.3: Illustrated depiction of uvulopalatopharyngoplasty

Uvulopalatopharyngoplasty (UPPP) is the most common surgical method used for the treatment of obstructive sleep apnea. The UPPP procedure involves the removal of the uvula, posterior palata, and potentially the tonsils.

1.3.6.4 – Unconventional and less common treatments

A recently developed therapy makes use of respiratory pressures to maintain airway patency during sleep. The expiratory positive airway pressure (EPAP) device is a one-way valve placed on the nostrils which allows for low inspiratory resistance during inhalation and, more importantly, high expiratory resistance during exhalation that keeps the airway open until the subsequent breath (Colrain, Brooks & Black 2008, Patel et al. 2011). Studies have established that usage of EPAP devices for the treatment of OSA leads to reductions in AHI score, improve snoring, and daytime somnolence (Rosenthal et al. 2009, Berry, Kryger & Massie 2011, Walsh et al. 2011). As a less cumbersome alternative to positive airway pressure treatments, EPAP devices have a high compliance rate compared to CPAP or APAP devices (De Dios, Brass 2012).

Obesity is very common independent risk factor for the development of OSA (Tishler et al. 2003, Woodson 2010). Reductions in body weight are associated with reductions in AHI score and improvements in daytime somnolence (Holty, Guilleminault 2010). Bariatric surgery is recommended for severely overweight OSA patients for whom conventional treatments are unsuccessful (Carvalho, Hsia & Capasso 2012). A meta-analysis of studies examining post-surgical outcomes related to OSA determined that bariatric surgery causes a significant decline in AHI score (Greenburg, Lettieri & Eliasson 2009). However, it is worth noting that the procedure does not completely alleviate the condition. Patients typically suffer from moderate apneas following bariatric surgery (Greenburg, Lettieri & Eliasson 2009), with a mean AHI score of 15 events per hour (mean AHI score of 51 events per hour before surgery) (Haines et al. 2007). While not a comprehensive treatment for OSA, bariatric surgery has important health benefits,

including postoperative reductions in body weight, adipose tissue deposition, cholesterol levels, blood pressure, as well as the risk of diabetic and cardiovascular complications (Marien, Rodenstein 2008). Similar to oral procedures, bariatric surgery is recommended as an adjunct to more conventional methods of treatment (Carvalho, Hsia & Capasso 2012).

1.3.7 – Obstructive sleep apnea and pregnancy

Pregnancy is associated with a number of anatomical and physiological changes that can affect respiratory function during gestation, including inflammation of nasal passages, decreased chest wall compliance, and elevation of the diaphragm (Morong, Hermsen & de Vries 2013). Furthermore, Pregnancy is known to cause narrowing of the upper airway. Taking together, these changes could presumably increase the risk of developing OSA or exacerbating existing conditions (Pien, Schwab 2004, Venkata, Venkateshiah 2009). It has been suggested that the prevalence of sleep-disordered breathing in pregnant women may be more than five-fold greater than the incidence rate for females overall (Louis, Auckley & Bolden 2012). Despite this, the incidence of OSA is likely underestimated in pregnancy (Morong, Hermsen & de Vries 2013).

Snoring, a common early-symptom of OSA, is reported to occur more frequently in women during pregnancy (Loube et al. 1996). However, it is only indicative of sleep-disordered breathing when considered in conjunction with other known associated symptoms, as snoring alone is not an ideal diagnostic measure for OSA. Studies have previously described the relationship between sleep-disordered breathing and fetal growth restriction (Bobrowski 2010a). In addition, OSA is associated with preeclampsia

during pregnancy and higher rates of admission in neonatal intensive care units post-delivery (Louis et al. 2012). The presence of OSA in women during pregnancy has previously been associated with low birthweight (Sahin et al. 2008, Champagne et al. 2010) and lower scores on the Apgar scale (Lin et al. 2004), which surveys the general health of children immediately following birth (Apgar 1953, Finster, Wood 2005). While there is symptomatic evidence to suggest that the rates of OSA during pregnancy may be higher than those measured for the overall population, the prevalence of the condition is not currently known (Chen et al. 2012). Many studies investigating the potential effect of OSA on offspring development have relied on a history of snoring or results from questionnaires as a screening tool (Loube et al. 1996, Franklin, Svanborg 2000, Ugur et al. 2012, Ko et al. 2013). This approach is problematic as assessment surveys lack the specificity in questioning to accurately determine the presence of OSA (Wilson et al. 2011, Fung et al. 2013). To date, there are no clinical data examining OSA treatment during pregnancy and potential benefits to fetal outcomes.

1.4 – FETAL GROWTH AND DEVELOPMENT

Fetal development entails a series of lengthy and dynamic changes related to offspring maturation. With a fetus that is continuously evolving to acquire new molecular and cellular functions during its development, neonatal growth is a symphony of complex processes. The structural and functional framework of all tissues of the body must be established during gestation. The early stages of pregnancy, which correspond to the embryonic period, involve a series of proliferation and differentiation steps for

constructing the foundation of all major organ systems. The remainder of pregnancy constitutes the fetal period, which includes the growth and maturation of organs and all other tissues within the body. As various systems are undergoing physical and functional changes simultaneously, fetal development requires a strict regulation of cellular growth, proliferation, and differentiation throughout pregnancy.

Ultrasound testing allows for physicians to estimate fetal weight changes during development and construct a fetal growth curve to predict birth weight (Dudley 2005). Tracking changes in fetal weight throughout pregnancy is useful for predicting fetal survival odds (Medchill et al. 1991). Furthermore, deviation from the expected growth curve may be a diagnostic indicator of perinatal morbidity and a potential risk of mortality (Barker 2007). In humans, the rate of fetal growth over the course of pregnancy is non-linear. Specifically, fetal growth follows a sigmoidal pattern (Olsen et al. 2010). Growth in the early stages of pregnancy is slow, with the fetus showing small increases in weight at the end of the first trimester. Fetal growth increases exponentially during the second and early third trimester, followed by a plateau in the final weeks of pregnancy (Harding, Bloomfield 2004). Fetal growth curves not only provide important information on the developmental progression of the offspring, but can also potentially indicate whether or not a fetus is receiving an adequate supply of nutrients from the mother. Throughout gestation, offspring rely on the maternal supply of resources for continued growth. During the embryonic period, the demand for resources is relatively low. The nutritional demand increases as the fetus continues to grow, with resource requirements peaking in the third trimester of pregnancy.

1.4.1 – Birthweight as a marker of fetal development

General health can be inferred by comparing growth curves to collected data from a large number of pregnancies within the region. Appropriate for gestational age (AGA) infants are those with a growth trajectory similar to the growth curves of the amalgamated data. Infants with a fetal weight during pregnancy or a birth weight at delivery above the 90th percentile are classified as large for gestational age (LGA). Infants with a fetal weight or birth weight below the 10th percentile are designated as small for gestational age (SGA) (Lubchenco et al. 1963, Battaglia, Lubchenco 1967). Divergence from the median data can reveal abnormalities in development and growth conditions, particularly for SGA infants. The World Health Organization has set a birth weight of 2500g (5.5 lbs) as the threshold for assessment of fetal development. Weights below the 10th percentile and/or below 2500g at birth are suggestive of impaired fetal growth and deficiencies in the intrauterine environment.

If a fetus grows too large during pregnancy, it may create difficulties during delivery and increase the risk of maternal or fetal mortality. Thus, the growth potential of a fetus is controlled based on the physique of the mother (Ounsted, Scott & Ounsted 2008, Lewis, Cleal & Hanson 2012). Larger women have larger babies, while smaller women have smaller babies. Embryo transfer studies in horses have given support to this idea by showing that embryos from small breeds are born larger when transferred into the womb of a larger breed, and embryos from large breeds are born smaller when transferred into the womb of a smaller breed (Allen et al. 2002). Similar findings have been reported In a human study of pregnancy following ovum transfer, with small

women giving birth to small babies following egg donation from large women (Brooks et al. 1995).

Based on these observations, the 10th percentile and low birth weight classifications, while often utilized for determining poor development, may not accurately quantify the percentage of infants that have experienced abnormal growth restriction during pregnancy. Though many infants will be correctly identified based on these classifications, it is conceivable that a significant proportion of growth-restricted offspring from larger women may still fall within the "normative range" of fetal growth. Constitutionally small infants may reach their growth potential and have a weight below the 10th percentile, while other infants may fall within the normal range but fail to reach their actual growth potential. Recently, efforts have been made to improve this classification system but creating customized fetal growth charts based on the inclusion of numerous factors that may alter fetal outcomes, including ethnicity, parity, and maternal size. Comparing the growth curve of a fetus to a specific population subset, rather than a collective dataset, allows for more accurate prediction of perinatal outcomes (Figueras et al. 2007).

1.5 – INTRAUTERINE GROWTH RESTRICTION

Low birth weight has been associated with a number of metabolic disorders in adult life, including glucose intolerance, insulin resistance, type II diabetes, and dyslipidemia (McCance et al. 1994, Barker 1998, Forsen et al. 2000, Phillips 2002, Kanaka-Gantenbein, Mastorakos & Chrousos 2003). Furthermore, epidemiological

studies have shown that low birth offspring have a higher prevalence of hypertension (Painter et al. 2006a, Stein et al. 2006) and coronary artery disease (Painter et al. 2006b). However, it is worth noting that metabolic and cardiovascular morbidities in adulthood are not a direct result of this one characteristic. During pregnancy fetal development follows an innate growth potential that, under healthy conditions, leads to the birth of an appropriate for gestational age offspring. Low birth weight is a consequence of suboptimal growth conditions in utero. An inadequate intrauterine environment typically constitutes a decrease in nutrient supply to the fetus, causing a slowing of growth during pregnancy (Barker 1997). This reduction in growth potential is associated with structural and functional changes within the offspring that dictate physiological consequences in adult life. In essence, low birth weight is a marker for a greater underlying problem.

The inability of a fetus to reach its growth potential due to suboptimal intrauterine conditions is termed intrauterine growth restriction (IUGR). A number of internal and environmental factors have been identified as potential causes of IUGR, including poor maternal nutrition (Thamotharan et al. 2005, Jansson et al. 2006), reduced blood flow to the fetus (Vuguin et al. 2004), smoking (Bassi et al. 1984), and hormonal imbalance (Ross, Beall 2008). During pregnancy, two categories of IUGR are possible: symmetric and asymmetric (Dashe et al. 2000). Symmetric IUGR occurs as a result of a nutritional insult to the intrauterine environment during the early stages of pregnancy, when major organ systems of the body are being established. Introduction of an insult through this period of growth leads to the fetus being underdeveloped as a whole. Conversely, assymetric IUGR occurs following a nutritional insult in the later stages of gestation,

when the fetus is undergoing rapid growth and maturation. Insults during the latter half of pregnancy result in a disproportional constraint on fetal development, such that certain organs display higher degrees of development and maturation than others (Halliday 2009). In symmetric IUGR offspring, brain size and body length correspond more accurately with body weight than with gestational age. In asymmetric IUGR offspring, brain size and body length correspond more accurately with gestational age than with body weight (Nardozza et al. 2012). This differential pattern in development between the two types of IUGR offspring is in part attributable to adaptive mechanisms initiated by the fetus in the later stages of pregnancy.

Resources for development are supplied via blood entering the fetus through the umbilical cord. The majority of blood entering the fetus is routed towards the liver. A fraction of blood (roughly 30 percent in humans) bypasses the liver through the ductus venosus and is directed to the upper body for perfusion of the heart and brain (Tchirikov et al. 1998, Kiserud, Acharya 2004). When challenged by an insufficient supply of nutrients in the later stages of gestation, the fetus is capable of initiating adaptive mechanisms to protect the growth and maturation of the most critical organs. During instances of limited nutrient availability, the fetus preferentially allocated resources to organs most vital for immediate survival: the brain and the heart (Desai et al. 1996). Doppler studies have determined that this is achieved by increasing the diameter of the ductus venosus, allowing a greater fraction of nutrient-rich blood to circumvent the liver and perfuse the heart and brain (Edelstone 1980, Reuss, Rudolph 1980). This adaptive mechanism, having been demonstrated in both human and animal studies, is often

associated with asymmetrical growth restriction (Paulick et al. 1990, Tchirikov et al. 1998, Kiserud, Rasmussen & Skulstad 2000, Tchirikov, Schroder & Hecher 2006).

Information regarding the impact of symmetric versus asymmetric IUGR and the relative incidence of these conditions is conflicting. Initial findings showed that approximately 20 to 30 percent of growth restricted offspring were a result of symmetric IUGR, while 70 to 80 percent of growth restricted offspring were a result of asymmetric IUGR (Lin, Su & River 1991). Furthermore, it was suggested that symmetric IUGR offspring have a higher incidence of neonatal morbidity and mortality compared to asymmetric IUGR offspring (Lin, Su & River 1991). Evidence in favour of the opposing argument, which states that asymmetric IUGR offspring are at greater neonatal risk, have also been described (Patterson, Pouliot 1987, Villar et al. 1990). A retrospective cohort study examined data from nearly 9,000 live-born singleton deliveries at the University of Texas Southwestern Medical Center over a period of almost 8 years (Dashe et al. 2000). Birth weight was used as a determining factor for classification of SGA and AGA infants. A head-to-abdomen circumference ratio with a 95th percentile threshold was used for determination of symmetry versus asymmetry, as initially described by Campbell and Thoms (Campbell, Thoms 1977). The cohort study found that 1364 (16 percent) infants were below the 10th percentile of weight and were categorized as SGA. Within this group, 274 (20 percent) infants had a head-to-abdomen circumference ratio above the 95th percentile and were classified as asymmetric SGA, while 1090 (80 percent) infants had ratios below the 95th percentile and were classified as symmetric SGA (Dashe et al. 2000). Asymmetric SGA infants were found to have significantly higher rates of admission to the neonatal intensive care unit, more frequent

requirement of intubation in the delivery room, and higher percentage of infants with an Apgar score below 3, which is considered a critical condition (Dashe et al. 2000).

1.6 – DEVELOPMENTAL ORIGINS OF ADULT DISEASE

Research in recent years has highlighted the importance of environmental influences on determining prenatal and postnatal health (Barker, Martyn 1992, Godfrey, Barker 2001, Nijland, Ford & Nathanielsz 2008, Suter, Anders & Aagaard 2013). While the framework for fetal growth is certainly derived from genetic components, the introduction of extrinsic elements during in utero development can have a major impact on fetal outcomes. A genotype (fixed) can give rise to an array of physiological phenotypes based on the influence of external factors (variable) on the fetus and surrounding intrauterine milieu (West-Eberhard 2005). This sensitivity to environmental effects is termed developmental plasticity, an ability to alter the structural and functional outcome of the offspring based on signals received by the fetus during pregnancy (Pigliucci 1998, Hochberg 2011, Phillips 2006). Developmental plasticity is considered a beneficial feature of prenatal growth, as it allows the moulding of a phenotype that matches the postnatal environment (Barker 2006). This would presumably be more suitable than producing a single phenotype for all environments. Developmental plasticity occurs in humans and animals, preparing offspring for a set of expected postnatal conditions based on communication between the fetus and mother during pregnancy (Gluckman et al. 2009a). For example, in situations where nutritional supply is limited, a fetus can adjust its growth rate and metabolism so that it will be better

suited to survive in environments with scarce resources (Barker 2006). While this mechanism does provide a developmental benefit, there is potential for severe consequences in the event of physiological insults being introduced to the fetus during this critical period of plasticity.

The Barker Hypothesis describes the relationship between fetal growth and postnatal health. More precisely, it postulates that adverse events in utero that impair fetal development greatly increase the risk of chronic diseases in adult life (Barker, Osmond & Law 1989, Barker 1990, Hales, Barker 1992, Godfrey et al. 1994). Early indications were provided in a study by studies describing an association between impaired fetal growth and higher rates of ischemic heart disease in English and Welsh populations (Barker, Osmond 1986). Additional support was provided by a report examining individuals born in a period of famine in the Netherlands during World War II (Ravelli et al. 1998). Data from this study demonstrated that pregnancies during the famine resulted in offspring that had significantly lower weights at birth and higher incidence of impaired glucose tolerance in adulthood. These findings are of particular significance as this was not observed in individuals from the same region that were born one year before or after the period of famine (Ravelli et al. 1998).

Introduction of a physiological insult during pregnancy can greatly impair the growth and functional development of a fetus, resulting in the birth of IUGR offspring. A number of epidemiological studies have demonstrated that growth restricted offspring have higher incidence of obesity, hypertension, dyslipidemia, impaired glucose tolerance, and type II diabetes (Barker 1992, Barker et al. 1993, Phipps et al. 1993, Leon et al. 1996, Jaquet et al. 2000, Gluckman et al. 2008). Specifically, using

birthweight as an index for IUGR, studies have demonstrated that offspring birthweight is inversely correlated with the prevalence of chronic diseases in adult life (Barker 2001). The relationship between IUGR and higher rates of chronic diseases is not a regional problem, but rather a global concern. For example, the association between low birthweight and incidence of heart disease has been described in many parts of the world, including North America, Europe, and South Asia (Stein et al. 2006, Forsen et al. 1997, Leon et al. 1998, Rich-Edwards et al. 1999, Newsome et al. 2003).

The result of this work has been a remarkable surge in the number of studies investigating the importance of fetal development and how it relates to long-term physiological outcomes in offspring. The Barker Hypothesis, while controversial when first introduced, has gained a high level of acceptance within the scientific community. The term "programming" was put forth to describe the impact of fetal alterations on postnatal health (Lucas 1991). Epidemiological studies investigating the relationship between impaired fetal growth and adult health have inspired a range of animal studies examining causes of IUGR and associated postnatal consequences. Animal models of IUGR include maternal protein restriction (Zhang et al. 2007, Cox et al. 2013, Vo et al. 2013), maternal high-fat diet (Luzzo et al. 2012, Fan et al. 2013), uterine artery ligation (Nusken et al. 2008, Thompson et al. 2011), chronic hypoxia (Bahtiyar et al. 2007, Bourque et al. 2013), smoking (Bruin, Gerstein & Holloway 2010, De Long et al. 2013), among others. Research surrounding fetal programming has generated an important knowledgebase for maternal-fetal medicine and aided in the understanding of how development during pregnancy can alter the risk of cardiovascular and metabolic diseases in adult life.
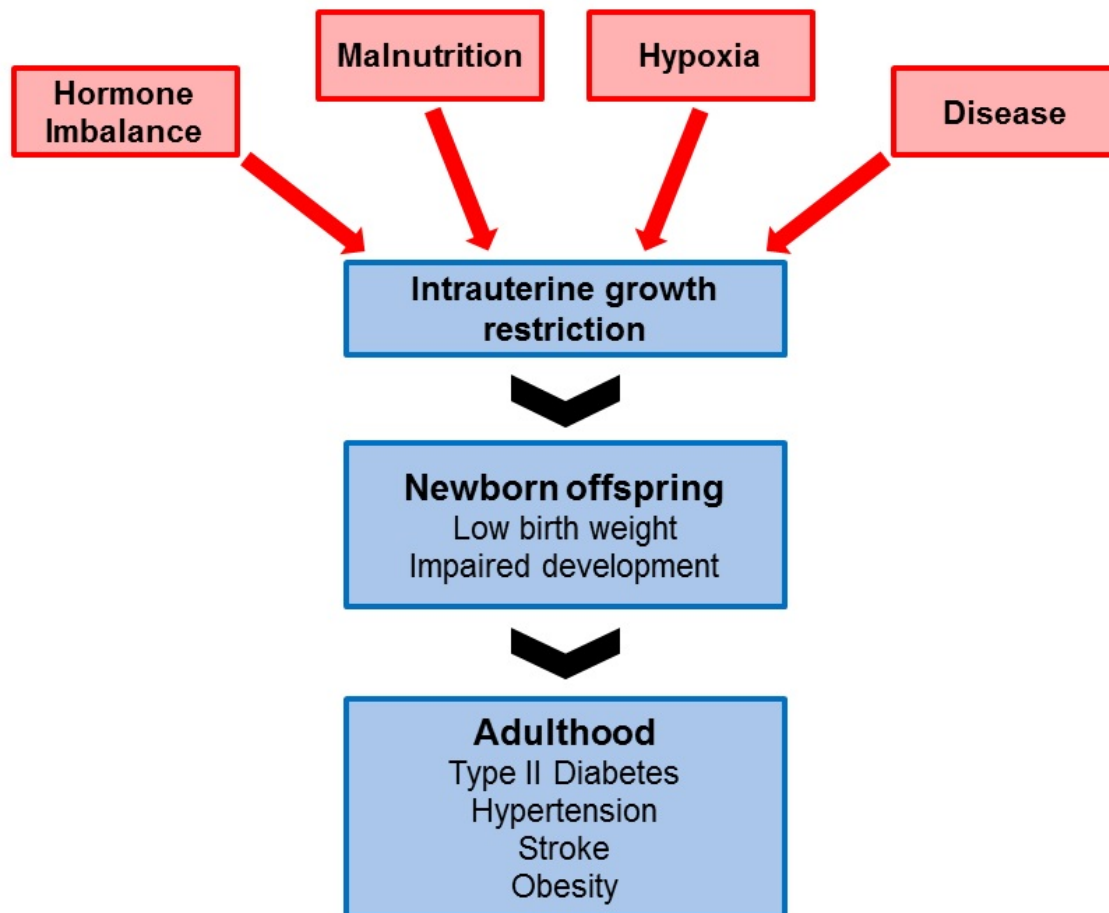
Figure 1.4: Physiological insults during pregnancy can alter the long-term health outcomes of offspring

An insult during gestation can hinder fetal development, resulting in intrauterine growth restriction. This alteration of the maternal intrauterine environment and fetal growth increases the propensity of offspring to chronic disease in adulthood, including a greater risk of cardiovascular and metablic morbidities.

1.7 – RATIONALE AND HYPOTHESIS

1.7.1 – Prevalence and economic impact of metabolic disorders

The prevalence of metabolic disorders such as obesity and diabetes has reached epidemic proportions, placing a major strain on public health resources and funds. Twenty-six percent of Canadian adults are obese with a body mass index greater than 30, and another 34 percent of adults are classified as overweight with a body mass index between 25 and 30. Additionally, the percentages of Canadian children that are overweight or obese are 20 and 12 percent, respectively (Statistics Canada, 2012). Roughly 9 percent of Canadian adults and 1 percent of Canadian children suffer from diabetes (Shaw, Sicree & Zimmet 2010), Public Health Agency of Canada 2011). The total healthcare costs for obese patients are, on average, 25 percent higher than the healthcare costs of normal weight individuals (Tarride et al. 2012). Furthermore, the annual healthcare costs of treating metabolic disorders such as obesity and diabetes in Canada are estimated to exceed 15 billion dollars per year (Puhl, Heuer 2009), Public Health Agency of Canada 2011). Interestingly, studies have demonstrated that in comparison to normal weight individuals, obese workers file twice as many workers' compensation claims, have 10 times as many lost work days, and nearly 8 times greater medical claims costs (Ostbye, Dement & Krause 2007, Neovius et al. 2009). The increasing strain on public healthcare funding and, more importantly, the severe health implications of these diseases have created a need for a better understanding of factors that increase the risk of developing metabolic disorders.

1.7.2 – Obstructive sleep apnea and intrauterine growth restriction

OSA is a chronic condition associated with metabolic comorbidities including obesity and diabetes. While a significant amount of research has investigated the consequences of OSA in adults, little is known about the cross-generational effects of this condition. Early studies have demonstrated that OSA during pregnancy results in IUGR, producing offspring with higher rates of perinatal morbidity. While there are preliminary data illustrating the effects of OSA on immediate postnatal outcomes, there are no studies describing the long-term consequences of OSA-induced fetal growth restriction. The physiological outcomes in adulthood and the associated risk of metabolic disorders have yet to be elucidated.

The Barker Hypothesis has established that adverse events in utero can impair development during pregnancy and result in IUGR. Furthermore, numerous epidemiological studies have reported that impaired fetal growth greatly increases the risk of developing metabolic disorders in adulthood. Thus, a series of studies were designed to investigate the relationship between OSA, IUGR, and potential consequences in adult life. The effects of OSA were examined through CIH, the underlying physiological state associated with this breathing disorder. A rat model of gestational CIH exposure was conceived to mimic OSA during pregnancy and elucidate the impact on the physiological wellbeing of offspring. It was hypothesized that gestational CIH exposure would induce changes in fetal physiology that affect organ growth and function, resulting in long-term metabolic consequences in adult life.

