10-3-2016 12:00 AM

Maternal Nutrient Restriction with Fetal Growth Restriction in Guinea Pigs Impacts Brain Development and Neuroimaging Correlates in Neonatal Offspring

Catherine Nevin
The University of Western Ontario

Supervisor
Dr. Bryan Richardson
The University of Western Ontario

Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

ABSTRACT

Aberrant brain development *in utero* accompanied by fetal growth restriction (FGR) increases the risk of neurodevelopmental disorders in later life. However there are limited non-invasive biomarkers in the brain for the early identification of said neurodevelopmental disorders in an animal model of FGR. Guinea pig sows were fed either ad libitum (Control) or 70% of the control diet pre-pregnancy, increasing to 90% at mid-pregnancy (MNR) creating appropriately grown (AGA) Control and FGR-MNR neonates, respectively. Three to four weeks corrected post-natal age, neonates were imaged using magnetic resonance imaging (MRI) and spectroscopy (MRS) techniques, and were killed 48-72 hours later for histological analysis. FGR-MNR neonates had smaller brain weights, whole brain volume, hippocampal volume and lateral ventricle volume, which correlate to histological findings. While there is a reduction in the hippocampal volume, there are no differences in hippocampus metabolite ratios between the AGA-Control and FGR-MNR neonates. Interestingly, there was a reduction in the width of the stratum oriens and stratum radiatum in the hippocampus proper, as well as the width of the polymorphic layer in the dentate gyrus, with no changes in pyramidal and granule cell number in the FGR-MNR neonates compared to AGA-Control neonates. In conclusion, MNR in guinea pigs produces FGR neonates that display catch up growth and structural differences in the brains while no changes in the metabolite levels in the hippocampal region of the brain. Together these results involve MRI and MRS as reliable imaging tools to detect the presence of brain injury for the future use of biomarkers for neurodevelopmental disorders and potential therapeutic interventions in the neonate period.

KEYWORDS

Fetal Growth Restriction, Maternal Nutrient Restriction, Brain Development, Magnetic Resonance Imaging, Magnetic Resonance Spectroscopy, Hippocampus, Nissl, N-acetyl aspartate.

CO-AUTHORSHIP

The following people contributed to the manuscripts contained within this thesis in the following ways:

Dr. B Richardson	Supervisor throughout all projects, provided grant funding to complete manuscripts, edited manuscript.
Dr. C M^{c}Kenzie	Magnetic resonance imaging support, advisory committee member, and edited manuscript.
Dr. R Bartha	Magnetic resonance spectroscopy advice and support.
B. Matushewski	Performed animal feeding and necropsy.
Dr. Y Maki	Performed animal feeding and necropsy.
K Nygard	Provided technical support for histology and image analysis, input into data analysis.
K Sinclair	Provided advice and support for Threshold Grower Tool for ImageJ analysis.
J Sahota	Performed Nissl stain and hippocampus and lateral ventricle histological measures
Dr. S Leung	Graduate Student Representative and member of advisory committee who provided advice and support throughout project.
Dr. J Ciriello	Advisory committee member who provided advice and support throughout project.

ACHKNOWLEDGMENTS

Thank you to my supervisor, Dr. Bryan Richardson for his constant guidance and ideas to make my thesis the best it can be. An additional thank you to Dr. Charlie McKenzie and Dr. Robert Bartha as they both provided me with support and guidance throughout my graduate career when it came to MRI and MRS work.

Thank you to everyone in the Richardson Perinatal Lab group, and everyone on the Robarts First Floor Imaging Unit who were constant aids as I learned about guinea pigs the new techniques of MRI and MRS.

Thank you to my family and friends who supported me through this different and interesting journey that is a Masters Degree. And last but not least, thank you to Dominique who always kept me on schedule and provided constant support no matter how good or bad of a day I had.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

~	Approximately
±	Plus or minus
+	Plus
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to
^{2}	Squared
^{3}	Cubed
%	Percent
*	Statistical Significance
μ m	Micrometers
aFGR	Asymmetrical Growth Restriction
AGR	Absolute Growth Rate
AGA	Appropriate for Gestational Age
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
Cho	Choline

Cr	Creatine
DG	Dentate Gyrus
et al.	et alia (and others)
FGR	Fetal Growth Restriction
FR	Fractional Growth Rate
g	Grams
Glu	Glutamate
GM	Grey Matter
IQ	Intelligence Quotient
LGA	Large for Gestational Age
LV	Lateral Ventricle
mm	Millimeters
MNR	Maternal Nutrient Restriction
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
Myo	Myo-inositol
NAA	N-acetyl aspartate
sFGR	Symmetrical Fetal Growth Restriction
WM	White Matter

Chapter 1
LITERATURE SEARCH

1. Fetal Growth Restriction

1.1.1 Fetal Growth Restriction Incidence and Classification

Fetal growth restriction (FGR) occurs in up to 5-10% of all babies born in Canada and is defined clinically, and in research, as birth weight below the 3rd, 5th, or 10th percentile for gestational age (Lackman *et al.*, 2001; Lausman *et al.*, 2012). FGR infants are considered to be pathologically small, unlike babies born constitutionally small or small for gestational age (SGA), due their inability to attain his or her *in utero* growth potential. FGR increases the risk of fetal mortality, neonatal morbidity and mortality, and later life health issues, with risk proportional to the severity of FGR (Rossavik *et al.*, 1996; Seeds & Peng, 1998; Doctor *et al.*, 2001). Due to the severity and increased risk of health issues with FGR babies, an early and accurate follow-up of FGR babies is essential.

FGR can be distinguished with the use of customized growth curves and Doppler velocimetry (Dubiel *et al.*, 2000, 2003; Verburg *et al.*, 2008). There are many resources available during pregnancy, and post-natally to assist in the determination of FGR. Pre-natally, a fetal weight standard growth curve is used with the assistance of ultrasound to monitor the gestational growth of the fetus. These growth curves are standardized to ensure accurate determination of FGR, SGA or appropriately grown fetuses and neonates. Such standardizations include maternal factors such as age, height, weight, and ethnicity to decrease confounding variables in the diagnosis and distinction of FGR (Arbuckle *et al.*, 1993). Environmental and socioeconomic factors are also included in the growth curve analysis which include maternal habits such as smoking, drug use, and health status including nutritional intake (Arbuckle *et al.*, 1993; Gardosi & Francis, 2009; Figueras & Gardosi, 2011). Post-natally, standard growth curves for growth of the neonate are continually monitored (de Jong *et al.*, 1998). The customized growth curves compared to whole population growth curves increase the likelihood of determining an FGR born versus an SGA born offspring and can increase assessment of perinatal morbidities and mortalities in FGR born offspring (de Jong *et al.*, 1998; Clausson *et al.*, 2001; Figueras *et al.*, 2007).

There are other prognosticators of fetal growth used as adjuncts to fetal growth curves.

Fetal ultrasound allows for measuring body proportions like fetal length, weight, head circumference, and femur length, also known as fetal biometry, as all of these measures can be indicators of delayed or altered fetal growth *in utero* (Verburg *et al.*, 2008). Amniotic fluid and placental ultrasound are used in conjunction with fetal biometry to further confirm FGR diagnosis (Youssef *et al.*, 1993; Thame *et al.*, 2004). Doppler assessment of the uterine artery and umbilical artery can also be used to further investigate whether a fetus is pathologically small or FGR, vs constitutionally small (Lausman *et al.*, 2012). An abnormal Doppler of the uterine artery waveforms, in both the first and second trimester, refines the risk of FGR due to placental insufficiency (Martin *et al.*, 2001; Papageorghiou *et al.*, 2001). The umbilical artery Doppler waveform in FGR cases is usually found to be abnormal with increased resistance to flow, and in the extreme with either absent or reversed end-diastolic flow velocity, due to a mal-developed terminal villous compartment (Krebs *et al.*, 1996; Macara *et al.*, 1996). Each of these measures has its own diagnostic value, however many abnormal measures are present in one case of FGR due to the many causes of FGR.

1.1.2 Causes of Fetal Growth Restriction

Normal fetal growth depends on the genetic potential of the fetus, the development and efficiency of the placenta, and the state of the maternal environment. Therefore the causes of FGR fall under three main categories: fetal, placental, and maternal. Fetal factors are the least common of the causes and can include various genetic factors, including fetal aneuploidy, congenital abnormalities, and infections such as cytomegalovirus (Demirci *et al.*, 2015). Placental causes include idiopathic placental insufficiency, abnormal umbilical cord insertion, nuchal cord, placental infarcts, placenta previa, and multiple gestations (Pollack & Divon, 1992). In each case the placenta, the nutritional and oxygen bridge between mother and fetus during pregnancy, is improperly developed and therefore offers insufficient nutrients and oxygen required for proper development and growth (Keswani *et al.*, 2015). Maternal causes of FGR include smoking, drug abuse, hypertension, and under- or malnourishment, which can then lead to improper placental development.

Maternal under-nutrition is a large problem in developing countries with limited food supply. However, under- and malnutrition are also issues in the developed world since many individuals experience food insecurity, consume food lacking specific macromolecules, as well as suffer from eating disorders (Tarasuk, 2001; Pike *et al.*, 2013). Undernourishment has specifically been shown to cause developmental changes in the structure and function of the placenta in both humans and rodent models, including the guinea pig (Sohlstrom *et al.*, 1998; Aplin, 2000; Roberts *et al.*, 2001*a*, 2001*b*). In humans, insufficient nutrient intake poses limitations to the placental metabolic and synthetic activity, which then interferes with the production of a functioning placenta with proper nutrient transport (Baschat, 2004).

1.1.2.1 Placental Insufficiency

The placenta is the interface between maternal and fetal compartments and it allows for proper delivery of oxygen and nutrients to the fetus. Placental development begins with the invasion and development of vascular connections and spiral arteries in the trophoblast, followed by formation of the maternal circulation and the intervillous space (Chaddha *et al.*, 2004). The placenta then further develops the maternal microvillous and the fetal basal layer for nutrient and oxygen delivery. These steps of placental development require sufficient nutrients to undergo angiogenesis and tissue protein synthesis; if nutrients are not available, there will be a reduction in placental growth, alterations in placental structure development, and a smaller placental size by the end of gestation (Chaddha *et al.*, 2004). Maintenance of placental transport is a highly energy intensive process such that the placenta consumes up to 40% of all oxygen and 70% of the glucose supplied by the mother to the placenta (Pardi *et al.*, 2002). Therefore nutrient and oxygen delivery to the placenta must exceed the placental demand so that the fetus can then receive nutrients and oxygen.

Proper blood flow across the placenta to the fetus is essential in the proper development of the fetus. Changes in vasculature, including abnormal trophoblastic invasion, and subsequent improper remodeling of the spiral arteries, are some of the changes that occur in placental insufficiency reducing umbilical venous blood flow suggesting a decrease in

fetal villous perfusion (Baschat, 2004; Chaddha *et al.*, 2004; Brett *et al.*, 2014). A subsequent decrease in umbilical artery end-diastolic velocity stimulates redistribution of blood flow in the fetal circulation (Baschat, 2004). Shunting of the umbilical venous blood to the heart, away from the liver elevates right ventricular afterload for preferential distribution of cardiac output to the heart and brain (Krebs *et al.*, 1996; Pardi *et al.*, 2002).

Deficient nutrient delivery such as a decrease in glucose, amino acids and fatty acids, can cause problems with placental metabolism and synthetic activity, which can then interfere with the endocrine feedback loops and active transport mechanisms responsible for nutrient delivery to the fetus and continued placenta development throughout gestation (Jansson & Persson, 1990; Jansson & Powell, 2007). All of the above defects can cause placental insufficiency such as reduced utero-placental blood flow, reduced placental size, and damage to placental structure; all responsible for the multisystem disorder of FGR.

1.1.2.2 Maternal Undernourishment

Maternal undernourishment plays a large role in the development of FGR. Early evidence of the effects of maternal undernourishment on newborn weight in humans occurred during the Dutch Famine. The famine occurred between the years of 1944 and 1945 but researchers also include 1946 cohorts as some of these women would have been exposed to the famine during pregnancy. The results of this naturally occurring study of maternal undernourishment varied depending on the time of onset of famine. Experiencing the famine at the time of conception and the first trimester, the famine did not affect birth weight or length, but demonstrated an increase in placental weight, suggesting some sort of compensatory mechanism in the placenta has occurred to ensure adequate oxygen and nutrient delivery to the fetus throughout pregnancy (Lumey, 1998; Stein *et al.*, 2004). If women were in their second or third trimester of pregnancy during the famine there were significant decreases in both birth weight and birth length, suggesting growth restriction occurred (Stein *et al.*, 2004). In addition to the changes seen in birth weight and length, follow up studies have demonstrated an increased risk for metabolic syndrome,

cardiovascular disease, and neurodevelopmental disorders in these individuals exposed to the famine *in utero* (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000; De Rooij *et al.*, 2006; Geier & Geier, 2012).

Growth restriction due to maternal undernourishment is very prominent in developing countries where there is upwards of 200 million children or 11% of all births that do not meet their genetic growth potential due to nutritional, socio-economic, and other health related factors that are associated with poverty (de Onis *et al.*, 1998; Walker *et al.*, 2007). Studies in these areas of the world have also demonstrated reductions in birth weight, and an increased prevalence of cognitive deficits, depression, and behavioral issues from the time of childhood to adulthood (Walker *et al.*, 2007).

As described above, maternal undernourishment produces growth-restricted offspring in humans and is a large contributor to fetal programming and later life disease outcomes. The placenta seems to be the modulator of nutrient and oxygen availability as it has been shown to compensate to the adverse maternal environments as described above, suggesting that it plays a great role in growth restriction in the maternal nutrient restriction (MNR) situation.

1.1.3 FGR Morbidity and Mortality

FGR is of much interest since there have been numerous studies published pertaining to the importance of a healthy *in utero* environment and the issues that can arise if the environment is suboptimal as discussed by the Developmental Origins of Health and Disease (DOHaD) (Haugen *et al.*, 2014). Many perinatal and later life consequences of FGR have been researched and can occur due to FGR. Prematurity is a factor which can cause issues as infants born FGR are 2-3 times more likely to be delivered spontaneously pre-term as assessed using fetal growth standards (Lackman *et al.*, 2001). In addition to the risk of prematurity, there are many other risks related to FGR. There is an increase in stillbirth associated with the severity of growth restriction and a 5-6 fold increased risk of perinatal death for both pre-term and full term fetuses with FGR (McIntyre *et al.*, 1999; Lackman *et al.*, 2001; Frøen *et al.*, 2004). There are also a number of short-term

morbidities that can occur with babies born FGR. Such morbidities include perinatal asphyxia, perinatal stroke, hypothermia, hypoglycemia, polycythemia and sepsis due to a compromised immune system (McIntyre *et al.*, 1999; Doctor *et al.*, 2001; Wu *et al.*, 2004).

1.1.4 Fetal Programming

Fetal programming is a process whereby an intrauterine insult has occurred that can have lasting or lifelong effects on the individual after birth (Lucas, 1994; Barker, 1998). This phenomenon has become known as the 'Barker Hypothesis' or DOHaD (Haugen *et al.*, 2014). Programming occurs during critical stages of development such as periods of rapid cell division that can lead to three different outcomes: 1) direct damage, such as early loss of a limb 2) induction, deletion, or impaired development of a somatic structure resulting from a stimulus or insult during a critical period or 3) physiological re-setting by an early stimulus or insult at a critical period with long term consequences for endocrine/ autocrine/ paracrine axes (Lucas, 1994).

Maternal under nutrition, as described above, is one of the insults that drives fetal programming to alter the structure of the placenta, redistribute blood flow, and alter metabolism through an increase in catabolism, and through the endocrine system by changing insulin levels and insulin like growth factor (Barker, 1998). Although these changes in programming are essential for survival of the fetus, they can be detrimental in later life if the environment does not stay constant and can permanently alter the growth of the neonate and leave the individual at an increases risk of developing later life health issues.

The idea of permanent fetal programming came about when it was first studied in babies of low birth weight who were seen to be hypertensive in adulthood and had an increased risk of developing cardiovascular disease (Osmond *et al.*, 1993; Barker *et al.*, 2005). Individuals born with low birth weight also had a higher risk of developing metabolic syndrome and type II diabetes, and becoming obese (Hales *et al.*, 1991; Law *et al.*, 1992; Forsen *et al.*, 2000). More recently, maternal undernourishment has been positively correlated with adverse brain development, abnormal behavior, and later mental disorders

Each of these can be attributed to fetal programming and provides further evidence for the theory of DOHaD whereby an insult that occurred due to an adverse intrauterine environment has lasting and detrimental effects later on in life (Morgane *et al.*, 2002; Rees & Harding, 2004; Geva *et al.*, 2006*a*, 2006*b*; Morsing *et al.*, 2011; Rees *et al.*, 2012; Abel *et al.*, 2013; Kallankari *et al.*, 2015; Starčević *et al.*, 2016). This information is of increasing importance to determine the flow of events from a poor intrauterine environment and begins to elucidate ways to decrease brain developmental events later in life.

1.1.5 FGR and Post-natal Growth

Catch-up growth, or the rapid post-natal growth displayed by FGR infants, has been shown to be a large contributor to the morbidity and mortality of the FGR neonate. The phenomenon of catch-up growth was studied early on in humans by Albertsson-Wikland et al (1993) although no negative outcomes were discussed in the relation to the remarkable rates of growth displayed by both the infant males and females born small for gestational age (Albertsson-Wikland *et al.*, 1993). More than 80% of infants born small for gestational age will catch up in growth within the first year of life (Karlberg & Albertsson-Wikland, 1995). However there are some instances such as in the 20% of SGA children that do not catch up by two years, of those, 50% will remain SGA throughout adulthood (Karlberg & Albertsson-Wikland, 1995). More recently, researchers have discovered the negative impacts that an accelerated post-natal growth could have on the morbidity and mortality of FGR infants. One study examined catch up growth after one year of age in babies who had restricted patterns of growth *in utero*, and found an increase in fat deposition at one year of age, although there were no changes in the metabolic profile at that time (Beltrand *et al.*, 2009). Additionally, another study has shown in humans that individuals who are born growth restricted and displayed catch up growth by seven years of age such that they were at an average or above average weight at 7 years old, had high rates of death from coronary artery disease in later life (Eriksson *et al.*, 1999). In sheep, low birth weight lambs had higher catch up growth in body weight than controls, with an increase in fat composition, while having smaller brain sizes after 2.3 years suggesting catch up growth is associated with FGR and can lead to problems in

body mass index, but it may be independent of brain development (Louey *et al.*, 2005). Rat pups born growth restricted but placed with a Control lactating dam showed increased catch-up growth rates but a reduction in their longevity demonstrating that growth restricted infants born into a post-natal environment with an abundance of nutrition availability have long term adverse consequences through increased growth (Hales & Ozanne, 2003). In contrast, rat pups born growth restricted that had slow post-natal growth due to a nutrient restricted lactating dam displayed an increase in longevity (Hales & Ozanne, 2003). At a more mechanistic level, the effects of placental insufficiency leading to FGR linger as an abundance of insulin receptors in the skeletal muscle after birth as a fetal adaptive mechanism, which is beneficial *in utero* (Morrison *et al.*, 2010). However this accelerates growth post-natally and puts the neonate at an increased risk for insulin resistance and visceral obesity as seen in young human adults who have been growth restricted at birth (Morrison *et al.*, 2010).

1.2 Animal Models of Fetal Growth Restriction

1.2.1 Animal Studies of FGR with Placental Insufficiency

Many clinical studies have examined the role of placental insufficiency as a contributor in the production of FGR. Aberrant placental vascularization and accompanying changes of umbilical and uterine artery blood flow are all factors that are related to placental insufficiency. Many animal models have been produced to further understand placental insufficiency and subsequently, FGR. These models produce placental insufficiency at a variety of different time points including pre-pregnancy, mid pregnancy, and late pregnancy, and through a variety of different methods. These methods are dependent on the animal model as well as the severity of placental insufficiency. Some of these methods include carunclectomy with the removal of endometrial tissue (caruncles) prior to pregnancy, hyperthermia environments from early pregnancy, placental embolization at mid-pregnancy, and uterine artery ligation/ablation at mid-gestation (Lafeber *et al.*, 1984; Harding *et al.*, 1985; Murotsuki *et al.*, 1997; Regnault *et al.*, 1999). In sheep, all of these methods have been successful in producing FGR with decreased fetal weight, increased brain to fetal weight and brain to liver weight ratios, and increases in hypoxemia and hypoglycemia through alterations of placental blood flow thus decreasing the oxygen and nutrients available to the fetus (Robinson *et al.*, 1979; Harding *et al.*,

1985; Murotsuki *et al.*, 1997; Regnault *et al.*, 1999). In rats, uterine artery ligation/ablation has been used to produce FGR and also displays brain injury in the neonates through a decrease in early reflexes and a decrease performance in open field testing (Nusken *et al.*, 2008; Black *et al.*, 2015). Lastly, the ligation and ablation model has also been used in the guinea pig to produce FGR and this model also shows a reduction in fetal and birth weight with evidence of brain injury and later life changes in metabolism (Jones & Parer, 1983; Mallard *et al.*, 2000; Turner & Trudinger, 2009). Although these methods are successful in producing FGR and the adverse conditions associated with FGR, there are still a number of drawbacks to each of these models. Specifically in relation to the ligation/ablation methods, increases in fetal demise and stillbirth occur which then decrease study numbers and the induction of FGR is inconsistent (Turner & Trudinger, 2009). In addition, this model induces placental insufficiency in an abrupt, invasive procedure, which does not accurately depict the human situation with the chronic insidious development of placental insufficiency.

1.2.2 Animal Studies of FGR with Maternal Undernourishment

The MNR model is another method for inducing FGR and studying the affects that an *in utero* insult has on fetal development, neonate morbidities and mortalities, and later life health outcomes. MNR alters placental development, which mimics placental insufficiency with a smaller placenta size and altered placental vascularization (Lumey, 1998; Sohlstrom *et al.*, 1998; Roberts *et al.*, 2001*a*, 2001*b*). Maternal undernourishment can be implemented at many different time points such as pre-conception, the time of conception, mid gestation, late gestation, or throughout the entire pregnancy. In addition, the MNR model can have varying levels of restriction with different types of restriction, either global or protein restriction, and each can range from mild restriction allowing 90% feed of what the *ad libitum* control group is eating, moderate restriction at 70% of what the ad libitum controls are eating, or severe restriction at ≤50% of what the ad libitum controls are eating.

MNR can also be studied in varying animal models ranging from rodents, including mice and rats, to guinea pigs, and can even be studied in a larger animal model like sheep.

MNR at the time of the pre-implantation embryo in rats studied by Kwong and colleagues (2000) displayed a delay in cell proliferation in the blastocyst which then gave rise to a decreased birth weight, delayed post-natal growth, and later onset hypertension at the age of 12 weeks post natal. This study demonstrates that a maternal food restriction at the pre-implantation stage of the embryo can alter fetal growth and development and can have lasting effects post-natally (Kwong *et al.*, 2000). Vieau and colleagues investigated the effects of a 50% MNR in the last week of gestation and during lactation in rats and noticed a decrease in hypothalamic-pituitary-adrenal axis function, an increase in glucocorticoids in the growth restricted neonates, as well as an increase in hyperactivity in the HPA axis in the adult rats (Vieau *et al.*, 2007). A mouse model of FGR through moderate MNR impairs physical growth, and increases mental disabilities such as increased anxiety and a decrease in overall cognitive function (Akitake *et al.*, 2015). A larger animal model of FGR in sheep found that the effects of a 50% reduction in food intake beginning in early gestation and continuing until mid-gestation caused a decrease in birth weight, cardiac ventricular hypertrophy, and an increased liver weight in the lamb neonates (Vonnahme, 2003). MNR has been used in many different animal models to produce FGR and is the *in utero* insult that will be used in this thesis using the guinea pig model.

1.2.3 Guinea Pig Model of Maternal Nutrient Restriction (MNR) Induced FGR

The guinea pig is an excellent model for the study of fetal development, as the guinea pig delivers precocious young after a relatively lengthy gestation of 67-68 days, so many critical developmental events occur *in utero* and can be easily delineated to the exact time period when they have occurred (Dobbing and Sand, 1970). The guinea pig MNR model of 70% food intake of the ad libitum controls, beginning pre-conception and continuing until mid-gestation, day 35, and then increasing to a 90% food restriction throughout the remainder of the study period has been shown to produce fetal growth restricted offspring and fetuses (Kind *et al.*, 2003, 2005; Elias *et al.*, 2016). The labyrinth placental structure of the guinea pig, as the main site of nutrient exchange, is more similar to the human placenta compared to other non-primate models and is affected by MNR to induce FGR. Maternal undernourishment in guinea pigs has been shown to cause reduced placental

weight at both mid-gestation and late-gestation with a reduced placental volume, as well as a reduction in labyrinth size in early gestation, compared to ad libitum-fed guinea pigs (Roberts *et al.*, 2001*a*). Consequently, a 60% reduction in placental surface area occurs in an undernourished guinea pig at the end of gestation as a consequence of the reduction in surface density and the proportion of the placenta that is occupied by labyrinth (Roberts *et al.*, 2001*a*). A positive correlation between fetal weight at the end of gestation and percent of placenta that was labyrinth was found in guinea pigs (Roberts *et al.*, 2001*a*). The placenta has a pivotal role in the development and growth of the fetus in the guinea pig, and if altered by MNR the changes that arise in the placenta can cause FGR.

MNR in the guinea pig produces asymmetrical growth restriction which is evident in the brain: liver weight ratios obtained and has also shown to cause increase fetal adiposity, an increase in plasma glucose with a decrease in glucose tolerance in addition to an increase in systolic blood pressure in later life (Kind *et al.*, 2002, 2003, 2005; Elias *et al.*, 2016).

1.2.4 Guinea Pig Brain Development

As guinea pigs are pre-natal brain developers over a long gestation, many critical neural developmental events and time points occur *in utero* which can be manipulated by creating an adverse intrauterine environment to produce altered brain development much like what occurs in human brain development as peri-natal brain developers (Dobbing & Sand, 1970; Piorkowska *et al.*, 2014). At the time of birth, both humans and guinea pigs have peaked in neuronal development compared to other animal models, including rodents, cats and dogs, whose brain development continues in the post-natal environment due to their short gestations and altricial young (Lennon *et al.*, 1980). Brain development for these animals will occur post-natally so many of the *in utero* insults will not affect brain development unless continued in the post-natal environment.

The guinea pig brain at birth is much further developed than the human brain at the end of gestation. Prenatal brain development in guinea pigs allows them to have much more neural function at the time of birth due to their peak neurogenesis occurring in utero in many brain areas to provide them with more motor skills and cognitive regulatory

processes than the human at birth (Dobbing & Sand, 1970). Due to this peak in neurogenesis occurring relatively early in the guinea pig brain, the guinea pig at birth has been compared to the same cognitive state as a human toddler. Therefore, many of the essential developmental events occur *in utero* for the guinea pig, which makes then an ideal animal model for studying brain development after a poor *in utero* environment.

1.3 FGR and the Brain

1.3.1 FGR and Neuroanatomical Outcomes

Injury to the brain can range throughout all structures however many FGR studies are interested in the hippocampus. The hippocampus may be one of the most vulnerable structures as it is almost entirely environmentally driven, as opposed to genetically driven, meaning the hippocampus is very vulnerable to an adverse in utero environment, and many of the cognitive deficits seen in FGR are brain tasks associated with the hippocampus (Lodygensky *et al.*, 2008). The hippocampus is a structure in the brain that is involved in the learning and memory processes and function of the brain. It is composed of four different subfields including the i) the subicular complex, ii) the entorhinal cortex, iii) the hippocampus proper composed of the cornu ammonis (CA) and lastly iv) the dentate gyrus (DG) (Amaral & Witter, 1989) (see Figure 1). The CA areas of the hippocampus proper can then be divided further into layers including the stratum oriens, streatum pyramidal, stratum radiatum, stratum lacunosum, and stratum moleculare (Anderson *et al.*, 2007). Each of these layers are composed of either cell types or fibers including pyramidal cells, granule cells, interneurons, and glial cells, axonal tracts, fibers, synapses, and dendritic trees. Similar to the CA in the hippocampus, the DG also has a layer arrangement and is composed of the polymorphic layer, the granule cell layer and the moleculare layer (Ribak *et al.*, 1985).

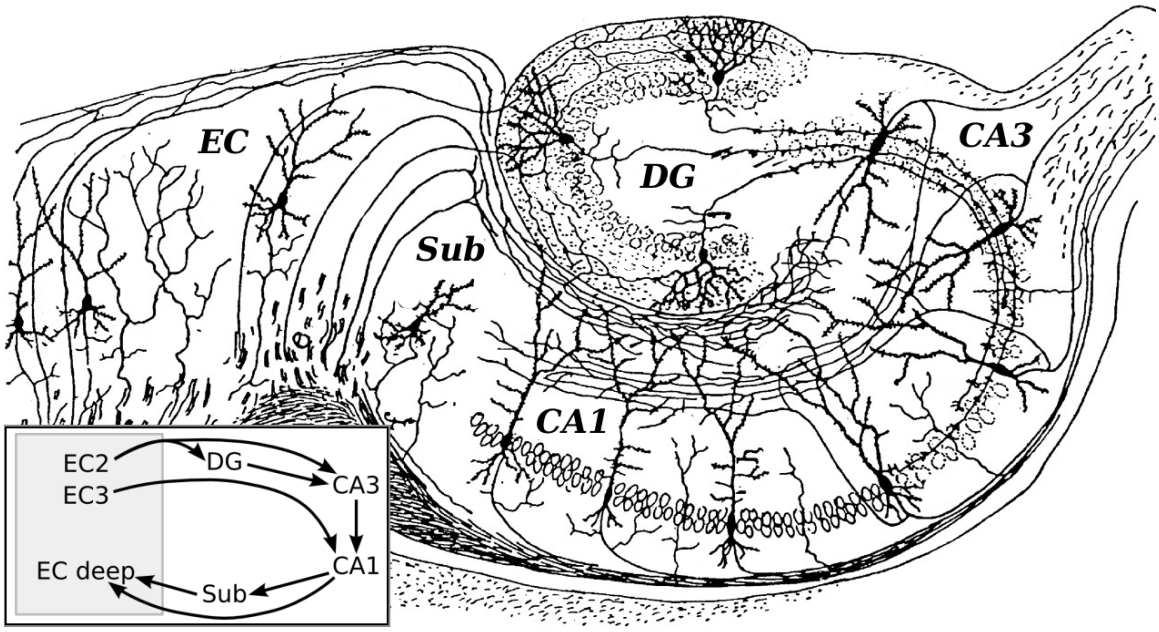


Figure 1: Modified schematic drawing of the neural circuitry of the rodent hippocampus. By Santiago Ramón y Cajal. Histologie du Systeme Nerveux de l'Homme et des Vertebretes, Vols. 1 and 2. A. Maloine. Paris. 1911.

The neural connections and processes extended throughout the hippocampus are very extensive and take up a vast majority of the hippocampus. The majority of the hippocampal input is through the Perforant Pathway, which begins at the entorhinal cortex (EC) where it continues down a unidirectional circuit throughout the subfields of the hippocampus (Amaral & Witter, 1989; Anderson *et al.*, 2007) (see Figure 1). Neuronal tracks from the entorhinal cortex feed into the excitatory DG granule cells, which project their mossy fibers into the CA3 pyramidal sub-area (Ribak *et al.*, 1985; Anderson *et al.,* 2007). The CA3 neurons then continue on to connect with the CA1 pyramidal neurons making their connection in the stratum radiatum in the CA1 sub region (Anderson *et al.,* 2007). These fibers then split to either exit into the subicular complex or back to the entorhinal cortex where they project to other areas of the brain via the hippocampus efferent fibers.

Alterations in the development of the hippocampus and the morphometry associated with proper hippocampal development have been associated with fetal growth restriction and pre-maturity (Lodygensky *et al.,* 2008). Specifically, a reduction in the grey matter

volume of the hippocampus and a positive correlation between birth weight and hippocampus volume were found through MRI in premature FGR infants (Lodygensky *et al.*, 2008). In an animal model, a reduction in the overall size of the hippocampus and an increase in lateral ventricle area have been shown in FGR fetal guinea pigs histologically (Mallard *et al.*, 1999, 2000). In addition, specific layers in the hippocampus proper, the stratum oriens, have been shown to be decreased in a ligation model of FGR in fetal guinea pigs suggesting that there is a decrease in pyramidal cell axonal projections or dendrites which could be related to the decrease in hippocampal volume (Mallard *et al.*, 1999). In the same model of uterine artery ligation in one week old guinea pig neonates, there was a reduction in pyramidal cell number and stratum oriens volume compared to the control animals (Mallard *et al.*, 2000). The loss of even small numbers of specific classes of cells in the hippocampus, such as the CA1, CA4 and dentate gyrus significantly affects particular neural functions and increases the risk of developing neurodevelopmental disorders later in life in both growth restricted rat pups and guinea pigs (Mallard *et al.*, 2000; Florian & Nunes, 2010). The tools to be able to accurately identify which FGR-born infants will develop neurobehavioural adversities later in life are being investigated with the use of brain biomarkers in a non-invasive and safe manner.

1.3.2 FGR and Neurobehavioural Outcomes

A number of developmental events occur in the brain of a fetus, which are energy requiring processes in order to develop properly. Initiation of myelination, axonal and dendritic growth, synaptogenesis, and proliferation of microglia and astrocytes are all examples of developmental events that can be disrupted or altered if nutrients and oxygen are not available, such as in a growth restricted fetus. The fetus has the capacity to adapt to an adverse environment by changing fetal blood flow distribution to shunt blood to the brain and other vital organs to maximize oxygen and nutrient delivery and minimize damage to the brain while shunting blood away from non-essential organs (Dubiel *et al.*, 2003; Poudel *et al.*, 2015). This adaptation is known as ‘brain sparing’, which produces a larger head in relation to the body and a smaller liver, and thereby asymmetrical growth in an FGR fetus. The disturbances or insults to the brain accompanied with FGR may be

overt such as a reduction in brain structure size like the hippocampus, or occult such as a disruption in the connections between axonal tracks and synapses throughout the brain (Lodygensky *et al.*, 2008).

Studies included in the literature search below did not always specify whether study participants were SGA or FGR therefore both will be included in the below information. SGA and FGR have been shown to increase the risk of developing poor neurological outcomes and disorders later in life. Cognitive deficits can become apparent from a very early age in infants born SGA and FGR such as impaired cognitive skills, and a compromised memory (Fattal-Valevski *et al.*, 2009; Løhaugen *et al.*, 2013). Learning difficulties, and weakened reading and writing skills have been shown to be more prominent and frequent in infants born below the 3rd percentile suggesting a pathological growth restriction having the biggest impact on neurological outcomes (O'Keeffe *et al.*, 2003). Behavior is also involved in the developmental differences in children born small for gestational age in both school age children and adolescents including increased inattentiveness, and restlessness in classrooms (Parkinson *et al.*, 1986; Pryor *et al.*, 1995). In addition, birth weight has been positively correlated with IQ score in children.

SES (socioeconomic status) can also play a role in the post-natal brain development, learning, and the level of cognition in young children. A higher SES results in improvement of children's cognition compared to children born in a low SES area (Christensen *et al.*, 2014). Therefore both the *in utero* environment and SES contribute to poor neurological outcomes of children born SGA and FGR.

FGR not only increases the risk of cognitive deficits and poor neurological outcomes but also increases the risk of developing later neurodevelopmental disorders such as attention deficit hyperactive disorder, autism, cerebral palsy, and schizophrenia with risk directly proportional to the severity of FGR (Mallard *et al.*, 1999; StrangKarlsson *et al.*, 2008; Haglund & Källén, 2011; Abel *et al.*, 2013; Dahlseng *et al.*, 2014).

1.3.3 Magnetic Resonance Imaging and the Brain

Magnetic Resonance Imaging (MRI) is a safe and reliable tool used to image and quantify the volume of many structures in the human anatomy, including the brain, at varying ages in both humans and animals (Woods, 2006). MRI has the capability of identifying macro- and microstructural changes of brain tissue in various physiological and pathological conditions. To accomplish this, MRI utilizes powerful magnetic fields to generate radiofrequency signals from hydrogen atoms throughout the body that can used to reconstruct an image (Rooney, 2003). The magnet, magnetic field gradients, and surface coil in the MRI machine capitalize on the relaxation and electromagnetic fields produced by the magnetized hydrogen atoms to produce different contrasts in the anatomy of interest (Rooney, 2003). A common type of MRI image contrast used in brain scans is a T2 weighted image, which displays liquids like cerebral spinal fluid and adipose rich white matter with high signal intensity. In comparison, the skull and grey matter in the brain of a T2 weighted image has lower signal intensity and are much darker on the T2 weighted MRI image. MRI is also used for morphometric measures of the various structures in the brain such as the hippocampus and lateral ventricles. These structures are easy to visualize due to their distinct boundaries and high contrast in MRI of the brain of humans, and many animal species including and guinea pigs. Due to its non-invasive nature and high sensitivity, MRI has been used in both clinical and research settings to study many disease-like states including Alzheimer's, Amyotrophic Lateral Sclerosis (ALS), cerebral palsy, autism, and schizophrenia. MRI has also been used to study the brain in infants born preterm and after perinatal asphyxia, and more recently, in FGR infants (Ellis *et al.*, 1999; Bax *et al.*, 2006; Desikan *et al.*, 2009; Yoshida *et al.*, 2009).

1.3.3.1 Neuroanatomical Correlates for MRI

Neuroanatomical correlates to MRI can be used to gain additional information on the microstructural changes going on in the brain that may provide additional information that the MRI might not be able to obtain. Haematoxylin and Eosin (H&E) staining and Nissl stains have been used on brain sections to gain more neuroanatomical information

on the structural make up of the brain that can be seen with MRI, and the microstructural neuron arrangement that can add to the MRI information. The Nissl stain in particular stains the Nissl bodies in neural tissue, which are components of the rough endoplasmic reticulum, with the amount, form and distribution of Nissl components varying in different types of neurons and glial cells (Kiernan, 2010; Garman, 2011). Nissl substance can also bind to clusters of DNA and RNA in the nuclei, which can be most prominently viewed in the neuron nucleolus. One type of Nissl stain that is popularly used is the Cresyl violet stain, which gives the neurons a violet colour leaving the background tissue, unstained (Kiernan, 2010). This stain provides excellent visualization of brain cell types including neurons, astrocytes, and glial cells and vivid contrast in the brain especially between the grey matter and white matter, and for differentiating cell layers and regions of the hippocampus.

Measurements of hippocampal layer width, grey matter and white matter width, cross-sectional area of various regions of the brain are all measurements that are useful in the determination of the brain development, arrangement, and neural connections present in the brain. Studies have looked at the histology of the FGR guinea pig brain, although these studies did not relate these histological findings to MRI work (Mallard *et al.*, 2000; Kim *et al.*, 2014; Piorkowska *et al.*, 2014). Therefore utilising both MRI and histological neuroanatomical correlates allows for more information to be obtained on the FGR brain in neonatal offspring.

1.3.4 Magnetic Resonance Spectroscopy and the Brain

Magnetic resonance spectroscopy (MRS) measures a spectrum of *in vivo* metabolites. MRS harnesses the same basic principles as MRI but uses a property known as chemical shift to distinguish different metabolites throughout the body. Chemical shift describes the range of frequencies that are produced from the electron clouds surrounding the proton nuclei in a metabolite due to the chemical structure (Bluml & Panigrahy, 2013). Each metabolite in the brain has a different chemical structure; therefore each metabolite will all have a different chemical shift (Bluml & Panigrahy, 2013). Analyzing the chemical shift of each metabolite allows researchers to assign the metabolite name to

each peak that is produced from MRS at the different frequencies that are expressed as parts per million (ppm) (Bluml & Panigrahy, 2013). Although the present study will use H^1 spectroscopy, there are other methods that can be used to measure metabolism such as P^{31} and C^{13} (Levine *et al.*, 1992; Morris & Bachelard, 2003).

The sensitivity of MRS to detect different metabolites depends on the strength of the MRI magnet that is being used. The lower the magnetic field strength, the more difficult it is to separate metabolite peaks as they have very similar shifts in the spectrum, however at higher magnetic fields it becomes much easier to separate the frequencies and therefore metabolites (Bluml & Panigrahy, 2013). MRS can be used throughout the body but has been used extensively in the brain. The spectrum in the brain usually visualized is composed of the lipid peak, creatine, choline, myo-inositol, N-acetyl aspartate (NAA), glutamate/glutamine, and lactate (Soares & Law, 2009).

The lipid peak can be used to assess induction of apoptosis through the movement of CH_3 molecules in the neuronal tissue as the lipids breakdown from the neuron lipid bi-layer (Ahn *et al.*, 2013).

The creatine peak is composed of both creatine and phosphocreatine, which are involved in energy metabolism through the production of ATP (Soares & Law, 2009; Story *et al.*, 2011). Creatine is relatively stable within cells with no changes in levels throughout gestation and shortly thereafter, therefore creatine is commonly used as the denominator in the relative quantification of other metabolites (Soares & Law, 2009; Story *et al.*, 2011). One drawback of using creatine in the quantification of metabolites is the vulnerability of creatine to ischemia reperfusion as it is an energy source for the brain (Levine *et al.*, 1992). Absolute concentrations of the metabolites can be used instead of creatine ratio quantification however the concentration of water per voxel is thought remain stable and uniform when calculating the absolute concentrations of the metabolites (Li *et al.*, 2003; Jansen *et al.*, 2006). For the purposes of this study, the levels of creatine were assumed to be stable therefore the concentrations of the metabolites are presented as a ratio over creatine.

Choline is comprised of signals from glycerophosphocholine, phosphocholine, and free choline. All of these are essential for membrane synthesis and degradation and are also intermediates for the synthesis of acetylcholine. Choline has been shown to decrease in the first 5 years of life due to myelination and the production of neuronal cell membranes while the levels of choline during fetal life are still under debate (Story *et al.*, 2011).

Myo-inositol (Myo) is a simple sugar that is absent from neurons. It is found in glial cells and almost exclusively in the astrocytes as an osmolyte or an astrocyte marker and has been known to be a breakdown product of myelin in the brain (Fisher & Agranoff, 1987).

Glutamate and glutamine (Glx) are two metabolites that are always present together in the brain with the same chemical shift and therefore make it difficult to separate the peaks in their spectrum. Glutamate (Glu) is an excitatory neurotransmitter and is the most abundant neurotransmitter in the brain (van den Pol *et al.*, 1990). Glutamine (Gln) is a precursor for glutamate and can be found in both glia cells and neurons as well as in the plasma (Pow & Crook, 1996). Glutamate can be made back into glutamine in the glial cell though an active process (Pow & Crook, 1996).

NAA is a peak that is composed of both NAA and N-acetylaspartylglutamate (NAAG). The acetate of the molecule is used for myelin synthesis while the aspartate is mostly used for energy production and protein synthesis (Moffett *et al.*, 2007). NAA is synthesized in neuronal mitochondria from acetyl coenzyme-A and aspartate by NAA transferases, which is dependant upon the energy state of the mitochondria (Moffett *et al.*, 2007). It is then transferred to the neuronal cytoplasm and is finally located in the oligodendrocyte to be broken down and used for cellular energy production (Bhakoo & Pearce, 2000). NAA has been shown to increase in the fetal brain with advancing gestation as more neurons develop and mature (Kreis *et al.*, 2002). Researchers believe this increase may be due to the synaptic complexity, increased myelination, and the metabolic status and development of neuronal mitochondria (Kreis *et al.*, 2002).

To date, there have been limited studies aimed at using MRS to understand the metabolism of the brain in FGR. One study that looked at the brain of a FGR human fetus

found decreased levels of NAA/Cr and NAA/Cho ratios, suggesting that FGR brains, as classified by altered cerebral blood redistribution, have altered neuronal and mitochondrial metabolism (Story *et al.*, 2011). Similarly, in two other human FGR fetal MRS studies, researchers found a decrease in NAA/Cho ratios in the central brain area (Azpurua *et al.*, 2008; Sanz-Cortes *et al.*, 2015). Additionally in an animal model, lower levels of NAA in fetal rabbit brain cortex and hippocampal areas have been found, suggesting neuronal metabolic impairment (Simoes *et al.*, 2015). These studies demonstrate altered neuronal metabolism and integrity in the FGR fetal brain in both humans and in animal models. To our knowledge, there are few studies of human neonates born small for gestational age (SGA) using MRS, with the majority of them being in premature FGR infants. One study looked at one-year old children who were born SGA and observed a significant increase in both the NAA/Cr and Glutamate/Cr ratios in the prefrontal cortex. Similarly, in a study of FGR premature neonates, there was an increase in absolute NAA levels (Lazeyras *et al.*, 2003; Roelants-Van Rijn *et al.*, 2004). The latter is very different finding from that in the fetal brains, which suggests that there are post-natal developmental changes that occur in human brains (Simões *et al.*, 2015). There are a limited number of studies in the field of MRS and FGR. Therefore, more research is required to explore abnormal brain development following an adverse *in utero* environment exposure in the neonate.

1.3.3.1 Neuro-anatomic Correlates for MRS

Metabolic correlates in histological tissue can be used to further investigate the molecular differences in the brain on a microscopic level. Different morphological stains can be used which can correlate to the metabolite values obtained by MRS, although this practise is not widely used. Nissl or H&E staining of the brain can provide morphological measures and cell number quantification for determining dendritic and axonal projections in the hippocampus, neuronal cell density, and arrangement, which could then correlate to the neuronal integrity as represented by NAA in MRS.

1.4 Summary

FGR is one of the leading causes of perinatal morbidity and mortality and increases the risk of developing later life health disorders. Although idiopathic placental insufficiency

is one of the major causes of FGR, MNR is still prevalent and produces alterations in the placenta that lead to decreased fetal growth and fetal programming as a compensatory mechanism. The adaptation to the adverse *in utero* environment through fetal programming attempts to allow for adequate fetal development, however brain development is altered despite 'brain sparing'.