1.8 – OBJECTIVES

To investigate the consequences of OSA during pregnancy, the physiological outcome of offspring was assessed through the following objectives:

1) Characterization of body and organ growth of rat offspring following exposure to CIH during pregnancy

2) Determine physiological and mechanistic changes associated with gluconeogenesis and glucose tolerance in adult rat offspring following gestational CIH

3) Examine alterations in cholesterol homeostasis and identify prospective regulators of lipid production/metabolism

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Chapter 2

Effect of Chronic Intermittent Hypoxia during Gestation on Offspring Growth in the Rat

2.1 – ABSTRACT

Objective: Obstructive sleep apnea, a breathing disorder caused by the repetitive collapse of the upper airway during sleep, results in a state of chronic intermittent hypoxia (CIH). While the etiology and consequences of CIH are extensively investigated in the adult, the developmental ramifications of this disease process are unknown.

Design: This study was done to investigate the effect of CIH during gestation on offspring development. Pregnant female Spraque-Dawley rats were exposed to daily CIH throughout the gestational period.

Results: Postnatal day-1 offspring from CIH mothers were asymmetrically growth restricted, with decreased body weights and elevated brain-weight:liver-weight ratios. Furthermore, CIH newborns had elevated heart- and brain-weight:body-weight ratios, and decreased liver-weight:body-weight ratios. By adulthood, body weights of growth restricted offspring were significantly greater, as were the liver-weight:body-weight ratios. CIH offspring also had greater body fat deposition, were hyperglycaemic and had elevated plasma levels of insulin during development into adults.

Conclusions: These data suggest that alteration of the maternal intrauterine environment by gestational CIH effects the long-term development of the offspring and increases the risk of the offspring to metabolic diseases in adulthood.

2.2 – INTRODUCTION

Obstructive Sleep Apnoea (OSA) is a breathing disorder caused by upper airway collapse during sleep, resulting in an intermittent reduction or complete blockage of airflow (Dempsey et al. 2010). Repetitive occurrences result in chronic intermittent hypoxia (CIH), characterized by frequent decreases in blood O_2 saturation (Dempsey et al. 2010). This hypoxic condition is corrected and breathing resumes following a surge in autonomic activity that awakens the sleeping individual and reopens the airway (Dempsey et al. 2010). OSA is associated with a variety of co-morbidities, including chronic hypertension, obesity, and insulin resistance (Dempsey et al. 2010). While recent efforts have placed importance on understanding the consequences of OSA and the underlying state of CIH, the cross-generational effects of this condition have yet to be investigated.

Data exist suggesting that developmental deficits during gestation increase the risk of morbidity and mortality from cardiovascular and metabolic diseases in adult life (Barker 2004, Barker 2007, Ananth, Vintzileos 2009). These deficits are thought to result from intrauterine growth restriction (IUGR), where the foetus does not reach its growth potential (Malamitsi-Puchner, Nikolaou & Puchner 2006, Rueda-Clausen, Morton & Davidge 2009). Although there are a variety of complications that can result in IUGR, the most common occurrence involves a decrease in the supply of O_2 to the foetus, that is vital for normal growth and development, which can be triggered by placental insufficiency, smoking, pulmonary diseases, and maternal hypoxic distress (Marsal 2002, Salihagić-Kadić et al. 2006). A chronic hypoxic stress during gestation creates a maternal intrauterine environment that does not adequately provide O_2 and

nutrients to the foetus. Thus, growth is restricted in an effort to create a balance between the decreased supply and consumption of O_2 (Peebles 2004). The low birth weight caused by IUGR represents a common pathological condition diagnosed during pregnancy (Ananth, Vintzileos 2009).

There is a well-described link between developmental deficits and increased incidence of perinatal mortality. The Barker hypothesis, highlighting the importance of prenatal development puts forth the idea that an inadequate supply of oxygen and nutrients creates an intrauterine environment that causes structural and functional adaptations during organogenesis (Barker 2004, Barker 2007). It is these changes that are believed to increase the susceptibility of an individual to cardiovascular and metabolic diseases later in life (Barker 2004, Barker 2007).

This study was done to examine the immediate and long-term changes in offspring development caused by suboptimal growth conditions resulting from CIH. This was carried out using a rodent model for investigating the effects of CIH exposure on pregnancy and offspring development which mimics the clinical changes in O_2 availability experienced by patients with OSA.

2.3 – METHODS AND MATERIALS

2.3.1 – Animal Handling

Adult female and male Sprague-Dawley rats (250g; n=14) were obtained from Charles River Canada (Saint-Constant, Quebec, Canada). All animals were housed individually in standard cages in rooms maintained at constant temperature (23°C) and humidity (65%) with a 12h light/dark cycle beginning at 0700h. Food and water were available to all animals *ad libitum*. All experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

All animals were allowed 1-wk acclimatization period following arrival during which the reproductive cycle of each female rat was followed. The stage of the oestrous cycle was confirmed daily through vaginal smear. Upon confirmation of the proestrous stage, the females were mated with one male for each female. Successful impregnation was confirmed by the presence of sperm in a vaginal smear taken the following morning.

2.3.2 – Gestational CIH exposure

The confirmation of pregnancy marked gestational day-0, at which point females were separated from males and housed individually. The following morning (gestational day-1), pregnant females (n=8) were exposed daily to CIH inside a hypoxia chamber with built-in plexiglass tubes (10cm diameter by 35cm length) for housing individual rats,

and each rat was exposed to alternating cycles of normoxic (room air; 120s) and hypoxic (6.5% O_2 nadir; 80s) conditions at a frequency of 18 cycles/h. Conditions within the chamber were isobaric (770±11 mmHg) and eucapnic (<0.1% CO_2). Control animals (n=6) were placed in an adjacent chamber that continuously cycled room air. The duration of exposure for both groups was 8h (1000h to 1800h) per day from gestational day-1 to day-20. Body weights of all rats were recorded prior and immediately following daily CIH exposure.

2.3.3 – Measurement of Food and Water Intake

All females and offspring were allowed standard rat chow for the duration of the study. Food and water intake measurements for each animal were obtained between 1800h and 1000h the next day and values were standardized to the daily pre-exposure body weight of each rat.

2.3.4 – Post-Delivery

After the final exposure to CIH, females were returned to the animal facility for an undisturbed delivery. Immediately following birth (postnatal day-1), offspring from each mother were counted and sexed within each litter using anogenital distance. In all cases, the litter size was then reduced to four males. All additional males from each litter were sacrificed for analysis of neonatal offspring development. The four males were housed with the mother until postnatal day-21, at which point the pups were

weaned and separated into pairs. All offspring had standard rat chow and water available *ad libitum* until adulthood (12-wk of age).

2.3.5 – Offspring Tissue Collection

Postnatal day-1 offspring were weighed and then sacrificed via decapitation. An incision was made along the midline to expose the thoracic and abdominal cavities to remove heart and liver which were immediately weighed and snap frozen in liquid nitrogen. An incision was made on the dorsal surface of the head to remove the skull and extract the brain which was immediately weighed and frozen on dry ice.

Four males from each litter were allowed to reach adulthood. From these offspring, two were sacrificed at 6-wk of age and the other two at 12-wk of age. All animals were fasted overnight prior to sacrifice. Under equithesin anaesthesia (0.4 mL/100g; ip), blood samples from each rat were collected via intra-cardiac puncture, and then the heart and liver were removed, weighed, and snap frozen in liquid nitrogen. Brains were extracted, weighed, and frozen on dry ice. Epididymal and retroperitoneal fat pads were removed bilaterally and weighed.

2.3.6 – Blood Glucose and Insulin Measurement

Blood collected from 6- and 12-wk old males was aliquoted into 1 mL centrifuge tubes containing 10 µL of 7% ethylenediaminetetraacetic acid (EDTA) dissolved in water. The samples were placed in a refrigerated centrifuge and spun at 10,000 RPM for 15 min at 4°C. The plasma layer was aspirated and stored at -80°C until assayed.

Fasting blood glucose was measured using a standard glucometer. Values were obtained at the time of sacrifice by sampling blood extracted from the heart via intra-cardiac puncture. Plasma insulin measurements were made using a rat-specific enzyme immunoassay for insulin (Cat.#80 INSRT-E01; ALPCO Diagnostics, Salem, NH). Absorbance readings of insulin assays were determined using a Spectramax M5 plate reader (Molecular Devices, Downingtown, PA).

Assay sensitivity was calculated as the mean plus 2 standard deviations for the zero standards in the insulin enzymatic immunoassay and determined to be 0.130ng/ml. Intra-assay variability was calculated as the mean of the coefficients of variation for all samples measured in the assay. The intra-assay variability of the insulin immunoassay was 5.2%.

2.2.7 – Intraperitoneal Glucose Tolerance Test

Glucose tolerance was measured in 6- and 12-wk old male offspring. Rats were fasted overnight prior to commencement of the test. Following a baseline measurement of blood glucose levels, rats were administered an injection of D-glucose (40% solution; 2 g/kg body weight, ip). Blood glucose measurements were taken at 5, 10, 15, 30, 60, and 120 min post-injection using a standard glucometer by sampling tail vein blood.

2.2.8 – Data and Statistical Analysis

All data are expressed as mean ± standard deviation. Maternal body weight and consumption data were analyzed using a two-way analysis of variance. Organ weights were measured in g and were expressed as a ratio standardized to body weight. All offspring results, glucose and insulin concentrations were compared using a two-tailed Student t-test to determine statistical significance. Glucose tolerance test data were analyzed using a two-way analysis of variance. In all comparisons, a minimum p-value of < 0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

2.4 – RESULTS

2.4.1 – Effect of CIH on Pregnant Females

During the first week pregnant females subjected to CIH treatment had no changes in body weight (Fig. 2.1A). However, from gestational day-8 to day-20, CIH mothers showed a daily increase in body weight (Fig. 2.1A). On the other hand, mothers exposed to normoxia during pregnancy showed increases in body weight from gestational day-1 to day-20 (Fig. 2.1A). The body weight of normoxic control mothers was significantly greater than that of CIH mothers from gestational day-11 to day-20. Furthermore, the cumulative weight gained by normoxic mothers during gestation was significantly greater than that of CIH mothers from gestational day-6 to day-20 (Figure 2.1B).

No differences in total daily caloric intake were observed throughout gestation between mothers exposed to CIH or normoxia (Fig. 2.1C). However, mothers exposed to CIH during the gestational period had significantly greater water intake compared to normoxic mothers (Fig. 2.1D).
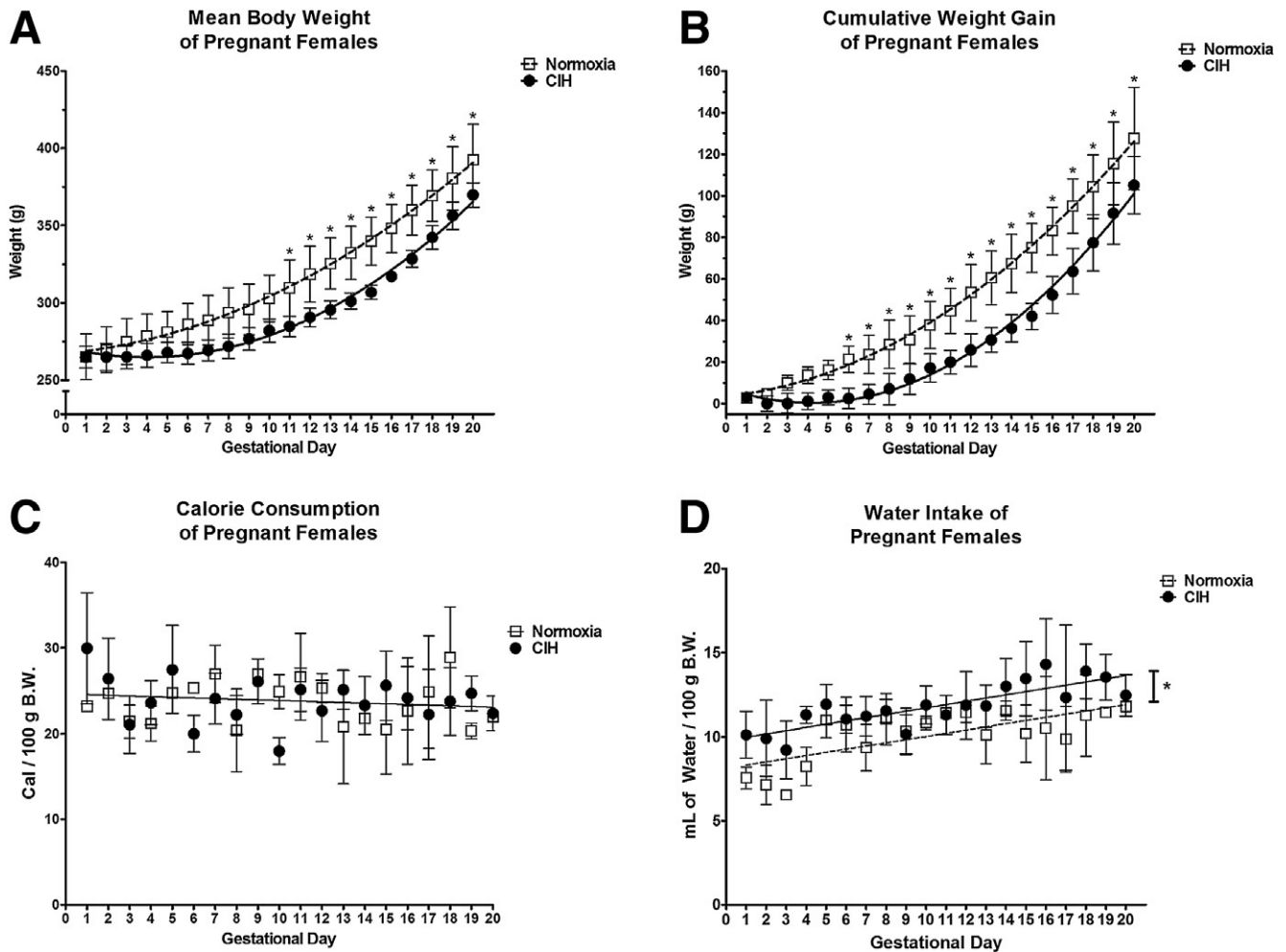
Figure 2.1: Pregnant mothers exposed to CIH during gestation have diminished weight gain in early pregnancy

Females were exposed to CIH from gestational day-1 to gestational day-20. Body weight measurements before each exposure period were used to determine (A) daily change in body weight and (B) cumulative weight gained over 20-day exposure period. (C) Daily total caloric intake is shown, whereas (D) shows water intake measurements standardized to pre-exposure body weight on corresponding day. Results were compared using a 2-way analysis of variance. Data are expressed as mean ± standard deviation. * $p < 0.05$

2.4.2 – Body Weight Changes During Exposure Period

Mothers subjected to 8h of CIH experienced significantly greater daily weight loss during exposure (Fig. 2.2). Additionally, weight loss data collected from control mothers was compared with a subset of pregnant females that were kept in their home cages and not placed within the chambers, but had no access to food and water for the 8h period. The weight loss experienced by mothers in the normoxic chamber was not different from that of mothers kept in their home cages.
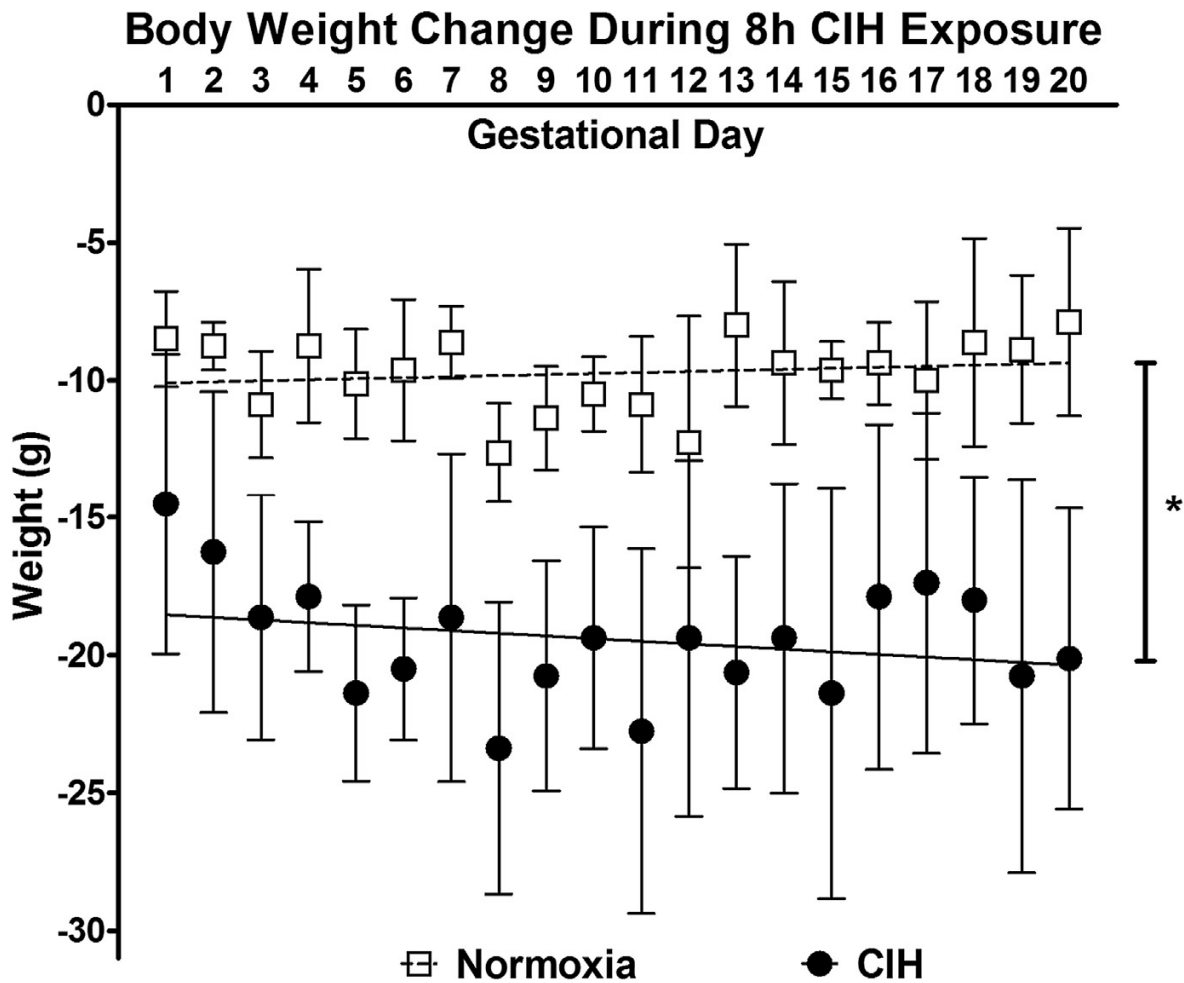
Figure 2.2: Pregnant mothers exposed to 8-hour cycle of CIH during gestation have greater daily weight loss

Females were exposed to CIH from gestational day-1 to gestational day-20. Body weight measurements immediately before and after the exposure cycle were used to determine total weight loss during 8-hour CIH or normoxia cycle. Results were compared using a 2-way analysis of variance. Data are expressed as mean ± standard deviation. * $p < 0.05$

2.4.3 – Effect of CIH on Offspring Litter Size, Body Weight and Organ Weights

There was no difference in the number of offspring in the litters of CIH and normoxic mothers (Fig. 2.3A). Furthermore, there was no difference in the number of male or female offspring produced in litters of either group. The litters of both CIH and normoxic mothers had a significantly greater proportion of female offspring in comparison to the number of male offspring (CIH, 9±1 females vs. 6±1 males; Normoxia, 8±1 females vs. 5±1 males).

Weight measurements taken on postnatal day-1 show that offspring from mothers exposed to CIH during gestation had significantly lower body weights compared to offspring from normoxic mothers (Fig. 2.3B). Brain weight was standardized to the liver weight within each offspring, a classical measurement for characterizing asymmetric growth restriction. CIH offspring were found to have significantly greater brain-weight:liver-weight ratios (Fig. 2.3C). The organ weights of the heart and brain were not different between the offspring of CIH and normoxic exposed mothers. The livers of CIH offspring weighed significantly less than the livers of normoxic offspring. When the organ weights of each offspring were standardized to its body weight, pups from mothers exposed to CIH had significantly greater heart-weight:body-weight and brain-weight:body-weight ratios (Fig. 2.3D and 2.3E, respectively). Furthermore, these offspring had lower liver-weight:body-weight ratio (Fig. 2.3F).

Figure 2.3: Pregnant mothers exposed to CIH during gestation give birth to growth restricted offspring

(A) Total litter size did not differ as a result of CIH exposure. (B) Data from postnatal day-1 offspring shows that pups from mothers exposed to CIH had significantly lower body weights. (C) Data from postnatal day-1 offspring shows that pups from mothers exposed to CIH had significantly greater brain weight-to-liver weight ratios. (D) Newborn male CIH offspring had significantly elevated heart weight-to-body weight. (E) Newborn male CIH offspring had significantly elevated brain weight-tobody weights. (F) Liver weight-to-body weight ratios were significantly decreased in newborn CIH offspring. Results were compared using a 2-tailed Student t test. Data are expressed as mean ± standard deviation. * p < 0.05

A Litter Size

D Standardized Heart Weight
of Newborn Offspring

B Mean Body Weight

E Standardized Brain Weight
of Newborn Offspring

C Brain Weight : Liver Weight Ratio

F Standardized Liver Weight
of Newborn Offspring
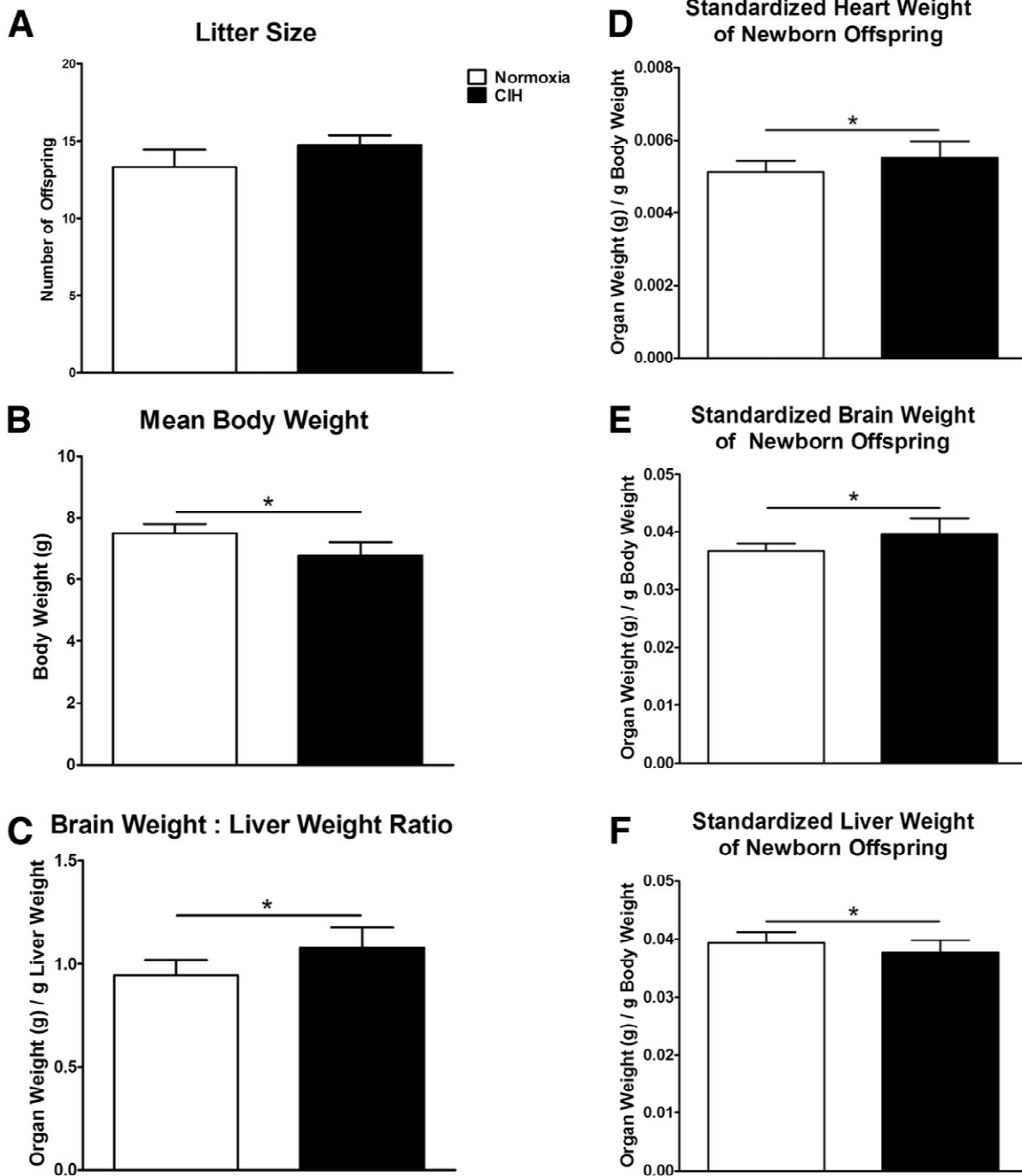
2.4.4 – Effect of CIH on Offspring Body Weight and Organ Weight at 6 Weeks of Age

At 6-wk of age, body weights of CIH offspring remained significantly lower (Fig. 2.4A). Standardized heart and brain weights were not different between the offspring of the CIH and normoxic mothers (Fig. 2.4B and 2.4C, respectively). Furthermore, there was no difference in the liver-weight:body-weight ratios between the two groups (Fig. 2.4D). However, CIH offspring had significantly greater epididymal and retroperitoneal fat mass compared to offspring of the normoxic mothers (Fig. 2.4E and 2.4F, respectively).