The brain is a very complex organ and requires high amounts of oxygen and nutrients in order to develop properly, and subsequently function properly. Structural changes in the brain of an FGR born individual have been described in literature when an inadequate amount of nutrients and oxygen are present in the *in utero* environment. The hippocampus is of particular interest as this is one structure in the brain most vulnerable to environmental stimuli and the least influenced by genetics and is the brain structure involved in many neurodevelopmental disorders that FGR neonates are at an increased risk of developing. The need for accurate and non-invasive biomarkers to determine the risk of developing such neurodevelopmental disorders later in life is necessary. Although these structures have been studied histologically, there are limited studies in both humans and animal models of FGR, particularly after MNR, to use MRI as a safe and reliable method to image brain injury and developmental abnormalities as potential biomarkers for later neurodevelopmental disorders. Although the gross structural differences are important for the analysis of risk, the metabolic state of the brain is an essential component to how it may function. The ability to utilize both MRI and MRS in the brain of a growth restricted neonate will build biomarkers in a crucial time of brain developmental plasticity for the potential to intervene with therapeutic targets to prevent later neurodevelopmental disorders. We have therefore studied moderate MNR in guinea pigs and investigated the neonatal outcomes after being born fetal growth restricted including growth profiles, organ weights, and potential brain biomarkers as indicators for later neurodevelopmental disorders using a non-invasive imaging technique of MRI and MRS.

1.5 References

Abel KM, Dalman C, Svensson a C, Susser E, Dal H, Idring S, Webb RT, Rai D & Magnusson C (2013). Deviance in fetal growth and risk of autism spectrum disorder. *Am J Psychiatry* **170,** 391–398.

Ahn SY, Yoo HS, Lee JH, Sung DK, Jung YJ, Sung SI, Lim KH, Chang YS, Lee JH, Kim KS & Park WS (2013). Quantitative in vivo detection of brain cell death after hypoxia ischemia using the lipid peak at 1.3 ppm of proton magnetic resonance spectroscopy in neonatal rats. *J Korean Med Sci* **28,** 1071–1076.

Akitake Y, Katsuragi S, Hosokawa M & Mishima K (2015). Moderate maternal food restriction in mice impairs physical growth , behavior , and neurodevelopment of offspring. *Nutr Res* **35,** 76–87.

Albertsson-Wikland K, Wennergren G, Wennergren M, Vilbergsson G & Rosberg S (1993). Longitudinal follow-up of growth in children born small for gestational age. *Acta Paediatr* **82,** 438–443.

Amaral DG & Witter MP (1989). The three-dimensional organization of the hippocampal formation: A review of anatomical data. *Neuroscience* **31,** 571–591.

Anderson P, Morris R, Amaral D, Bliss T & O'Keefe J (2007). *The hippocampus book*.

Aplin J (2000). Maternal influences on placental development. *Semin Cell Dev Biol* **11,** 115–125.

Arbuckle TE, Wilkins R & Sherman GJ (1993). Birth weight percentiles by gestational age in Canada. *Obstet Gynecol* **81,** 39–48.

Azpurua H, Alvarado A, Mayobre F, Salom T, Copel J & Guevara-Zuloaga F (2008). Metabolis Assessment of the brain using proton magnetic resonance spectroscopy in a growth restricted human fetus: case report. *Am J Perinatol* **25,** 305–309.

Barker DJ (1998). In utero programming of chronic disease. *Clin Sci (Lond)* **95,** 115–128.

Barker DJP, Osmond C, Forsén TJ, Kajantie E & Eriksson JG (2005). Trajectories of growth among children who have coronary events as adults. *N Engl J Med* **353,** 1802–1809.

Baschat AA (2004). Fetal responses to placental insufficiency: An update. *BJOG An Int J Obstet Gynaecol* **111,** 1031–1041.

Bax M, Tydeman C & Flodmark O (2006). Clinical and MRI correlates of cerebral palsy: the European Cerebral Palsy Study. *JAMA* **296,** 1602–1608.

Bejjani A, O'Neill J, Kim JA, Frew AJ, Yee VW, Ly R, Kitchen C, Salamon N, McCracken JT, Toga AW, Alger JR & Levitt JG (2012). Elevated glutamatergic compounds in pregenual anterior cingulate in pediatric autism spectrum disorder demonstrated by1H MRS and 1H MRSI. *PLoS One*; DOI: 10.1371/journal.pone.0038786.

Beltrand J, Nicolescu R, Kaguelidou F, Verkauskiene R, Sibony O, Chevenne D, Claris O & Lévy-Marchal C (2009). Catch-up growth following fetal growth restriction promotes rapid restoration of fat mass but without metabolic consequences at one year of age. *PLoS One*; DOI: 10.1371/journal.pone.0005343.

Bhakoo KK & Pearce D (2000). In vitro expression of N-acetyl aspartate by oligodendrocytes: implications for proton magnetic resonance spectroscopy signal in vivo. *J Neurochem* **74,** 254–262.

Black AM, Armstrong EA, Scott O, Juurlink BJH & Yager JY (2015). Broccoli sprout supplementation during pregnancy prevents brain injury in the newborn rat following placental insufficiency. *Behav Brain Res* **291,** 289–298.

Bluml S & Panigrahy A (2013). *MR Spectroscopy of Pediatric Brain Disorders*. Available at: http://link.springer.com/10.1007/978-1-4419-5864-8.

Brett K, Ferraro Z, Yockell-Lelievre J, Gruslin A & Adamo K (2014). Maternal–Fetal Nutrient Transport in Pregnancy Pathologies: The Role of the Placenta. *Int J Mol Sci* **15,** 16153–16185.

Chaddha V, Viero S, Huppertz B & Kingdom J (2004). Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med* **9,** 357–369.

Christensen DL, Schieve LA, Devine O & Drews-Botsch C (2014). Socioeconomic status, child enrichment factors, and cognitive performance among preschool-age children: Results from the Follow-Up of Growth and Development Experiences study. *Res Dev Disabil* **35,** 1789–1801.

Clausson B, Gardosi J, Francis A & Cnattingius S (2001). Perinatal outcome in SGA births defined by customised versus population-based birthweight standards. *Br J Obstet Gynaecol* **108,** 830–834.

Dahlseng MO, Andersen GL, Irgens LM, Skranes J & Vik T (2014). Risk of cerebral palsy in term-born singletons according to growth status at birth. *Dev Med Child Neurol* **56,** 53–58.

Demirci O, Selçuk S, Kumru P, Asoğlu M, Mahmutoğlu D, Boza B, Türkyılmaz G, Bütün Z, Arısoy R & Tandoğan B (2015). Maternal and fetal risk factors affecting perinatal mortality in early and late fetal growth restriction. *Taiwan J Obs Gynecol* **54,** 700–704.

Desikan RS, Cabral HJ, Hess CP, Dillon WP, Glastonbury CM, Weiner MW, Schmansky NJ, Greve DN, Salat DH, Buckner RL & Fischl B (2009). Automated MRI measures identify individuals with mild cognitive impairment and Alzheimers disease. *Brain* **132,** 2048–2057.

Dobbing J & Sand J (1970). Growth and development of the brain and spinal cord of the guinea pig. *Brain Res* **17,** 115–123.

Doctor BA, O'Riordan MA, Kirchner HL, Shah D & Hack M (2001). Perinatal correlates and neonatal outcomes of small for gestational age infants born at term gestation. *Am J Obstet Gynecol* **185,** 652–659.

Dubiel M, Breborowicz GH & Gudmundsson S (2003). Evaluation of fetal circulation redistribution in pregnancies with absent or reversed diastolic flow in the umbilical artery. *Early Hum Dev* **71,** 149–156.

Dubiel M, Breborowicz GH, Marsal K & Gudmundsson S (2000). Fetal adrenal and middle cerebral artery Doppler velocimetry in high-risk pregnancy. *Ultrasound Obstet Gynecol* **16,** 414–418.

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Ellis CM, Simmons A, Jones DK, Bland J, Dawson JM, Horsfield MA, Williams SC & Leigh PN (1999). Diffusion tensor MRI assesses corticospinal tract damage in ALS. *Neurology* **53,** 1051–1058.

Eriksson JG, Forsén T, Tuomilehto J, Winter PD, Osmond C & Barker DJ (1999). Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *BMJ* **318,** 427–431.

Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R & Harel S (2009). Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *J Child Neurol* **24,** 846–851.

Figueras F, Figueras J, Meler E, Eixarch E, Coll O, Gratacos E, Gardosi J & Carbonell X (2007). Customised birthweight standards accurately predict perinatal morbidity. *Arch Dis Child Fetal Neonatal Ed* **92,** F277-80.

Figueras F & Gardosi J (2011). Intrauterine growth restriction: new concepts in antenatal surveillance, diagnosis, and management. *Am J Obstet Gynecol* **204,** 288–300.

Fisher SK & Agranoff BW (1987). Receptor Activation and Inositol Lipid Hydrolysis in Neural Tissues.

Florian ML & Nunes ML (2010). Effects of intra-uterine and early extra-uterine malnutrition on seizure threshold and hippocampal morphometry of pup rats. *Nutr Neurosci* **13,** 265–273.

Forsen T, Eriksson J, Tuomilehto J, Reunanen A, Osmond C & Barker D (2000). The fetal and childhood growth of persons who develop type 2 diabetes. *Ann Intern Med* **133,** 176–182.

Frøen JF, Gardosi JO, Thurmann A, Francis A & Stray-Pedersen B (2004). Restricted fetal growth in sudden intrauterine unexplained death. *Acta Obstet Gynecol Scand* **83,** 801–807.

Gardosi J & Francis A (2009). A customized standard to assess fetal growth in a US population. *Am J Obstet Gynecol* **201,** 25.e1-25.e7.

Garman RH (2011). Histology of the central nervous system. *Toxicol Pathol* **39,** 22–35.

Geier MR & Geier D a (2012). Neurodevelopmental Disorders after. **147,** 213–216.

Geva R, Eshel R, Leitner Y, Fattal-Valevski A & Harel S (2006*a*). Memory functions of children born with asymmetric intrauterine growth restriction. *Brain Res* **1117,** 186–194.

Geva R, Eshel R, Leitner Y, Valevski AF & Harel S (2006*b*). Neuropsychological outcome of children with intrauterine growth restriction: a 9-year prospective study. *Pediatrics* **118,** 91–100.

Haglund NGS & Källén KBM (2011). Risk factors for autism and Asperger syndrome. Perinatal factors and migration. *Autism* **15,** 163–183.

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

Hales CN & Ozanne SE (2003). The dangerous road of catch-up growth. *J Physiol* **547,** 5–10.

Harding JE, Jones CT & Robinson JS (1985). Studies on experimental growth retardation in sheep. The effects of a small placenta in restricting transport to and growth of the fetus. *J Dev Physiol* **7,** 427–442.

Haugen a. C, Schug TT, Collman G & Heindel JJ (2014). Evolution of DOHaD: the impact of environmental health sciences. *J Dev Orig Health Dis*1–10.

Jansen JFA, Backes WH, Nicolay K & Kooi ME (2006). 1H MR Spectroscopy of the Brain: Absolute Quantification of Metabolites1. *Radiology* **240,** 318–332.

Jansson T & Persson E (1990). Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Pediatr Res* **28,** 203–208.

Jansson T & Powell TL (2007). Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)* **113,** 1–13.

Jones CT & Parer JT (1983). The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea-pig. *J Physiol* **343,** 525–537.

de Jong CL, Gardosi J, Dekker G a, Colenbrander GJ & van Geijn HP (1998). Application of a customised birthweight standard in the assessment of perinatal outcome in a high risk population. *Br J Obstet Gynaecol* **105,** 531–535.

Kallankari H, Kaukola T, Ols??n P, Ojaniemi M & Hallman M (2015). Very preterm birth and foetal growth restriction are associated with specific cognitive deficits in children attending mainstream school. *Acta Paediatr* **104,** 84–90.

Karlberg J & Albertsson-Wikland K (1995). Growth in full-term small-for-

gestational-age infants: from birth to final height. *Pediatr Res* **38,** 733–739.

Keswani SG, Balaji S, Katz AB, King A, Omar K, Habli M, Klanke C & Crombleholme TM (2015). Intraplacental Gene Therapy with Ad-IGF-1 Corrects Naturally Occurring Rabbit Model of Intrauterine Growth Restriction. *Hum Gene Ther* **26,** 172–182.

Kiernan J a (2010). Staining Sections of the Central Nervous System. *Connection*60–67.

Kim J, Choi I-Y, Dong Y, Wang W-T, Brooks WM, Weiner CP & Lee P (2014). Chronic fetal hypoxia affects axonal maturation in guinea pigs during development: A longitudinal diffusion tensor imaging and T 2 mapping study. *J Magn Reson Imaging*n/a-n/a.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS & Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS & Owens J a (2005). Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol* **288,** R119–R126.

Kind KL, Simonetta G, Clifton PM, Robinson JS & Owens J a (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Exp Physiol* **87,** 469–477.

Krebs C, Macara LM, Leiser R, Bowman AW, Greer IA & Kingdom JCP (1996). Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. *Am J Obstet Gynecol* **175,** 1534–1542.

Kreis R, Hofmann L, Kuhlmann B, Boesch C, Bossi E & Hüppi PS (2002). Brain metabolite composition during early human brain development as measured by quantitative in vivo 1H magnetic resonance spectroscopy. *Magn Reson Med* **48,** 949–958.

Kwong WY, Wild a E, Roberts P, Willis a C & Fleming TP (2000). Maternal

undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* **127,** 4195–4202.

Lackman F, Capewell V, Richardson B, DaSilva O & Gagnon R (2001). The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol* **184,** 946–953.

Lafeber H, Rolph T & Jones C (1984). Studies in the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol* **6,** 441–459.

Lausman A, McCarthy FP, Walker M & Kingdom J (2012). Screening, diagnosis, and management of intrauterine growth restriction. *J Obstet Gynaecol Can* **34,** 17–28.

Law CM, Barker DJ, Osmond C, Fall CH & Simmonds SJ (1992). Early growth and abdominal fatness in adult life. *J Epidemiol Community Health* **46,** 184–186.

Lazeyras F, Chen A, Henry F, Borradori-Tolsa C, Magistretti P & Huppi P (2003). Quantitative 1H-MRS shows prominent increase of lactate in cerebral white matter in premature infants with intrauterine growth restriction (IUGR). *Proc Intl Soc Mag Reson Med*.

Lennon A, Francon J, Fellous A & Nunez J (1980). Rat, mouse, and guinea pig brain development and microtubule assembly. *J Neurochem* **35,** 804–813.

Levine S, Helpern J, Welch K, Vande Linde A, Sawaya K, Brown E, Ramadan N, Deveshwar R & Ordidge R (1992). Human focal cerebral ischemia: evaluation of brain pH and energy metabolism with P-31 NMR spectroscopy. *Radiology* **185,** 537–544.

Li BSY, Wang H & Gonen O (2003). Metabolite ratios to assumed stable creatine level may confound the quantification of proton brain MR spectroscopy. *Magn Reson Imaging* **21,** 923–928.

Lodygensky G a., Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F & Hüppi PS (2008). Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr Res* **63,** 438–443.

Løhaugen GCC, Østgård HF, Andreassen S, Jacobsen GW, Vik T, Brubakk A-M, Skranes J & Martinussen M (2013). Small for gestational age and intrauterine growth restriction decreases cognitive function in young adults. *J Pediatr* **163,** 447–453.

Louey S, Cock ML & Harding R (2005). Long term consequences of low birthweight on postnatal growth, adiposity and brain weight at maturity in sheep. *J Reprod Dev* **51,** 59–68.

Lucas A (1994). Role of nutritional programming in determining adult morbidity. *Arch Dis Child* **71,** 288–290.

Lumey LH (1998). Compensatory placental growth after restricted maternal nutrition in early pregnancy. *Placenta* **19,** 105–111.

Macara L, Kingdom JCP, Kaufmann P, Kohnen G, Hair J, More IAR, Lyall F & Greer IA (1996). Structural analysis of placental terminal villi from growth-restricted pregnancies with abnormal umbilical artery Doppler waveforms. *Placenta* **17,** 37–48.

Mallard C, Loeliger M, Copolov D & Rees S (2000). Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* **100,** 327–333.

Mallard EC, Rehn A, Rees S, Tolcos M & Copolov D (1999). Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: Implications for the aetiology of schizophrenia. *Schizophr Res* **40,** 11–21.

Martin AM, Bindra R, Curcio P, Cicero S & Nicolaides KH (2001). Screening for pre-eclampsia and fetal growth restriction by uterine artery Doppler at 11-14 weeks of gestation. *Ultrasound Obstet Gynecol* **18,** 583–586.

McIntyre D, Bloom S, Casey B & Leveno K (1999). Birth Weight in Relation to Morbidity and Mortality Among Newborn Infants. *N Engl J Med* **340,** 1234–1238.

Moffett JR, Ross B, Arun P, Madhavarao CN & Namboodiri a. M a (2007). N-Acetylaspartate in the CNS: From neurodiagnostics to neurobiology. *Prog Neurobiol* **81,** 89–131.

Morgane PJ, Mokler DJ & Galler JR (2002). Effects of prenatal protein malnutrition

on the hippocampal formation. *Neurosci Biobehav Rev* **26,** 471–483.

Morris P & Bachelard H (2003). Reflections on the application of 13C-MRS to research on brain metabolism. *NMR Biomed* **6,** 303–312.

Morrison JL, Duffield J a, Muhlhausler BS, Gentili S & McMillen IC (2010). Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol* **25,** 669–677.

Morsing E, Åsard M, Ley D, Stjernqvist K & Maršál K (2011). Cognitive Function After Intrauterine Growth Restriction and Very Preterm Birth. *Pediatrics* **127,** e874–e882.

Murotsuki J, Challis JR, Han VK, Fraher LJ & Gagnon R (1997). Chronic fetal placental embolization and hypoxemia cause hypertension and myocardial hypertrophy in fetal sheep. *Am J Physiol* **272,** R201-7.

Nusken KD, Dotsch J, Rauh M, Rascher W & Schneider H (2008). Uteroplacental insufficiency after bilateral uterine artery ligation in the rat: Impact on postnatal glucose and lipid metabolism and evidence for metabolic programming of the offspring by sham operation. *Endocrinology* **149,** 1056–1063.

O'Keeffe MJ, O'Callaghan M, Williams GM, Najman JM & Bor W (2003). Learning, cognitive, and attentional problems in adolescents born small for gestational age. *Pediatrics* **112,** 301–307.

de Onis M, Blossner M & Villar J (1998). Levels and patterns of intrauterine growth restardation in developin countries. *Eur J Clin Nutr* **52,** 5–15.

Osmond C, Barker DJ, Winter PD, Fall CH & Simmonds SJ (1993). Early growth and death from cardiovascular disease in women. *BMJ* **307,** 1519–1524.

Papageorghiou AT, Yu CKH, Bindra R, Pandis G & Nicolaides KH (2001). Multicenter screening for pre-eclampsia and fetal growth restriction by transvaginal uterine artery Doppler at 23 weeks of gestation. *Ultrasound Obstet Gynecol* **18,** 441–449.

Pardi G, Marconi AM & Cetin I (2002). Placental-fetal interrelationship in IUGR fetuses - A review. *Placenta* **23,** 136–141.

Parkinson CE, Scrivener R, Graves L, Bunton J & Harvey D (1986). Behavioural

differences of school-age children who were small-for-dates babies. *Dev Med Child Neurol* **28,** 498–505.

Pike KM, Dunne PE & Addai E (2013). Expanding the boundaries: Reconfiguring the demographics of the "typical" eating disordered patient. *Curr Psychiatry Rep*; DOI: 10.1007/s11920-013-0411-2.

Piorkowska K, Thompson J, Nygard K, Matushewski B, Hammond R & Richardson B (2014). Synaptic development and neuronal myelination are altered with growth restriction in fetal guinea pigs. *Dev Neurosci* **36,** 465–476.

van den Pol a N, Wuarin JP & Dudek FE (1990). Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. *Science* **250,** 1276–1278.

Pollack R & Divon M (1992). Intrauterine Growth Retardation: Definition, Classification, and Etiology. **35,** 99–107.

Poudel R, McMillen IC, Dunn SL, Zhang S & Morrison JL (2015). Impact of chronic hypoxemia on blood flow to the brain, heart, and adrenal gland in the late-gestation IUGR sheep fetus. *Am J Physiol Regul Integr Comp Physiol* **308,** R151-62.

Pow D V & Crook DK (1996). Direct immunocytochemical evidence for the transfer of glutamine from glial cells to neurons: use of specific antibodies directed against the d-stereoisomers of glutamate and glutamine. *Neuroscience* **70,** 295–302.

Pryor J, Silva PA & Brooke M (1995). Growth, development and behaviour in adolescents born small-for-gestational-age. *J Paediatr Child Health* **31,** 403–407.

Ravelli ACJ, van der Meulen JHP, Osmond C, Barker DJP & Blecker OP (1999). Obesity at the age of 50y in men and women exposed to famine prenatally. *Am J Clin Nutr* **70,** 811–816.

Rees S & Harding R (2004). Brain development during fetal life: Influences of the intra-uterine environment. *Neurosci Lett* **361,** 111–114.

Rees S, Harding R & Walker D (2012). The biological basis of injury and neuroprotection in the fetal and enonatal brain. *Changes* **29,** 997–1003.

Regnault TRH, Orbus RJ, Battaglia FC, Wilkening RB & Anthony R V. (1999). Altered arterial concentrations of placental hormones during maximal placental growth

in a model of placental insufficiency. *J Endocrinol* **162,** 433–442.

Ribak CE, Seress L & Amaral DG (1985). The development, ultrastructure and synaptic connections of the mossy cells of the dentate gyrus. *J Neurocytol* **14,** 835–857.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001*a*). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA (2001*b*). Altered placental structure induced by maternal food restriction in guinea pigs: A role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22,** 77–82.

Robinson JS, Kingston E., Jones C. & Thornburn G. (1979). Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *J Dev Physiol* **1,** 379–398.

Roelants-Van Rijn AM, Van Der Grond J, Stigter RH, De Vries LS & Groenendaal F (2004). Cerebral structure and metabolism and long-term outcome in small-for-gestational-age preterm neonates. *Pediatr Res* **56,** 285–290.

De Rooij SR, Painter RC, Roseboom TJ, Phillips DIW, Osmond C, Barker DJP, Tanck MW, Michels RPJ, Bossuyt PMM & Bleker OP (2006). Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. *Diabetologia* **49,** 637–643.

Rooney W (2003). MRI: From Picture to Proton,. *Health Phys* **85,** 504–505.

Roseboom TJ, van der Meulen JH, Osmond C, Barker DJ, Ravelli a C, Schroeder-Tanka JM, van Montfrans G a, Michels RP & Bleker OP (2000). Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart* **84,** 595–598.

Rossavik IK, Piper JM, Xenakis EM, McFarland M, Elliott BD, Berkus MD & Langer O (1996). Do growth-retarded premature infants have different rates of perinatal morbidity and mortality than appropriately grown premature infants? *Obstet Gynecol* **87,** 169–174. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/8559517\nhttp://www.ncbi.nlm.nih.gov/pubmed/8649694.

Sanz-Cortes M, Simoes R V., Bargallo N, Masoller N, Figueras F & Gratacos E (2015). Proton magnetic resonance spectroscopy assessment of fetal brain metabolism in late-onset "small for gestational age" versus "intrauterine growth restriction" fetuses. *Fetal Diagn Ther* **37,** 108–116.

Seeds JW & Peng T (1998). Impaired growth and risk of fetal death: is the tenth percentile the appropriate standard? *Am J Obstet Gynecol* **178,** 658–669.

Simões R V., Cruz-Lemini M, Bargalló N, Gratacós E & Sanz-Cortes M (2015). Brain Metabolite Differences in One-Year-Old Infants Born Small at Term and its Association with Neurodevelopmental Outcome. *Am J Obstet Gynecol*1–11.

Simoes R V, Munoz-Moreno E, Carbajo RJ, Gonzalez-Tendero A, Illa M, Sanz-Cortes M, Pineda-Lucena A & Gratacos E (2015). In vivo detection of perinatal brain metabolite changes in a rabbit model of Intrauterine Growth restriction (IUGR). *PLOS One (In Press*1–16.

Soares DP & Law M (2009). Magnetic resonance spectroscopy of the brain: review of metabolites and clinical applications. *Clin Radiol* **64,** 12–21.

Sohlstrom A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS & Owens JA (1998). Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *Am J Physiol* **274,** E410-6.

Starčević M, Predojević M, Butorac D, Tumbri J, Konjevoda P & Kadić AS (2016). Early functional and morphological brain disturbances in late-onset intrauterine growth restriction. *Early Hum Dev* **93,** 33–38.

Stein AD, Zybert PA, van de Bor M & Lumey LH (2004). Intrauterine famine exposure and body proportions at birth: The Dutch Hunger Winter. *Int J Epidemiol* **33,** 831–836.

Story L, Damodaram MS, Allsop JM, Dcr R, Mcguinness A, Appsc BA, Patel A, Wylezinska M, Hagberg H, Kumar S & Rutherford MA (2011). Brain metabolism in fetal intrauterine growth restriction : a proton magnetic resonance spectroscopy study. *YMOB* **205,** 483.e1-483.e8.

StrangKarlsson S, Raikkonen K, Pesonen a K, Kajantie E, Paavonen EJ, Lahti J, Hovi P,

Heinonen K, Jaervenpaeae a L, Eriksson JG & Andersson S (2008). Very low birth weight and behavioral symptoms of attention deficit hyperactivity disorder in young adulthood: The Helsinki study of very- low-birth-weight adults. *AmerJ Psychiat* **165,** 1345–1353.

Tarasuk V (2001). A critical examination of community-based responses to household food insecurity in Canada. *Health Educ Behav* **28,** 487–499.

Thame M, Osmond C, Bennett F, Wilks R & Forrester T (2004). Fetal growth is directly related to maternal anthropometry and placental volume. *Eur J Clin Nutr* **58,** 894–900.

Turner a. J & Trudinger BJ (2009). A Modification of the Uterine Artery Restriction Technique in the Guinea Pig Fetus Produces Asymmetrical Ultrasound Growth. *Placenta* **30,** 236–240.

Verburg BO, Steegers EAP, De Ridder M, Snijders RJM, Smith E, Hofman A, Moll HA, Jaddoe VW V & Witteman JCM (2008). New charts for ultrasound dating of pregnancy and assessment of fetal growth: Longitudinal data from a population-based cohort study. *Ultrasound Obstet Gynecol* **31,** 388–396.

Vieau D, Sebaai N, Léonhardt M, Dutriez-Casteloot I, Molendi-Coste O, Laborie C, Breton C, Deloof S & Lesage J (2007). HPA axis programming by maternal undernutrition in the male rat offspring. *Psychoneuroendocrinology* **32,** 16–20.

Vonnahme KA (2003). Maternal Undernutrition from Early- to Mid-Gestation Leads to Growth Retardation, Cardiac Ventricular Hypertrophy, and Increased Liver Weight in the Fetal Sheep. *Biol Reprod* **69,** 133–140.

Walker SP, Wachs TD, Meeks Gardner J, Lozoff B, Wasserman G a, Pollitt E & Carter J a (2007). Child development: risk factors for adverse outcomes in developing countries. *Lancet* **369,** 145–157.

Woods TO (2006). *MRI Safety*. Available at: http://dx.doi.org/10.1002/9780471740360.ebs1374.

Wu Y, March W, Croen L, Grether J, GJ. E & Newman T (2004). Perinatal Stroke in Children With Motor Impairment: A Population-Based Study. *Pediatrics* **114,** 612–619.

Yoshida T, McCarley RW, Nakamura M, Lee K, Koo MS, Bouix S, Salisbury DF, Morra

L, Shenton ME & Niznikiewicz MA (2009). A prospective longitudinal volumetric MRI study of superior temporal gyrus gray matter and amygdala-hippocampal complex in chronic schizophrenia. *Schizophr Res* **113,** 84–94.

Youssef AA, Abdulla SA, Sayed EH, Salem HT, Abdelalim AM & Devoe LD (1993). Superiority of amniotic fluid index over amniotic fluid pocket measurement for predicting bad fetal outcome. *South Med J* **86,** 426–429.

Chapter 2
RATIONALE, OBJECTIVES AND HYPOTHESES

2.1 Rationale

Fetal growth restriction (FGR) occurs when a fetus does not reach their genetic growth potential, and is therefore pathologically small versus constitutionally small. FGR is one of the most common antenatal complications in developed countries contributing to 5-30 times increased risk for fetal/neonatal morbidity and mortality compared to appropriately grown infants (Ghidini, 1996; Lackman *et al.*, 2001). Depending on the type of morbidity or complications, FGR infants may become a large burden to the health care system as many FGR born infants end up in the neonatal intensive care unit and require a great deal of medical assistance and intervention. This health care burden continues throughout their lifespan as FGR born individuals could undergo catch up growth which puts them at an increased risk of developing later life adversities, more so than just FGR on its own, including cardiovascular disease, metabolic syndrome, type 2 diabetes and neurodevelopmental disorders (Hales *et al.*, 1991; Law *et al.*, 1992; Barker *et al.*, 2005; Abel *et al.*, 2013; Lui, Jing, Wang, Xiao-Feng, Yan, 2014).

FGR has been studied extensively in the fetus in many animal models to gain a better understanding of the determination of FGR *in utero* and adaptive responses from the fetus to an adverse intrauterine environment. Models include uterine caruncletomy in sheep, uterine artery ligation/ablation in rats and guinea pigs and protein or nutrient restriction in rats and guinea pigs. Moderate Maternal Nutrient Restriction (MNR) in the guinea pig is a robust means of producing FGR in an animal model (Elias et al., 2016) that mimics human FGR, through a chronic and non-invasive insult throughout gestation, which more closely parallels the human situation with moderate to severe FGR (Jones & Parer, 1983). This model restricts maternal guinea pigs through a moderate global caloric restriction of 70% of the ad libitum caloric intake pre-pregnant to mid-pregnancy increasing to 90% thereafter until the end of gestation producing asymmetrical FGR, analogous to human FGR. Additionally, the fetuses produced by this model are hypoglycemic and polycythemic (Elias *et al.*, 2016). MNR in guinea pigs can alter the placental structure, through a decrease in placental and trophoblast size, and function through changes in the placental transporters and vessel invasion, which mimics placental insufficiency in humans (Jones & Parer, 1983; Jansson & Persson, 1990; Dwyer *et al.*, 1992; Roberts *et al.*, 2001). This model can also be used to study catch up growth as this is a phenomenon

that occurs frequently with FGR born individuals, and may play a role in the neonate organ development, although this has not been studied before (Kind *et al.*, 2003, 2002). Additionally, brain development in the guinea pig occurs primarily *in utero* as they are pre-natal brain developers which is more similar to human brain development as they are peri-natal brain developers (Dobbing & Sands, 1970; Kolb, 1989; Kolb *et al.*, 2000). The guinea pig model is more analogous to human brain development compared to other rodent models, such as rats and mice, that have post-natal brain development whereby the majority of the developmental events *in utero* would not be as representative in the offspring (Dobbing & Sands, 1970; Lennon *et al.*, 1980). After such an insult as MNR, our lab has identified markers of hypoxia in the brains of FGR fetal brains, as well as in the liver, kidney, and placenta (unpublished data). Moderate MNR, in guinea pigs leading to FGR has been studied for both the fetal growth characteristics, and the later life adversities that are associated with FGR (Kind *et al.*, 2003, 2005; Elias *et al.*, 2016). Although the fetal and placental growth characteristics of the guinea pig after MNR have been well studied, there are limited studies that characterize post-natal development after FGR in the guinea pig and how the brain is affected after a nutrient restricted intrauterine environment.

Numerous developmental events occur throughout gestation, including, but not limited to, the development of neurons and neural connection, glial cells, axonal maturation, myelination, and synaptic pruning, all of which require a significant amount of energy and oxygen (Kato *et al.*, 1997; Hua & Smith, 2004). An inadequate in utero environment, such as maternal nutrient restriction or idiopathic placental insufficiency, leads to FGR and could lead to adverse development of these processes such as an increase in necrosis, or apoptosis leading to decreased neuron cell number, changes in dendritic and axonal growth, and altered brain metabolism leading to abnormal brain function. As such, FGR with maternal nutrient restriction could impair such energy dependent processes leading to altered brain development and potentially later brain developmental disorders. In addition to the extensive growth and development in the intrauterine environment, there is a vast amount of growth in an FGR born individual, commonly known as catch-up growth, which has the potential to negatively affect the neonate (Ong *et al.*, 2000; Hales

& Ozanne, 2003; Beltrand *et al.*, 2009; Fattal-Valevski *et al.*, 2009).Although guinea pig animal studies focus on a poor intrauterine environment through MNR (Mallard *et al.*, 2000; Kind *et al.*, 2002, 2003), many do not continue this adverse environment into the post-natal years of development which mimics the human situation of maternal nutrient restriction and the effect of nutrient restriction on milk production (Rasmussen, 1992).

FGR increases the risk of developing cognitive impairments at school age and future difficulties in language memory and learning (Parkinson *et al.*, 1986; O'Keeffe *et al.*, 2003; Tanis *et al.*, 2012; Christensen *et al.*, 2014). Additionally, the risk of developing later mental disorders such as attention deficit hyperactive disorder, autism, and schizophrenia intensifies with increasing severity of FGR (Walker & Marlow, 2008; Halliday, 2009; Indredavik *et al.*, 2010). As these developmental disorders can occur after FGR, the brain structural abnormalities involved in these disorders, such as a decrease in hippocampal volume and an increase in lateral ventricle volume, and a decrease in grey matter volume are likely to be involved in FGR (Mallard *et al.*, 1999; McAlonan *et al.*, 2005; Yoshida *et al.*, 2009). The need for better identification of children at risk of neurodevelopmental impairment early in neonatal life by targeting the brain structures known to be involved in neurodevelopmental disorders could assist in targeting those FGR-born who would benefit from specific interventions.

Magnetic Resonance Imaging (MRI) is a safe and reliable tool used in many brain disorders including schizophrenia, Alzheimer's and cerebral palsy (Hüppi *et al.*, 1998; Tolsa *et al.*, 2004; Bax *et al.*, 2006; Desikan *et al.*, 2009; Yoshida *et al.*, 2009). Additionally, there has been an increased usage of MRI in perinatal asphyxia and pre-term birth in brain by imaging the structures and metabolites in the brain to look at grey matter, white matter and the hippocampus and to examine the potential neurological outcomes of these children (Baenziger *et al.*, 1993; Foster-Barber *et al.*, 2001; L'Abee *et al.*, 2005; Howard *et al.*, 2006). As MRI has become increasingly popular for perinatal asphyxia and preterm birth, imaging for FGR has begun to emerge in the MRI field, although there are still few studies to date. MRI has the ability to image the entire brain to target specific brain structural abnormalities and can therefore be used in brains of FGR-born individuals. Specific to MRI, growth restricted preterm infants have been imaged

using structural MRI, and show a decrease in hippocampus volume compared to controls (Lodygensky *et al.*, 2008). This study provided insight into the hippocampus as a vulnerable target in the brain to adverse intrauterine environments and therefore will be studied in this thesis (Lodygensky *et al.*, 2008). Additionally, one study in fetal FGR guinea pigs found a decrease in the hippocampus with an accompanied increase in lateral ventricle volume histologically, therefore these two structures may be related to each other in the overall brain development in FGR and should be compared to MRI through neuroanatomical correlates (Mallard *et al.*, 1999) and will be studied in the present study. Magnetic resonance spectroscopy has also been used to study some aspects of FGR, including neuronal integrity through N-acetyl aspartate (NAA) in human fetuses although there is still little evidence in the neonate (Story *et al.*, 2011; Sanz-Cortes *et al.*, 2015; Simões *et al.*, 2015) and in animal models and histological correlates associated with NAA, such as neuron counts. Using MRI-MRS in the neonate FGR brain in the guinea pig to identify potential brain biomarkers with further investigation into the histology of these brains would provide more insight into the developmental changes in an FGR brain with hopes of it being used in the development of potential therapeutic interventions and decrease later neurodevelopmental diseases in the future.

2.2 Governing Hypothesis

Magnetic resonance imaging and spectroscopy can identify the presence of brain developmental abnormalities and biomarkers in the structure and metabolism of growth restricted neonate guinea pigs.

2.2.1 Specific Hypotheses

1. Neonatal guinea pigs born growth restricted after moderate MNR will demonstrate post-natal catch-up growth compared to Appropriate for Gestational Age (AGA)-Control neonates with inter-organ differences in growth.

2. These FGR-MNR neonates will have a reduced hippocampal volume and an increased lateral ventricle volume compared to the AGA-Control neonates, which will be detectable

using non-invasive MRI. These MRI findings will be consistent with comparable histological findings in the same brains.

3. These FGR-MNR neonates will display altered metabolite levels in the hippocampus compared to the AGA-Control neonates, specifically a decrease in N-acetyl-aspartate (NAA) concentration indicating a decrease in neuronal integrity which will be detectable using non-invasive MRS. These MRS findings will be consistent with comparable histological findings in the same brains.

2.3 Aims/ Research Objectives

Aim 1: To characterize the neonate guinea pig growth patterns both whole body and organ specific of FGR offspring after *in utero* moderate MNR.

Aim 2: To determine the presence and extent of structural brain injury that can be detected in growth restricted guinea pig neonates using noninvasive T2 MRI along with neuro-anatomic correlates as a means of predicting adverse neurodevelopmental outcomes.

Aim 3: To determine the presence and extent of brain metabolite alterations that can be detected in growth restricted guinea pig neonates using noninvasive MRS along with neuro-metabolite correlates as a means of predicting adverse neurodevelopmental outcomes.

2.4 References

Abel KM, Dalman C, Svensson a C, Susser E, Dal H, Idring S, Webb RT, Rai D & Magnusson C (2013). Deviance in fetal growth and risk of autism spectrum disorder. *Am J Psychiatry* **170,** 391–398.

Baenziger O, Martin E, Steinlin M, Good M, Largo R, Burger R, Fanconi S, Duc G, Buchli R & Rumpel H (1993). Early pattern recognition in severe perinatal asphyxia: a prospective MRI study. *Neuroradiology* **35,** 437–442.

Barker DJP, Osmond C, Forsén TJ, Kajantie E & Eriksson JG (2005). Trajectories of growth among children who have coronary events as adults. *N Engl J Med* **353,** 1802–1809.

Bax M, Tydeman C & Flodmark O (2006). Clinical and MRI correlates of cerebral palsy: the European Cerebral Palsy Study. *JAMA* **296,** 1602–1608.

Beltrand J, Nicolescu R, Kaguelidou F, Verkauskiene R, Sibony O, Chevenne D, Claris O & Lévy-Marchal C (2009). Catch-up growth following fetal growth restriction promotes rapid restoration of fat mass but without metabolic consequences at one year of age. *PLoS One*; DOI: 10.1371/journal.pone.0005343.

Christensen DL, Schieve LA, Devine O & Drews-Botsch C (2014). Socioeconomic status, child enrichment factors, and cognitive performance among preschool-age children: Results from the Follow-Up of Growth and Development Experiences study. *Res Dev Disabil* **35,** 1789–1801.

Desikan RS, Cabral HJ, Hess CP, Dillon WP, Glastonbury CM, Weiner MW, Schmansky NJ, Greve DN, Salat DH, Buckner RL & Fischl B (2009). Automated MRI measures identify individuals with mild cognitive impairment and Alzheimers disease. *Brain* **132,** 2048–2057.

Dobbing J & Sands J (1970). Growth and development of the brain and spinal cord of the guinea pig. *Brain Res* **17,** 115–123.

Dwyer C, Madgwick A, Crook A & Strickland N (1992). The effect of maternal undernutrition on the growth and development of the guinea pig placenta. *J Dev Physiol* **18,** 295–302.

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R & Harel S (2009). Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *J Child Neurol* **24,** 846–851.

Foster-Barber A, Dickens B & Ferriero DM (2001). Human perinatal asphyxia: Correlation of neonatal cytokines with MRI and outcome. *Dev Neurosci* **23,** 213–218.

Ghidini A (1996). Idiopathic fetal growth restriction: a pathophysiologic approach. *Obs Gynecol Surv* **51,** 376–832.

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

Hales CN & Ozanne SE (2003). The dangerous road of catch-up growth. *J Physiol* **547,** 5–10.

Halliday HL (2009). Neonatal management and long-term sequelae. *Best Pract Res Clin Obstet Gynaecol* **23,** 871–880.

Howard K, Sc B & Inder TE (2006). Neonatal MRI to Predict Neurodevelopmental Outcomes in Preterm Infants. 685–694.

Hua JY & Smith SJ (2004). Neural activity and the dynamics of central nervous system development. *Nat Neurosci* **7,** 327–332.

Hüppi PS, Warfield S, Kikinis R, Barnes PD, Zientara GP, Jolesz F a, Tsuji MK & Volpe JJ (1998). Quantitative magnetic resonance imaging of brain development in premature and mature newborns. *Ann Neurol* **43,** 224–235.

Indredavik MS, Vik T, Evensen KAI, Skranes J, Taraldsen G & Brubakk A (2010). Perinatal Risk and Psychiatric Outcome in Preterm Vlbw and Term Sga Adolescents. 1–24.

Jansson T & Persson E (1990). Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Pediatr Res* **28,** 203–208.

Jones CT & Parer JT (1983). The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea-pig. *J Physiol* **343,** 525–537.

Kato T, Nishina M, Matsushita K, Hori E, Mito T & Takashima S (1997). Neuronal

maturation and N-acetyl-L-aspartic acid development in human fetal and child brains. *Brain Dev* **19,** 131–133.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS & Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS & Owens J a (2005). Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol* **288,** R119–R126.

Kind KL, Simonetta G, Clifton PM, Robinson JS & Owens J a (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Exp Physiol* **87,** 469–477.

Kolb B (1989). Brain development, plasticity, and behavior. *Am Psychol* **44,** 1203–1212.

Kolb B, Gibb R & Gorny G (2000). Cortical plasticity and the development of behavior after early frontal cortical injury. *Dev Neuropsychol* **18,** 423–444.

L'Abee C, De Vries LS, Van Der Grond J & Groenendaal F (2005). Early diffusion-weighted MRI and 1H-magnetic resonance spectroscopy in asphyxiated full-term neonates. *Biol Neonate* **88,** 306–312.

Lackman F, Capewell V, Richardson B, DaSilva O & Gagnon R (2001). The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol* **184,** 946–953.

Law CM, Barker DJ, Osmond C, Fall CH & Simmonds SJ (1992). Early growth and abdominal fatness in adult life. *J Epidemiol Community Health* **46,** 184–186.

Lennon A, Francon J, Fellous A & Nunez J (1980). Rat, mouse, and guinea pig brain development and microtubule assembly. *J Neurochem* **35,** 804–813.

Lodygensky G a., Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F & Hüppi PS (2008). Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr Res* **63,** 438–443.

Lui, Jing, Wang, Xiao-Feng, Yan W (2014). Long-Term Adverse Outcomes of Fetal Growth Restriction. **93,** 1–5.

Mallard C, Loeliger M, Copolov D & Rees S (2000). Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* **100,** 327–333.

Mallard EC, Rehn A, Rees S, Tolcos M & Copolov D (1999). Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: Implications for the aetiology of schizophrenia. *Schizophr Res* **40,** 11–21.

McAlonan GM, Cheung V, Cheung C, Suckling J, Lam GY, Tai KS, Yip L, Murphy DGM & Chua SE (2005). Mapping the brain in autism. A voxel-based MRI study of volumetric differences and intercorrelations in autism. *Brain* **128,** 268–276.

O'Keeffe MJ, O'Callaghan M, Williams GM, Najman JM & Bor W (2003). Learning, cognitive, and attentional problems in adolescents born small for gestational age. *Pediatrics* **112,** 301–307.

Ong KK, Ahmed ML, Emmett PM, Preece MA & Dunger DB (2000). Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *BMJ* **320,** 967–971.

Parkinson CE, Scrivener R, Graves L, Bunton J & Harvey D (1986). Behavioural differences of school-age children who were small-for-dates babies. *Dev Med Child Neurol* **28,** 498–505.

Rasmussen K (1992). The influence of maternal nutrition on lactation. *Annu Rev Nutr*. Available at: http://www.annualreviews.org/doi/pdf/10.1146/annurev.nu.12.070192.000535.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Sanz-Cortes M, Simoes R V., Bargallo N, Masoller N, Figueras F & Gratacos E (2015). Proton magnetic resonance spectroscopy assessment of fetal brain metabolism in late-onset "small for gestational age" versus "intrauterine growth restriction"

fetuses. *Fetal Diagn Ther* **37,** 108–116.

Simões R V., Cruz-Lemini M, Bargalló N, Gratacós E & Sanz-Cortes M (2015). Brain Metabolite Differences in One-Year-Old Infants Born Small at Term and its Association with Neurodevelopmental Outcome. *Am J Obstet Gynecol*1–11.

Story L, Damodaram MS, Allsop JM, Dcr R, Mcguinness A, Appsc BA, Patel A, Wylezinska M, Hagberg H, Kumar S & Rutherford MA (2011). Brain metabolism in fetal intrauterine growth restriction : a proton magnetic resonance spectroscopy study. *YMOB* **205,** 483.e1-483.e8.

Tanis JC, van der Ree MH, Roze E, Huis in 't Veld AE, van den Berg PP, Van Braeckel KNJ a. & Bos AF (2012). Functional outcome of very preterm-born and small for gestational age children at school age. *Pediatr Res* **72,** 641–648.

Tolsa CB, Zimine S, Warfield SK, Freschi M, Rossignol AS, Lazeyras F, Hanquinet S, Pfizenmaier M & Hüppi PS (2004). Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. *Pediatr Res* **56,** 132–138.

Walker D-M & Marlow N (2008). Neurocognitive outcome following fetal growth restriction. *Arch Dis Child Fetal Neonatal Ed* **93,** F322-5.

Yoshida T, McCarley RW, Nakamura M, Lee K, Koo MS, Bouix S, Salisbury DF, Morra L, Shenton ME & Niznikiewicz MA (2009). A prospective longitudinal volumetric MRI study of superior temporal gyrus gray matter and amygdala-hippocampal complex in chronic schizophrenia. *Schizophr Res* **113,** 84–94.

Chapter 3
MATERNAL NUTIENT RESTRICTION IN GUINA PIGS AS AN ANIMAL MODEL FOR STUDYING GROWTH RESTRICTED NEONATAL OFFSPRING

3.1 Introduction

Fetal growth restriction (FGR) with infants small for their gestational age when born, is a major contributor to perinatal morbidity and mortality and for later adverse health outcomes, including cardiovascular disease, diabetes, and neurodevelopmental disability (Kramer *et al.*, 1990; Pryor *et al.*, 1995; Rossavik *et al.*, 1996; Lackman *et al.*, 2001*a*). This has led to the notion that the intrauterine environment during fetal life can "programme" the development of risk factors for these later adverse outcomes and an increasing number of human and animal based studies examining mechanisms underlying this relationship support this concept (Barker, 1998; Godfrey & Barker, 2000; Armitage *et al.*, 2004; Fowden *et al.*, 2006). Moreover, dietary conditions and feeding activity in the early post-natal period are likely to impact this relationship with FGR offspring showing acceleration of growth for their size, termed "catch-up" growth, at further risk for later health impairment as well as obesity (Colle *et al.*, 1976; Eriksson *et al.*, 1999; Beltrand *et al.*, 2009).