2.4.5 – Effect of CIH on Offspring Body Weight and Organ Weight at 12 Weeks of Age

By 12-wk of age, offspring of the CIH mothers had outgrown the normoxic offspring and had a higher mean body weight (Fig. 2.5A). Standardized heart and brain weights were not different between CIH and normoxic offspring (Fig. 2.5B and 2.5C, respectively). Offspring of CIH exposed mothers had significantly greater liver-weight:body-weight ratios (Fig. 2.5D). In addition, CIH group offspring had significantly greater epididymal and retroperitoneal fat mass compared to normoxic offspring (Fig. 2.5E and 2.5F, respectively).

Figure 2.4: Offspring exposed to CIH during gestation have elevated fat deposition at 6 weeks of age

(A) Body weights of CIH offspring were significantly lower. No differences were seen in (B) heart weight-to-body weight, (C) brain weight-to-body weight, or (D) liver weight-to-body weight ratios. Despite having a lower body weight, CIH offspring showed greater (E) epididymal fat and (F) retroperitoneal fat pad weights compared with normoxic offspring. Results were compared using a 2-tailed Student t test. Data are expressed as mean ± standard deviation. * $p < 0.05$

A Mean Body Weight of Males at 6 Weeks

B Standardized Heart Weight of Males at 6 Weeks

C Standardized Brain Weight of Males at 6 Weeks

D Standardized Liver Weight of Males at 6 Weeks

E Standardized Epididymal Fat of Males at 6 Weeks

F Standardized Retroperitoneal Fat of Males at 6 Weeks
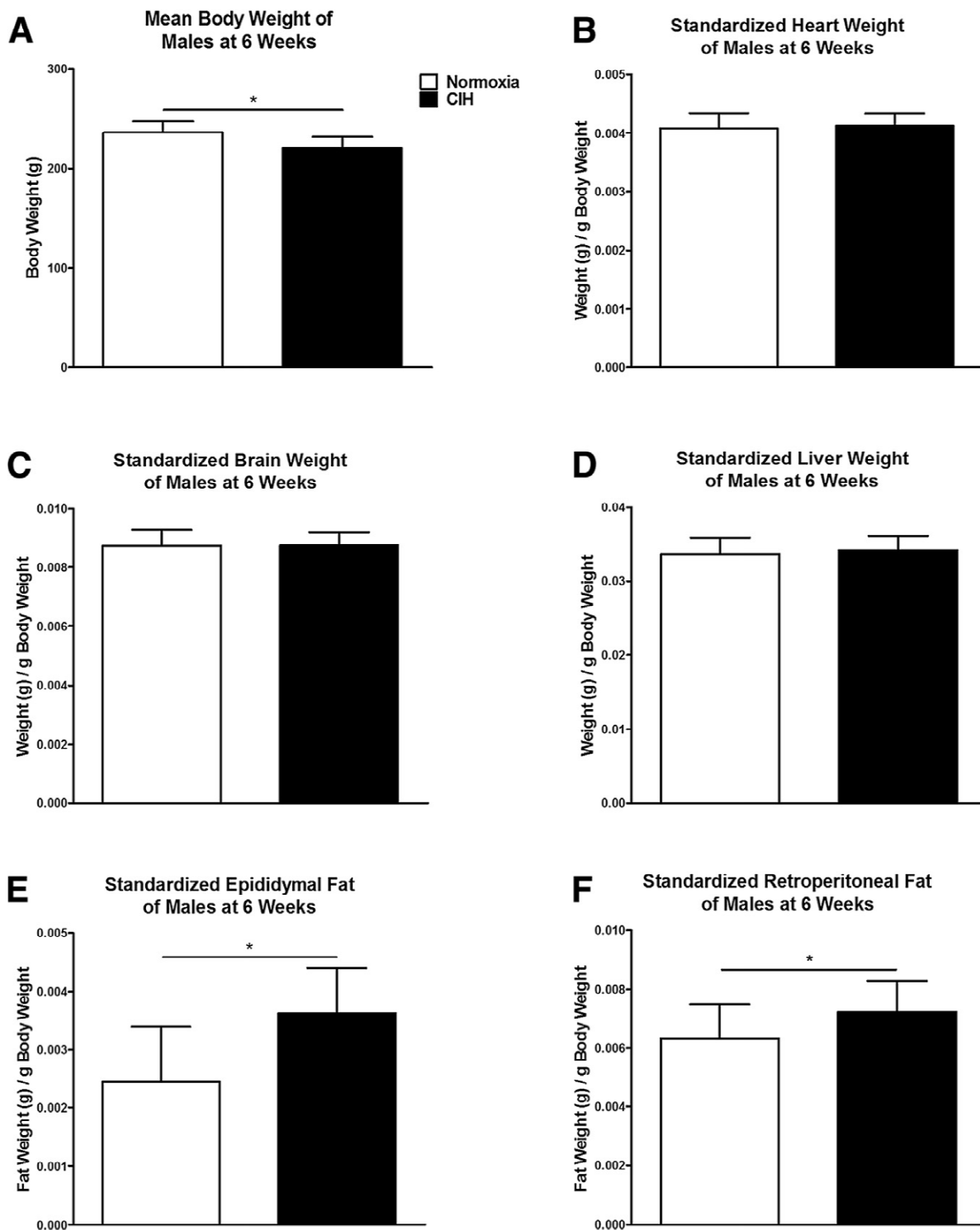
Figure 2.5: Offspring exposed to CIH during gestation have elevated fat deposition at 12 weeks of age

(A) Body weights of CIH offspring were significantly greater than normoxic offspring. No differences were seen in (B) heart weight-to-body weight or (C) brain weight-to-body weight. (D) Liver weight-tobody weight ratios were elevated in CIH offspring. CIH offspring showed greater (E) epididymal fat and (F) retroperitoneal fat pad weights compared with normoxic offspring. Results were compared using a 2-tailed Student t test. Data are expressed as mean ± standard deviation. * $p < 0.05$

A Mean Body Weight of Males at 12 Weeks

B Standardized Heart Weight of Males at 12 Weeks

C Standardized Brain Weight of Males at 12 Weeks

D Standardized Liver Weight of Males at 12 Weeks

E Standardized Epididymal Fat of Males at 12 Weeks

F Standardized Retroperitoneal Fat of Males at 12 Weeks
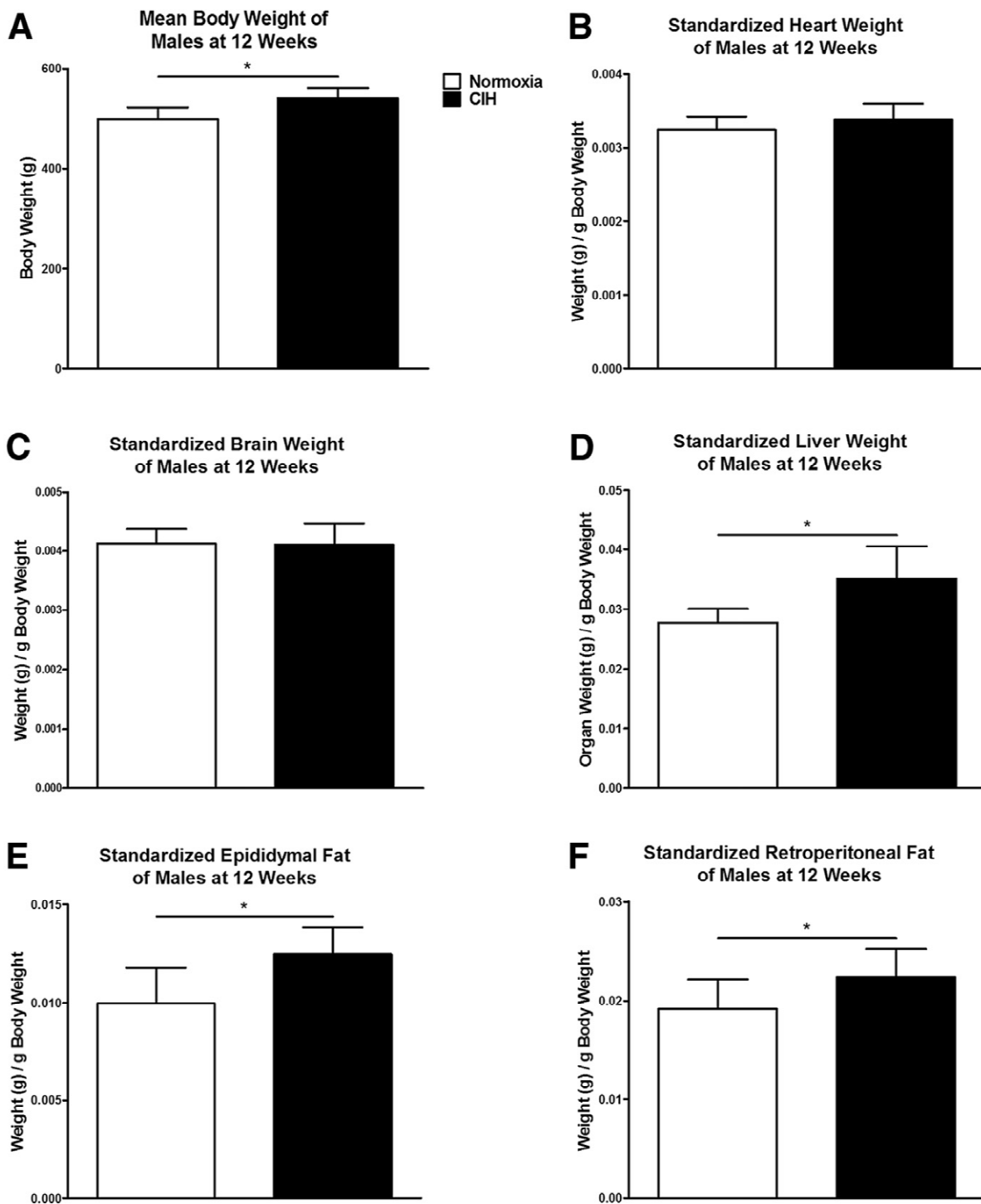
2.4.6 – CIH Effects on Blood Glucose and Insulin Concentrations

CIH offspring had significantly greater fasting blood glucose levels at 6-wk of age (Fig. 2.6A), though plasma insulin concentrations between the two groups were not different (Fig. 2.6B). The fasting blood glucose of CIH group offspring was significantly greater at 12-wk of age (Fig. 2.6C). These CIH offspring also had significantly greater plasma insulin concentration (Fig. 2.6D).

2.4.7 – CIH Effects on Glucose Tolerance

At 6-wk of age, CIH offspring displayed trends in glucose tolerance similar to those seen in normoxic control offspring. Baseline and 5-min post-injection blood glucose levels were greater in CIH offspring. There were no differences at any other post-injection time point (Fig. 2.7A)

CIH offspring at 12-wk of age had elevated baseline blood glucose levels. Furthermore, glucose was increased at 5- and 10-min post-injection. The CIH group offspring had significantly lower blood glucose levels 60-min post-injection (Fig. 2.7B). There were no differences in endpoint blood glucose levels (T=120-min) in either age group.

Figure 2.6: Gestational CIH offspring are hyperglycemic in adulthood

(A) Fasting blood glucose concentration was significantly greater in CIH male offspring, whereas no differences were observed in (B) plasma insulin concentration at 6 weeks of age. (C) Fasting blood glucose concentration was significantly greater in CIH male offspring, as was the (D) plasma insulin concentration. Results were compared using a 2-tailed Student t test. Data are expressed as mean ± standard deviation. * $p < 0.05$

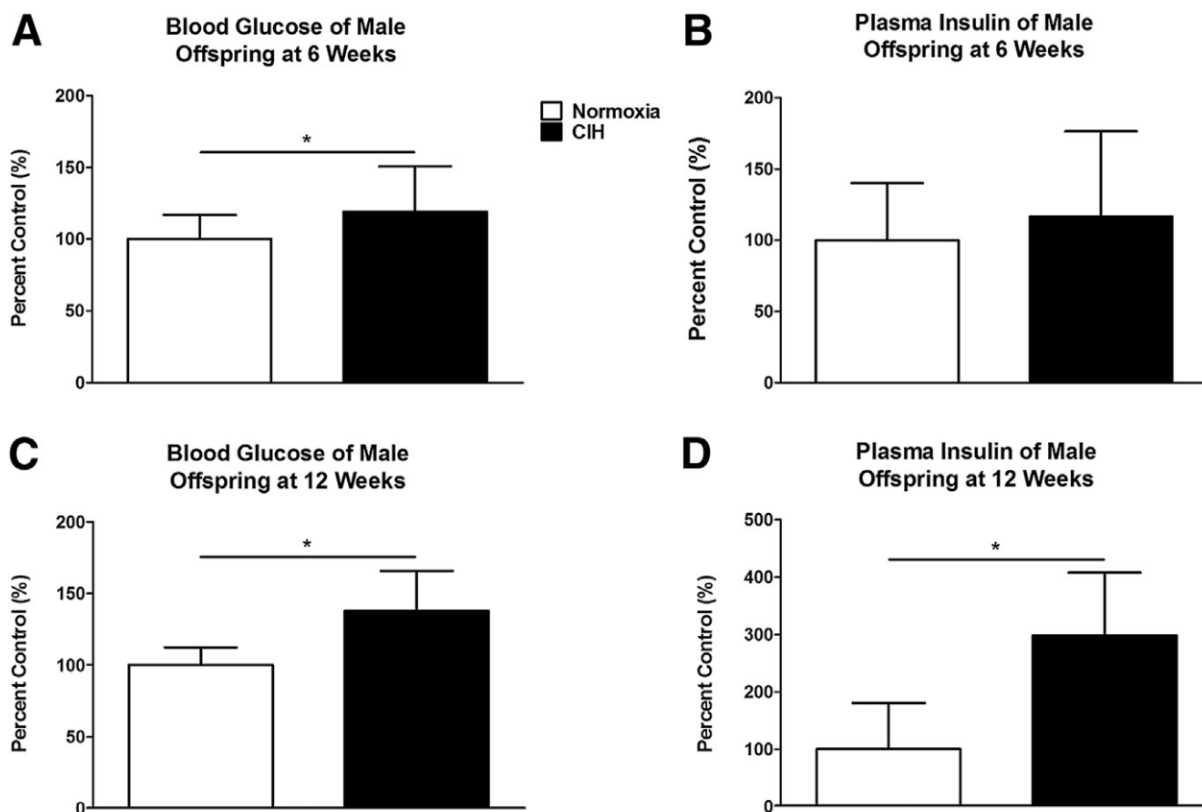**Figure 2.7: Offspring exposed to CIH during gestation show impaired early-stage glucose tolerance at 12 weeks of age**

(A) Differences were seen in blood glucose concentrations at only 5-minute time point in the 6 weeks old rats. However, (B), at 12 weeks of age, CIH offspring had elevated blood glucose concentrations 5- and 10-minute postinjection. Results were compared using a 2-way analysis of variance. Data are expressed as mean ± standard deviation. * $p < 0.05$

A Glucose Tolerance Test in Male Offspring at 6 Weeks of Age

B Glucose Tolerance Test in Male Offspring at 12 Weeks of Age
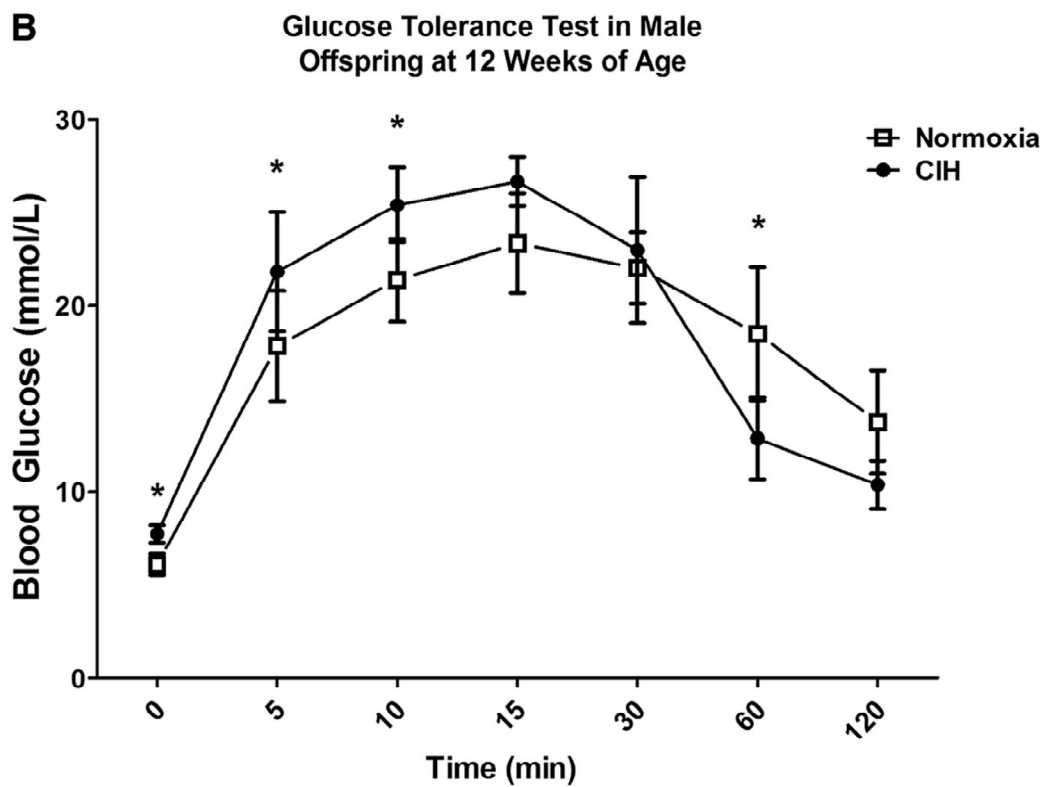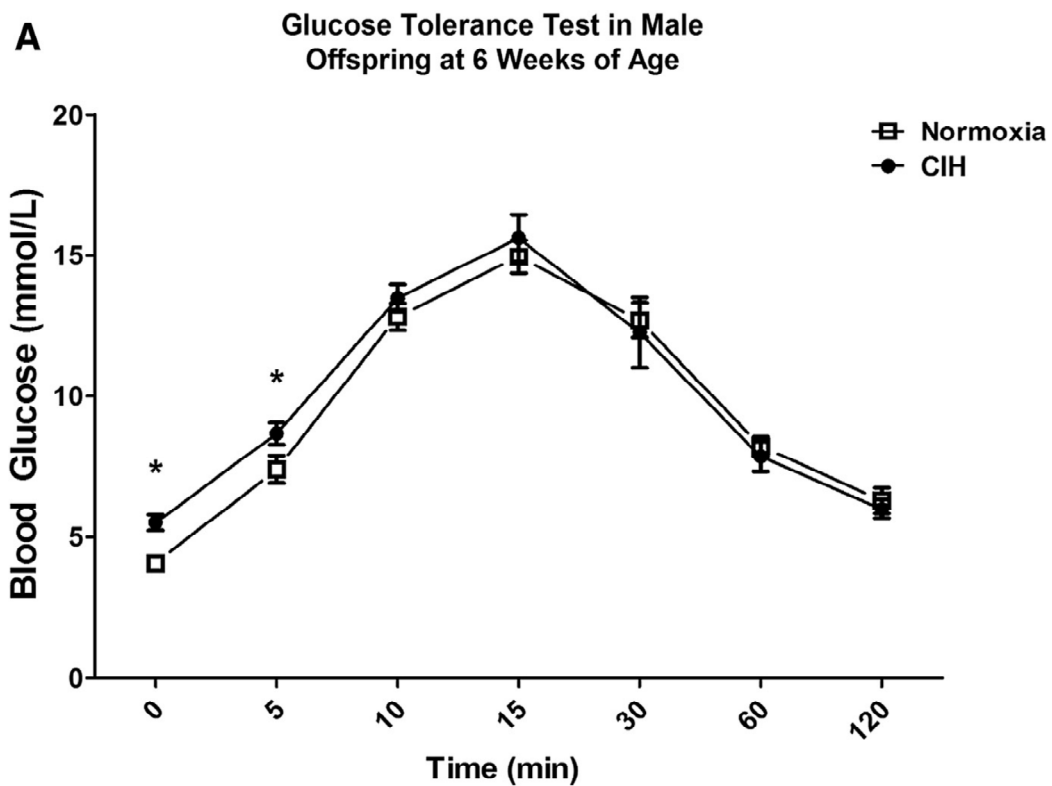
2.5 – DISCUSSION

This study has provided the first direct evidence that maternal CIH, as observed in patients with OSA, can lead to changes in offspring growth and development. Current *in vivo* models of CIH are primarily focussed on deleterious effects in adult subjects, and have shown that CIH can lead to induction of a variety of symptoms associated with the metabolic syndrom. Several obesogenic and diabetic factors have been suggested as potential catalysts for the changes observed in metabolic function during CIH, including changes in adrenergic and inflammatory pathways (Volgin, Kubin 2006), glucose tolerance (Chiu et al. 2004), pancreatic dysfunction (Pallayova, Lazurova & Donic 2011), sensitivity changes in the hypothalamic-pituitary-adrenal axis (Ma et al. 2008), and disruption of central leptin signalling pathways (Yang et al. 2011). Although several studies have examined the effects of CIH directly on developing offspring, there are no studies investigating the consequences of CIH exposure during gestational development of offspring (Reeves, Gozal 2006, Cai et al. 2010, Julien, Joseph & Bairam 2010). This study was done to characterize some of the outcomes observed in offspring exposed to CIH exclusively during gestational development.

The observation of lower birth weight offspring from CIH mothers was consistent with the findings of other studies investigating the effects of abnormal gestational environments. Decreased offspring birth weights have been reported in human IUGR models of maternal chronic hypoxia (Giussani et al. 2001, Moore 2003), and in animal IUGR models utilizing maternal protein restriction (Alexandre-Gouabau et al. 2011, Ramadan, Alshiraihi & Al-Karim 2012) or uterine artery ligation (Wigglesworth 1974). The elevated brain weight- and heart weight-to-body weight ratios and decreased liver

weight-to-body weight ratios in CIH offspring suggest a noticeable difference in growth rates of these organs during pregnancy. The brain-sparing effect is observed during hypoxia-induced IUGR and involves the reallocation of nutrients (Richardson, Bocking 1998, Peebles 2004, Malamitsi-Puchner, Nikolaou & Puchner 2006). In response to hypoxic stress, there is a redistribution of blood flow to ensure sufficient supply and sustained growth of the most vital organs, the brain and heart. This occurs at the expense of the liver, which is subject to hypoperfusion (McMillen et al. 2001, Malamitsi-Puchner, Nikolaou & Puchner 2006). Maternal protein restriction has been reported to decrease heart weight and cardiomyocyte number in newborn offspring (Corstius et al. 2005). In this study, CIH offspring had a greater heart weight-to-body weight ratio compared to normoxic offspring. Growth restriction leading to a decrease in cardiomyocyte number has the potential for severe cardiovascular consequences in adult life, as cardiomyocyte number is fixed at birth (Fernandez-Twinn, Ozanne 2006). The different patterns of organ development observed in the two groups of newborns and emphasis on growth of the heart during gestation could presumably exempt CIH offspring from such potential consequences in adult life. While this adaptive mechanism may optimize the immediate chance of survival, the functional capabilities of heart, brain and liver in adulthood remain to be determined.