Guinea pigs deliver precocial young after a relatively long pregnancy with many developmental events occurring during fetal life similar to that in humans (Carter, 2007). Accordingly, moderate maternal nutrient restriction (MNR) in guinea pigs at 70% of an ad libitum diet from 4 weeks pre-conception until mid-pregnancy increasing to 90% thereafter, has been utilized for inducing FGR and studying maternal, placental and fetal growth characteristics. We (Elias *et al.*, 2016) and others (Roberts *et al.*, 2001*b*; Kind *et al.*, 2002, 2005) have shown that this experimental paradigm in guinea pigs leads to moderate to severe FGR with fetal weights decreased by 30 to 40% near term, and with aberrant placental development, asymmetrical growth, polycythemia, hypoglycemia, and evidence of chronic hypoxia in visceral tissues. These findings support the utility of this model for inducing FGR with many similarities to that in humans with moderate to severe growth restriction whether resulting from maternal undernourishment or placental insufficiency (Sohlstrom *et al.*, 1998; Roberts *et al.*, 2001; Kind *et al.*, 2003, 2005). Moderate MNR as outlined also targets the insult pre-conception and throughout pregnancy (Elias *et al*., 2016) analogous to the human situation with moderate to severe FGR, where intrauterine deprivation is likely to be early in onset (Kramer *et al.*, 1990; Pardi *et al.*, 2002).

Moderate MNR in guinea pigs has also been utilized for studying offspring outcomes, with hyperinsulinemia seen in FGR-MNR animals as postnatal young adults and suggestive of insulin resistance as seen in humans born growth restricted (Hales *et al.*, 1991; Kind *et al.*, 2003; De Blasio *et al.*, 2007*a*). However, while fetal growth characteristics have been well described with moderate MNR in guinea pigs, there has been limited study of growth characteristics in FGR offspring and the impact on organ weights during the early neonatal period and through to adulthood. In the one study by Kind et al (2003), absolute growth rates and fractional growth rates were unchanged in FGR offspring for the first month post-natal after moderate MNR before and through pregnancy. However, only nine FGR animals were studied due to pregnancy losses and fractional growth rate was increased the smaller the birth weight in all offspring combined supporting the concept of "catch-up growth" in pups that were small at birth (Kind *et al.*, 2002). We have therefore studied moderate MNR in guinea pigs and report on our pregnancy outcomes in animals delivering spontaneously near or at term, and the distribution of newborn weights and means for denoting FGR to further characterize the utility of this model. We have also determined the impact on absolute and fractional growth rates through the neonatal period and on select organ weights at neonatal necropsy, hypothesizing that MNR-FGR animals will show "catch-up growth" as often seen in human infants with growth restriction, but with organ-specific differences which might then have implications for later disease risk (Albertsson-Wikland *et al.*, 1993; Eriksson *et al.*, 1999; Hales & Ozanne, 2003; Beltrand *et al.*, 2009).

3.2 Material and Methods

3.2.1 Animal Feeding, Breeding and Pregnancy

A previously established model of moderate MNR in guinea pigs (Sohlstrom *et al.*, 1998) was used with all experimental procedures approved by The University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. The same guinea pigs were used for all three results chapters in this thesis and an over view of the general protocol can be referenced in Figure 1. Nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were housed in individual cages in a dedicated small animal

care facility with a 12 hour light/dark cycle and temperature at 25ºC. Animals were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked (Lilley *et al.*, 1997).

Thirty-nine guinea pig sows were randomly assigned to either a Control group fed ad libitum or an MNR group fed 70% of the average food intake per kilogram of body weight of the ad libitum fed animals as described by Sohlstrom et al (1998). After 2 weeks of adaptation to respective feeding regimens, animals began the breeding cycle. A female found to be in estrous was placed in a cage with a male for 48-72 hours and removed when the vaginal membrane was again closed. Animal pregnancies were confirmed by ultrasound 14-21 days later with conception taken to be the day prior to membrane closure and thereby day zero of gestation. Animals that were not pregnant were rebred at their next estrous cycle. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the Control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the Control animals per kilogram body weight. Throughout the experiment, daily food intake and body weight of the animals were monitored 3-4 times per week and the dietary intake of the MNR animals adjusted as needed to maintain their food intake at 70% or 90% of the average food intake per kilogram of body weight of the ad libitum fed animals.

3.2.2 Pupping, Post-Natal Feeding and Growth Measurements

Sows were allowed to continue their pregnancies and deliver spontaneously near or at term which is normally at 67 – 68 day`s gestation in guinea pigs. The number of live born and demised newborns was noted with weights (g) obtained on all newborns and with body length from the tip of the nose to the rump (cm), and abdominal circumference (cm) additionally obtained on all live born pups. Newborns were considered to be appropriate for gestational age (AGA) if > 95 g and FGR if < 85 g, which was extrapolated from past fetal studies using moderate MNR (Elias *et al.*, 2016).

Control and MNR sows remained on their respective diets until 15 days post-natal to mimic the human situation where undernourished mothers are likely to remain undernourished through the lactation period (Brown *et al.*, 1986; Emmett & Rogers,

1997). However, at this time MNR sows were placed on 100% of the average food intake per kilogram body weight of the ad libitum fed animals since pups were beginning to wean and were noted to be eating the mother`s food allocation.

Neonatal weights were measured every other day to minimize distress caused by animal handling. Absolute growth rates (g/day) were then calculated from birth to the end of week three post-natal as a measure of early neonatal growth up until weaning (Kind *et al.*, 2002, 2003). Fractional growth rates (%/day) were additionally calculated as the absolute growth rate for 0 – 21 days divided by the weight at birth which may be a better reflection of the anabolic state in animals over this time period (Kind *et al.*, 2002, 2003).

3.2.3 Neonatal Necropsy and Tissue Collection

Neonatal necropsies were undertaken between 23 – 27 days corrected post-natal age so that post-conceptual age was comparable for all neonates at the time of necropsy. Only AGA newborns from Control group litters and FGR newborns from MNR group litters were subjected to neonatal necropsy with no more than 1-2 males and 1-2 females to a maximum of 3 offspring per litter utilized. Remaining animals meeting the AGA-Control and FGR-MNR birth weight criteria were allowed to continue up to young adulthood for later study and will be reported on separately. Prior to necropsy, animals were weighed and then euthanized with 0.3 cc intra-peritoneal injection of Euthanol (sodium pentobarbital, MTC Pharmaceuticals, Cambridge, ON, Canada). Cardiac puncture was then carried out to obtain ~ 2 cc of blood in a heparinized syringe which was cold centrifuged with the plasma then stored at -80° C for later analysis. This was followed by dissection and weighing of the brain, heart, liver, kidneys, pancreas and gonads, and extraction of skeletal muscle and peri-renal adipose tissue. These organs/tissues were similarly partitioned in all animals and immersion fixed in 4% paraformaldehyde and/or flash frozen in liquid nitrogen for later analysis.

3.2.4 Data Acquisition and Statistical Analysis

Litter size and weight were based on live born and demised newborns noted at birth. Overall Control and MNR growth characteristics included data from all Control sows and their live born newborns, and all MNR sows and their live born newborns, excluding data from animals that failed to conceive. Select AGA-Control and FGR-MNR

growth characteristics included data from all AGA-Control and FGR-MNR newborns who were live born and met the birth weight criteria noted, excepting organ weights which were limited to those offspring undergoing neonatal necropsy. Maternal and newborn/neonatal characteristic findings are presented as group means ± SEM. Overall Control and MNR population characteristics and select AGA-Control and FGR-MNR growth and necropsy characteristics were compared using unpaired Student`s t-test for group differences, analysis of variance for sex differences, and correlation analysis between FR and birth weight (Graphpad Software, San Diego, CA). For all analysis, statistical significance was assumed for $p<0.05$.

3.3 Results

3.3.1 Breeding and Pregnancy Outcomes

Of the thirty-nine guinea pig sows, 18 were bred under ad libitum feeding conditions and 21 under MNR feeding conditions assuming breeding and pregnancy outcomes would be more adverse for the MNR animals. Two or 11% of the animals bred under ad libitum feeding conditions and 4 or 19% of the animals bred under MNR feeding conditions failed to become pregnant despite up to four breeding attempts. The 16 pregnant Control sows delivered at 67.9 ± 0.3 days gestation (range 67 to 71 days) with 44 live born and 9 newborn demises, which formed the overall Control population. The 17 pregnant MNR sows delivered a day earlier on average at 66.6 ± 0.4 days gestation (range 64 to 71 days) ($p<.05$) with 52 live born and 6 newborn demises which formed the overall MNR population.

3.3.2 Maternal and Newborn Population Characteristics

The overall maternal and newborn population characteristics from all ad libitum fed control pregnancies and all MNR pregnancies are shown in Table 1. These data are presented for all newborns to indicate the population variance and justify the use of birth weight thresholds for categorizing AGA-Control and FGR-MNR cohorts as we have previously reported for fetal study in MNR guinea pigs (Elias *et al.*, 2016). While maternal weights were not different at conception averaging ~ 830 g, at delivery MNR sows were 12% lighter at 1094 ± 18 g than Control sows at 1247 ± 41 g ($p<.01$). Food consumption for both animal groups increased through pregnancy as maternal weight

increased, with the actual food consumption of MNR sows at conception and at delivery, being 30 ± 1 g and 38 ± 1g, respectively, while Control sows consumed 39 ± 2 and 50 ± 6 g respectively. While litter size did not differ between the two study groups averaging 3-4, the combined newborn weight per litter was 21% less for the MNR animals at 271 ± 9 g than that of the Control animals at 342 ± 28 g ($p<0.05$). This was due to newborn weights at delivery for all live born being 25% less in the MNR pregnancies at 79 ± 2 g than in the Control pregnancies at 105 ± 2 g ($p<.001$). The 44 live born Control newborns ranged in weight from 132 g to 80 g with the 50^{th} and 10^{th} percentiles being ~ 105 g and 87 g, respectively, while the 52 live born MNR newborns ranged in weight from 126 g to 58 g with the 50^{th} and 10^{th} percentiles being ~ 77 g and 62 g, respectively (Figure 2). Body lengths were also decreased in the live born MNR newborns by ~ 10% at 15.2 ± 0.2 cm versus that of the Controls at 16.8 ± 0.2 cm ($p<.001$), but less than the decrease in body weights. As such, body weight/length as a measure of leaness was also decreased in the MNR newborns by 17% at 5.2 ± 0.1 g/cm versus that of the Control newborns at 6.3 ± 0.1 g/cm ($p<0.001$). Abdominal circumference as an additional measure of leaness was also decreased in the MNR newborns by ~ 8% at 9.9 ± 0.2 cm versus the Control newborns at 10.8 ± 0.2 cm ($p<.001$).

While MNR newborns were smaller than Control newborns on average, there was overlap in the population weight distributions as seen in Figure 1, since litter size, number of fetuses per uterine horn, and fetal position within the horn are also known to impact fetal growth (Detmer & Carter, 1992; Turner & Trudinger, 2000; Piorkowska *et al.*, 2014). We therefore chose to establish a select cohort of AGA born offspring from the Control group pregnancies and a select cohort of FGR born offspring from the MNR group pregnancies for more in-depth comparative study of growth-related parameters. As noted, we used > 95 g and < 85 g as our thresholds for categorizing these respective cohorts at delivery which was close to the 20^{th} and 10^{th} percentiles for the population weight distribution of the live born Control newborns at ~ 97 g and 87 g. Accordingly, 37 of 44 or 84% of all live born Control newborns were deemed to be AGA, while 41 of 52 or 79% of all live born MNR newborns were deemed to FGR. While all newborn weights in MNR pregnancies were decreased 25% on average compared to Control pregnancies, FGR-MNR newborn weights were decreased by 32% at 74 ± 1g compared to that of the

AGA-Control newborns at 109 ± 1g ($p<.001$) (Table 1). Not surprisingly, body weight/length and abdominal circumference as measures of leaness were also decreased to a greater extent in the FGR-MNR newborns compared to the AGA-Controls by 22% at 5.0 ± 0.1 g/cm versus 6.4 ± 0.1 g/cm, and by 12% at 9.6 ± 0.1cm versus 10.9 ± 0.1cm, respectively (both $p<.001$) (Table 1). After sexing live born animals, it was determined there were 19 Control males of whom 15 were AGA-Controls and 25 Control females of whom 22 were AGA-Controls; and 20 MNR males of whom 16 were FGR-MNR and 28 MNR females of whom 21 were FGR-MNR, with 4 MNR offspring unsexed due to early post-natal demise. There were no sex differences evident for any of these maternal and newborn population characteristics as assessed using analysis of variance.

3.3.3 Neonatal Growth Measurements

While all AGA-Control newborns survived out to 3 weeks for neonatal growth measurements, 4 of the FGR-MNR newborns succumbed during the first week post-natal precluding their study. Newborn and weekly neonatal weights for remaining offspring over the first three weeks post-natal along with associated growth rates are shown in Table 2. FGR-MNR newborn weights for these animals were again decreased by ~ 32% at 75 ± 1g compared to that of the AGA-Control newborns at 109 ± 1g ($p<.001$) and while both animal groups showed increased weight through week 1 post-natal, FGR-MNR offspring continued to be ~ 32% smaller. However, by the end of week 2 post-natal FGR-MNR offspring were now only 26% smaller, and by week 3 post-natal this weight difference had narrowed to 21% with FGR-MNRs at 227 ± 4g versus the AGA-Controls at 289 ± 6g ($p<.001$). Absolute growth rates over the first 3 weeks post-natal were decreased in the FGR-MNR offspring at 7.3 ± 0.2 g/day compared to that of the AGA-Controls at 8.6 ± 0.2 g/day ($p<.001$). However, fractional growth rates over this time period were increased in the FGR-MNR offspring compared to that of the AGA-Controls by 23% at 9.7 ± 0.2 %/day versus 7.9 ± 0.2 %/day ($p<.001$). Moreover, fractional growth rates showed a modest negative correlation to birth weights in the FGR-MNR offspring, $r = -0.38$ ($p<.05$), but not in the AGA-Control offspring, $r = -0.16$ (NS), as assessed using correlation analysis, shown in Figure 3. There were no sex differences evident for any of the neonatal growth rate measurements as assessed using analysis of variance excepting for absolute growth rates where males were found to have higher values independent of

the effect of MNR (p=.03), AGA-Control males vs females, 9.1 ± 0.5 g/day vs 8.2 ± 0.2 g/day, and FGR-MNR males vs females, 7.4 ± 0.2 g/day vs 7.1 ± 0.2 g/day; and the week 3 weights where males were again found to have higher values independent of the effect of MNR (p=.03), AGA-Control males vs females, 301 ± 11 g vs 280 ± 5 g, and FGR-MNR males vs females, 233 ± 6 g vs 223 ± 6 g.

3.3.4 Neonatal Necropsy Measurements

Eighteen AGA-Control offspring (8 males and 10 females) and 18 FGR-MNR offspring (9 males and 9 females) were selected for neonatal necropsy measurements. These animals were representative of the mean newborn and neonatal weights for their respective groups with birth weights and neonatal necropsy weights shown in Table 3. While birth weights in these FGR-MNR animals were decreased 35% compared to the AGA-Controls at 71 ± 2g versus 110 ± 2g (p<.001), at the time of neonatal necropsy ~ 25 days later, FGR-MNR weights were only decreased by 16% at 259 ± 10g versus 309 ± 7g (p<.01). Mean organ weights obtained at necropsy were all decreased in the FGR-MNR neonates compared to that of the AGA-controls, but this was variable with brain weights decreased ~ 6% at 3.13 ± 0.05g versus 3.32 ± 0.05g (p<.05), heart weights decreased ~ 24% at 1.46 ± 0.08g versus 1.91 ± 0.06g (p<.001), liver weights decreased ~ 10% at 11.6 ± 0.6g versus 13.0 ± 0.5g (NS), kidney weights decreased ~ 16% at 2.55 ± 0.09g versus 3.03 ± 0.09g (p<.01), pancreas weights decreased ~ 28% at 0.57 ± 0.04g versus 0.79 ± 0.06g (p<.01), testes weights decreased ~ 24% at 0.57 ± 0.04g versus 0.75 ± 0.06g (p<.05), and ovary weights decreased ~ 26% at 0.11 ± 0.01g versus 0.15 ± 0.01g (p<.05). Organ weights as a percentage of body weight were also assessed as a measure of individual organ size relative to that for the body with findings again variable. Whereas FGR-MNR brain/body weights were increased compared to the AGA-Controls by ~ 14% at 1.23 ± 0.05% versus 1.08 ± 0.03% (p<.05), liver/body weights and kidney/body weights remained little changed at ~ 4.3% and 1.0%, respectively, while heart/body weights were decreased by ~ 10% at 0.56 ± 0.01% versus 0.62 ± 0.02% (p<.05), as were pancreas/body weights by ~ 12% at 0.23 ± 0.02% versus 0.26 ± 0.02, although this was not significant due to population variance. There were no sex differences evident for any of the neonatal necropsy measurements as assessed using analysis of variance excepting for pancreatic weights where there was an interactive effect (p=.005), with the decrease in

FGR-MNR neonates from AGA-Controls, much more in females, 0.48 ± 0.05 g vs 0.91 ± 0.11 g, than in males, 0.66 ± 0.06 g vs 0.67 ± 0.06.

3.4 Discussion

In the present study we have further characterized pregnancy and neonatal-e outcomes in guinea pigs subjected to moderate nutrient restriction prior to conception, throughout pregnancy, and continuing during the early post-natal period, as a useful model for studying FGR offspring with post-natal catch-up growth. Moderate MNR at 70% of the ad libitum diet beginning at least 30 days pre-pregnancy and increasing to 90% of the ad libitum intake at mid-pregnancy, resulted in a decrease in maternal weights by 15% on the last gestational day, and a decrease in maternal weight after giving birth, but with no differences between maternal weights at conception, similar to that found in the MNR fetal study (Elias *et al.*, 2016). This pattern was also seen in a study conducted by Kind et al., (2003) who noted no differences between maternal weights at the time of mating, however they did note a 6% reduction in maternal weight at the time of delivery. Likewise, the actual decrease in food consumption at 23% and 32% in MNR sows at conception and pupping, respectively, was somewhat less than that reported by Roberts *et al* (2001) at 37% and 36% in their MNR sows which may be attributable to their smaller animals. Litter weight was decreased in the present study in the MNR group by 20% which is similar to findings by Kind et al (2003) who found a 31% decrease in litter weight in their MNR pups (Kind *et al.*, 2003). Interestingly, the present study showed no changes in the litter number between the AGA-control and FGR-MNR groups, therefore litter number is not a confounding variable in this study however, it is a different result to what has been documented in other MNR guinea pig papers describing a decrease in the number of pups per litter in the MNR litter (Kind *et al.*, 2002, 2003). These differences between studies could be attributed to the differences between the strain of guinea pig used and the differences in maternal weights prior to breeding (Sohlstrom *et al.*, 1998; Roberts *et al.*, 2001*a*).

Time of pupping for the MNR sows was earlier than the Control sows at 66.6 days and 67.9 days respectively, and of not is a finding similar to that of the human situation where the risk of pre-term birth is increased with FGR (Lackman *et al.*, 2001). Birth weights

were decreased by 25% on average for all MNR pregnancies in the present study. This finding is different from previous studies where there was only a decrease in both weight between the male Control and MNR guinea pig neonates (Kind *et al.*, 2003). This sex dependent difference in birth weights was shown by a dramatic decrease in MNR male birth weights compared to the Control males, while no differences between female MNR and Control birth weight, although the animal numbers in the study are less than those in the present study (Kind *et al.*, 2003). In addition to birth weights to characterize FGR, birth weight to length is commonly used as a measure of leanness and asymmetrical growth in addition to the use of abdominal circumference; with the present study demonstrating a 17% decrease in the weight/length ratio and a 12% decrease in the abdominal circumference in the MNR pups. Similar findings have been shown in fetal MNR guinea pig studies (Kind *et al.*, 2005; Elias *et al.*, 2016), and in neonate MNR guinea pig studies (Kind *et al.*, 2002, 2003), as well as human FGR studies whether derived from maternal undernourishment or idiopathic placental insufficiency (Kramer *et al.*, 1990).

To further study the post-natal population of neonates, we set a threshold of < 85g at birth for pups born from the MNR sows and >95g at birth for pups born from Control sows for categorizing the AGA-Control and FGR-MNR neonate cohorts. These thresholds are an extrapolation from previous fetal work that our lab (Piorkowska *et al.*, 2014; Elias *et al.*, 2016) and others (Jansson & Persson, 1990) have used for categorizing AGA and FGR fetal weights at 60 days of gestation. In the present study, the cutoffs of 85g and 95g fell close to the 10^{th} and 20^{th} percentiles for the population weight distribution of the live born Control newborns at ~ 87g and 97g, respectively, further justifying their use. All newborns that met these weight criteria were studied for neonatal growth pattern analysis with representative animals additionally selected for study of neonate necropsy values. Of note, four FGR-MNR neonates succumbed to demise within the first two weeks of life. This pattern of neonatal mortality is described in sheep and in the human situation where FGR neonates are at an increased risk for neonatal mortality and morbidity (Lackman *et al.*, 2001*a*; De Blasio *et al.*, 2007*b*).

From the time of birth, weights in the FGR-MNR group were decreased by ~ 32%, however at week 3, weights in the FGR-MNR group were only 21% less than the AGA-

Control animals, indicating that some degree of post-natal catch up growth has occurred. FGR-MNR neonates displayed a decreased AGR over the first three weeks post-natal. A decreased AGR is not surprising as the MNR sows maintained their 90% intake of the ad libitum Control fed diet until day 14 post-natal suggesting that the AGR might be a result of changes to the MNR sows milk production and composition and how this can limit the post-natal growth of the FGR-MNR neonates (Brown *et al.*, 1986; Grigor *et al.*, 1987; Emmett & Rogers, 1997). Guinea pig studies that have allowed *ad libitum* food intake post-natally after a moderate MNR through pregnancy did not show a difference in the AGR between FGR born and Control neonates at three weeks of age demonstrating that the post-natal environment is a strong contributor to the post-natal growth, as is true in humans (Kind *et al.*, 2002; Løhaugen *et al.*, 2013; Christensen *et al.*, 2014). Additionally, there was found to be a sex effect independent of MNR grouping suggesting that males were growing more than females. This finding is similar to the findings found in growth restricted sheep after caruncletomy whereby the males grew more per day than did the females independent of the experimental group (De Blasio *et al.*, 2007*b*). These findings are similar to the human situation where male FGR children from 5 years of age to 10 years of age show an increased rate of catch up growth compared to the growth restricted females (Knops *et al.*, 2005). Fractional growth rate has been known to be a parameter which better represents the anabolic state of the animal (Kind *et al.*, 2002), and the present study suggests that the FGR-MNR neonates have a higher anabolic rate and are therefore building and growing more for their weight in comparison to the AGA-Control neonates (Kind *et al.*, 2002; De Blasio *et al.*, 2007*b*). This finding is different from that studied by Kind et al (2003) whereby there were no differences between the FGR neonates and the Control neonates (Kind *et al.*, 2003). Of note, there is a spectrum of growth that is determined by birth weight as is shown through a correlational analysis between birth weights FR at birth suggesting that a smaller weight at birth has programmed the body to gain more weight for compensation through insulin programming, as described in human and animal literature (Colle *et al.*, 1976; Desai *et al.*, 2005; De Blasio *et al.*, 2007*a*; Muhlhausler *et al.*, 2009). Additionally, differences in feeding patterns as seen in FGR born sheep neonates as studied by De Blasio et al. (2007)

and may contribute to the extent of post-natal catch up growth seen in FGR-MNR neonates (De Blasio *et al.*, 2007*a*).