This study has demonstrated a number of long-term metabolic impairments in offspring exposed to CIH conditions during gestation, including elevated fat deposition and impaired glucose tolerance. The epididymal and retroperitoneal fat pads provide an index of overall adiposity that is commonly used to determine changes in fat accumulation within the body (Rueda-Clausen et al. 2011). The greater weight of these

fat pads at 6- and 12-wks of age suggests that CIH offspring are prone to developing and maintaining an obese phenotype from a very early age. Similar observations have previously been reported in IUGR models examining maternal protein restriction and postnatal catch-up growth (Bol et al. 2009).

Excess accumulation of adipose tissue is associated with hepatic insulin resistance (Miyazaki et al. 2002). Free fatty acids (FFA) released from visceral adipose tissue have been suggested to play an important role in the overproduction of glucose in the liver (Bergman, Ader 2000). Increases in adiposity are associated with increased FFA concentration in the circulation. The result of this is greater FFA uptake by the liver (Girard, Lafontan 2008), and hepatic insulin resistance through FFA-induced inhibition of the suppressive effects of insulin on hepatic glucose production (González-Manchón, Ayuso & Parrilla 1989). At 12-wks of age, fasted CIH offspring were hyperglycemic and hyperinsulinemic. Furthermore, IGTT results indicate that adult CIH offspring exhibit poor short-term tolerance, but are still able to respond during the peak hyperglycemic state 15-30 min post-injection. Taken together, these findings suggest that adult CIH offspring have a decreased sensitivity to insulin, but have not developed a complete resistance to its signalling effects. Several potential methods of FFA-induced decreases in hepatic insulin sensitivity potentially include disruption of the insulin receptor substrate-1 signalling pathway through activation of protein kinase C-δ and inhibitor of kappa B kinase-β (Boden et al. 2005), p38 MAP kinase induced activation of PEPCK and G6Pase (Wang et al. 2006), JNK pathway activation through mixed-lineage protein kinases (Jaeschke, Davis 2007), although the exact mechanism for the changes in insulin sensitivity remains unknown.

If this change in liver metabolic function is indeed occurring, potential consequences could extend beyond the control of gluconeogenic pathways, into the regulation of lipogenesis. In obese individuals, the rates of adipocyte lipolysis are higher, resulting in elevated levels of circulatory fatty acids (Greenberg et al. 2011). With the accumulation of visceral adipose tissue in obese individuals, the excess transport for fatty acids to the liver can cause selective resistance to the effects of insulin. In such instances, a resistance develops to the actions of insulin in regulating hepatic gluconeogenesis, yet the liver retains sensitivity to the lipogenic actions of insulin (Brown, Goldstein 2008). It has been suggested that hyperglycemia and hyperinsulinemia (as seen in 12-wk old CIH offspring) may induce lipogenesis within hepatocytes, leading to an accumulation of lipids within the liver and further attenuating the effects of insulin on gluconeogenic pathways in the liver (Foufelle, Ferré 2002).

2.6 – REFERENCES

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Chapter 3

Gestational Chronic Intermittent Hypoxia Impairs Hepatic Glucose Homeostasis via Reduced Liver X Receptor Regulation of Gluconeogenic Enzymes and Glucocorticoid Signalling

3.1 – ABSTRACT

Chronic intermittent hypoxia (CIH) is the underlying pathophysiological condition seen in individuals with obstructive sleep apnea. Exposure of pregnant females to CIH results in offspring with increased fat deposition that are hyperglycemic, hyperinsulinemic, and have impaired early-stage glucose tolerance in adulthood. However, the underlying mechanisms are unknown. This study was done to determine changes in glucoregulatory function of the liver in offspring as a result of CIH exposure during gestational development. Female Sprague-Dawley rats were mated and exposed daily to CIH from gestational day-1 to day-20. Offspring were sacrificed at postnatal day-1 or 6-wks of age to determine immediate and long-term changes in liver function. By 6 wks of age, male CIH offspring had higher plasma corticosterone concomitant with higher liver protein expression of 11β-hydroxysteroid dehydrogenase type I and glucocorticoid receptor (GR), and this was associated with lower liver LXR protein expression. Analysis of both GR and LXR-direct target genes in gluconeogenesis showed that glucose-6-phosphatase expression was higher in CIH offspring, while no changes in phosphoenolpyruvate carboxykinase protein levels were observed. These data suggest that gestational exposure to CIH results in long-term alteration of hepatic glucose homeostasis via increased glucocorticoid signalling and decreased regulation of gluconeogenic enzymes by LXR.

3.2 – INTRODUCTION

Chronic intermittent hypoxia (CIH) is the underlying pathophysiological condition in individuals with obstructive sleep apnea (OSA), a breathing disorder characterized by repetitive closing of the upper airway during sleep (Dempsey et al. 2010). These blockages limit the availability of oxygen to the body, causing cyclical declines in blood oxygen saturation. Studies have established links between OSA and hypertension, obesity, and metabolic dysfunction (Weitzenblum et al. 1988, Wittels, Thompson 1990, Babu et al. 2005). However, the potential for multigenerational consequences is not well understood. Additionally, how this condition may affect offspring development and, ultimately, physiological phenotypes have not been elucidated.

Studies in recent years have revealed the importance of normal prenatal development on offspring health both in early life and adulthood (Hales 1993, Ozanne et al. 1996a, Ozanne et al. 1996b, Barker 2007). A number of physiological insults have been identified as having long-term metabolic consequences on low birth weight offspring including chronic hypoxia (Reeves, Gozal 2006), protein deficiency (Ramadan, Alshiraihi & Al-Karim 2012), and decreased blood supply (Wigglesworth 1974). Intrauterine growth restriction (IUGR) caused by an abnormal maternal environment during pregnancy has been associated with reduced glucose uptake from the circulation (Ozanne et al. 2001, Ozanne et al. 2003), decreased hepatic responsiveness to insulin (Ozanne et al. 1996b), type 2 diabetes (Desai, Hales 1997), and increased incidence of insulin resistance (Hales 1993). Furthermore, growth restricted offspring have shown impaired glucose tolerance in adulthood (Chamson-Reig et al. 2009, Vo et al. 2013) and permanent changes in hepatic enzyme activity, including genes involved in hepatic

glucose homeostasis (Desai et al. 1995). These findings suggest that changes to the maternal-fetal environment can exert long-term consequences on offspring metabolic phenotype. Thus, the possibility exists that the cyclic changes in oxygen availability during OSA that occurs in a pregnant female exposed to CIH has the potential to alter offspring development and metabolism. However, the consequences of OSA during pregnancy and the role of CIH in altering development during pregnancy have not been fully elucidated.

Recently, we have demonstrated the growth and metabolic consequences of CIH during gestation in Sprague-Dawley rats (Iqbal, Ciriello 2013). The hypoxic insult during pregnancy resulted in asymmetrically growth restricted offspring, characterized by lower birth weight and higher brain-to-liver weight ratios (Iqbal, Ciriello 2013). In adulthood, male offspring from mothers exposed to CIH during pregnancy had higher visceral fat deposition, and higher body weights compared to normoxic offspring. Furthermore, adult male offspring had higher fasting blood glucose levels and impaired early-stage glucose tolerance. (Iqbal, Ciriello 2013). However, the underlying molecular mechanisms for these physiological abnormalities are unknown.

Given the role of the nuclear receptor Liver X Receptors (LXRα and LXRβ) in regulating lipid (Schultz et al. 2000), cholesterol (Repa, Mangelsdorf 2000) and glucose (Mitro et al. 2007) homeostasis in adults, they are likely candidates to govern the molecular mechanisms underlying the etiology of the metabolic syndrome. LXR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily (Stulnig et al. 2002a, Cao et al. 2003). Existing in two isoforms, LXRα is primarily expressed in liver, intestine, and adipose tissue, while LXRβ is ubiquitously expressed

(Willy et al. 1995, Repa, Mangelsdorf 1999, Lu, Repa & Mangelsdorf 2001). Oxysterols, derivatives of cholesterol, were identified as the physiological ligand for LXR (Janowski et al. 1996, Lehmann et al. 1997), which led to the elucidation of the critical role of LXR in regulating metabolism and transport of cholesterol (Peet et al. 1998, Schwartz, Lawn & Wade 2000, Edwards, Kennedy & Mak 2002), and lipid metabolism (DeBose-Boyd et al. 2001, Laffitte et al. 2001, Tobin et al. 2002). Recent studies have also established a governing role for LXR in hepatic gluconeogenic pathways, directly impairing the expression and activity of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), enzymes critical for glucose production within the liver (Stulnig et al. 2002b, Cao et al. 2003, Vo et al. 2013). Also, LXR has been shown to inhibit the expression and activity of 11β-hydroxysteroid dehydrogenase type I (11β-HSD1), the enzyme involved in converting inactive glucocorticoids (cortisone in humans, 11-dehydrocorticosterone in rodents) into active glucocorticoids (cortisol in humans, corticosterone in rodents) (Stulnig et al. 2002a). Furthermore, studies using LXR agonists have demonstrated that LXR inhibits glucocorticoid receptor (GR) expression in rodents (Liu et al. 2006). This is of particular importance, as GR has been shown, via a transactivation mechanism, to mediate gluconeogenic pathways in the liver by activating G6Pase (Renga et al. 2012). In response to elevations in blood glucose, transport of glucose into hepatocytes in increased via glucose transporters (GLUT2) (Shiota, Magnuson 2008). Additionally, LXR has been shown to positively regulate GLUT2 expression through indirect mechanisms (Zitzer et al. 2006). These findings suggested an important role for LXR in regulating glucose homeostasis in adulthood and perinatal life. Recent studies have implicated that alterations in LXR activity during

abnormal fetal development led to symptoms of the metabolic syndrome including hypercholesterolemia and impaired glucose tolerance/homeostasis in adulthood (Sohi et al. 2011, Osumek et al. 2013, Vo et al. 2013).

This study was done to investigate the changes in LXR-regulated gluconeogenic pathways within the liver and determine how they may contribute to the metabolic dysfunction observed in adult CIH offspring. Understanding the role of LXR in mediating these developmental abnormalities can aid us in developing better strategies for preventing the onset of adult diseases. Our earlier results demonstrated that offspring from CIH mothers have elevated circulating glucose levels and impaired glucose tolerance (Iqbal, Ciriello 2013). Thus, it was hypothesized that gestational CIH exposure impairs glucose homeostasis through decreased regulation of gluconeogenic pathways in the liver both directly and indirectly by LXR.

3.3 – METHODS AND MATERIALS

3.3.1 – Animal Handling

Adult Sprague-Dawley rats were obtained from Charles River Canada (Saint-Constant, Quebec, Canada). All animals were housed in standard cages within the university animal care facility with rooms maintained at a constant temperature and humidity (23°C and 65%, respectively) on a 12h light/dark cycle beginning at 0700h. Food and water were available to all animals *ad libitum*. All experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental

Animals by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Western Ontario.

3.3.2 – Gestational CIH exposure

Pregnant rats were exposed to CIH as previously described (Iqbal, Ciriello 2013). In brief, female rats (250g; n=14) were mated and successful pregnancy was confirmed by the presence of sperm in a vaginal smear taken the following morning. The dams were placed in a hypoxia chamber and exposed to 200 s cycles of alternating hypoxic (6.5% O_2 nadir; 80 s) and normoxic (room air; 120 s) conditions at a frequency of 18 cycles (hypoxic episodes)/h (Iqbal, Ciriello 2013). The pregnant females were exposed to CIH for 8h daily (1000h to 1800h) during the light cycle (rodent sleep cycle) from gestational day-1 to gestational day-20. Control dams were placed in an adjacent chamber that cycled room air continuously during the 8h exposure period.

3.3.3 – Post-delivery

Following delivery, a subset of male offspring was immediately sacrificed for postnatal day-1 measurements. Litters were then culled to four males, which were housed with the mother throughout the lactation period (postnatal day-1 to day-21) and housed in pairs post-weaning.

3.3.4 – Offspring Tissue Collection

Postnatal day-1 offspring were weighed and sacrificed via decapitation. Hearts and livers were extracted and snap frozen in liquid nitrogen. Brains were extracted and frozen on dry ice. Adult male offspring were fasted overnight prior to sacrifice. Rats

were anesthetized with equithesin (0.4 ml/100g body weight; ip) (Iqbal, Ciriello 2013); adult hearts and liver were extracted and snap frozen in liquid nitrogen. Brains were extracted and frozen on dry ice.

3.3.5 – Blood Collection

Blood samples were collected from adult male offspring at the time of sacrifice. Blood was obtained via intra-cardiac puncture using a syringe with an 18 g needle and collected in 1 ml aliquots in centrifuge tubes containing 10 µl of 7% ethylenediaminetetraacetic acid (EDTA) dissolved in water. Plasma was isolated by spinning the blood samples in a refrigerated centrifuge at 10,500 g for 15 min at 4°C. Physiological levels of corticosterone follow a diurnal pattern of variation. To minimize differences in corticosterone concentration as a result of daily physiological changes, all animals were sacrificed at the same time of day (1200h).

3.3.6 – Triglyceride Measurement

Triglyceride levels were measured using an enzymatic colorimetric assay for total triglycerides. In this assay, enzymatic hydrolysis of triglycerides produces fatty acids and glycerol. Glycerol is phosphorylated into glycerol-3-phosphate, which is then oxidized to form dihydroxyacetone phosphate and hydrogen peroxide. Along with 4-aminophenazone and 4-chlorophenol, hydrogen peroxide undergoes reaction in the presence of peroxidase. Absorbance of the resulting pigmented product is determined at a wavelength of 500 nm, providing a measurement of total triglycerides in the plasma

sample. Absorbance readings of triglyceride assays will be determined using the COBAS Mira S Analyzer (Roche Diagnostics, Laval, Quebec, Canada).

3.3.7 – Corticosterone Measurement

Frozen liver tissue samples were weighed and prepared in centrifuge tubes containing 0.5 ml of a precipitating solution (0.05 g/ml $ZnSO_4$ and Methanol; 1:1 v/v) using an electric pestle (Cat.#47747-370; VWR International, Mississauga, Canada) until full dispersion of the tissue was achieved in the solution. Following 30 s on a vortex, the resulting mixtures were incubated at 4 °C overnight with continuous shaking. Samples were centrifuged at 18,000 g at 4°C for 10 min and the supernatant was collected into new centrifuge tubes. Samples were air dried and re-suspended in assay buffer for determination of corticosterone concentration.

Plasma and liver corticosterone levels were measured using an enzymatic immunoassay (Cat. #ADI-900-097; Enzo Life Sciences, Farmingdale, NY) according to manufacturer's instructions. This competitive immunoassay utilizes a polyclonal antibody to bind corticosterone in samples or a solution containing alkaline phosphatase molecules with covalently bound corticosterone. Following a simultaneous incubation with sample and the alkaline phosphatase solution, a substrate was added to the plate and the pigmented product was read at 405 nm. Absorbance readings of corticosterone were determined using a Spectramax M5 plate reader (Molecular Devices, Downingtown, PA).

Assay sensitivity was calculated as the mean ± two standard deviations for the zero standards in the corticosterone enzymatic immunoassays. Sensitivity of the corticosterone immunoassay was calculated as 0.017 ng/ml. Intra-assay variability was calculated as the mean of the coefficients of variation for all samples measured in the assay. The intra-assay variability for the corticosterone assay was 2.9%.

3.3.8 – Western Blot Analysis

Frozen liver tissue samples were homogenized using an electric pestle in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1% TritonX-100, 1 mM EDTA, 0.2 M sodium orthovanadate, 0.1 M sodium fluoride, 0.1 M β-glycerophosphate) with a dissolved protease inhibitor cocktail (Cat.#11836153001; Roche Diagnostics, Laval, Canada). After incubation on ice for 15 min, homogenized samples were centrifuged for 10 min at 1,000 g at 4 °C. The supernatant was collected and centrifuged for 20 min at 18,000 g at 4 °C. The supernatant was collected and stored as the final protein isolate. Protein concentrations were standardized using the BIO-RAD detergent compatible protein assay, a colorimetric assay for quantification of protein content (Cat. #500-0111; Bio-Rad Laboratories, Mississauga, Canada). Proteins were separated through SDS-PAGE on graded polyacrylamide gels (Cat. #EC60755BOX; Life Technologies, Burlington, Canada) and transferred to polyvinylidene fluoride membrane for probing. Blots were probed for LXR (1:1000; Cat.#sc-13068, Santa Cruz), 11β-HSD1 (1:1000; Cat.#sc-20175; Santa Cruz Biotechnology, Santa Cruz, CA), glucocorticoid receptor (GR, 1:1000; Cat.#sc-8992, Santa Cruz), glucose-6-phosphatase (G6Pase, 1:1000; Cat.#sc-25840, Santa Cruz),

phosphoenolpyruvate carboxykinase (PEPCK, 1:1000; Cat.#sc-32879, Santa Cruz), glucose transporter 1 (GLUT1, 1:1000; Cat.#sc-7903, Santa Cruz), and glucose transporter 2 (GLUT2, 1:1000; Cat.#sc-9117, Santa Cruz). Primary antibodies were diluted in 5% powdered milk-0.05% Tris-buffered saline-Tween 20 (TBS-T) buffer. Horseradish peroxidase-conjugated donkey-anti-rabbit IgG (1:10,000; Cat. #711035152, Jackson ImmunoResearch Laboratories, West Grove, PA) was utilized as a secondary antibody diluted in 5% powdered milk-0.05% TBS-T buffer. Membranes were incubated in primary antibodies overnight at $4°C$, while secondary antibody incubations were 1h at room temperature. Protein expression was standardized to β-actin (1:50,000; Cat. #A3854, Sigma-Aldrich, Oakville, Canada). Immunoreactive bands were visualized using a chemiluminescence detection system (Cat. #WBLUF0100, Millipore, Toronto, Canada).

3.3.9 – Data and Statistical Analysis

All data are expressed as mean ± standard deviation. Corticosterone measurements and western blot results were compared between normoxic and CIH groups using a two-tailed Student t-test to determine statistical significance. In all comparisons, a minimum p-value of < 0.05 was taken to indicate statistical significance (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

3.4 – RESULTS

As previously reported, male offspring from mothers exposed to gestational CIH had changes in glucose homeostasis and impaired glucose tolerance in adulthood (Iqbal, Ciriello 2013). CIH offspring had higher fasting blood glucose levels (Normoxia: 4.2 ± 0.61 mmol/L vs. CIH: 6.41 ± 0.64 mmol/L) and higher levels of fasting plasma insulin (Normoxia: 0.358 ± 0.20 ng/ml vs. CIH: 1.11 ± 0.50 ng/ml).

3.4.1 – Gestational CIH exposure lowers LXR protein expression in male offspring

As previously reported, LXR has been shown to regulate expression of glucocorticoid and gluconeogenic markers in the liver (Figure 3.1). Based on this regulatory role, western blots were performed on liver samples from male offspring at postnatal day-1, 6 weeks, and 12 weeks of age to investigate changes in protein expression of LXR. Newborn CIH offspring were found to have significantly lower hepatic LXR protein at postnatal day-1 (Fig. 3.2A). Similarly, hepatic LXR protein levels were lower in CIH offspring livers at both 6 weeks and 12 weeks of age (Fig. 3.2B and 3.2C, respectively).
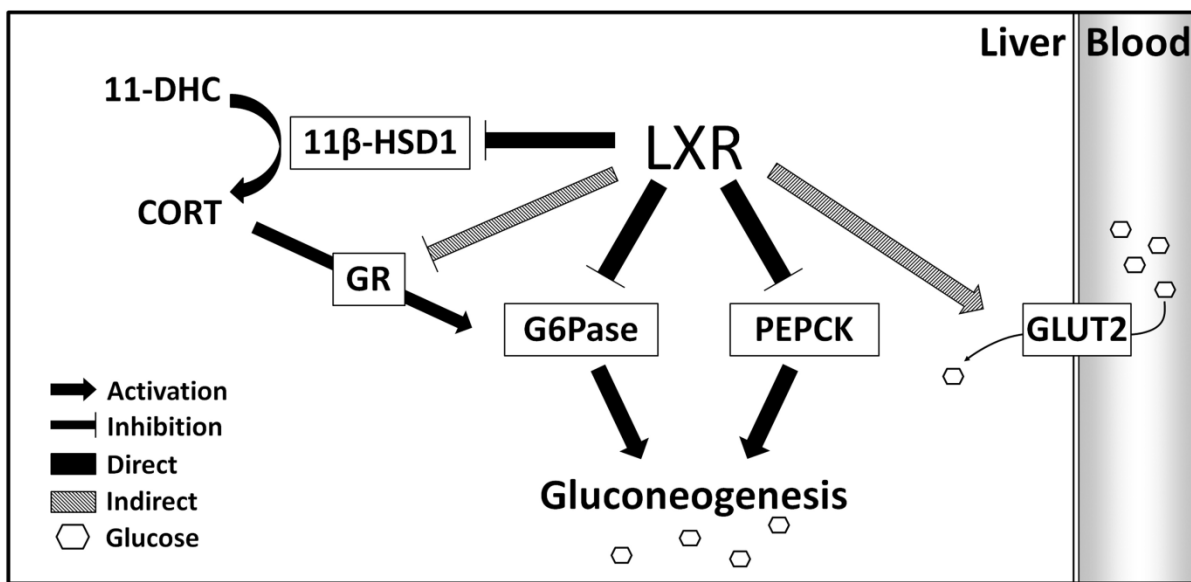
Figure 3.1: Schematic diagram of direct and indirect hepatic glucoregulatory mechanisms for LXR
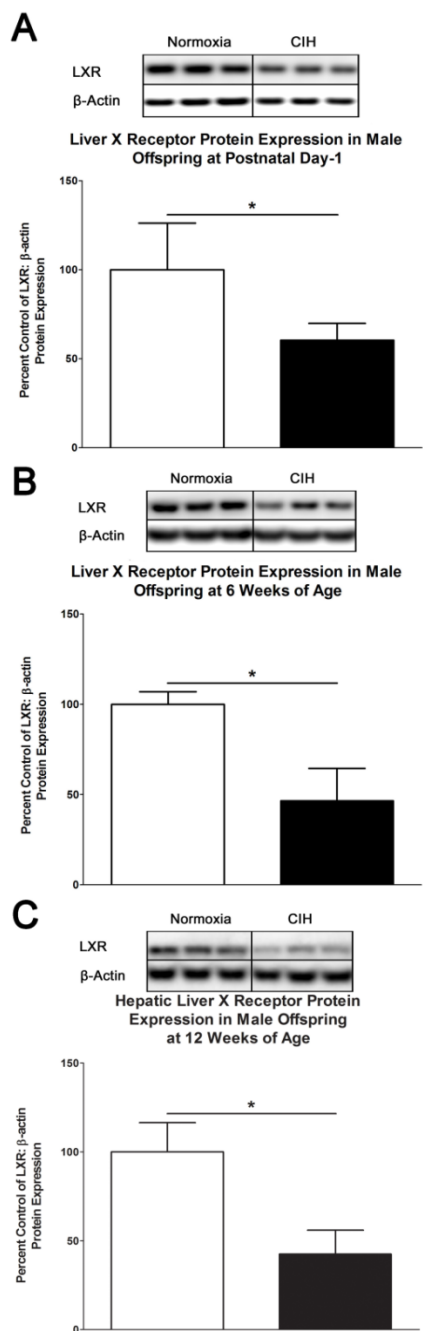
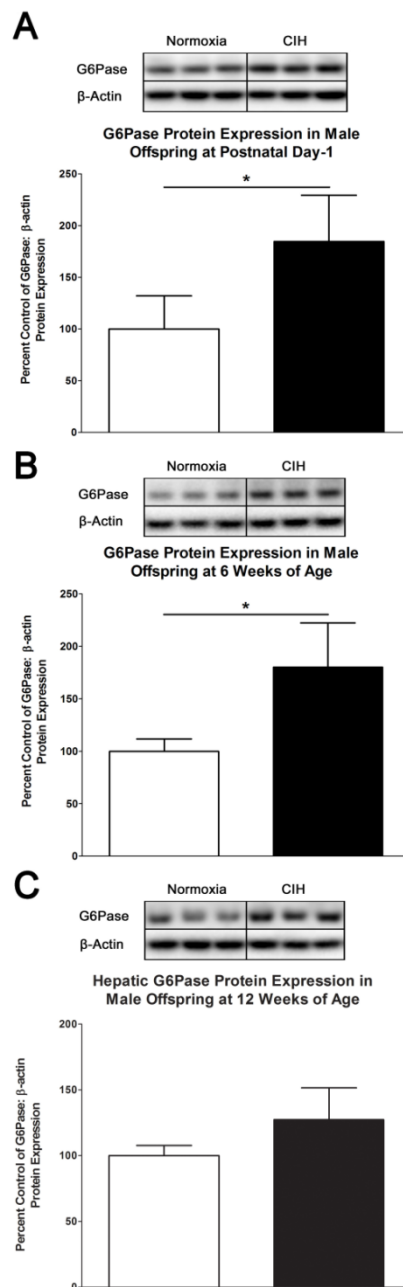**Figure 3.2: Gestational CIH exposure lowers LXR protein expression in livers of male offspring**

Western blot analysis of LXR in the livers of male offspring. CIH male offspring were found to have significantly lower hepatic LXR protein expression at (A) postnatal day-1, (B) 6 weeks of age, and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

3.4.2 – Gestational CIH-induced changes in LXR protein expression result in higher protein expression of the gluconeogenic marker G6Pase in male offspring

To examine LXR-target enzymes directly involved in inhibiting gluconeogenesis within the liver, G6Pase and PEPCK protein expression were next measured (Fig. 1). CIH male offspring had significantly greater hepatic G6Pase protein expression at postnatal day-1 (Fig. 3.3A). Similarly, adult male CIH offspring had higher G6Pase protein levels at 6 weeks and 12 weeks of age (Fig. 3.3B amd 3.3C, respectively). No differences in PEPCK protein expression were observed between CIH and normoxic offspring (Fig. 3.4A-C).