Weight and growth data are essential in FGR-MNR populations as studies indicate the rate of post-natal growth is programmed throughout gestation after placental insufficiency, and accelerated by the post-natal food availability. Normalized growth of FGR born infants is beneficial for long term health and lifespan, although accelerated growth as described by "catch up" growth has been shown to decrease lifespan, increase adiposity, and alter insulin sensitivity, which have implications for obesity, cardiovascular disease, and type 2 diabetes in humans (Law *et al.*, 1992; Hediger *et al.*, 1998; Ong *et al.*, 2000; Beltrand *et al.*, 2009). In both human and animal studies there has been an abundance of evidence showing an increase in fat deposition at varying ages of FGR born individuals. Sheep and guinea pig FGR offspring have an increased ability to consume nutrients in excess of lean tissue growth requirements, and increase adipose deposition, leading to an increase in post-natal growth (Greenwood *et al.*, 1998; Kind *et al.*, 2003, 2005; Louey *et al.*, 2005; De Blasio *et al.*, 2007*a*). The exact mechanisms of catch up growth in FGR infants have been increasingly studied in both humans and animal models. Programing in the growth restricted fetus, caused by placental insufficiency throughout the gestational period influences the degree of growth in the first six months of age due to an increase in insulin release (Colle *et al.*, 1976). As insulin is an anabolic regulator of growth in infants, insulin and insulin receptors are involved in accelerated growth. Higher levels of insulin action and insulin receptors due to an early emergence of increased insulin sensitivity before insulin resistance to glucose metabolism, circulating amino acids and free fatty acids have been shown to negatively correlated with birth weight and contribute to the catch-up growth and early-onset visceral obesity in growth restricted sheep (De Blasio *et al.*, 2007*a*; Muhlhausler *et al.*, 2009). Therefore there seems to be a relationship between the *in utero* programming of insulin and its contribution to adipose deposition, insulin sensitivity to glucose, amino acids, and free fatty acids which over a period of time will cause accelerated catch up growth as demonstrated by the present study, and could lead to an increased risk for cardiovascular disease, type 2 diabetes, and obesity.

The extent to which prenatal under nutrition and postnatal growth affects organ growth and development has been studied in this paper, and has not been previously studied in guinea pigs with moderate MNR. At the time of necropsy, ~23-27 days corrected post-natal age, the FGR-MNR population of animals displayed a 16% decrease in overall body weight, a 6% decrease in brain weight, and a 12% increase in the brain to body weight ratio. There were no differences in the liver weight, a 24% decrease in heart weight, a 16% decrease in kidney weight, and no differences in pancreas weight compared to the AGA-Control neonate population. Ovary and Testes were both decreased in the FGR-MNR neonates by 26% and 24%, respectively.

The decrease in brain weight is of interest as moderate MNR studies completed on fetuses at 60 days gestation displayed an ~12% decrease in brain weight in the MNR compared to the Control fetuses, while only a 6% decrease in the present neonates (Kind *et al.*, 2005; Elias *et al.*, 2016). The difference in brain weight from the time of the fetus to the neonate, although at different study periods but with identical study protocols, suggests that there is post-natal catch-up growth occurring in the brain in these FGR-MNR offspring, although these brains continued to be smaller. This could be due to the effects of blood shunting to the brain *in utero* that has continued post-natally, in addition to the post-natal environment, thereby contributing to increased substrate delivery and brain growth (Bellotti et al 2004, Haugen et al., 2005).

The liver of the FGR-MNR neonates did not show any significant difference in weight compared to the AGA-Control neonates. However, when compared to the fetal liver values in earlier studies with moderate MNR there is a 40% decrease in liver weight in the FGR-MNR fetuses compared to AGA-Control fetuses (Kind *et al.*, 2005; Elias *et al.*, 2016). This suggests that there is immense post-natal catch up growth in the liver in the first three to four weeks of post-natal life in these FGR-MNR neonates. This rapid increase in post-natal weight in the liver and thereby growth could be caused by an increase in glycogen deposition and lipid accumulation as seen with other guinea pig and rat models of FGR (Lafeber *et al.*, 1984; Joshi *et al.*, 2003) as well as an increase in blood supply to this organ causing increased nutrient availability, hepatic nutrient inter-conversion, and subsequent growth (Haugen *et al.*, 2005; Baschat & Harman, 2006).

The kidneys and heart of the FGR-MNR neonates are shown to both be decreased in size in comparison to the AGA-Control neonates, although the kidney/body ratio is unchanged, the heart/body ratio is decreased suggesting that the catch up growth for the kidney is similar to that of the body, while there is less catch up growth for the heart. This finding is quite different from that in the moderate MNR fetal study where the heart/body ratio was increased in the FGR-MNR fetus group compared the AGA-Control fetuses (Elias *et al.*, 2016). This is an interesting finding as hypertension and cardiovascular disease are both later life disease risks of FGR and both the kidneys and heart are essential organs in the maintenance of blood pressure and body fluid volume (Osmond *et al.*, 1993; Kind *et al.*, 2002; Barker *et al.*, 2005). The decrease in pancreas, size in the FGR-MNR neonate compared to the AGA-Control neonate may provide some evidence into the early origins of insulin resistance as there may be less alpha and beta cells present in the pancreas, leading to the smaller size, and loss of proper control of blood glucose homeostasis in FGR-born individuals, therefore causing diabetes (Hales *et al.*, 1991; Kind *et al.*, 2003; De Rooij *et al.*, 2006). Testes and ovary weights are significantly decreased in the FGR-NR offspring, which may contribute to increased risk for premature gonadal failure in FGR offspring as shown in animal and human studies (De Bruin *et al.*, 1998; Godfrey & Barker, 2000; Zambrano *et al.*, 2005).

3.5 Conclusion

Moderate MNR in guinea pigs has been studied extensively in the fetus, placenta, and mother for modeling human FGR. The number of studies that focus on the neonate post-natal age group after moderate MNR is limited and the present study has now added to the findings to further characterize the neonate model of FGR, the growth characteristics associated, and organ weight information and how these values related to other neonate findings and past fetal findings.

This study highlights newborn outcomes after MNR in the guinea pig which is in conjunction with other studies (Kind *et al.*, 2002, 2003). We confirm that FGR-MNR neonates are smaller and leaner at birth as is often seen in human infants with moderate growth restriction whether resulting from maternal undernourishment or placental insufficiency (Kramer *et al.*, 1990; Lumey, 1998; Lackman *et al.*, 2001*b*). We also

provide justification for using a neonate weight threshold for categorizing AGA-Control and FGR-MNR cohorts which both fall at the 10th and 20th percentiles of the Control population and decrease the coefficient of variation which could be driven by the position of the fetus in the uterine horn, and number of pups per litter. The impact of the post-natal environment on the growth of FGR born guinea pig neonates was studied and found to be different than other studies that provide an *ad libitum* diet to MNR sows after pupping although still reflects the phenomenon of accelerated catch up growth as seen in humans (Ong *et al.*, 2000; Kind *et al.*, 2002, 2003; Hales & Ozanne, 2003). This information, in conjunction with the other neonate organ weight differences, provides insight into the post-natal changes that occur after being born FGR. Differences in organ weights are representative of what is seen in placental insufficiency models and most human FGR cases, and provide follow up information to fetal FGR studies after MNR. This model can contribute to future study of possible indicators of the later life health issues that arise if born FGR and be a model for future interventions.

3.6 REFERENCES

Albertsson-Wikland K, Wennergren G, Wennergren M, Vilbergsson G & Rosberg S (1993). Longitudinal follow-up of growth in children born small for gestational age. *Acta Paediatr* **82,** 438–443.

Armitage J a, Khan IY, Taylor PD, Nathanielsz PW & Poston L (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol* **561,** 355–377.

Barker DJ (1998). In utero programming of chronic disease. *Clin Sci (Lond)* **95,** 115–128.

Barker DJP, Osmond C, Forsén TJ, Kajantie E & Eriksson JG (2005). Trajectories of growth among children who have coronary events as adults. *N Engl J Med* **353,** 1802–1809.

Baschat AA & Harman CR (2006). Venous Doppler in the assessment of fetal cardiovascular status. *Curr Opin Obstet Gynecol* **18,** 156–163.

Beltrand J, Nicolescu R, Kaguelidou F, Verkauskiene R, Sibony O, Chevenne D, Claris O & Lévy-Marchal C (2009). Catch-up growth following fetal growth restriction promotes rapid restoration of fat mass but without metabolic consequences at one year of age. *PLoS One*; DOI: 10.1371/journal.pone.0005343.

De Blasio MJ, Gatford KL, McMillen IC, Robinson JS & Owens JA (2007*a*). Placental restriction of fetal growth increases insulin action, growth, and adiposity in the young lamb. *Endocrinology* **148,** 1350–1358.

De Blasio MJ, Gatford KL, Robinson JS & Owens J a (2007*b*). Placental restriction of fetal growth reduces size at birth and alters postnatal growth, feeding activity, and adiposity in the young lamb. *Am J Physiol Regul Integr Comp Physiol* **292,** R875–R886.

Brown KH, Akhtar N a, Robertson a D & Ahmed MG (1986). Lactational capacity of marginally nourished mothers: relationships between maternal nutritional status and quantity and proximate composition of milk. *Pediatrics* **78,** 909–919.

De Bruin JP, Dorland M, Bruinse HW, Spliet W, Nikkels PGJ & Te Velde ER (1998). Fetal growth retardation as a cause of impaired ovarian development. *Early Hum*

Dev **51,** 39–46.

Carter AM (2007). Animal Models of Human Placentation - A Review. *Placenta* **28,** S41–S47.

Christensen DL, Schieve LA, Devine O & Drews-Botsch C (2014). Socioeconomic status, child enrichment factors, and cognitive performance among preschool-age children: Results from the Follow-Up of Growth and Development Experiences study. *Res Dev Disabil* **35,** 1789–1801.

Colle E, Schiff D, Andrew G, Bauer CB & Fitzhardinge P (1976). Insulin responses during catch-up growth of infants who were small for gestational age. *Pediatrics* **57,** 363–371.

Desai M, Gayle D, Babu J & Ross MG (2005). Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *Am J Physiol Regul Integr Comp Physiol* **288,** R91–R96.

Detmer A & Carter A (1992). Factors influencing the outcome of ligating the uterine artery and vein in a guinea pig model of intrauterine growth retardation. *Scand J Lab Anim Sci* **19,** 9–16.

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Emmett PM & Rogers IS (1997). Properties of human milk and their relationship with maternal nutrition. *Early Hum Dev* **49 Suppl,** S7–S28.

Eriksson JG, Forsén T, Tuomilehto J, Winter PD, Osmond C & Barker DJ (1999). Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *BMJ* **318,** 427–431.

Fowden AL, Giussani DA, Forhead AJ, Fowden AL, Dino A & Forhead AJ (2006). Intrauterine Programming of Physiological System Causes and consequences.pdf. 29–37.

Godfrey K & Barker D (2000). Fetal Nutrition and Adult Disease. *Am J Clin Nutr* **71,** 1344s–1352s.

Greenwood PL, Hunt a S, Hermanson JW, Bell a W, Greenwood PL, Hunt AS, Hermanson JW & Bell AW (1998). Effects of birth weight and postnatal nutrition on

neonatal sheep : I . Body growth and composition , and some aspects of energetic efficiency The online version of this article , along with updated information and services , is located on the World Wide . *J Anim Sci*2354–2367.

Grigor MR, Allan JE, Carrington JM, Carne a, Geursen a, Young D, Thompson MP, Haynes EB & Coleman R a (1987). Effect of dietary protein and food restriction on milk production and composition, maternal tissues and enzymes in lactating rats. *J Nutr* **117,** 1247–1258.

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

Hales CN & Ozanne SE (2003). The dangerous road of catch-up growth. *J Physiol* **547,** 5–10.

Haugen G, Hanson M, Kiserud T, Crozier S, Inskip H & Godfrey KM (2005). Fetal liver-sparing cardiovascular adaptations linked to mother's slimness and diet. *Circ Res* **96,** 12–14.

Hediger ML, Overpeck MD, Kuczmarski RJ, McGlynn a, Maurer KR & Davis WW (1998). Muscularity and fatness of infants and young children born small- or large-for-gestational-age. *Pediatrics* **102,** E60.

Jansson T & Persson E (1990). Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Pediatr Res* **28,** 203–208.

Joshi S, Garole V, Daware M, Girigosavi S & Rao S (2003). Maternal Protein Restriction before pregnancy affects vital organs of offspring in wistar rats. *Metabolism* **52,** 13–18.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS & Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS & Owens J a (2005). Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol* **288,** R119–R126.

Kind KL, Simonetta G, Clifton PM, Robinson JS & Owens J a (2002). Effect of maternal

feed restriction on blood pressure in the adult guinea pig. *Exp Physiol* **87,** 469–477.

Knops NBB, Sneeuw KC a, Brand R, Hille ETM, den Ouden a L, Wit J-M & Verloove-Vanhorick SP (2005). Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. *BMC Pediatr* **5,** 26.

Kramer MS, Olivier M, McLean FH, Willis DM & Usher RH (1990). Impact of intrauterine growth retardation and body proportionality on fetal and neonatal outcome. *Pediatrics* **86,** 707–713.

Lackman F, Capewell V, Richardson B, DaSilva O & Gagnon R (2001*a*). The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol* **184,** 946–953.

Lackman F, Capewell V, Richardson B, DaSilva O & Gagnon R (2001*b*). The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. *Am J Obstet Gynecol* **184,** 946–953.

Lafeber H, Rolph T & Jones C (1984). Studies in the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol* **6,** 441–459.

Law CM, Barker DJ, Osmond C, Fall CH & Simmonds SJ (1992). Early growth and abdominal fatness in adult life. *J Epidemiol Community Health* **46,** 184–186.

Lilley KG, Epping RJ & Hafner LM (1997). The guinea pig estrous cycle: correlation of vaginal impedance measurements with vaginal cytologic findings. *Lab Anim Sci* **47,** 632–637.

Løhaugen GCC, Østgård HF, Andreassen S, Jacobsen GW, Vik T, Brubakk A-M, Skranes J & Martinussen M (2013). Small for gestational age and intrauterine growth restriction decreases cognitive function in young adults. *J Pediatr* **163,** 447–453.

Louey S, Cock ML & Harding R (2005). Long term consequences of low birthweight on postnatal growth, adiposity and brain weight at maturity in sheep. *J Reprod Dev* **51,** 59–68.

Lumey LH (1998). Compensatory placental growth after restricted maternal nutrition in

early pregnancy. *Placenta* **19,** 105–111.

Muhlhausler BS, Duffield J a, Ozanne SE, Pilgrim C, Turner N, Morrison JL & McMillen IC (2009). The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. *J Physiol* **587,** 4199–4211.

Ong KK, Ahmed ML, Emmett PM, Preece MA & Dunger DB (2000). Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *BMJ* **320,** 967–971.

Osmond C, Barker DJ, Winter PD, Fall CH & Simmonds SJ (1993). Early growth and death from cardiovascular disease in women. *BMJ* **307,** 1519–1524.

Pardi G, Marconi AM & Cetin I (2002). Placental-fetal interrelationship in IUGR fetuses - A review. *Placenta* **23,** 136–141.

Piorkowska K, Thompson J, Nygard K, Matushewski B, Hammond R & Richardson B (2014). Synaptic development and neuronal myelination are altered with growth restriction in fetal guinea pigs. *Dev Neurosci* **36,** 465–476.

Pryor J, Silva PA & Brooke M (1995). Growth, development and behaviour in adolescents born small-for-gestational-age. *J Paediatr Child Health* **31,** 403–407.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001*a*). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA (2001*b*). Altered placental structure induced by maternal food restriction in guinea pigs: A role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22,** 77–82.

De Rooij SR, Painter RC, Roseboom TJ, Phillips DIW, Osmond C, Barker DJP, Tanck MW, Michels RPJ, Bossuyt PMM & Bleker OP (2006). Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. *Diabetologia* **49,** 637–643.

Rossavik IK, Piper JM, Xenakis EM, McFarland M, Elliott BD, Berkus MD & Langer O (1996). Do growth-retarded premature infants have different rates of perinatal

morbidity and mortality than appropriately grown premature infants? *Obstet Gynecol* **87,** 169–174. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8559517\nhttp://www.ncbi.nlm.nih.gov/pubmed/8649694.

Sohlstrom A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS & Owens JA (1998). Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *Am J Physiol* **274,** E410-6.

Turner AJ & Trudinger BJ (2000). Ultrasound measurement of biparietal diameter and umbilical artery blood flow in the normal fetal guinea pig. *Comp Med* **50,** 379–384.

Zambrano E, Rodríguez-González GL, Guzmán C, García-Becerra R, Boeck L, Díaz L, Menjivar M, Larrea F & Nathanielsz PW (2005). A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. *J Physiol* **563,** 275–284.

Table 1. Maternal and Newborn Population Characteristics

	Control (16 sows / 44 live born)	MNR (17 sows / 52 live born)	AGA-Control (37 live born)	FGR-MNR (41 live born)
Maternal wt (g)				
Conception	812±16	842±11		
Delivery	1247±41	1094±18**		
Food (g/day)				
Conception	38.5±2	30±1		
Delivery	56.1±6	38±1		
Litter size	3.3±0.3	3.4±0.2		
Total litter wt (g)	342±28	271±9*		
Newborn wt (g)	105±2	79±2***	109±1	74±1***
Body length (cm)	16.8±0.2	15.2±0.2***	17.0±0.2	14.9±0.2***
Body wt/length (g/cm)	6.3±0.1	5.2±0.1***	6.4±0.1	5.0±0.1***
Abd circumference (cm)	10.8±0.2	9.9±0.2***	10.9±0.1	9.6±0.1***

Data presented as means ± SEM; * p <.05, ** p <.01, *** p <.001 vs corresponding Control group value analyzed using unpaired Student's t-test; MNR = maternal nutrient restriction; AGA = appropriate for gestational age; FGR = fetal growth restriction; GA = gestational age.

Table 2. Neonatal Growth Measurements

	AGA-Control (37 neonates)	FGR-MNR (37 neonates)
Newborn wt (g)	109±1	75±1***
Week 1 wt (g)	150±4	100±2***
Week 2 wt (g)	220±6	162±4***
Week 3 wt (g)	289±6	227±4***
AGR (g/day)	8.6±0.2	7.3±0.2***
FR (%/day)	7.9±0.2	9.7±0.2***

Data presented as means ± SEM; *** $p < .001$ vs corresponding AGA-Control group value analyzed using unpaired Student's t-test; AGA = appropriate for gestational age; FGR = fetal growth restricted; MNR = maternal nutrient restricted; AGR = absolute growth rate; FR = fractional growth rate.

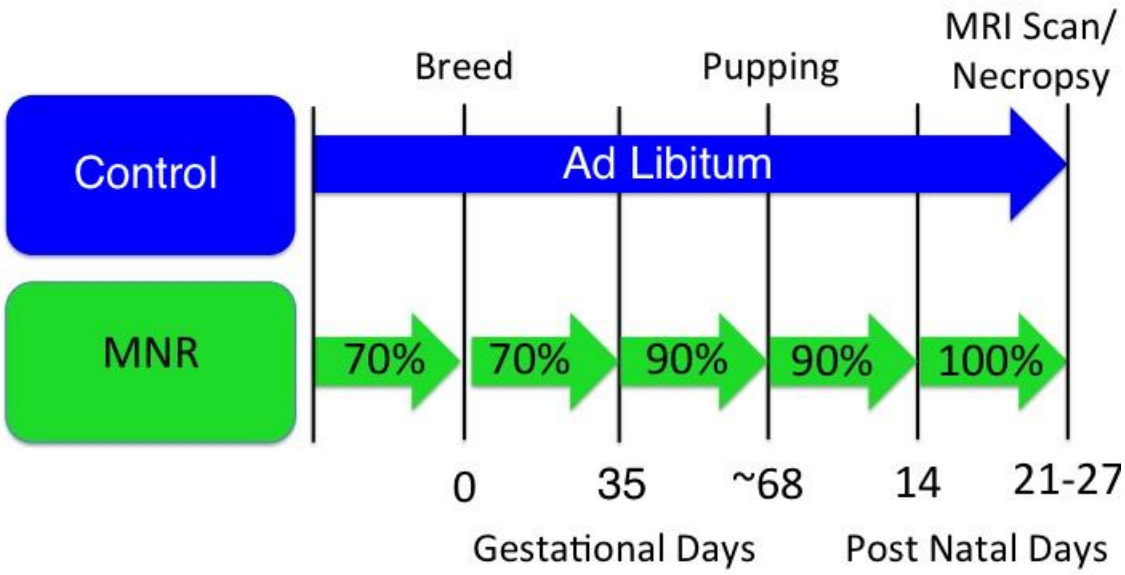
Table 3. Neonatal Necropsy Measurements

	AGA-Control (8 males/10 females)	FGR-MNR (9 males/9 females)
Newborn wt (g)	110±2	71±2***
Necropsy wt (g)	309±7	259±10**
Brain wt (g)	3.32±0.05	3.13±0.05*
Heart wt (g)	1.91±0.06	1.46±0.08***
Liver wt (g)	13.0±0.5	11.6±0.6
Kidney wt (g)	3.03±0.09	2.55±0.09**
Pancreas wt (g)	0.79±0.06	0.57±0.04**
Testes wt (g)	0.75±0.06	0.57±0.04*
Ovary wt (g)	0.15±0.01	0.11±0.01*
Brain/body wt (%)	1.08±0.03	1.23±0.05*
Heart/body wt (%)	0.62±0.02	0.56±0.01*
Liver/body wt (%)	4.2±0.1	4.4±0.1
Kidney/body wt (%)	0.98±0.02	0.99±0.03
Pancreas/body wt (%)	0.26±0.02	0.23±0.02

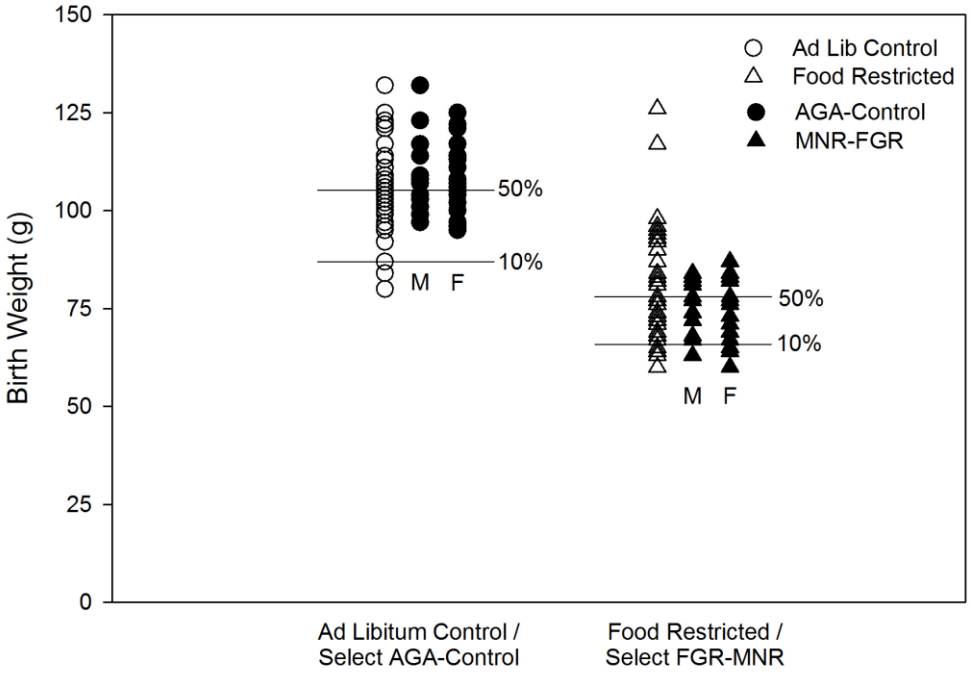
Data presented as means ± SEM; * $p < .05$, ** $p < .01$, *** $p < .001$ vs corresponding AGA-Control group value analyzed using unpaired Student's t-test; combined weights are shown for paired organs; AGA = appropriate for gestational age; FGR = fetal growth restricted; MNR = maternal nutrient restricted.