3.4.3 – Gestational CIH increases hepatic glucose transport in young adult male offspring

To investigate changes in glucose uptake, GLUT protein expression was measured in the livers of offspring. GLUT1 protein levels are highest following birth and decrease significantly during postnatal maturation. GLUT2 expression is at maximal levels in adult life (Bell et al. 1990). No difference in GLUT1 protein expression was observed between offspring at postnatal day-1 (Fig. 3.5A). Male CIH offspring had significantly lower GLUT2 protein expression at 6 weeks of age (Fig. 3.5B). At 12 weeks of age, GLUT2 protein levels were not different (Fig. 3.5C).

Figure 3.3: Young adult male CIH offspring have decreased hepatic G6Pase protein expression

Western blot analysis of LXR target gene G6Pase in the livers of male offspring. CIH males had significantly higher hepatic G6Pase protein expression than normoxic male offspring at (A) postnatal day-1 and (B) 6 weeks of age. (C) G6Pase protein levels were not different at 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

A PEPCK Protein Expression in Male Offspring at Postnatal Day-1

B PEPCK Protein Expression in Male Offspring at 6 Weeks of Age

C Hepatic PEPCK Protein Expression in Male Offspring at 12 Weeks of Age
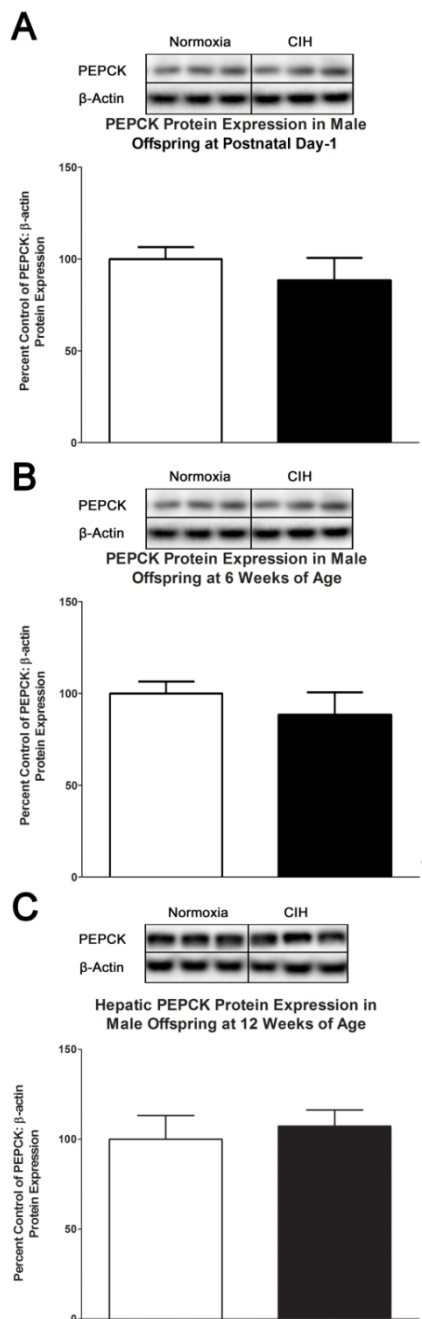
Figure 3.4: Gestational CIH does not alter PEPCK protein expression

Western blot analysis of LXR target gene PEPCK in the livers of male offspring. No differences in hepatic PEPCK protein expression were observed between CIH and normoxic offspring in any age group. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

Figure 3.5: Young adult male CIH offspring have elevated hepatic GLUT2 protein expression

Western blot analysis of LXR target gene GLUT in the livers of male offspring. (A) No differences in GLUT protein expression were observed at postnatal day-1. (B) CIH male offspring had lower hepatic GLUT protein expression compared to normoxic offspring at 6 weeks of age. (C) GLUT protein expression was not different at 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

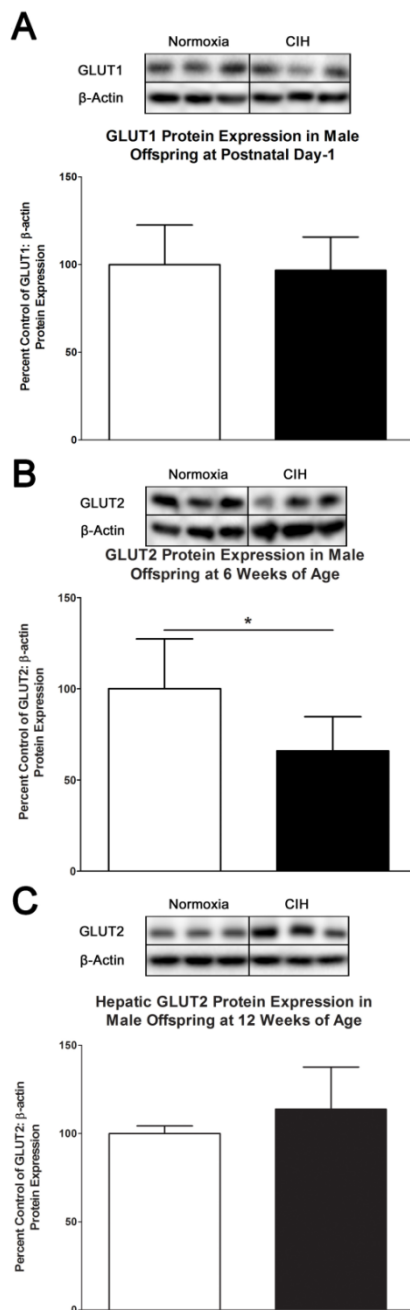### 3.4.4 – Gestational CIH exposure increases glucocorticoid signalling in the livers of male offspring

LXR indirectly influences gluconeogenesis by inhibiting the expression of 11β-HSD1, the critical enzyme in the conversion of inactive corticosteroids to bioactive corticosteroids involved in glucose production (Fig. 1) (Stulnig et al. 2002a). Thus, circulating and hepatic glucocorticoids were measured in CIH offspring at 6 weeks and 12 weeks of age. Furthermore, Western blots were performed on livers samples from male offspring to examine protein expression of 11β-HSD1 and GR. While there was no difference in the expression level of 11β-HSD1 in postnatal day-1 offspring (Fig. 3.6A), by 6 weeks of age, CIH male offspring had significantly greater 11β-HSD1 levels compared to normoxic offspring (Fig. 3.6B). Similarly, adult male CIH offspring had higher 11β-HSD1 protein expression at 12 weeks of age (Fig. 3.6C). The elevated enzyme protein expression was associated with significantly greater plasma corticosterone levels in CIH offspring compared to normoxic offspring at both 6 weeks and 12 weeks of age (Fig. 3.7A and 3.8C, respectively). Hepatic corticosterone levels were not different between groups at 6 weeks or 12 weeks of age (Fig. 3.7B and 3.7D, respectively). At postnatal day-1, CIH offspring has significantly lower GR expression levels (Fig. 3.8A). Conversely, higher GR protein expression was observed in CIH offspring at 6 weeks and 12 weeks of age (Fig. 3.8B and 3.8C, respectively).
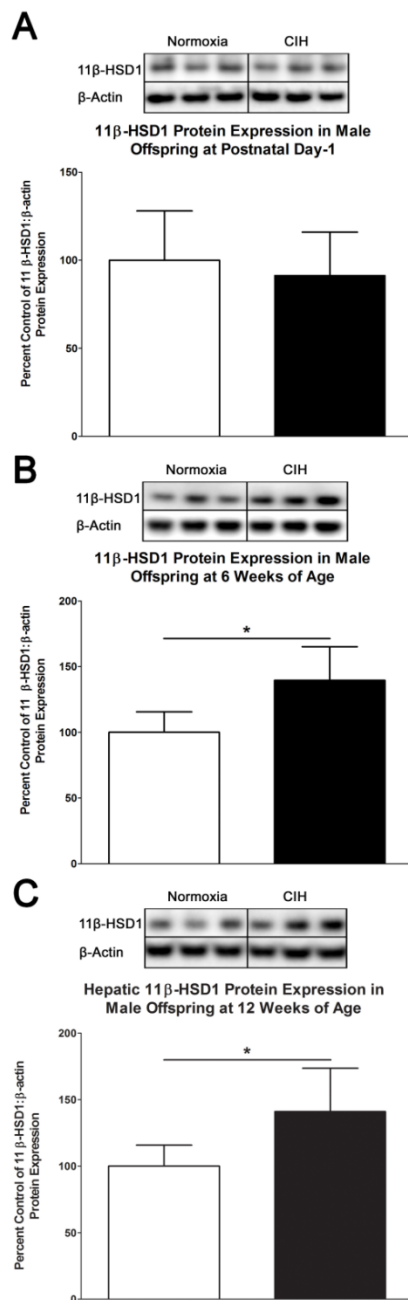
Figure 3.6: Gestational CIH exposure increases hepatic 11β-HSD1 protein expression in male offspring

Western blot analysis of LXR target gene 11β-HSD1 in the livers of male offspring. (A) No differences in 11β-HSD1 protein expression were observed at postnatal day-1. (B) CIH male offspring had significantly higher hepatic 11β-HSD1 protein expression compared to normoxic offspring at both (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8
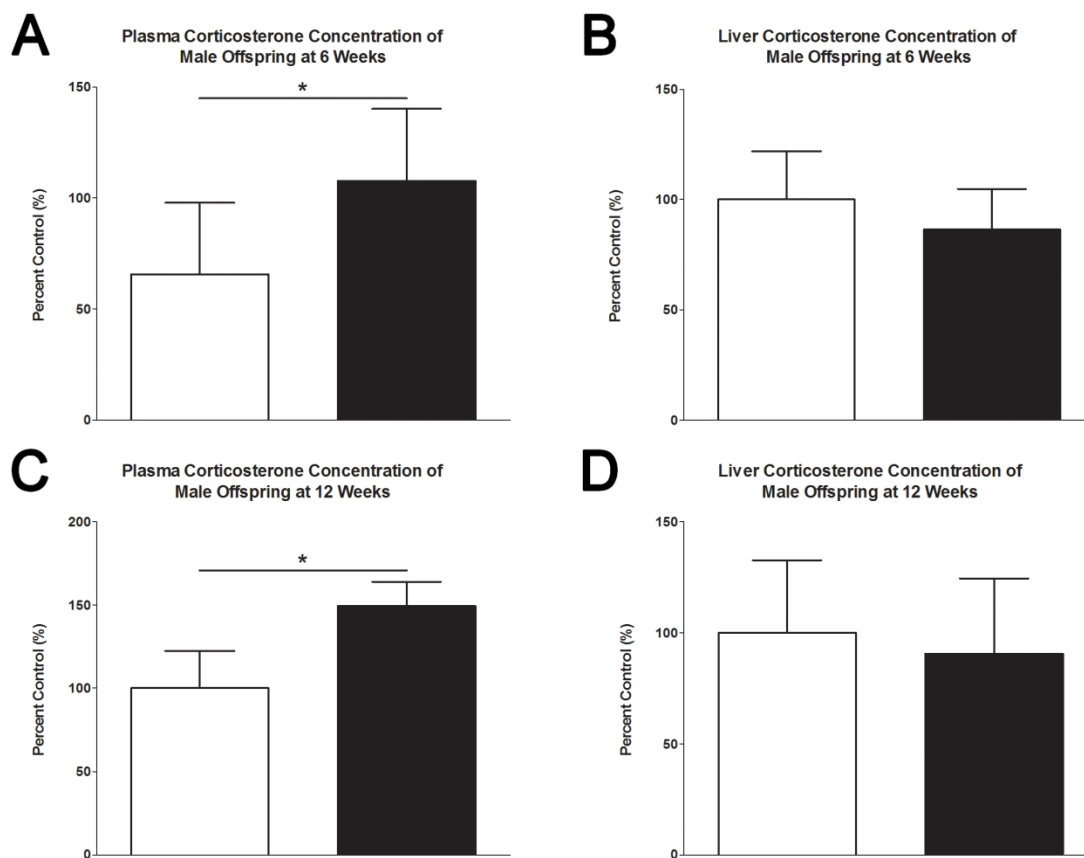
Figure 3.7: Gestational CIH increases glucocorticoid signalling in male offspring

Plasma and hepatic corticosterone concentrations were measured using an enzymatic immunoassay. (A) CIH offspring had higher plasma corticosterone concentration at 6 weeks of age. (B) Hepatic corticosterone concentrations were not different between groups at 6 weeks. (C) CIH offspring had significantly greater plasma corticosterone concentrations at 12 weeks of age. (D) Hepatic corticosterone concentrations were not different between offspring groups at 12 weeks. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8
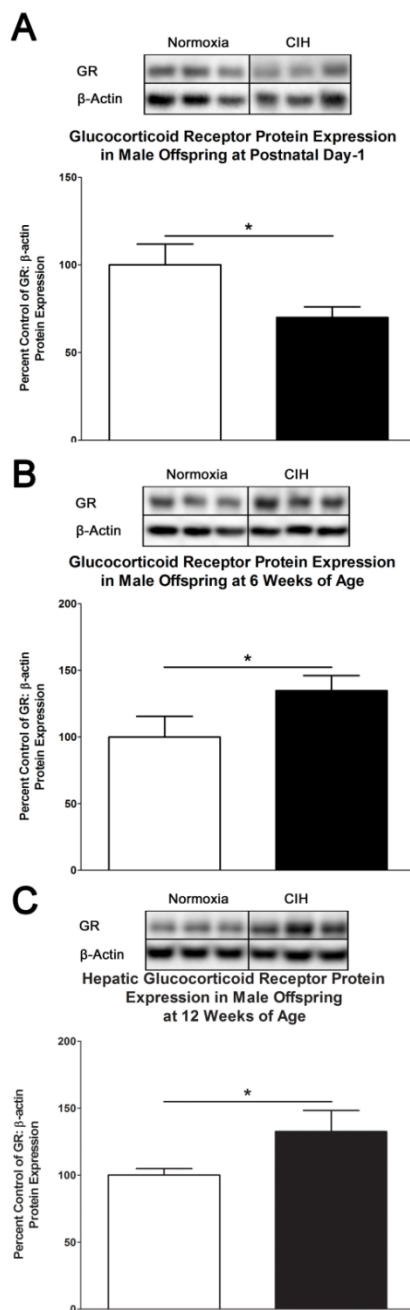
Figure 3.8: Adult male CIH offspring have elevated hepatic GR protein expression

Western blot analysis of LXR target gene GR in the livers of male offspring. (A) CIH offspring had significantly lower hepatic GR protein expression at postnatal day-1. At both (B) 6 weeks and (C) 12 weeks of age, CIH male offspring had higher GR protein levels compared to normoxic offspring. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

Total Plasma Triglyceride Concentrations in Male Offspring at 6 Weeks of Age

Total Plasma Triglyceride Concentrations in Male Offspring at 12 Weeks of Age

Figure 3.9: Adult male CIH offspring have higher circulating triglyceride concentrations

Total circulating triglyceride concentrations were measured using an enzymatic immunoassay. Adult male CIH offspring were found to have significantly greater total circulating triglyceride concentrations compared to normoxic offspring at both 6-weeks and 12-weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 8; Normoxia: n = 8

3.5 – DISCUSSION
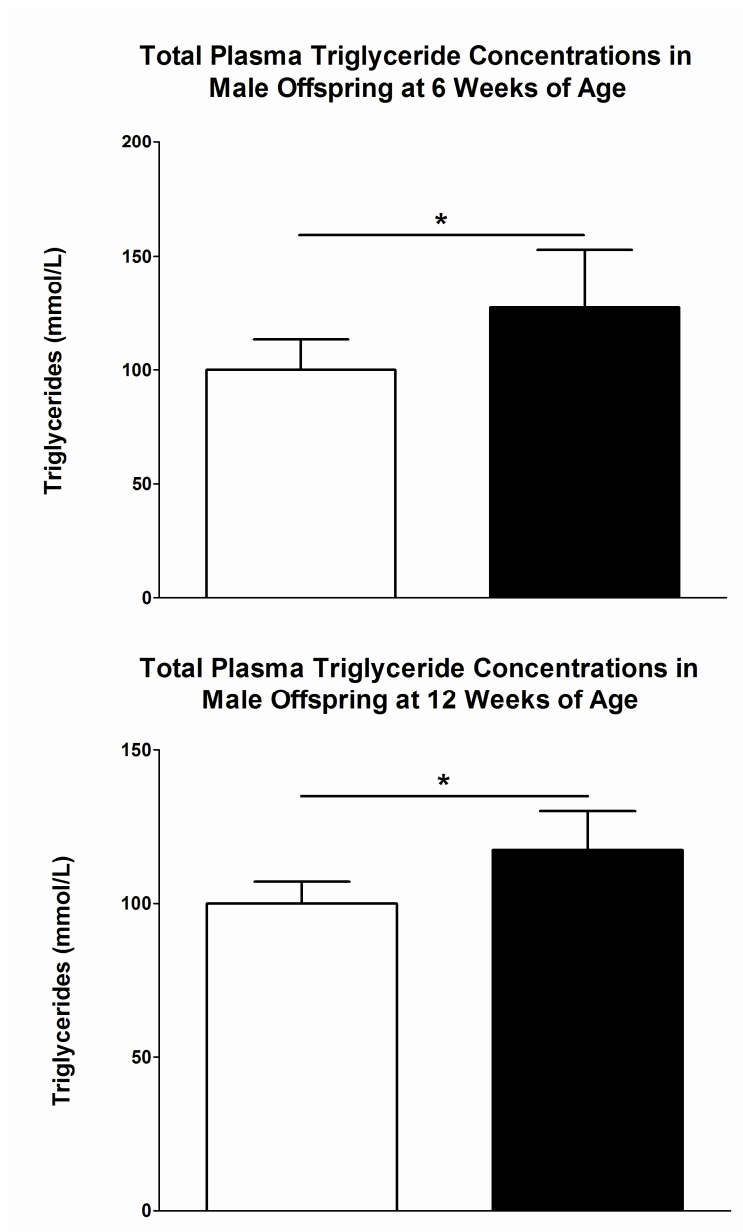
Recently, we have demonstrated the negative implications of exposure to CIH during gestational development on the metabolic physiology of offspring (Iqbal, Ciriello 2013). Despite having lower birth-weights, offspring exposed to CIH during pregnancy had higher body weights and greater visceral fat deposition in adulthood (Iqbal, Ciriello 2013). Furthermore, adult CIH offspring were hyperglycemic, hyperinsulinemic, and had impaired glucose tolerance compared to normoxic offspring (Iqbal, Ciriello 2013). The observations reported in this study further elucidate the physiological consequences of altering the maternal intrauterine environment during pregnancy by exposure to CIH during pregnancy.

Adult male CIH offspring were found to have higher circulating corticosterone levels compared to young adult male normoxic offspring. This was associated with higher protein expression of 11β-HSD1 and GR in the adult offspring. Interestingly, LXR expression was significantly lower in the livers of CIH offspring at birth which was maintained into adulthood. Young adult male CIH offspring had higher expression of the critical gluconeogenic enzyme, G6Pase. Furthermore, CIH offspring had decreased expression of GLUT2. Taken together, these data suggest that gestational exposure to CIH has programmed a long-term hyperglycemia state in the adult male offspring mediated by greater hepatic glucose production and reduced internalization of glucose from the circulation.

LXR regulates gluconeogenic enzymes and glucose production in the liver (Stulnig, Steffensen et al. 2002). LXR agonist studies have demonstrated using db/db

mice and Zucker fatty rats, that activation of LXR pathways in the liver lowers blood glucose levels (Cao, Liang et al. 2003). Additionally, LXR agonists dose-dependently decrease expression of G6Pase and PEPCK in the liver (Cao, Liang et al. 2003). While we did not observe any differences in PEPCK expression in offspring at postnatal day-1 or in adulthood, the higher G6Pase expression is of notable importance, as this enzyme catalyzes the hydrolysis of glucose-6-phosphate to glucose, the final step of gluconeogenesis from a glycerol precursor. GLUT2 protein expression was lower in the young adult CIH offspring, limiting the glucose uptake ability of the liver, a transport process that typically occurs at a high rate following increases in blood glucose concentrations (Shiota, Magnuson 2008). As LXR has previously been demonstrated to positively regulate GLUT2 protein expression (Zitzer, Wente et al. 2006), the lower hepatic GLUT2 expression correlates well with the lower hepatic LXR protein levels observed in young adult CIH offspring. The findings of this study are similar to those recently published by Vo et al. (2013), which described glucose intolerance and hyperglycemia in offspring using a rodent maternal protein restriction model (Vo, Revesz et al. 2013). These authors reported lower hepatic expression and activity of LXR, associated with augmented expression of G6Pase in adult offspring, while no changes in PEPCK protein levels were observed (Vo, Revesz et al. 2013). This protein expression pattern is consistent with the findings of the current study.

Our recent findings described the hyperglycemic and hyperinsulinemic state of adult male CIH offspring (Iqbal, Ciriello 2013). The inability of insulin to effectively regulate blood glucose levels are consistent with the findings of Ozanne et al. (1996), where they demonstrated that growth restricted offspring had dramatically higher rates

of insulin uptake and degradation compared to control offspring (Ozanne, Smith et al. 1996). The development of an insulin resistant state certainly warrants further investigation, as this could be an important contributor to the metabolic dyshomeostasis observed in CIH offspring. Investigating the responsiveness to insulin may also provide indications of functional changes in other tissues, as glucose uptake by skeletal muscle and adipose tissue is diminished in the insulin resistant state. Another possible explanation for these observations is the potential interaction between insulin and the LXR signalling pathways. It has recently been suggested that the regulatory effects of insulin in the liver may, at least in part, be mediated through LXR (Chen, Liang et al. 2004, Tobin, Ulven et al. 2002). Indeed, activation of the LXR pathways results in improved glucose tolerance and insulin sensitivity (Cao, Liang et al. 2003). The lower hepatic LXR protein expression reported in this study may explain the lower responsiveness to insulin observed.

Higher plasma corticosterone levels were found in CIH offspring at 6-weeks and 12-weeks of age. Concomitantly, liver corticosterone concentrations were not different between groups, suggesting that glucocorticoids produced in excess are likely being immediately transported out of the liver. This hypothesis is supported by the higher expression of 11β-HSD1 observed in the liver of CIH offspring, which would produce more glucocorticoid conversion from 11-dehydrocorticosterone to corticosterone. Higher receptor protein expression in adulthood, as observed in male CIH offspring, suggests a likely greater hepatic responsiveness to corticosterone. The increase in hepatic 11β-HSD1, GR, and bioactive corticosteroids is important to the phenotype observed in CIH offspring given that glucocorticoids have been demonstrated to induce gluconeogenesis

by transcriptionally activating G6Pase (Lin, Morris et al. 1998, Lu, Xiong et al. 2013). Thus, the higher circulating corticosterone levels may be an important mediator of the hyperglycemic state we have observed in adult CIH offspring. The higher GR protein expression in adult CIH offspring correlates well with the lower LXR protein expression observed in these animals, as LXR is known to negatively regulate GR expression (Liu, Yan et al. 2006). However, it is likely the GR protein is also being regulated by other sources, as suggested by the lower GR expression in CIH offspring at postnatal day-1. Interestingly, recent studies have established a critical role for hepatic glucocorticoid receptors in promoting postnatal growth (Tronche, Opherk et al. 2004). Decreased GR signalling in hepatocytes during early postnatal life may result in poor development due to impaired growth hormone signalling (Tronche, Opherk et al. 2004). The lower GR protein expression observed in postnatal day-1 offspring could have severe consequences for early life development of CIH offspring. In particular, lower GR signalling may have long-term consequences on liver functionality, as hepatic differentiation and development are still ongoing during the first weeks of postnatal life.