FIGURE LEGENDS

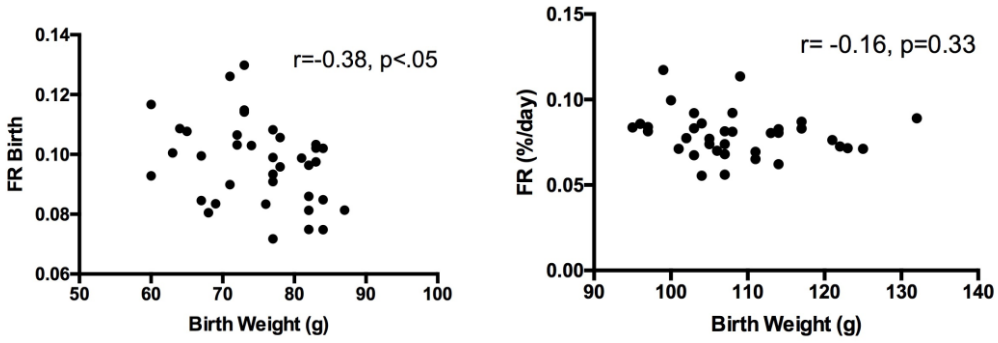
1. Guinea Pig breeding, feeding and imaging protocol for the entirety of the study as adapted by Sohltrom et al., 1998.



2. Scatter plot showing the fetal weights for all 44 liveborn Control neonates (open circles) and all 52 liveborn maternal nutrient restricted (MNR) neonates (open triangles) along with the 50th and 10th percentiles for each of these cohort populations. Additionally shown are the distribution of neonate weights for the select 37 AGA-Control neonates (closed circles, M=males, F=females) and 41 FGR-MNR neonates (closed triangles, M=males, F=females).



3. Fractional Growth Rate correlation with birth weight for the 41 FGR-MNR (left), and the 37 AGA-Control (right) neonates. FR= Fractional Growth Rate, r=Pearson coefficient.



Chapter 4
MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS LEADING TO GROWTH RESTRICTED NEONATAL OFFSPRING: IMPACT ON T2 MAGNETIC RESONANCE IMAGING OF THE BRAIN AND NEUROANATOMICAL CORRELATES

Maternal Nutrient Restriction in Guinea Pigs Leading to Growth Restricted Neonatal Offspring: Impact on T2 Magnetic Resonance Imaging of the Brain and Neuroanatomical Correlates

4.1 INTRODUCTION

Fetal growth restriction (FGR) with infants small for their gestational age when born, is a major contributor to perinatal morbidity and mortality and for later adverse health outcomes. FGR is a known risk factor for neurological disorders later in life, which include schizophrenia, cerebral palsy, autism and attention deficit hyperactive disorder (StrangKarlsson *et al.*, 2008; Haglund & Källén, 2011; Lui, Jing, Wang, Xiao-Feng, Yan, 2014). The risk of developing these disorders is directly proportional to the severity and onset of FGR. Neurocognitive evaluations involving human neonates and young adults who were born growth restricted demonstrate poorer neurodevelopmental outcomes than those performed on non-growth restricted neonates (Fattal-Valevski *et al.*, 2009; Løhaugen *et al.*, 2013). This indicates the occurrence of an *in utero* adverse event with variable degrees of insult with lifelong consequences. The latter suggests structural and functional changes in FGR brains (Haugen *et al.*, 2014).

Guinea pigs deliver precocial young after a relatively long pregnancy with many developmental events occurring during fetal life similar to that in humans (Carter, 2007). Accordingly, moderate maternal nutrient restriction (MNR) in guinea pigs at 70% of an ad libitum diet from 4 weeks pre-conception until mid-pregnancy increasing to 90% thereafter, has been utilized for inducing FGR and studying maternal, placental and fetal growth characteristics. We (Elias *et al.*, 2016) and others (Roberts *et al.*, 2001*a*; Kind *et al.*, 2005) have shown that this experimental paradigm in guinea pigs leads to moderate to severe FGR with fetal weights decreased by 30 to 40% near term, and with aberrant placental development, asymmetrical growth, polycythemia, hypoglycemia, and evidence of chronic hypoxia in visceral tissues. These findings support the utility of this model for inducing FGR with many similarities to that in humans with moderate to severe growth restriction whether resulting from maternal undernourishment or placental insufficiency (Sohlstrom *et al.*, 1998; Roberts *et al.*, 2001*b*; Kind *et al.*, 2003, 2005). Moderate MNR

as outlined also targets the insult pre-conception and throughout pregnancy analogous to the human situation with moderate to severe FGR, where intrauterine deprivation is likely to be early in onset (Kramer *et al.*, 1990; Pardi *et al.*, 2002; Elias *et al.*, 2016).

Safe and non-invasive technologies such as Magnetic Resonance Imaging (MRI) have been developed to better study the impact of an adverse, intrauterine environment as well as prematurity on the structure and function of the developing brain (Hüppi *et al.*, 1998; Tolsa *et al.*, 2004; Lodygensky *et al.*, 2008). MRI can be used to study the anatomy in the brain, specifically the grey matter, white matter, and hippocampus, and to assist in the determination of potential future neurodevelopmental abnormalities. A reduction in hippocampus volume has been shown in premature, FGR human neonates (Lodygensky *et al.*, 2008). Additionally, Tolsa *et al.* (2004) found a reduction in both total cerebral volume in preterm FGR neonates as compared to term appropriately grown human neonates.

Previous histological research has shown a reduction in hippocampal volume, stratum oriens thickness, and the number of pyramidal neurons in hippocampal sections of one-week old guinea pig neonates after a mid-pregnancy insult of uterine artery ligation causing FGR (Mallard *et al.*, 2000). Similar findings, including decreases in hippocampal neuron cell numbers in the CA2, CA4 and Dentate Gyrus (DG), were discovered in growth restricted rat neonates at two weeks of age following maternal undernourishment (Florian & Nunes, 2010). Other growth restricted guinea pig fetal studies discovered a decrease in axon outreach, maturation and growth in the hippocampal layers, thus leading to a decrease in hippocampal volume and layer thickness with an accompanying increase in ventricle volume (Mallard *et al.*, 1999, 2000). The hippocampus and lateral ventricle volume have been found to be involved in the brain structure of individuals and animal models of schizophrenia and autism, which are neurological disorders associated with FGR (Mallard *et al.*, 1999; McAlonan *et al.*, 2005; Yoshida *et al.*, 2009; Haglund & Källén, 2011). Additional research regarding the impact of an adverse intrauterine environment on *in utero* brain development in growth restricted neonate guinea pigs after

MNR is needed to better understand the neuroanatomical correlates associated with FGR and later neurological disorders.

The present study utilizes T2 weighted MRI in an MNR model to identify brain developmental abnormalities and biomarkers in the brain of growth restricted neonate guinea pigs and correlate these findings with the same brains through neuroanatomical histology study. We hypothesize a decrease in hippocampus volume and an increase in lateral ventricle volume to be present in the FGR-MNR guinea pig neonates on MRI as seen in the FGR human situation and neurodevelopmental disorders associated with FGR.

4.2 MATERIALS AND METHODS

4.2.1 The Animal Model

A previously established model of moderate MNR in guinea pigs (Sohlstrom *et al.*, 1998) was used with all experimental procedures approved by The University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. Nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were housed in individual cages in a dedicated small animal care facility with a 12 hour light/dark cycle and temperature at 25ºC. Animals were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked (Lilley *et al.*, 1997). The guinea pigs used in this Chapter are the same used for Chapter 3.

Thirty-nine guinea pig sows were randomly assigned to either a Control group fed ad libitum or an MNR group fed 70% of the average food intake per kilogram of body weight of the ad libitum fed animals as described by Sohlstrom et al (1998). After 2 weeks of adaptation to respective feeding regimens, animals began the breeding cycle. A female found to be in estrous was placed in a cage with a male for 48-72 hours and removed when the vaginal membrane was again closed. Animal pregnancies were confirmed by ultrasound 14-21 days later with conception taken to be the day prior to membrane closure and thereby day zero of gestation. Animals that were not pregnant

were rebred at their next estrous cycle. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the Control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the Control animals per kilogram body weight. Throughout the experiment, daily food intake and body weight of the animals were monitored 3-4 times per week and the dietary intake of the MNR animals adjusted as needed to maintain their food intake at 70% or 90% of the average food intake per kilogram of body weight of the ad libitum fed animals.

Sows were allowed to continue their pregnancies and deliver spontaneously near or at term which is normally at 67 – 68 day`s gestation in guinea pigs. Newborns were considered to be appropriate for gestational age (AGA) if > 95 g and FGR if < 85 g, which was extrapolated from past fetal studies using moderate MNR (Elias *et al.*, 2016).

Control and MNR sows remained on their respective diets until 15 days post-natal to mimic the human situation where undernourished mothers are likely to remain undernourished through the lactation period (Brown *et al.*, 1986; Emmett & Rogers, 1997). However, at this time MNR sows were placed on 100% of the average food intake per kilogram body weight of the ad libitum fed animals since pups were beginning to wean and were noted to be eating the mother`s food allocation. On post-natal day 21-25, one female and one male neonate from each AGA-Control and FGR-MNR litter underwent MRI studies.

4.2.2 Animal Handling

Each animal was handled in the Animal Preparation Facility alongside the 9.4T magnet in the Robarts Imaging Facility by an animal technician. The guinea pig was initially anesthetized using 4% isofluorane, 1.5L/min oxygen in a chamber, and then was decreased for continued sedation at 1.8% through a nose cone for the duration of testing. The guinea pig was placed on a warm water pad to maintain core body temperature measured through a rectal thermometer throughout the MRI scan. Heart rate and oxygen saturation were all monitored through a foot monitor. After the scan was complete, the

guinea pig was taken out of the scanner, injected with 1cc saline and placed under a heat lamp for recovery. Animals were allowed to recover for 48-72 hours before necropsy.

4.2.3 Neonate Imaging Protocol

MRI scans were obtained at Western University in the Robarts Research Institute Centre for Metabolic Mapping. Scanning was performed on a 9.4 T magnet (9.4 Tesla Varian MR Systems) using a head surface radiofrequency (RF) coil. The protocol consisted of a T2 Weighted 2-Dimensional Fast Spin Echo (FSE) pulse sequence consisting of 51 coronal slices with a 500 micron slice thickness, in plane resolution of 150x150 microns, repetition time (TR) of 6000 ms, and echo time (TE) of 75 ms with 8 averages overall. The total scan time for this protocol was 20 minutes.

4.2.4 Imaging Analysis

Analysis of the MRI stacked images consisted of one blinded observer completing the measurements of hippocampal and lateral ventricular volumes. For each MRI data set the hippocampal and ventricular volumes were quantified using ImageJ version 1.6 image analysis software, specifically using the region of interest (ROI) measurement tool for hippocampal volume, and the Connected Threshold Grower tool for the ventricular volume quantification.

4.2.4.1 Hippocampal Volume Quantification

Hippocampal tissue was defined as brain tissue arranged in the C-shaped horn displayed on a coronal brain section. All images were inspected to ensure the use of the scale of 6.667 pixels/mm. Manual segmentation was completed on all images using the region of interest segmentation tool. This area was then multiplied by the image slice thickness (0.5mm) to calculate volume.

4.2.4.2 Ventricular Volume Quantification

Prior to image analysis and volume quantification, all raw images underwent a processing stage in order to increase the quality of the images by improving the contrast between tissues. Images were further standardized to a 16-bit type image dynamic and the distance between pixels was set at 1.00 pixel/mm to suit the software requirements. The

ventricular space included the lateral ventricle volumes and was defined as the higher brightness threshold on the T2 weighted image located continuously throughout the brain.

4.2.5 Neonate Brain Histology for Neuroanatomical Correlates

4.2.5.1 Tissue Processing and Embedding

After neonate MRI, guinea pigs were allowed to recover fully for 48-72 hours after which they underwent a full necropsy. Brains were extracted from the skull after decapitation following an intra-peritoneal injection of Euthanol. The brain was sectioned at the cerebral peduncles and the rostral two thirds of the brain was placed in 4% paraformaldehyde for immersion fixation, three rinses of Phosphate Buffer Saline (PBS) over 72 hours, and 70% Ethanol for 2-3 weeks. The rostral two thirds of the brain was then dissected at the optic chiasm for further sectioning of the brain in the rostral section, optic chiasm and forward, and middle or hippocampal section from the cerebral peduncles to the optic chiasm. These two blocks were embedded in paraffin wax for histological analysis.

4.2.5.2 Tissue Sectioning of Rostral and Hippocampal Block

Both the rostral block and hippocampal block were sectioned for histological analysis. The rostral block was sectioned at 5um thickness for 2 sections with one corresponding to section 720, and a second corresponding to section 760 in the guinea pig brain atlas for ventricular cross sectional areas (Welker *et al.*, 2010) (Figure 1).

The hippocampal block was sectioned at 5um thickness for 3 sections corresponding to sections 800, 840 and 880 in the guinea pig brain atlas (Welker *et al.*, 2010). All of the hippocampal slides were used for, hippocampal cross-sectional area, and lateral ventricle cross sectional area (Figure 2).

4.2.5.3 Tissue Staining and Histological Analysis

Nissl Stain of Guinea Pig Brain Tissues

To visualize neurons and examine the morphology of the coronal brain sections, Nissl staining was performed on these paraffinized sections to stain the Nissl bodies using

0.1% cresyl violet (The British Drug Houses Ltd., Poole, England). All sections were first deparaffinized in two changes of xylene and sequentially hydrated in 100%, 95% and 70% alcohol, followed by rinsing twice in double distilled water. The sections were immediately stained in 0.1% cresyl violet for 5 minutes, after which they were rinsed quickly in double distilled water and differentiated in 95% ethyl alcohol for 15 minutes. The sections were then dehydrated in 100% alcohol, cleared in xylene, and slides were mounted with permanent mounting medium.

Each brain slide was scanned at 2.5x magnification on the Zeiss AxioImager Z1 Brightfield microscope and captured with the Zen Pro 2012 software. Measurements of the brain regions were completed using Image-Pro Premier, v9.1 made by MediaCybernetics.

4.2.7 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism. Data are presented as the mean ± the standard error of the mean (SEM). Hippocampal and whole brain volumes were statistically analyzed using a two-tailed Student t-test. Ventricular volumes were analyzed using a two-tailed Students t-test. All histological measures were compared between groups using a Students t-test. Histological data are presented as % Difference from Control as a composite measure of all data obtained from all 5-brain sections. Correlational analyses between measures were compared on an individual animal level, and a Pearson correlation coefficient was generated. Values for $P<0.05$ were considered statistically significant.

4.3 RESULTS

4.3.1 Population Characteristics

Eighteen AGA-Control (8 males and 10 females) and 18 FGR-MNR (9 males and 9 females) neonates were studied at the age of 21-25 days post-natal. FGR-MNR neonates were 30% smaller at birth compared to AGA-Control neonates, while at the time of MRI and necropsy 48-72 hrs later, body weights were only 15% smaller compared to AGA-Control neenates ($p<0.05$). Brain weights at necropsy in the FGR-MNR group were 5%

smaller than the AGA-Control neonates with values of 3.13+/- 0.05 and 3.32+/- 0.05, respectively ($p<0.05$). There were no sex differences in the results shown.

4.3.2 MRI Analysis

Seventeen AGA-Control and 18 FGR-MNR neonates were included in the study for MRI analysis. One AGA-Control MRI could not be analyzed due to non-compatible file size and type for the software.

FGR-MNR neonates who underwent MRI displayed a 6% decrease in whole brain volume with a value of 2614 ± 30 mm^3 for the FGR-MNR animals and 2787 ± 30 mm^3 for the AGA-Control animals ($p<0.05$), as shown in Table 1. Brain volume was also positively correlated with birth weight with an R value of 0.55, $p<0.001$ as shown in Figure 1. The brain weight, obtained 48-72 hours after MRI, and brain volume measures were positively correlated with an R value of 0.66, $p<0.001$ as shown in Figure 2. FGR-MNR neonates had a 15% decrease in total hippocampal volume compared to the AGA-Control neonates with values of 141.5 ± 3.8 mm^3 and 120.6 ± 3.3 mm^3 ($p<0.05$), as shown in Table 1, with a positive correlation of hippocampal volume to birth weight ($r=0.5$, $p<0.05$) as shown in Figure 1. The hippocampal volume to whole brain volume ratios showed a 9% decrease in the FGR-MNR neonates ($p<0.05$) (Table 1).

Lateral ventricle volume was decreased in the FGR-MNR neonates by 28% compared to the AGA-Control neonates with values of 14.8 ± 0.9 mm^3 for the FGR-MNR group and 20.7 ± 1.4 mm^3 for the AGA-Control neonates ($p<0.05$) (Table 1). Additionally, the lateral ventricle volume to whole brain volume ratio was decreased by 24% with values of 0.0059 ± 0.001 for the FGR-MNR neonates and 0.0075 ± 0.001 for the AGA-Controls ($p<0.05$), as shown in Table 1. There were no sex differences in the results shown.

4.3.3 Neuro-anatomical Correlates

The neuroanatomical correlates were analyzed at two levels in the rostral section of the brains and at three levels of the hippocampus section of the brains. .

Values obtained for all 5 brain levels were converted to a % change from Control and compiled to make a composite measure for each brain parameter to allow for comparison with the MRI neuro-anatomic measurements and are shown in Table 2. Results for this analysis were only available from 12 of the AGA-Control neonates and 9 of the FGR-MNR neonates due to initial difficulty with sectioning the hippocampal blocks. This compilation of data demonstrates a 14% decrease in hippocampus in the FGR-MNR neonates compared to AGA-Control neonates, as shown in Table 2. The lateral ventricle area was also reduced by 20% in the FGR-MNR neonates compared to the AGA-Controls (Table 2). There were no sex differences in the results shown.

4.3.4 Correlation between Histology and MRI Analysis

A correlation analysis was completed between the MRI hippocampal volumes and the composite hippocampal cross sectional areas measured histologically. Similarly the MRI lateral ventricle volumes were compared to the composite lateral ventricle cross sectional areas measured histologically. The hippocampal correlations had an R value of 0.44 with a p value of 0.06 as shown in Figure 5. The lateral ventricle correlations had an R value of 0.54 with a p value of 0.01 as shown in Figure 5.

Additionally, a comparison of the coefficient of variation for the MRI measurements and the histology measurements was conducted. The coefficient of variation for the hippocampus MRI measures was 11% while the coefficient of variation for the hippocampus histology measures was 18%.

4.4 Discussion

Overall this study identified brain developmental abnormalities in the growth-restricted neonate with the use of MRI and confirms the findings through neuroanatomical correlates in histology. An overall decrease in whole brain volume, hippocampus volume, and lateral ventricle volume is evident in the growth-restricted neonate suggesting that there is some degree of brain developmental disparities between the AGA-Control and FGR-MNR neonates. One study conducted in human FGR and preterm infants at two weeks of life and at term showed a reduction in intracranial volume and cortical grey matter, and a less mature school in behavioural assessments in the FGR infants at term

(Tolsa *et al.*, 2004). This finding is similar to that of the present study as we noted a decrease whole brain volume, and provides insight into the functional and behavioural aspects of the FGR born individuals that our study did not complete. Of note, FGR-MNR neonates that have a much lower birth weight also have decreased hippocampus volume and whole brain volume as these values are positively correlated with birth weight, and this could be used for future reference to brain developmental abnormalities. One paper that studied FGR and the effect it has on a premature infant's hippocampus demonstrated a reduction in MRI hippocampus volume and positively correlated hippocampus volume to birth weight (Lodygensky *et al.*, 2008). Our results are very similar to those found in the Lodygensky *et al*. (2008) study and demonstrate the importance of birth weight as a factor for determining neonate brain development in addition to its importance in determining the risk of neonate morbidity and mortality (Lackman *et al.*, 2001). Lateral ventricle volumes were decreased in the present study, which was different from that hypothesized. Although our results differed from our hypothesis, this study demonstrates new findings in relation to the model of maternal nutrient restriction in the neonate. One study conducted in premature FGR infants showed no differences in cerebral spinal fluid volume through MRI, which is a different finding than that seen in the present study (Tolsa *et al,* 2004). The use of MRI for FGR fetuses and neonates is a relatively new field that is growing in popularity and has proven its importance in the present study, as studies that utilize MRI as a measure of brain development in autism and schizophrenia have found similar findings to those in the present study (McAlonan *et al.*, 2005; Yoshida *et al.*, 2009; Haglund & Källén, 2011; Abel *et al.*, 2013). Therefore more studies in this model are necessary to build on the results of the MRI findings in the present study to assist in the determination of biomarkers for later neuro-developmental disorders.

Histological studies in the guinea pig neonate are sparse, however one study that investigates the hippocampus of an FGR neonate noted a decrease in hippocampus area, neuron number, stratum oriens area, and a decrease in white matter area in the cerebral cortex (Mallard *et al.*, 2000). These findings are similar to those seen in the present study; however, the cause of FGR was by MNR, and not by uterine artery ligation in the Mallard et al. (2000) study, although both insults have shown to affect the placenta

resulting in an insult to the brain (Turner & Trudinger, 2009). Another study investigating the hippocampus and lateral ventricles in the brain by histology was completed in an FGR guinea pig fetus; the group noted a decrease in hippocampus area and an increase in the lateral ventricle volumes in the growth restricted fetuses (Mallard *et al.*, 1999). Although the hippocampus volume is similar to that found in the present study, the lateral ventricle volume was opposite of the present study. One such explanation for the difference could be the insult to the fetus to produce FGR as the one in the present study was a chronic moderate MNR while the study cited created FGR by uterine artery ligation. Uterine artery ligation is an acute, surgical restriction of blood flow to the fetus, and therefore a much different onset of FGR, which could affect brain development differently than MNR and potentially alter lateral ventricle volume (Jones & Parer, 1983; Lafeber *et al.*, 1984; Turner & Trudinger, 2009). Additionally, the age of the animal at study can also have an effect on the brain structure size, as one focused on the fetus while the preset study focused on the neonate (Mallard *et al.*, 1999). The guinea pig brain at the time of fetal study has a much smaller weight, 2.39g (Elias *et al.*, 2016), in comparison to the neonate guinea pig FGR brain at 3.13g, suggesting that there is post-natal brain growth in the growth restricted offspring which could account for the differences in lateral ventricle volume between the fetus and the neonate as the increase in overall brain tissue growth and development could fill lateral ventricle volume. Lastly, studies in FGR sheep have shown a decrease in hippocampus volume. To our knowledge, the present study is the first to positively correlate MRI structural findings to histological findings in a fetal growth restricted neonate guinea pig model. This paper provides insight into the use of MRI and the reliability of this tool for its future use into early detection of biomarkers to decrease later neurodevelopmental disorders.