Another potential contributor to the higher plasma corticosterone levels is a greater supply from peripheral sources, the likely candidate being visceral adipose tissue, which was higher in CIH offspring at 6-weeks and 12-weeks of age. Interestingly, 11β-HSD1 knockout models have demonstrated a reduction in hepatic glucose production and resistance to developing obesity (Morton, Holmes et al. 2001). Also, 11β-HSD1 activity is associated with increased expression of the gluconeogenic liver enzyme G6Pase, which was up-regulated in the livers of 6-week old CIH offspring. Increased G6Pase protein expression results in greater amounts of free glucose

(Nordlie, Sukalski 1985). The free glucose molecules are then transported to the circulation via glucose transporters in the liver (Bell, Kayano et al. 1990). Masuzaki et al. (2003) have reported that rodents overexpressing 11β-HSD1 develop obesity, have elevated circulating corticosteroid levels and develop type-II diabetes (Masuzaki, Yamamoto et al. 2003). Visceral adipose tissue has been shown to have a high concentration of glucocorticoid receptors (Rebuffe-Scrive, Bronnegard et al. 1990). As a result, the increased concentration of corticosterone in the circulation could further exacerbate the obesogenic phenotype of CIH offspring through interaction with visceral adipose tissue, which was higher in males at 6-weeks and 12-weeks of age. Expression of 11β-HSD1 is negatively regulated by LXR in the liver and LXR activation results in more than a 50 percent decrease in 11β-HSD1 enzymatic activity (Stulnig, Oppermann et al. 2002). As mentioned previously, LXR agonists have been shown to inhibit GR expression in rodents (Liu, Yan et al. 2006). The higher 11β-HSD1 protein levels, glucocorticoid receptor expression, and corticosterone concentrations correlate well with the lower protein expression of LXR, observed in adult CIH offspring.

In summary, these data have provided evidence of altered hepatic mechanistic glucose homeostasis as a result of exposure to CIH during gestational development. These represent the first observations of higher glucocorticoid signalling and lower regulation of hepatic gluconeogenic pathways by LXR in offspring of dames exposed to CIH during pregnancy. Taken together, these data suggest that alteration of the maternal intrauterine environment by this intermittent hypoxic insult can have severe consequences on liver function, resulting in long-term glucose dyshomeostasis.

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Chapter 4

Gestational Chronic Intermittent Hypoxia Causes Hypercholesterolemia Due to Impairments In LXR-mediated Cholesterol Metabolism and Transport

4.1 – ABSTRACT

Chronic intermittent hypoxia (CIH) is the underlying physiological condition of obstructive sleep apnea (OSA), a progressively worsening sleep-breathing disorder. CIH exposure during pregnancy causes fetal growth restriction and long-term metabolic dyshomeostasis in offspring. Adult male CIH offspring have impaired regulation of gluconeogenic pathways in the liver due to reduced hepatic Liver X Receptor (LXR) protein expression. This study was done to determine the impact of diminished LXR expression on cholesterol homeostasis in the liver of CIH offspring. Female Sprague-Dawley rats were exposed daily to CIH during pregnancy. Offspring were sacrificed at postnatal day-1, 6 weeks, and 12 weeks of age to assess early life and long-term alterations in liver function. At both 6 weeks and 12 weeks of age, male CIH offspring had significantly higher total circulating cholesterol levels compared to normoxic offspring. Western blot analysis showed that adult male CIH offspring had lower protein expression of the cholesterol metabolism enzyme cholesterol 7α-hydroxylase (CYP7A1), the cholesterol excretion transporter ATP-binding cassette transporter G8 (ABCG8), and the low density lipoprotein receptor (LDLR) at both 6 weeks and 12 weeks of age. These data indicate that gestational CIH exposure-induced hypercholesterolemia results from diminished expression of LXR target genes associated with cholesterol breakdown, excretion, and removal from the circulation.

4.2 – INTRODUCTION

Obstructive sleep apnea (OSA) is a chronic breathing disorder involving repetitive interruptions in respiration during sleep (Dempsey et al. 2010). Disturbances in breathing are a result of a complete blockage of the airway (apnea) or a partial constriction of the airway leading to a dramatic reduction in airflow (hypopnea). Normal breathing resumes following a transient surge in sympathetic nervous system activity, which expels an individual from the deeper stages of sleep and reopens the airway. The severity of OSA is classified according to the apnea-hypopnea index (AHI), which calculates the frequency of apneic and/or hypopneic episodes per hour of sleep. An AHI between 5 and 15 is categorized as mild OSA, between 15 and 30 as moderate OSA, and an AHI greater than 30 is considered severe sleep apnea (Epstein et al. 2009). Each apneic or hypopneic episode causes a decrease in blood-oxygen saturation, leading to reduced O_2 availability throughout the body (Lavie, Polotsky 2009).The cyclical decline in oxygen levels is termed chronic intermittent hypoxia (CIH), the underlying physiological condition experienced by sufferers of OSA (Tamisier et al. 2009).

Our studies have shown that exposure to CIH during pregnancy leads to intrauterine growth restriction (IUGR). CIH newborns were found to have lower birthweights with higher brain-to-liver weight ratios, suggesting that offspring experienced asymmetric growth restriction during gestational development (Iqbal, Ciriello 2013). In adulthood, CIH offspring were found to have significantly higher bodyweight as well as greater epididymal and retroperitoneal fat deposits (Iqbal, Ciriello 2013), which are common markers of visceral adiposity (Rueda-Clausen et al. 2011).

Additionally, adult male CIH offspring were hyperglycemic, hyperinsulinemic, and showed impaired glucose tolerance. Alteration of the maternal intrauterine environment is associated with a number of chronic, adult-onset diseases including obesity, type II diabetes, and cardiovascular disease (Desai, Ross 2011, Thorn et al. 2011, Xu et al. 2013). Furthermore, clinical and animals studies have determined a link between IUGR and functional alteration of liver metabolism leading to hypercholesterolemia in adulthood (Nusken et al. 2008, Sohi et al. 2011, Malo et al. 2013).

Recently, we have demonstrated that adult male offspring exposed to CIH during gestation have altered regulation of gluconeogenic pathways in the liver (Iqbal et al. 2013). CIH offspring have decreased hepatic protein expression of the Liver X Receptor (LXR) and increased expression of its negatively-regulated target genes glucose-6-phosphatase (G6Pase), 11beta-hydroxysteroid dehydrogenase type I (11β-HSD1), and glucocorticoid receptor (GR) (Iqbal et al. 2013). LXR, a ligand-activated transcription factor, is an important regulator of hepatic metabolic function (Schultz et al. 2000, Repa, Mangelsdorf 2000). Early studies of this nuclear receptor established the role of LXR in regulating cholesterol transport and metabolism (Peet et al. 1998). Cholesterol is an important structural component of cells throughout the body and is a precursor to a number of important metabolic and endocrine molecules, including bile acids, steroids, sterols, and vitamins (Repa, Mangelsdorf 1999). The primary method of cholesterol removal within the body is the conversion to bile acids within the liver (Makishima 2005). Through transcriptional activation of the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1), LXR promotes the conversion of cholesterol into bile acids within the liver (Cohen 1999). Following conversion, bile acids are stored in the gallbladder and

released into the intestinal tract to aid in digestion (Bahar, Stolz 1999). The conversion to bile acids is of particular importance, as this mechanism is responsible for the majority of cholesterol removal from the body (Hylemon, Stravitz & Vlahcevic 1994).

In addition to cholesterol breakdown, the liver also regulates circulating cholesterol concentrations by modulating the synthesis, influx and efflux of cholesterol from hepatocytes (Plosch et al. 2006). The primary supply of cholesterol to the body is from de novo synthesis within cells and absorption from dietary sources (Carulli, Tripodi & Carubbi 1989). De novo cholesterol synthesis within the liver accounts for nearly 50% of cholesterol produced for the body (Repa, Mangelsdorf 2000). An important target for hepatic cholesterol synthesis is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), an intermediate rate-limiting enzyme in the pathway that produces cholesterol from Acetyl-CoA (Wang et al. 1994). Cholesterol is transported throughout the circulation and supplied to target tissues by lipoproteins – large complexes of apolipoproteins, cholesterol, and triglycerides (Chan, Barrett & Watts 2004). The low-density lipoprotein receptor (LDLR) mediates the absorption of cholesterol-rich lipoproteins into hepatocytes (Brown, Goldstein 1999). Suppression of LDLR expression is known to be associated with hypercholesterolemia (Brown, Goldstein 1999). ATP-binding cassette transporter G8 (subfamily G, member 8; ABCG8) is also important for cholesterol efflux, as it has been shown to play a role in the removal of cholesterol from the body via biliary cholesterol secretion (Yu et al. 2002). Cholesterol is packaged by ATP-binding cassette transporter A1 (subfamily A, member 1; ABCA1), which is responsible for assembling and secreting high-density lipoproteins (HDL), the primary source of cholesterol in rodents (Bovenberg et al. 2010). Knockout models and agonist studies

have established a role for LXR as a regulator of ABCG8 and ABCA1 expression in the liver (Fitzgerald, Moore & Freeman 2002). Furthermore, LXR has been shown to regulate the expression of apolipoprotein E (ApoE), the primary acceptor of effluxed cholesterol during the formation of HDL particles (Tall 2008). The liver plays a critical role in managing cholesterol levels within the body, as dysregulation of cholesterol homeostasis is associated with atherosclerosis and dyslipidemia (Goldstein, Brown 1990).

Based on early indications of metabolic dysfunction in adult CIH offspring (Iqbal, Ciriello 2013) Iqbal et al. 2013), this study was done to determine the potential changes in LXR-regulated cholesterol metabolism within the liver. Further elucidating the role of LXR, a master regulator of hepatic metabolic function, will provide a greater understanding of disease processes associated with CIH and potential risks to individuals with OSA. Given that adult male CIH offspring have decreased hepatic LXR protein expression, it was hypothesized that gestational CIH exposure will cause hypercholesterolemia due to impaired regulation of hepatic cholesterol metabolism and transport by LXR.

4.3 – METHODS AND MATERIALS

4.3.1 – Animal Handling

Adult male and female Sprague-Dawley rats were obtained from Charles River Canada (Saint-Constant, QC, Canada). Animals were housed in standard rat cages within the university housing facility with *ad libitum* access to food and water. Animal

rooms were maintained at a constant temperature and humidity (23°C and 65% respectively) and kept on a 12h light/dark cycle beginning at 0700 h. All experimental procedures for this study were performed in agreement with the Care and Use of Experimental Animals by the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Western Ontario

4.3.2 – Gestational CIH Exposure

Female rats were exposed to pregnancy as previously described (Iqbal, Ciriello 2013). Briefly, adult female (250g; n = 14) rats were mated with one male per dam. Confirmation of pregnancy was done by vaginal smear the following morning, marking gestational day-0. Dams were exposed to alternating periods of hypoxic (6.5% O^2 nadir; 80s) and normoxic (room air; 120s) conditions at a frequency of 18 hypoxic episodes per hour. Pregnant females were exposed to CIH for 8h daily during the light cycle (sleep cycle; 1000h to 1800h) from gestational day-1 to day-20. Control dams were placed in an adjacent chamber during the exposure periods that continuously cycled room air. Following the final day of exposure, the dams were returned to the housing facility for an undisturbed delivery.

4.3.3 – Post-delivery

Following delivery, a subset of male offspring was sacrificed immediately for measurements at postnatal day-1. Litters were culled to four males and housed with the

mother during the lactation period (21 days). Post-weaning, male offspring were housed in pairs.

4.3.4 – Offspring Tissue Collection

Postnatal day-1 offspring were sacrificed via decapitation. Brains were extracted and frozen on dry ice. Hearts and livers were extracted and snap frozen in liquid nitrogen. Adult male offspring were fasted overnight prior to sacrifice at 6 weeks of age. Adult rats were anesthetized using equithesin (3 mL/kg, ip). Following extraction, brains were frozen on dry ice, while hearts and livers were snap frozen in liquid nitrogen. Blood samples were acquired from adult offspring at the time of sacrifice via intra-cardiac puncture with an 18 g needle. Blood was collected in 1 mL aliquots in centrifuge tubes containing 10 µL of 7% ethyldiaminetetraacetic acid (EDTA) dissolved in ddH$_2$O. Samples were then spun at 10,500 g for 15 min at 4°C for isolated of plasma. The supernatant was collected and stored at -80°C for future serological analysis.

4.3.5 – Cholesterol Measurement

Total circulating cholesterol levels were determined using Cholesterol E (Wako Diagnostics; Richmond, VA, USA), an enzymatic colorimetric assay for total cholesterol. In this assay, cholesterol is oxidized to cholest-4-en-3-one and hydrogen peroxide. The produced hydrogen peroxide causes an oxidative condensation reaction between 3,5-Dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline sodium salt (DAOS) and 4-

aminoantipyrine catalyzed by peroxidase. Absorbance of the resulting pigmented product is determined at a wavelength of 600 nm, providing a measurement of total cholesterol in the plasma sample. Absorbance readings of cholesterol assays were determined using a COBAS Mira S Analyzer (Roche Diagnostics, Laval, QC, Canada).

4.3.6 – Western Blot Analysis

Using an electric pestle, frozen liver tissue samples were homogenized in radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris-HCl, 150 mM sodium chloride, 1% TritonX-100, 1 mM EDTA, 0.2 M sodium orthovanadate, 0.1 M sodium fluoride, 0.1 M β-glycerophosphate) with a dissolved protease inhibitor cocktail tablet (Cat. #11836153001; Roche Diagnostics). Homogenized samples were incubated on ice for 15 min, followed by centrifugation at 1,000 g for 10 min at 4°C. The supernatant was collected and spun at 18,000 g for 20 min at 4°C. The supernatant was once again collected and stores at -80°C as the final protein extraction product. The Bio-Rad detergent compatible protein assay, a colorimetric assay for protein quantification, was utilized for standardization of sample protein concentrations (Cat. #500-0111; Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins were separated using SDS-PAGE on 7.5% polyacrylamide gels and transferred to polyvinylidine fluoride (PVDF) membranes (Cat. #EC60755BOX; Life Technologies, Burlington, ON, Canada) for probing. Blots were probed for CYP7A1 (1:1000; Cat. #, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ABCA1 (1:1000; Cat. #, Santa Cruz), ABCG8 (1:1000; Cat. #, Santa Cruz), Apo B100 (1:1000; Cat. #, Santa Cruz) Apo E (1:1000; Cat. #, Santa Cruz), LDLR (1:1000;

Cat. #, Santa Cruz), and HMGCR (1:1000; Cat. #, Santa Cruz). Primary antibodies were diluted in 5% powdered milk-0.05% Tris-buffered saline-Tween 20 (TBS-T) buffer. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10,000; Cat. #711035152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as a secondary antibody diluted in 5% powdered milk-0.05% TBT-T buffer. Membranes were incubated overnight in primary antibody at 4°C, while secondary antibody incubations were 1h at room temperature. Both incubations were done under constant agitation. Measured protein levels were standardized to β-actin (1:50,000; Cat. #A3854, Sigma-Aldrich, Oakville, ON, Canada).A chemiluminescence detection system was utilized for visualization of immunoreactive bands (Cat. #WBLUF0100, Millipore, Toronto, ON, Canada).

4.3.7 – Data and Statistical Analysis

All data are presented as mean ± standard deviation. Total cholesterol measurements and western blot data were compared between CIH and normoxic groups using a two-tailed Student's t-test to determine statistical significance. A minimum p-value of < 0.05 was chosen to indicate statistical significance in all comparisons (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

4.4 – RESULTS

4.4.1 – Gestational CIH exposure results in higher total circulating cholesterol levels in male offspring

Total plasma cholesterol levels were measured using a commercially available enzymatic immunoassay kit (Wako Diagnostics). At 6 weeks of age, adult male CIH offspring had significantly greater total circulating cholesterol concentrations compared to normoxic offspring (Fig. 4.1A). Similarly, CIH offspring had higher total cholesterol levels at 12 weeks of age (Fig. 4.1B).

As previously described, male offspring from mothers exposed to gestational CIH have altered LXR protein expression (Iqbal et al. 2013). Adult male CIH offspring had significantly lower hepatic LXR protein levels compared to normoxic offspring (Iqbal et al. 2013). To assess the contribution of LXR-mediated pathways to the hypercholesterolemia observed in adult CIH offspring, western blots were performed to examine protein expression of LXR target genes.
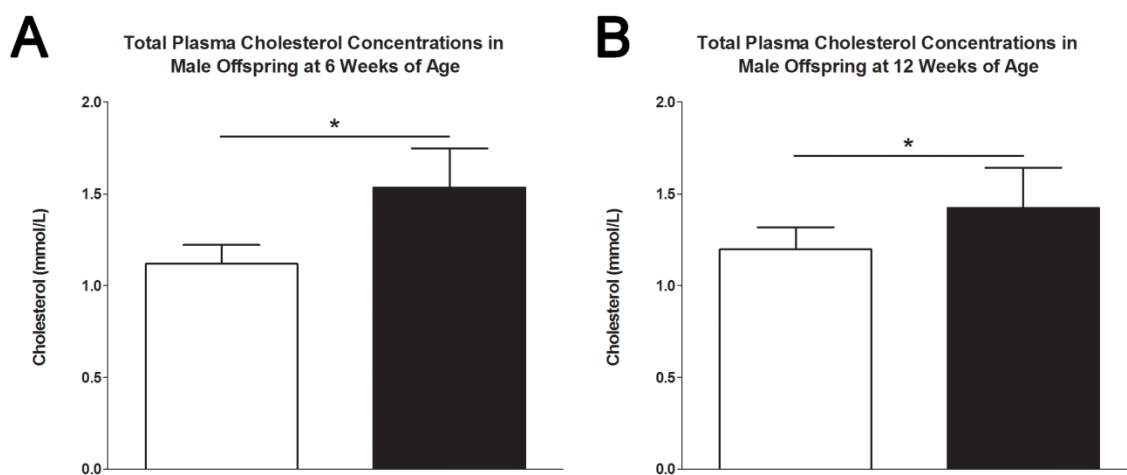
**Figure 4.1: Gestational CIH increases total circulating cholesterol levels in adult male offspring**

CIH male offspring had significantly greater circulating total cholesterol concentrations compared to normoxic offspring at both (A) 6 weeks and (B) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6
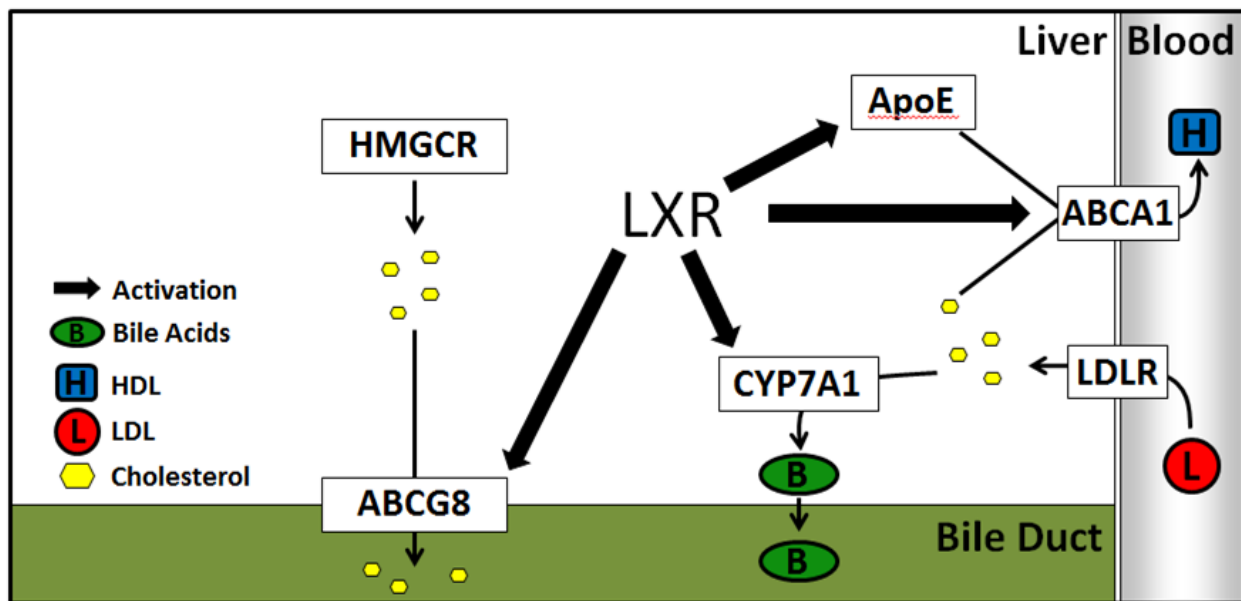
Figure 4.2: Schematic diagram of hepatic cholesterol regulation mechanisms for LXR

4.4.2 – Gestational CIH exposure results in lower hepatic protein expression of the cholesterol conversion enzyme CYP7A1 in male offspring

LXR directly influences cholesterol metabolism by transcriptionally activating CYP7A1, the rate-limiting enzyme in the conversion of cholesterol into bile acids within the liver Western blot analysis showed that male CIH offspring have significantly lower protein expression of CYP7A1 at postnatal day-1 (Fig 4.3A). Furthermore, adult male CIH offspring had lower hepatic CYP7A1 protein levels at both 6 weeks (Fig. 4.3B) and 12 weeks of age (Fig. 4.3C).
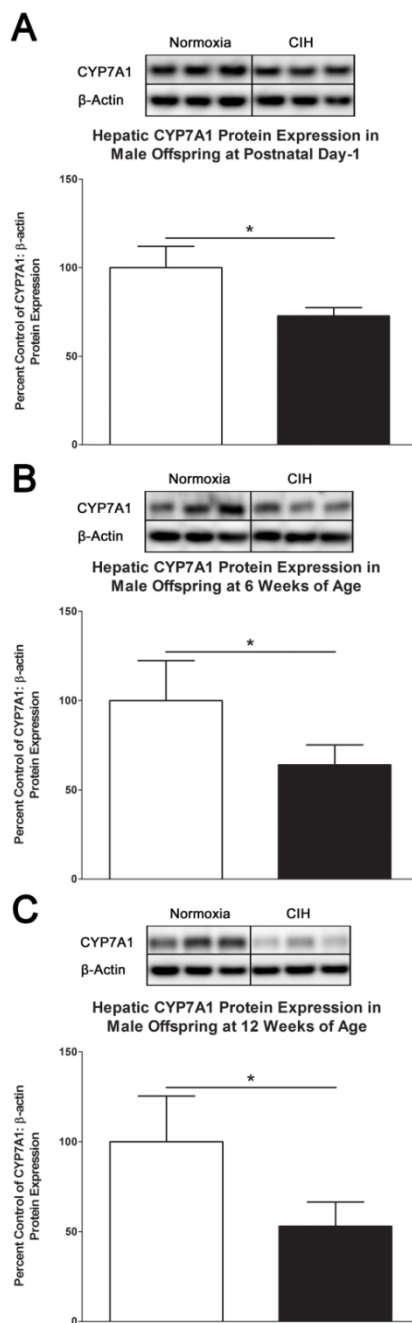
Figure 4.3: Gestational CIH exposure lowers hepatic protein expression of the LXR target gene CYP7A1

Western blot analysis of LXR target gene CYP7A1 in the livers of male offspring. CIH males had significantly lower hepatic CYP7A1 protein expression compared to normoxic offspring at (A) postnatal day-1, (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6

4.4.3 – Gestational CIH exposure lowers hepatic protein expression of cholesterol excretion and efflux markers in male offspring

LXR has been shown to positively regulate expression of ABCG8 to promote biliary cholesterol excretion. Furthermore, LXR promotes packaging of cholesterol into HDL via increased expression of ABCA1 and ApoE. At postnatal day-1, ABCG8 protein expression was not different between groups (4.4A). At 6 weeks of age, adult male CIH offspring were found to have lower hepatic protein expression of ABCG8 (Fig. 4.4B). CIH offspring also had lower hepatic ABCG8 protein expression at 12 weeks of age (Fig. 4.4C). No differences in ABCA1 protein expression were observed at postnatal day-1 (Fig 4.5A). At both 6 weeks and 12 weeks of age, adult male CIH offspring had significantly lower hepatic protein expression of ABCA1 (Fig. 4.5B and 4.5C, respectively). Correspondingly, CIH offspring showed no differences in ApoE protein levels at postnatal day-1 (Fig 4.6A), but had lower protein expression of ApoE at both 6 weeks (Fig. 4.6B) and 12 weeks of age (Fig. 4.6C).
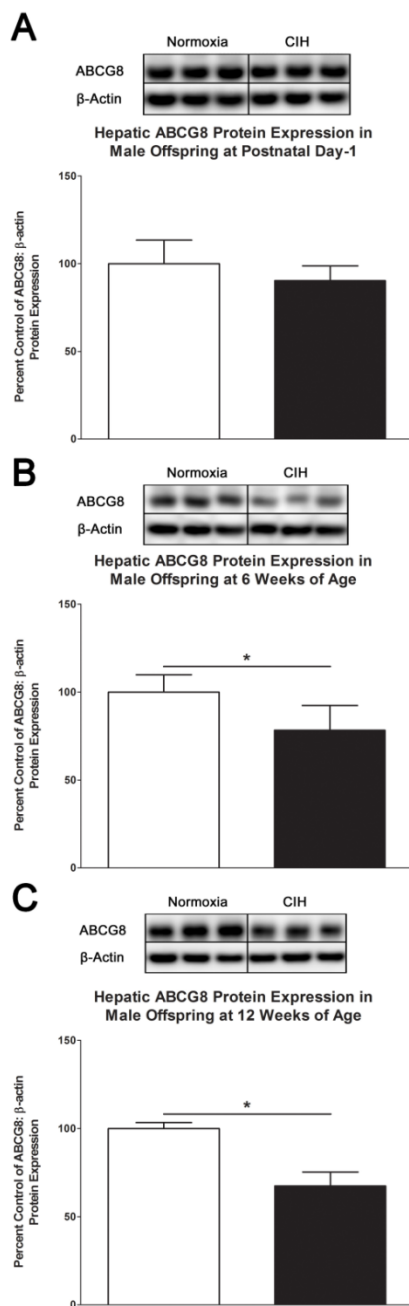
Figure 4.4: Gestational CIH exposure lowers hepatic protein expression of the LXR target gene ABCG8

Western blot analysis of LXR target gene ABCG8 in the livers of male offspring. (A) Protein levels of ABCG8 were not different at postnatal day-1. CIH male offspring had significantly lower hepatic ABCG8 protein expression at both (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6
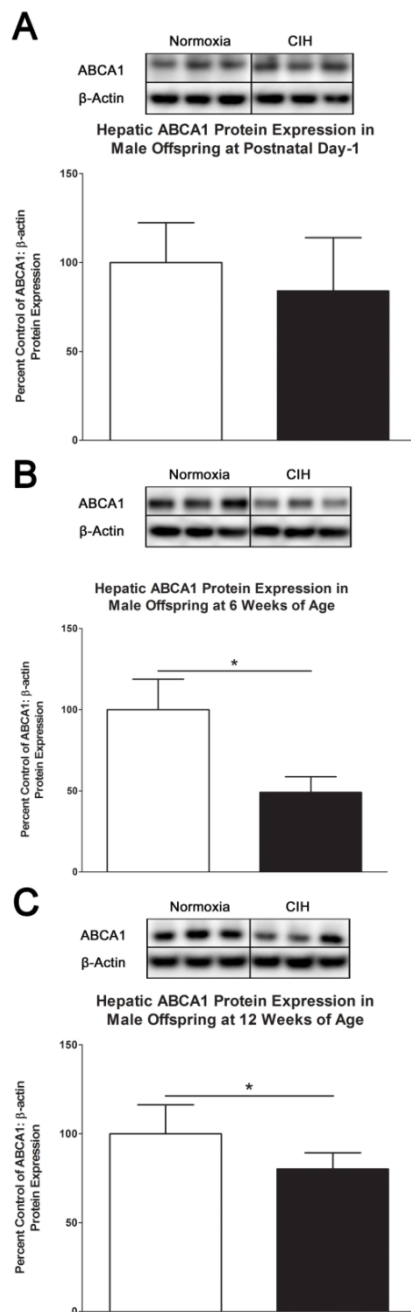
Figure 4.5: Adult male CIH offspring have lower hepatic ABCA1 protein expression

Western blot analysis of LXR target gene ABCA1 In the livers of male offspring. (A) Protein levels of ABCA1 were not different at postnatal day-1. CIH male offspring had significantly lower hepatic ABCA1 protein expression at both (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * p < 0.05. CIH: n = 6; Normoxia: n = 6
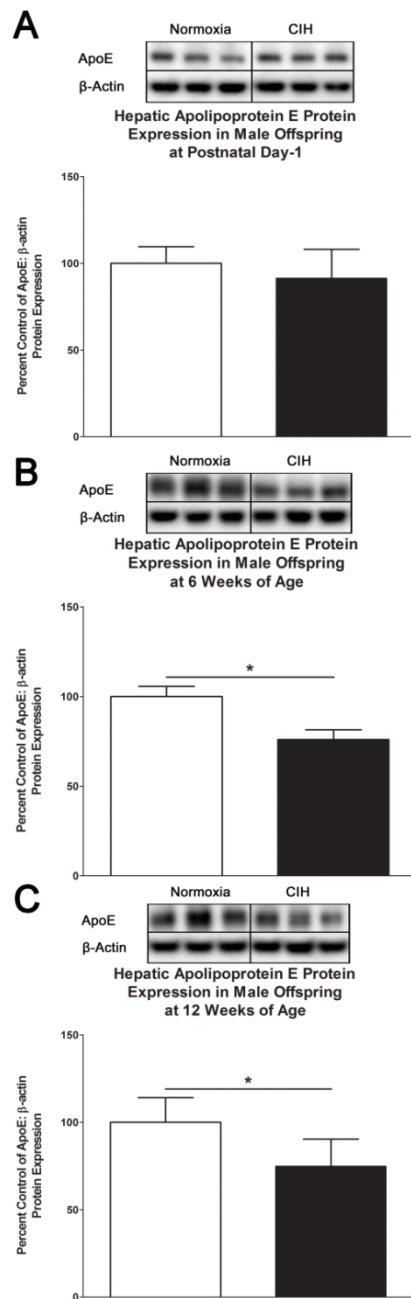
Figure 4.6: Male CIH offspring have lower hepatic ApoE protein expression in adulthood

Western blot analysis of LXR target gene ApoE in the livers of male offspring. (A) Protein levels of ApoE were not different at postnatal day-1. CIH male offspring had significantly lower hepatic ApoE protein expression at both (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6

4.4.4 – Gestational CIH exposure lowers LDLR protein expression in adult male offspring

To determine potential changes to the internalization of circulating cholesterol by the liver, protein levels of LDLR were measured. LDLR protein expression was not different between CIH and normoxic offspring at postnatal day-1 (Fig 4.7A). Adult male CIH offspring were found to have significantly lower hepatic protein expression of LDLR at 6 weeks (Fig. 4.7B) as well as 12 weeks of age (Fig. 4.7C).

4.4.5 - Gestational CIH exposure does not alter hepatic cholesterol synthesis

To assess the endogenous production of cholesterol within the liver, we measured the protein levels of HMGCR, the rate-limiting enzyme in hepatic cholesterol synthesis (Wang et al. 1994). No differences in hepatic HMGCR protein expression were observed between CIH and normoxic offspring in any age group (Fig. 4.8A-C).

Figure 4.7: Gestational CIH exposure lowers LDLR protein expression in the livers of adult male offspring

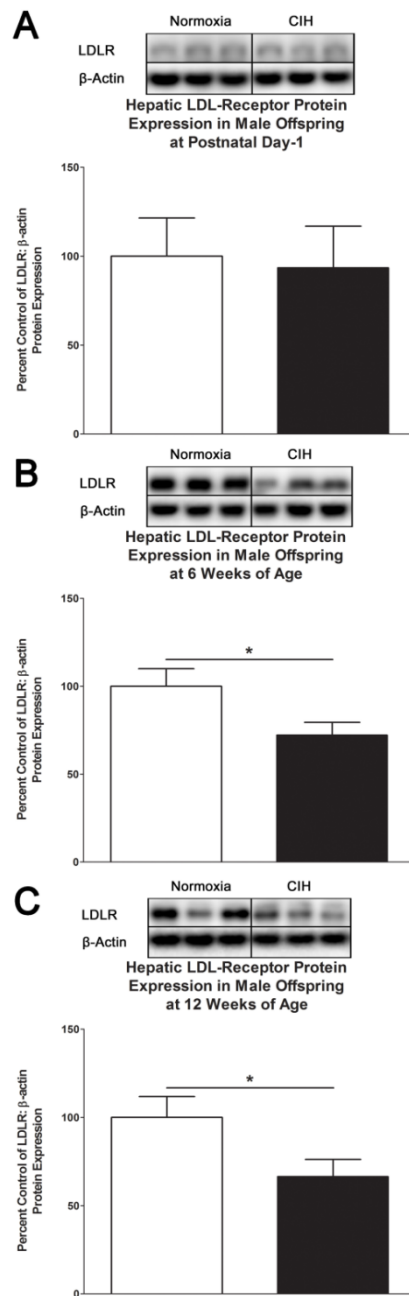Western blot analysis of the LDL-receptor in the livers of male offspring. (A) Protein levels of LDLR were not different at postnatal day-1. CIH male offspring had significantly lower hepatic LDLR protein expression at both (B) 6 weeks and (C) 12 weeks of age. Data are presented as mean ± standard deviation. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6
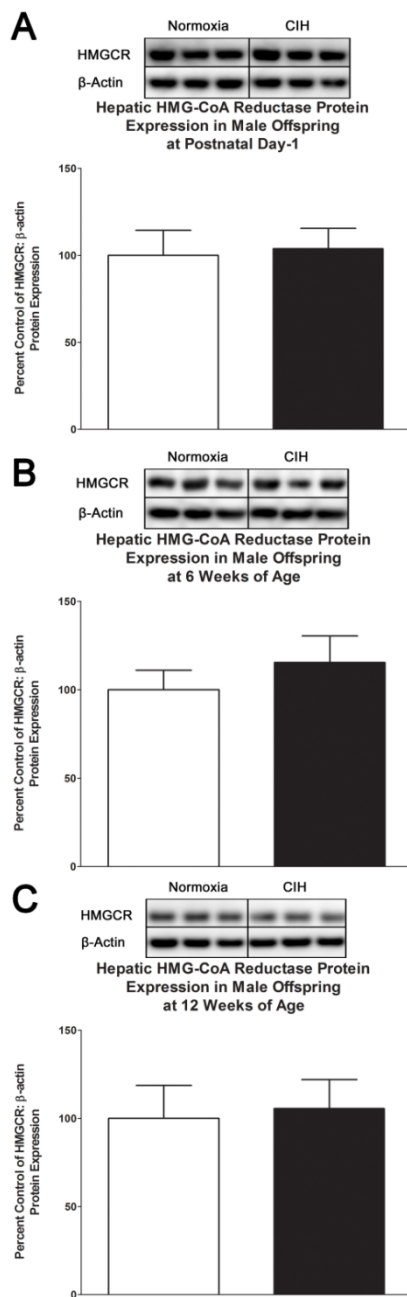
Figure 4.8: Gestational CIH exposure does not alter hepatic HMGCR protein expression

Western blot analysis of the HMG-CoA Reductase in the livers of male offspring. No differences in hepatic HMGCR protein expression were observed between CIH and normoxic offspring in any age group. * $p < 0.05$. CIH: n = 6; Normoxia: n = 6

4.5 – DISCUSSION

Our recent studies have shown that exposure to CIH during pregnancy causes IUGR, resulting in low birthweight offspring with significantly lower liver weights (Iqbal, Ciriello 2013). Newborn and adult male CIH offspring were found to have lower hepatic LXR protein expression compared to normoxic offspring, as well as higher G6Pase, 11β-HSD1, and GR protein expression, providing mechanistic evidence for the hyperglycemia and impaired glucose tolerance observed in CIH offspring (Iqbal et al. 2013). The findings of this study on gestational CIH exposure provide additional understanding of how a hypoxic insult during pregnancy can impact offspring physiology and have functional consequences on LXR-regulated metabolic pathways in the liver.

Adult male offspring from mothers exposed to CIH during gestation were found to have significantly higher total circulating cholesterol concentrations compared to normoxic offspring. Corresponding with the previously reported differences in hepatic LXR protein expression, CIH offspring had lower protein expression of CYP7A1, the rate-limiting enzyme in the conversion of cholesterol into bile acids within the liver (Cohen 1999). Adult CIH offspring were also found to have significantly lower hepatic protein expression of LXR target genes ABCG8, ABCA1, and ApoE. Additionally, CIH offspring were found to have lower hepatic protein levels of LDLR compared to normoxic offspring, while no changes were observed in HMGCR expression. Examining protein expression in newborn rats, CIH offspring had lower hepatic CYP7A1 protein expression. No differences in ABCG8, ABCA1, ApoE, LDLR, or HMGCR protein expression were observed between groups at postnatal day-1.

LXR plays a critical role in cholesterol homeostasis. As a transcription factor that controls cholesterol transport and metabolism within the liver, LXR is able to regulate to circulating levels of cholesterol within the body (Repa, Mangelsdorf 2000). Studies investigating the role of LXR in cholesterol homeostasis have demonstrated that LXR increases the expression and activity of CYP7A1 to promote the metabolism of cholesterol in the liver through the neutral bile acid synthesis pathway (Peet et al. 1998). Similar findings have also been demonstrated by agonist studies, which have shown increases in CYP7A1 activity and expression, as well as the corresponding increase in bile acid excretion following administration of the synthetic LXR agonist T0901317 (Menke et al. 2002, Sato, Kamada 2011). Furthermore, rodent models have shown an association between the loss of LXR function and cholesterol homeostasis, as evidences by elevated circulating cholesterol levels in LXR null mice (Peet et al. 1998). As conversion to bile acids accounts for a significant proportion of cholesterol metabolism within the body, the decreased protein expression of LXR and its target gene CYP7A1 are likely major contributing factors to the higher cholesterol levels observed in adult male CIH offspring.

Biliary cholesterol secretion is a hepatic mechanism that facilitates the elimination of cholesterol from the body via excretion from gastrointestinal system (Dikkers, Tietge 2010). ABCG8, along with ABCG5, has been shown to mediate the removal of cholesterol from hepatocytes via biliary cholesterol secretion (Yu et al. 2002). Studies examining cholesterol efflux have revealed that overexpression of ABCG8 dramatically increases biliary cholesterol secretion, ultimately leading to greater excretion of cholesterol in fecal waste (Yu et al. 2002). Additionally, mutations in ABCG

transporters are associated retention of cholesterol and higher circulating levels of LDL (Su et al. 2012). Lower hepatic ABCG8 protein expression would presumably result in lower rates of secretion, leading to retention of cholesterol and contribute to the higher circulating cholesterol levels measured in adult male CIH offspring. Hypercholesterolemia and subsequent accumulation of cholesterol within the liver has been associated with the onset of non-alcoholic fatty liver disease (NAFLD) (Subramanian et al. 2011a). Interestingly, ABCG transporters have been shown to prevent the development of NAFLD through their promotion of biliary cholesterol secretion (Van Rooyen et al. 2011). LXR is a direct transcriptional regulator of ABCG8 expression in the liver (Repa, Dietschy & Turley 2002). Remarkably, agonist studies have shown that administration of the synthetic LXR agonist T0901317 causes a reduction in hepatic cholesterol levels of wild-type rodents, but does not decrease cholesterol levels in ABCG transporter knockout mice (Yu et al. 2003). These findings further highlight the importance of biliary cholesterol secretion as a mechanism for cholesterol regulation. The lower hepatic LXR protein expression and associated lower ABCG8 protein expression observed in CIH offspring likely limit the ability of the liver to eliminate cholesterol through gastrointestinal excretion. Indirectly, the decreased protein expression of CYP7A1 and ABCG8 may also be contributing to the glucose dyshomeostasis that we have previously reported in CIH offspring (Iqbal, Ciriello 2013) Iqbal et al. 2013). Elevations in cholesterol have been shown to initiate the unfolded protein response (also referred to as the ER stress response) (Devries-Seimon et al. 2005), leading to the development of NAFLD and insulin resistance (Malhi, Kaufman 2011).

Activation of LXR has been shown to increase expression of ABCA1 (Higham et al. 2013) as well as ApoE (Namjoshi et al. 2013). Activity of these hepatic markers is associated with efflux of HDL particles from hepatocytes (Tall 2008). Several studies have demonstrated that mutation of the ABCA1 transporter leads to reductions in circulating HDL levels (Kim et al. 2011, Fasano et al. 2012). LXR regulates cholesterol levels within hepatocytes by promoting the packaging of cholesterol with ApoE, a process that is achieved via the function of ABCA1 (Suon et al. 2010). The newly constructed particle is then released in the form of HDL, often referred to as "good cholesterol" (Krieger 1999). Male CIH offspring show higher total circulating cholesterol levels in adulthood. The decreased hepatic protein expression of ABCA1 and ApoE suggest that the greater cholesterol concentrations are a result of higher LDL levels in the blood, often referred to as "bad cholesterol".

This hypothesis is supported by the lower ABCG8 protein expression in CIH offspring, which has previously been associated with higher circulating concentrations of LDL (Su et al. 2012). Additional support for this premise is derived from the differences in LDLR protein expression, as male CIH offspring had significantly lower hepatic LDLR levels compared to normoxic offspring. We did not observe any changes in HMGCR protein expression. While some studies have suggested that HMGCR is downregulated in response to elevations in cholesterol (Wang et al. 1994), the lack of a change is not entirely surprising as the suppression of HMGCR expression has been shown to be mediated by the activity of LDLR, which was found to be lower in adult male CIH offspring. The role of LDLR in cholesterol homeostasis is well established, acting to regulate cholesterol levels by binding LDL in the circulation and absorbing the particles

into hepatocytes via endocytosis mechanisms (Goldstein, Brown 2009). Lower hepatic LDLR protein expression suggests that male CIH offspring likely have a limited ability to regulate circulating LDL levels, further contributing to the hypercholesterolemia observed in adulthood.

This study has provided further evidence of compromised hepatic function due to an insult during pregnancy. The presented findings provide the first indication of deficiencies in LXR-mediated metabolism and transport in adult offspring caused by gestational exposure to CIH, the underlying physiological condition of OSA. Taken together, these data suggest that alteration of the maternal intrauterine environment during pregnancy can have severe long-term consequences on liver function, resulting in impaired homeostatic regulation of cholesterol.

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Chapter 5

Summary and Future Directions

The studies outlined in this thesis were designed with the goal of establishing a rodent model to investigate the developmental origins of metabolic disease following fetal growth restriction associated with maternal OSA. There have been a number of studies linking OSA with cardiovascular and metabolic comorbidities in adulthood (Hamilton, Naughton 2013, Kim et al. 2013, Vrints et al. 2013). However, foundation of evidence related to the impact of OSA on pregnancy and fetal outcomes is very limited. Clinical studies investigating the relationship between this breathing disorder and fetal development have thus far focussed on early-life outcomes and characterizing relative differences in neonatal growth (Sahin et al. 2008, Bobrowski 2010, Champagne et al. 2010). In essence, these early studies have demonstrated that maternal OSA leads to fetal growth restriction. However, the fundamentals of fetal programming are based in the idea that adverse events in utero not only affect early-life outcomes, but also the continuing health of offspring. Thus, the objectives of this thesis were to establish an animal model for investigating the physiological consequences of OSA-related insult during gestation on the long-term well-being of offspring.

While there have been previous investigations into the consequences of decreased oxygen availability during pregnancy, these studies have primarily focussed on the outcomes associated with chronic hypoxia exposure (Rueda-Clausen, Morton & Davidge 2009, Bourque et al. 2013) or permanently lowered blood supply through arterial ligation (Lueder, Ogata 1990, Ernst et al. 1993). Though these models provide a wealth of information regarding the consequences on fetal outcome following reduced oxygen supply and placental insufficiency, we believed that a persistent insult would not accurately reflect the conditions expected in OSA. As such, we utilized a CIH paradigm

to mimic the changes in oxygen availability during OSA, which have been clinically demonstrated to be more transient in nature, rather than a chronic insult.

5.1 – SUMMARY OF FINDINGS

5.1.1 – Gestational CIH Exposure and Offspring Growth

The primary objective of this thesis was to characterize the phenotype of rat offspring following exposure to CIH during gestation. Before examining any potential metabolic outcomes, we first sought to track the growth of offspring at the level of the organs and body as a whole. Female Sprague-Dawley rats were mated and exposed to CIH during the first 20 days of gestation. Dams were exposed for 8 hours per day during the rat sleep cycle at a frequency of 18 hypoxic episodes per hour, which would be categorized as a moderate sleep apnea according to the AHI. Following delivery, offspring measurements were recorded at postnatal day-1, 6 weeks of age, and 12 weeks of age. Parallel to observations from clinical studies examining OSA during pregnancy, offspring from mothers exposed to CIH during pregnancy were found to have significantly lower birthweights. Specifically, CIH offspring had greater heart-to-body and brain-to-body weight ratios as well as lower liver-to-body weight ratios. Along with the significantly higher brain-to-liver weight ratios in CIH offspring, these observations collectively suggest that gestational CIH exposure results in asymmetrical growth restriction. At 6 weeks of age, male CIH offspring still had lower body weights compared to normoxic offspring. Despite this, CIH offspring had greater amounts of epididymal and retroperitoneal fat deposition (standardized to body weight), common indices of overall visceral adiposity. Standardized organ weights were not different

between groups for the heart, liver, and brain. At 12 weeks of age, male CIH offspring had higher overall body weights as well as greater amounts of epididymal and retroperitoneal fat deposits. Interestingly, the livers of CIH offspring, which were found to have lower weights at birth, now had significantly higher weights than the livers of normoxic offspring (standardized to body weight).

5.1.2 – Glucose Homeostasis in Adult Offspring

Western blots and enzymatic immunoassays were utilized for the assessment of factors associated with glucose homeostasis. Blood samples were collected from fasted animals for serological measurement of circulating factors. Fasting blood glucose measurements taken at the time of sacrifice revealed that male CIH offspring had significantly higher blood glucose concentrations compared to normoxic offspring at both 6 weeks and 12 weeks of age. Plasma insulin concentrations, as measured by enzymatic immunoassay, were not different between groups at 6 weeks of age. Interestingly, CIH offspring had nearly 3-fold higher plasma insulin concentrations than normoxic offspring at 12 weeks of age. Furthermore, circulating corticosterone levels were significantly greater in adult male CIH offspring, while no differences in hepatic corticosterone concentrations were observed between groups. Intraperitoneal glucose tolerance tests were performed in offspring at 6 weeks and 12 weeks of age. Glucose tolerance in CIH offspring was similar to that of normoxic offspring at 6 weeks. CIH offspring at 12 weeks of age showed impaired short-term glucose tolerance, as evidenced by the higher blood glucose levels at the early time points, but were able to respond to the glucose load at maximal levels. The hyperglycemia coupled with

hyperinsulinemia suggests a potential resistance to the effects of insulin in CIH offspring. Taken together with the glucose tolerance test data, the results signify that the effects of insulin are diminished at basal levels and in response to small increases in blood glucose. However, the response during maximal glucose levels and effective clearance in the latter half of the glucose tolerance tests show that CIH offspring are still able to respond when challenged by very high blood glucose levels. These results indicate that, at 12 weeks of age, male CIH offspring have altered sensitivity to insulin as well as a higher blood glucose threshold to initiate effective clearance from the circulation.

Based on observed changes in blood glucose, insulin, and corticosterone concentrations, we focussed our attention on the liver, an organ known to play a critical role in the regulation of all three circulating factors. Our primary target of interest was the Liver X Receptor (LXR), a master regulator of the liver's role in metabolism. LXR had previously been shown to regulate glucose homeostasis by transcriptionally suppressing the expression of several gluconeogenic markers in the liver. Thus, we used western blots to assess the expression levels of LXR in the liver as well as its downstream targets involved in glucose homeostasis. Adult male CIH offspring were found to have lower LXR protein expression in newborn and adult offspring. Corresponding with these lower LXR levels, CIH offspring had greater hepatic protein expression of the gluconeogenic marker glucose-6-phosphatase. LXR had also previously been shown to suppress the expression of 11β-hydroxysteroid dehydrogenase type I and the glucocorticoid receptor, two essential components of glucocorticoid signalling. As expected with the lower LXR expression, CIH offspring

showed higher hepatic protein levels of 11β-HSD1 and glucocorticoid receptor. The changes in glucocorticoid signalling were significant, as corticosterone had previously been shown to increase gluconeogenesis by activating glucose-6-phosphatase. The observed pattern of protein expression was consistent with the findings of previous models of fetal growth restriction and highlighted the importance of hepatic LXR-mediated pathways in the regulation of glucose levels. The hyperglycemia, hyperinsulinemia, higher circulating corticosterone concentrations, and higher expression of gluconeogenesis promoting markers in the livers of adult CIH offspring all suggest that exposure to CIH during gestation results in glucose dyshomeostasis.

5.1.3 – Cholesterol Homeostasis in Adult Offspring

Based on the observed changes in hepatic LXR protein expression, we investigated the potential impact of gestational CIH on cholesterol levels. This follow up study was based on the idea that the originally identified function of LXR was that of regulating cholesterol homeostasis in the liver. Enzymatic immunoassays were used to measure the total circulating cholesterol concentrations in offspring at 6 weeks and 12 weeks of age. Adult male CIH offspring were found to have significantly higher total cholesterol levels than normoxic offspring in both age groups. As we had already observed changes in LXR protein levels, we expanded on our previous findings to investigate LXR-mediated mechanisms involved in hepatic cholesterol regulation. Our first target of interest was cholesterol 7α-hydroxlyase (CYP7A1), the expression of which is increased following transcriptional activation by LXR. CYP7A1 is a well-characterized hepatic marker that functions as the rate-limiting enzyme in the

conversion of cholesterol into bile acids, a mechanism responsible for the majority of cholesterol removal within the body. Corresponding with the decreased LXR protein expression, adult male CIH offspring were found to have significantly lower CYP7A1 protein levels. This result strengthened the idea that reduced LXR expression was an important contributor to the metabolic dysfunction we were seeing in CIH offspring.

We next examined the hepatic markers associated with cholesterol transport and excretion. Adult CIH offspring were found to have lower protein expression of ABCG8, a transporter critical for removal of cholesterol from the body via biliary cholesterol secretion. This expression corresponded with the lower LXR protein levels, as LXR has previously been shown to transcriptionally activate ABCG8. The activation of ABCA1 and ApoE by LXR had also been previously demonstrated, describing a mechanism for cholesterol removal from hepatocytes through packaging and release of HDL particles. The lower hepatic protein expression of ABCA1 and ApoE indicate that the hypercholesterolemia observed in adult CIH offspring is not likely a result of higher HDL production. The role of the LDL-receptor in mediating circulating cholesterol levels has been well-established, as over 1000 mutations of this receptor have thus far been identified as a contributor to familiar hypercholesterolemia. The LDL-receptor functions to regulate circulating cholesterol levels by internalizing LDL particles from the blood into hepatocytes. Adult CIH offspring had lower hepatic LDL-receptor expression compared to normoxic offspring. Taken together with the expression changes in ABCA1 and ApoE, these results suggest that the hypercholesterolemia observed in CIH offspring likely results from accumulation of LDL particles within the circulation, and not HDL particles.

Female Sprague-Dawley rats were exposed to chronic intermittent hypoxia during pregnancy from gestational day-1 to day-20
It was hypothesized that gestational CIH exposure would cause functional changes in offspring, leading to long-term metabolic consequences

Male offspring from mothers exposed to CIH during pregnancy were found to have:

Objective #1	**Objective #2**	**Objective #3**
Newborn offspring ↓ Birth weight ↑ Heart weights ↑ Brain weights ↓ Liver weights ↑ Brain-to-liver weight ratio **Adult offspring** ↑ Body weight ↑ Epididymal fat ↑ Retroperitoneal fat ↑ fasting blood glucose ↑ plasma insulin Impaired early-stage glucose tolerance in adulthood	↓ LXR protein expression **Hepatic markers of gluconeogenesis** ↑ G6Pase expression ↔ PEPCK expression **Hepatic markers of glucocorticoid signalling** ↑ 11β-HSD1 expression ↑ GR expression **Circulating factors** ↑ Corticosterone levels ↑ Total triglyceride levels	**Circulating factors** ↑ Total cholesterol levels **Cholesterol conversion to bile acids** ↓ CYP7A1 expression **Cholesterol excretion** ↓ ABCG8 expression **HDL production/release** ↓ ABCA1 expression ↓ ApoE expression **LDL uptake** ↓ LDL-receptor expression **Cholesterol synthesis** ↔ HMGCR reductase expression

Table 5.1: Table highlighting the findings of this thesis, as described in chapters 2 through 4

5.2 – MALE VERSUS FEMALE OFFSPRING

The studies described in this thesis assessed the physiological outcomes of male offspring following gestational CIH exposure. We decided to focus specifically on male offspring based on previous suggestions of sexually dimorphic responses to intrauterine growth restriction (Mueller, Bale 2008, Alexander 2009, Duffield et al. 2009, Moritz et al. 2010, Katkhuda et al. 2012). In recent years, a number of studies have demonstrated that males and female respond differently to in utero insults, based on observed differences in postnatal development, endocrine function, as well as cardiovascular and metabolic outcomes (Vickers et al. 2011, Fournier et al. 2013, Gray et al. 2013, Kundakovic et al. 2013, Wadley et al. 2013). Fetal growth restriction models investigating metabolic health in rat offspring have shown sex-specific programming effects, including impaired glucose tolerance, increased visceral adiposity and hypercholesterolemia observed only in male offspring (Guan et al. 2005, Chamson-Reig et al. 2009, Sohi et al. 2011). Interestingly, studies utilizing the aromatase knockout model, which is unable to produce estrogen, have shown hypercholesterolemia in female offspring (Hewitt et al. 2003), supporting previous suggestions of a protective role for estrogen in male and female adult health (Cho et al. 2003, Wang et al. 2010, Bernelot Moens et al. 2012, Xue, Johnson & Hay 2013). In the first study, we measured fasting blood glucose concentrations in female offspring at 6 weeks and 12 weeks of age and found no differences between CIH offspring and normoxic offspring. Based on the findings of numerous previous studies assessing metabolic health in male and female offspring, as well as our measurement of blood glucose levels in female offspring, we decided to focus specifically on the metabolic outcomes of male offspring.

5.3 – FUTURE DIRECTIONS

The findings of chapters 2 – 4 improve our understanding of the relationship between in utero insults and the risk of metabolic diseases in adulthood. The described mechanisms in chapters 3 and 4 provide insight to the alterations in liver function that contribute to glucose and cholesterol dyshomeostasis. While a number of exciting observations have been reported, there are many questions that remain to be addressed.

The first important avenue of research relates to the mechanisms affecting gene expression in the liver. As observed in male offspring, there are permanent, long-term changes in important hepatic regulatory markers, in particular LXR. It would be advantageous to investigate the mechanisms responsible for altering the expression of hepatic genes involved in glucose and cholesterol homeostasis. It has been previously reported that epigenetic modification of DNA structures can play an important role in mediating long-term changes in gene expression (Jaenisch, Bird 2003). Epigenetics relates to control of transcriptional activity to dictate patterns of gene expression without altering the gene sequence. Common examples of epigenetic modification include DNA methylation, which represses gene expression, and DNA acetylation, which promotes gene expression (Wolffe, Matzke 1999). The first of two major sites of epigenetic modification is CpG domains in the promoter region, which regulates access of transcription factors to the promoter of target genes. The second major site is histones which, through epigenetic modification, can alter to chromatin structure to permit (acetylation) or restrict (methylation) access of transcriptional machinery to the genes of interest (Jaenisch, Bird 2003, Gicquel, El-Osta & Le Bouc 2008). Previous reports have

described the link between adaptive epigenetic mechanisms during fetal development and the onset of cardiovascular and metabolic diseases in adulthood (Gluckman et al. 2009b, Pinney, Simmons 2010). Furthermore, adverse in utero events resulting in epigenetic modification of hepatic genes have previously been described for the glucocorticoid receptor (Begum et al. 2013) and CYP7A1 (Sohi et al. 2011), both of which had altered expression in CIH offspring. Studying the role of epigenetics in mediating the long-term changes in hepatic gene expression would certainly provide important information related to mechanistic changes occurring at the time of the gestational insult.

A number of epidemiological and animal studies have described an association between abnormal fetal development and cardiovascular disease in adulthood (Barker, Osmond & Law 1989b, Huxley, Shiell & Law 2000, McMillen, Robinson 2005, Morrison 2008, Watkins et al. 2010). In a preliminary study, we examined the impact of gestational CIH-induced IUGR on cardiovascular health in adult offspring. Blood pressure was recorded in male CIH and normoxic offspring at 12 weeks of age. Measurements were made using a non-invasive tail cuff recording device (CODA Non-invasive blood pressure system, Kent Scientific Corporation, Torrington, CT, USA). A minimum of 40 cycles were measured in each rat during each recording session, with every cycle measuring systolic, diastolic, and mean arterial pressure, as well as heart rate. No significant differences were observed between groups for any of the measured cardiovascular parameters. There are two potential approaches based on these initial findings. The first would be to follow-up with subsequent blood pressure recordings later on in life. The argument for this study would be that changes in cardiovascular health

may not manifest as early as 12 weeks of age. This is not inconceivable as cardiovascular events are often precipitated by metabolic dysfunction, which is progressively worsening in the CIH offspring between 6 weeks and 12 weeks of age. Based on the changes in metabolic health that have been observed, there may be the potential for cardiovascular impairments later on in the life of the rat. The other potential approach would be to assess the susceptibility to cardiovascular events in the CIH offspring. It has previously been suggested that the introduction of a second physiological insult postnatally (the first insult occurring during gestational development – protein restriction, blood flow reduction, CIH, etc.) can greatly increase the risk of cardiovascular pathophysiological outcomes. A commonly utilized postnatal insult for fetal growth restriction models is the high-fat diet (Mela et al. 2012, Claycombe et al. 2013, Su et al. 2013). Though we have not observed any differences in blood pressure or heart rate as of yet, there are certainly many questions regarding cardiovascular function that should be addressed.
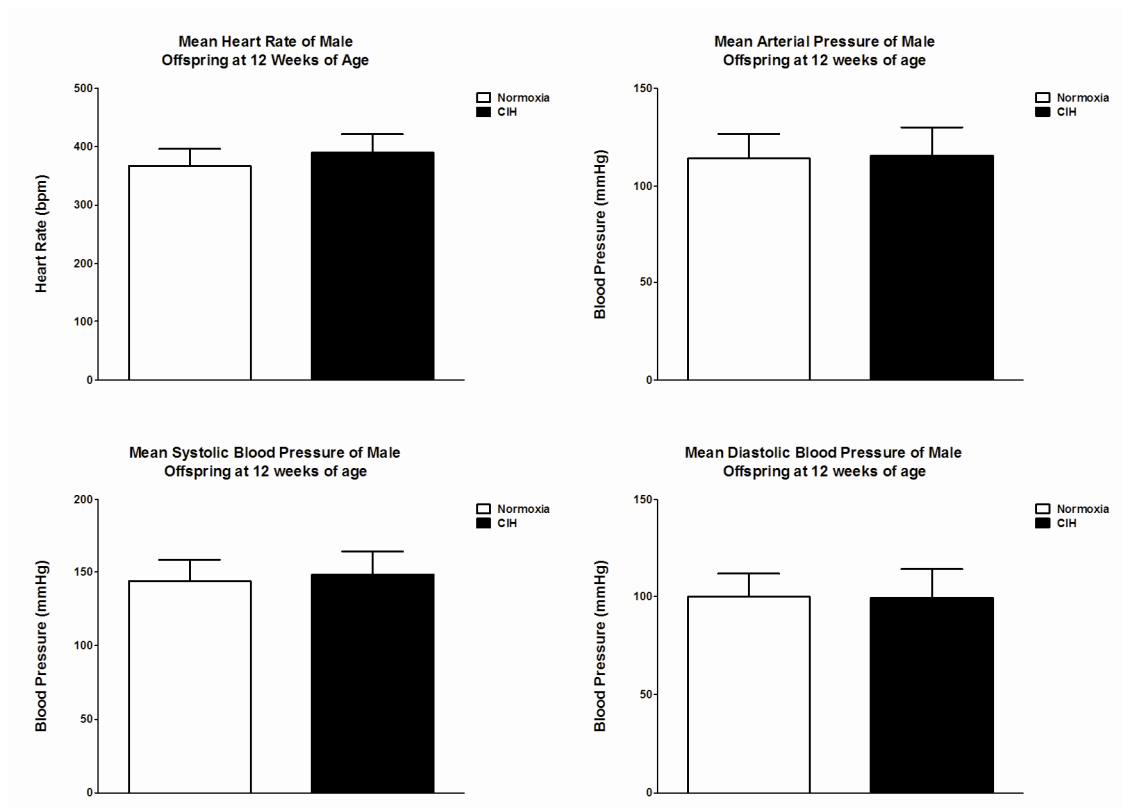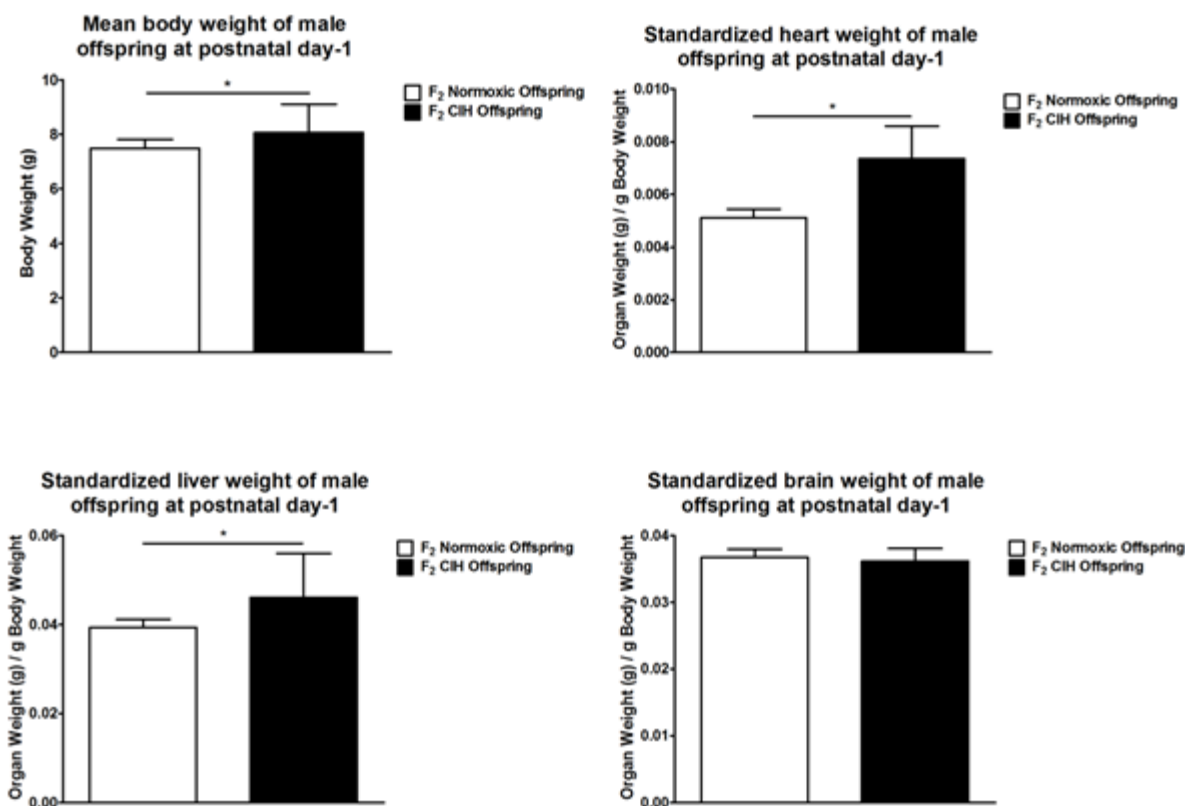
Figure 5.1: Gestational CIH offspring do not show cardiovascular consequences at 12-weeks of age

Blood pressure was measured in male offspring at 12-weeks of age using a non-invasive tail-cuff method. No differences were observed between normoxic and CIH offspring in mean arterial pressure, systolic blood pressure, diastolic blood pressure, or heart rate. CIH: n=8; Normoxia; n=6

Another interesting area of research would be to investigate the heritability of altered hepatic function. It has been suggested that the programming effects observed in offspring following gestational insult may not only have severe consequences in that generation, but also subsequent generations. Epidemiological studies indicate that abnormal fetal programming may have an intergenerational effect (Emanuel et al. 1992, Hennessy, Alberman 1998, Collins, Wu & David 2002). Furthermore, animal models of fetal programming have shown multigenerational effects related to offspring growth, endocrine function, and metabolism (Pinto, Shetty 1995, Francis et al. 1999, Martin et al. 2000). Epigenetic modification has been implicated as a mediator of intergenerational effects stemming from an initial in utero insult (Roemer et al. 1997, Morgan et al. 1999, Reik et al. 2003), resulting in the multigenerational inheritance of cardiovascular and metabolic diseases (Kaati, Bygren & Edvinsson 2002, Pembrey 2002). Preliminary results from our model suggest that impairments in glucose regulation may in fact be transmitted to subsequent generations after gestational CIH insult. Following exposure during pregnancy, male and female CIH offspring (F1 generation) from different litters were mated upon reaching adulthood. Similarly, F1 males and female from normoxic mothers were mated. For the second round of pregnancy (F1 mothers), dams were not exposed to CIH. At 12 weeks of age, male F2 offspring from gestational CIH parents were found to have significantly higher body weights, retroperitoneal fat deposition, and greater fasting blood glucose levels compared to F2 offspring from normoxic parents. These initial results indicate that a single adverse pregnancy may impact the metabolic health of multiple subsequent generations. It would be interesting to examine the metabolic profile of these F2

offspring to determine the mechanisms responsible for the altered glucose and lipid regulation.

Figure 5.2: Second generation pups from gestational CIH offspring have higher birth weights

CIH male and CIH female offspring were mated in adulthood. Following an undisturbed pregnancy, F_2 generation offspring were found to have significantly higher mean body weights at postnatal day-1. Furthermore, F_2 CIH male offspring had greater heart-to-body weight ratios and liver-to-body weight ratios. Brain-to-body weight ratios were not different between groups. * $p < 0.05$. CIH: n=6; Normoxia: n=6
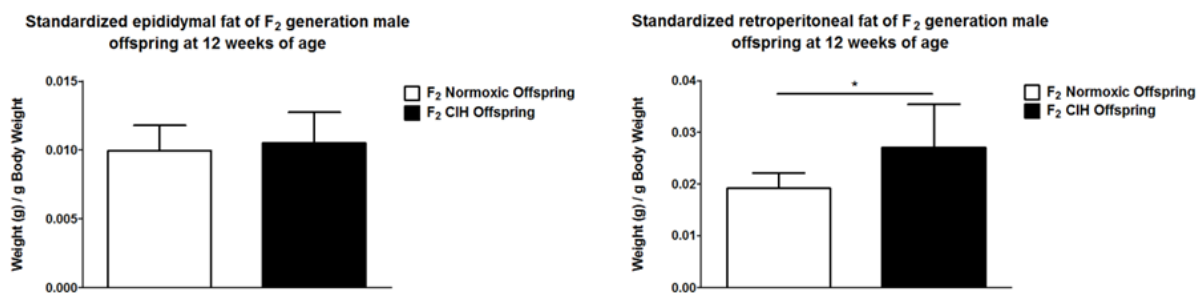
Figure 5.3: Second generation pups from gestational CIH offspring have higher body weights in adulthood

CIH male and CIH female offspring were mated in adulthood. At 12-weeks of age, F_2 generation offspring were found to have significantly higher mean body weights compared to F_2 generation pups from normoxic offspring. * $p < 0.05$. CIH: n=6; Normoxia: n=6

Figure 5.4: Second generation pups from gestational CIH offspring have greater fat deposits in adulthood

CIH male and CIH female offspring were mated in adulthood. At 12-weeks of age, F_2 generation offspring were found to have significantly greater retroperitoneal fat deposits compared to F_2 generation pups from normoxic offspring. Epididymal fat deposits were not different between groups. * $p < 0.05$. CIH: n=6; Normoxia: n=6

Figure 5.5: Second generation pups from gestational CIH offspring have higher fasting blood glucose levels in adulthood

CIH male and CIH female offspring were mated in adulthood. At 12-weeks of age, fasting blood glucose concentrations were measured using a standard glucometer. F_2 CIH offspring were found to have significantly higher blood glucose levels compared to F_2 normoxic offspring. * $p < 0.05$. CIH: n=6; Normoxia: n=6

5.4 – SIGNIFICANCE OF FINDINGS

The conclusions of the work outlined in this thesis provide insight into the long-term metabolic consequences associated with alteration of the maternal environment by exposure to CIH during pregnancy. By utilizing a CIH paradigm, this animal model mimics the clinically described changes in oxygen availability that sufferers of OSA typically experience during sleep. Early clinical results have shown that OSA during pregnancy results in lower birthweight offspring due to fetal growth restriction. However, the long-term physiological outcomes have not been described in human or animal studies. While there are certainly many aspects of metabolic regulation that remain to be investigated, the results of this thesis have established a rodent model of OSA that describes persistent changes in glucose and cholesterol homeostasis, important precursors to type II diabetes and cardiovascular disease. Perhaps most importantly, these findings have highlighted that OSA requires significant consideration in the assessment of maternal health during pregnancy and expected outcomes for offspring. As OSA is known to be a treatable disorder, alleviating this condition during pregnancy may have a significant impact on the long-term health of future generations.

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Appendix
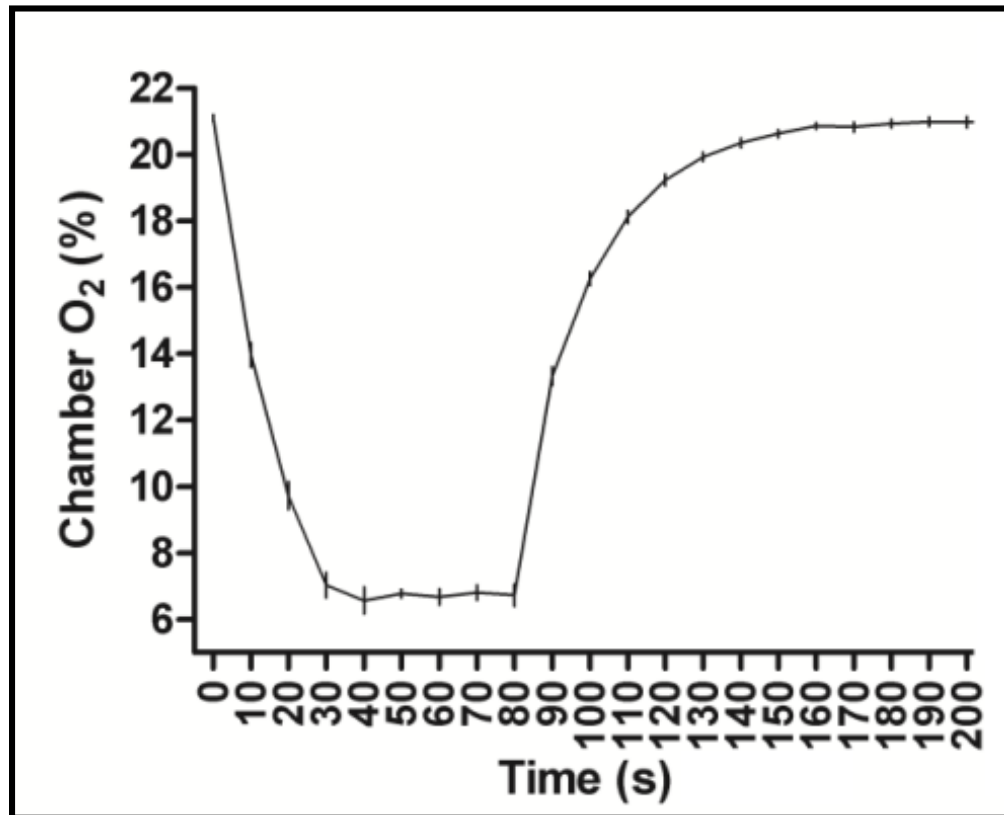
Graph depicting the changes in oxygen levels within the hypoxia chamber during a single cycle. During each cycle, the oxygen level was reduced within the chamber using a nitrogen gas infusion. The oxygen percentage was decreased to 6.5% nadir for 50 seconds, before returning to normoxic levels. The oxygen levels were brought back to 21% by flushing the chamber with room air. During the gestational CIH exposure, this cycle (total cycle time: 200 seconds) was repeated at a frequency of 18 cycles per hour for 8 hours per day from gestational day-1 to gestational day-20

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