4.5 Conclusion

In conclusion, MNR in guinea pigs leads to FGR neonates, with smaller brains compared to the age matched AGA-Controls despite a degree of "brain sparing". These neonates have decreased hippocampus volume and hippocampal/brain volume ratios on MRI, indicating selective hippocampal vulnerability as a possible biomarker for later cognitive impairment. Contrary to our hypothesis, lateral ventricle volumes were variably

decreased relative to brain volumes, suggesting that other brain areas might be undergoing reactive increases in growth as additional markers for aberrant brain development in these FGR neonates, and more research is needed in this area. The histological study showed similar results compared to the MRI results indicating that MRI is a reliable method for obtaining brain anatomical data however, the variation seen in histology is much greater than that seen in MRI.

In summary, MRI has the capability of discovering structural differences in the developing brain of a fetal growth restricted guinea pig neonate, and can accurately determine brain volume that reflects the neonate brain weight. This information is useful in the understanding and continued development of biomarkers in an animal model for predicting future neurodevelopmental disorders however, more research is needed in the guinea pig to determine the correlation between various biomarker findings and neurodevelopmental sequelae so that this imaging could be used in humans.

4.6 References

Abel KM, Dalman C, Svensson a C, Susser E, Dal H, Idring S, Webb RT, Rai D & Magnusson C (2013). Deviance in fetal growth and risk of autism spectrum disorder. *Am J Psychiatry* **170,** 391–398.

Brown KH, Akhtar N a, Robertson a D & Ahmed MG (1986). Lactational capacity of marginally nourished mothers: relationships between maternal nutritional status and quantity and proximate composition of milk. *Pediatrics* **78,** 909–919.

Carter AM (2007). Animal Models of Human Placentation - A Review. *Placenta* **28,** S41–S47.

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Emmett PM & Rogers IS (1997). Properties of human milk and their relationship with maternal nutrition. *Early Hum Dev* **49 Suppl,** S7–S28.

Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R & Harel S (2009). Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *J Child Neurol* **24,** 846–851.

Florian ML & Nunes ML (2010). Effects of intra-uterine and early extra-uterine malnutrition on seizure threshold and hippocampal morphometry of pup rats. *Nutr Neurosci* **13,** 265–273.

Haglund NGS & Källén KBM (2011). Risk factors for autism and Asperger syndrome. Perinatal factors and migration. *Autism* **15,** 163–183.

Haugen a. C, Schug TT, Collman G & Heindel JJ (2014). Evolution of DOHaD: the impact of environmental health sciences. *J Dev Orig Health Dis*1–10.

Hüppi PS, Warfield S, Kikinis R, Barnes PD, Zientara GP, Jolesz F a, Tsuji MK & Volpe JJ (1998). Quantitative magnetic resonance imaging of brain development in premature and mature newborns. *Ann Neurol* **43,** 224–235.

Jones CT & Parer JT (1983). The effect of alterations in placental blood flow on the growth of and nutrient supply to the fetal guinea-pig. *J Physiol* **343,** 525–537.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS &

Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS & Owens J a (2005). Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol* **288,** R119–R126.

Kramer MS, Olivier M, McLean FH, Willis DM & Usher RH (1990). Impact of intrauterine growth retardation and body proportionality on fetal and neonatal outcome. *Pediatrics* **86,** 707–713.

Lafeber H, Rolph T & Jones C (1984). Studies in the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol* **6,** 441–459.

Lilley KG, Epping RJ & Hafner LM (1997). The guinea pig estrous cycle: correlation of vaginal impedance measurements with vaginal cytologic findings. *Lab Anim Sci* **47,** 632–637.

Lodygensky G a., Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F & Hüppi PS (2008). Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr Res* **63,** 438–443.

Løhaugen GCC, Østgård HF, Andreassen S, Jacobsen GW, Vik T, Brubakk A-M, Skranes J & Martinussen M (2013). Small for gestational age and intrauterine growth restriction decreases cognitive function in young adults. *J Pediatr* **163,** 447–453.

Lui, Jing, Wang, Xiao-Feng, Yan W (2014). Long-Term Adverse Outcomes of Fetal Growth Restriction. **93,** 1–5.

Mallard C, Loeliger M, Copolov D & Rees S (2000). Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* **100,** 327–333.

Mallard EC, Rehn A, Rees S, Tolcos M & Copolov D (1999). Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: Implications for the aetiology of schizophrenia. *Schizophr Res* **40,** 11–21.

McAlonan GM, Cheung V, Cheung C, Suckling J, Lam GY, Tai KS, Yip L, Murphy

DGM & Chua SE (2005). Mapping the brain in autism. A voxel-based MRI study of volumetric differences and intercorrelations in autism. *Brain* **128,** 268–276.

Pardi G, Marconi AM & Cetin I (2002). Placental-fetal interrelationship in IUGR fetuses - A review. *Placenta* **23,** 136–141.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001*a*). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA (2001*b*). Altered placental structure induced by maternal food restriction in guinea pigs: A role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22,** 77–82.

Sohlstrom A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS & Owens JA (1998). Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *Am J Physiol* **274,** E410-6.

StrangKarlsson S, Raikkonen K, Pesonen a K, Kajantie E, Paavonen EJ, Lahti J, Hovi P, Heinonen K, Jaervenpaeae a L, Eriksson JG & Andersson S (2008). Very low birth weight and behavioral symptoms of attention deficit hyperactivity disorder in young adulthood: The Helsinki study of very- low-birth-weight adults. *AmerJ Psychiat* **165,** 1345–1353.

Tolsa CB, Zimine S, Warfield SK, Freschi M, Rossignol AS, Lazeyras F, Hanquinet S, Pfizenmaier M & Hüppi PS (2004). Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. *Pediatr Res* **56,** 132–138.

Turner a. J & Trudinger BJ (2009). A Modification of the Uterine Artery Restriction Technique in the Guinea Pig Fetus Produces Asymmetrical Ultrasound Growth. *Placenta* **30,** 236–240.

Welker W, Johnson J & Noe A (2010). Cell Stain Brain Atlas of the Domesticated Guinea Pig. Available at: http://neurosciencelibrary.org/specimens/rodentia/guineapig/sections/thumbnail.html .

Yoshida T, McCarley RW, Nakamura M, Lee K, Koo MS, Bouix S, Salisbury DF, Morra L, Shenton ME & Niznikiewicz MA (2009). A prospective longitudinal volumetric MRI study of superior temporal gyrus gray matter and amygdala-hippocampal complex in chronic schizophrenia. *Schizophr Res* **113,** 84–94.

Table 1: MRI Brain region Volumetric Analysis

Data presented as means ± SEM; n values were 17 and 18 for neonate AGA-Control and FGR- MNR brain volumetric analysis, respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted, mm^3 = mm^3, *p<.05, **p<.01, ***p<.001.

Brain Region	AGA-Control N=17	FGR-MNR N=18
Brain Volume (mm^3)	2787 ± 29.9	2614 ± 30.9 ***
Hippocampus Volume (mm^3)	141.5 ± 3.7	120.8 ± 3.2 ***
Hippocampus/Brain Volume	0.051 ± 0.001	0.046 ± 0.001 *
Lateral Ventricle Volume (mm^3)	20.7 ± 1.4	14.8 ± 0.9 **
Lateral Ventricle/Brain Volume	0.0075± 0.001	0.0057 ± 0.001 **

Table 2: Histological Analysis of Neuroanatomical Correlates

Data presented as % Difference of AGA-Control; n values as shown in Table for neonate AGA-Control and FGR- MNR neuro-anatomic analysis, respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted, *p<.05.

Histological Composite Brain Region	AGA-Control N=12	FGR-MNR N=9
Hippocampus Cross-Sectional Area	100%	86% *
Lateral Ventricle Cross-Sectional Area	100%	80% *

Figure 1: Correlation analysis of 17 AGA-Control and 18 FGR-MNR neonates between brain volumes obtained by MRI and birth weight (Left) and Hippocampus volume and birth weight (Right).

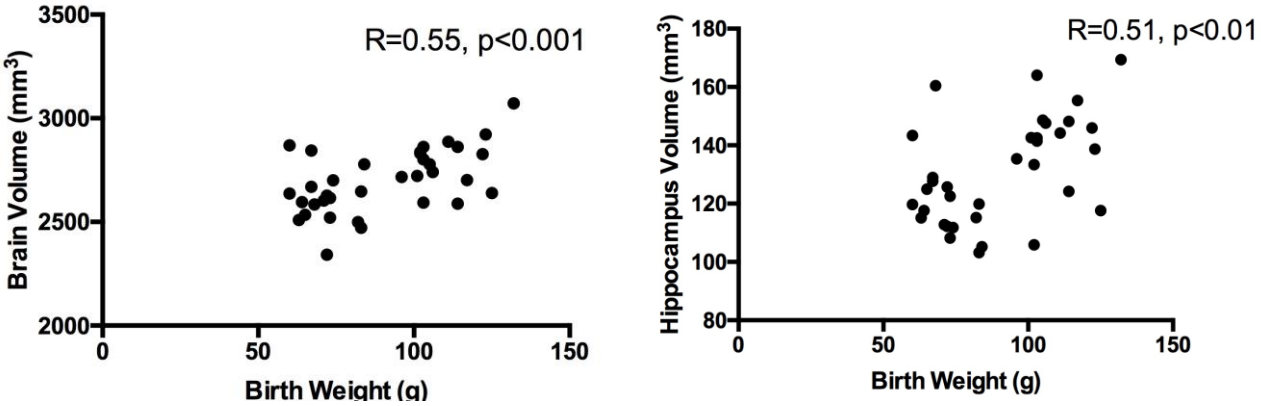
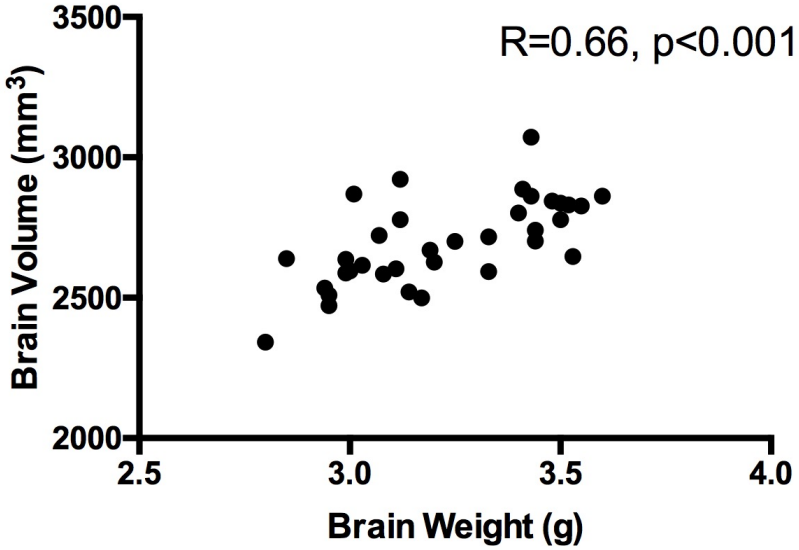


Figure 2: Correlation analysis of 17 AGA-Control and 18 FGR-MNR neonate brain weights obtained at necropsy and brain volumes obtained at MRI scan.



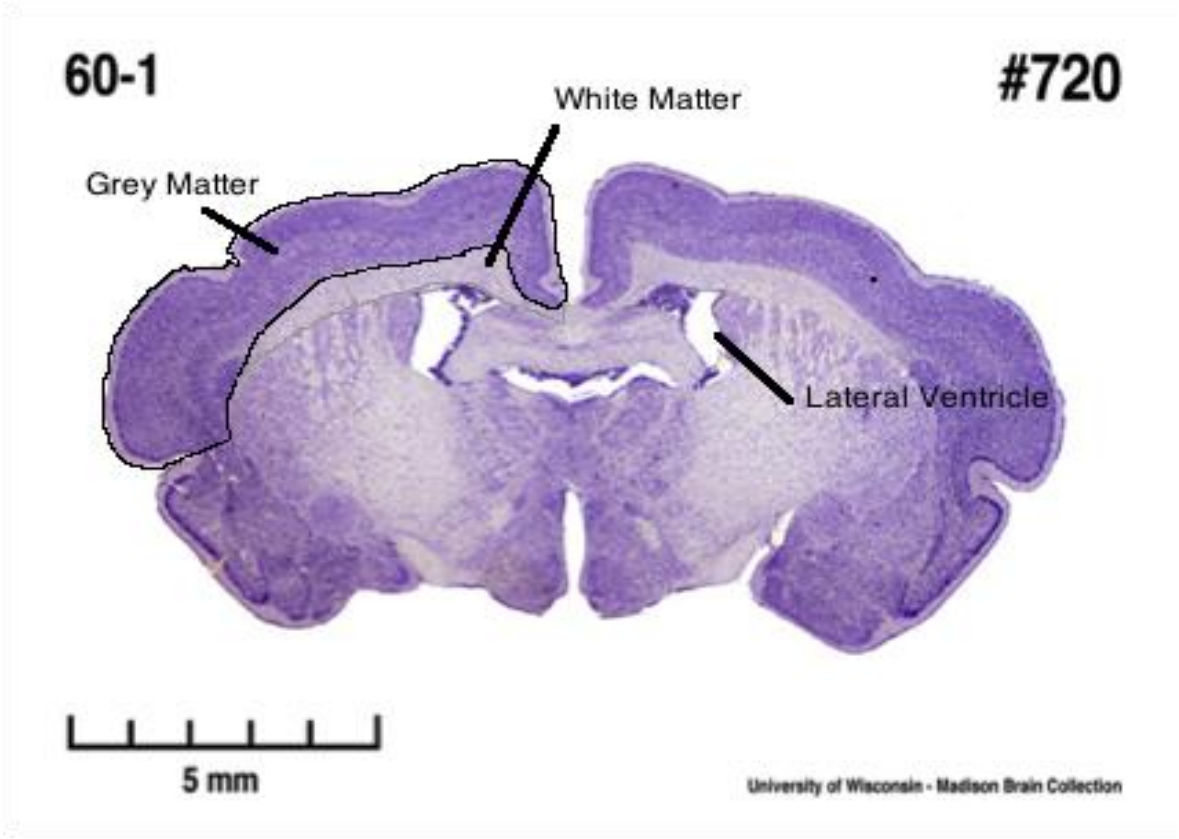


Figure 3: Representative image of rostral brain level, modified from Online Guinea Pig Brain Atlas (Welker *et al.*, 2010) with labels corresponding to regions quantified for analysis.

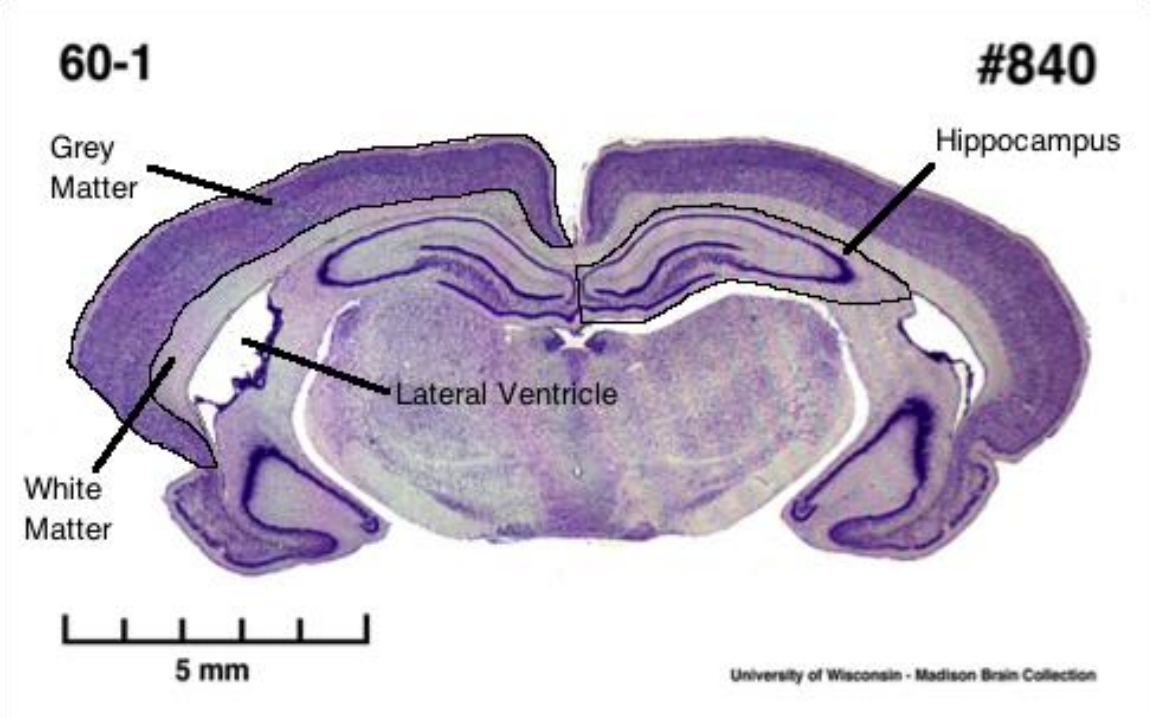


Figure 4: Representative image of a hippocampal brain level, modified from Online Guinea Pig Brain Atlas (Welker *et al.*, 2010) with labels corresponding to regions quantified for analysis.

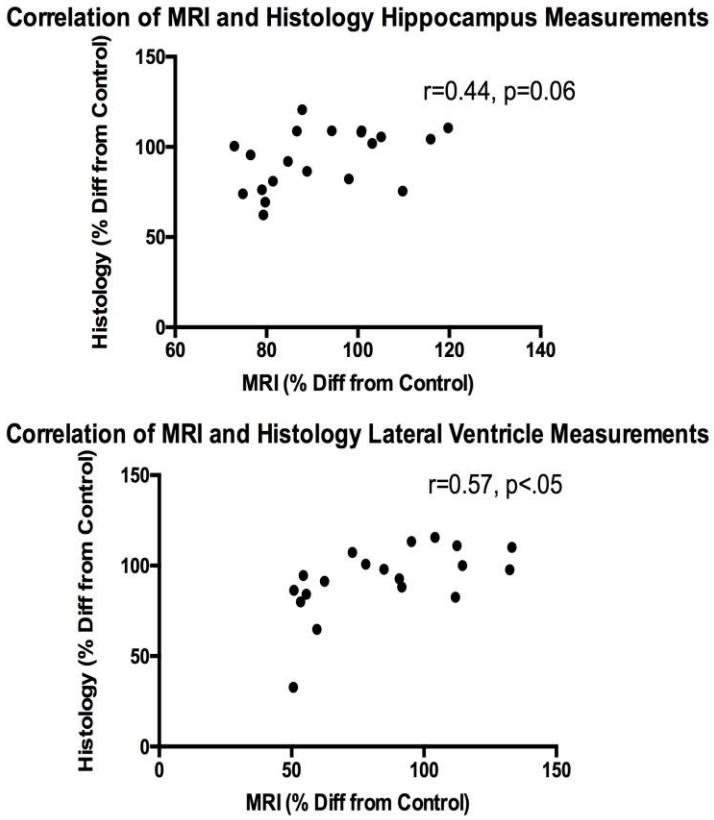


Figure 5: Correlation of AGA-Control and FGR-MNR MRI data with histological data for both the hippocampus (Above) and lateral ventricle data (Below). MRI = Magnetic Resonance Imaging.

Chapter 5

MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS LEADING TO GROWTH RESTRICTED NEONATAL OFFSPRING: IMPACT ON HIPPOCAMPUS MAGNETIC RESONANCE SPECTROSCOPY AND NEUROANATOMICAL CORRELATES

Maternal Nutrient Restriction in Guinea Pigs Leading to Growth Restricted Neonatal Offspring: Impact on Hippocampus Magnetic Resonance Spectroscopy and Neuroanatomical Correlates

5.1 INTRODUCTION

Fetal growth restriction (FGR) with infants small for their gestational age (SGA) when born is a major contributor to perinatal morbidity and mortality and for later adverse health outcomes. FGR increases the risk of neurological decline, cognitive deficits, as well as developing disorders such as autism, attention deficit hyperactive disorder, and schizophrenia (StrangKarlsson *et al.,* 2008; Haglund & Källén, 2011; Lui, Jing, Wang, Xiao-Feng, Yan, 2014).

Cognitive deficits, such as impaired cognitive skills and compromised memory, can become apparent at a very young age in infants born SGA (Geva *et al.*, 2006*a*; Fattal-Valevski *et al.,* 2009; Løhaugen *et al.,* 2013). Learning difficulties, decreased intelligence quotient, and weakened reading and writing skills have been shown to be more prominent and frequent in infants born below the 3rd percentile for weight (O'Keeffe *et al.*, 2003; Morsing *et al.,* 2011). In addition, SGA school children display increased inattentiveness and restlessness in the classroom (Parkinson *et al.*, 1986; Pryor *et al.*, 1995). These deficits can all persist until the age of adolescence and, more recently, have also been found to persevere into young adulthood (O'Keeffe *et al.*, 2003; Geva *et al.*, 2006*a*, 2006*b*; Morsing *et al.*, 2011; Løhaugen *et al.,* 2013). These abnormalities have been associated with changes in specific neural networks and pathways involved in learning and memory such as the hippocampal-prefrontal network, parahippocampal complex and the striatum (Geva *et al.*, 2006*a*, 2006*b*; Eichenbaum *et al.*, 2007). Although these behavioural changes are evident in children born FGR, there are few non-invasive technologies able to detect the presence and extent of brain injury that could later lead to cognitive deficits and neurodevelopmental disorders.

Magnetic resonance imaging (MRI) and other techniques, such as magnetic resonance spectroscopy (MRS), are safe and reliable tools that have been used to further the

understanding of brain development and neurological disorders. MRI has been used in the study of FGR to determine structural changes in human and animal brains, with specific focus on the hippocampus and its reduction in size following preterm birth of growth-restricted infants (Lodygensky *et al.*, 2008). More recently, studies are using MRS in the brain by measuring specific brain metabolites to determine metabolic biomarkers in the FGR brain. Many metabolites can be measured using MRS which can help in the assessment of the *in vivo* metabolic state of the brain, which has been shown to be altered in FGR (Moxon-Lester *et al.*, 2007). In the FGR fetal brain, decreased levels of N-acetyl aspartate (NAA)/Creatine (Cr) and decreased NAA/Choline (Cho) have been found, suggesting that FGR brains, as classified by altered cerebral blood redistribution, have altered neuronal integrity and mitochondrial metabolism (Azpurua *et al.*, 2008; Story *et al.*, 2011; Sanz-Cortes *et al.*, 2015). To our knowledge, there are limited studies of human neonates born SGA using MRS with the majority of studies focusing on FGR preterm neonates. One-year old children born SGA had a significant increase in both the NAA/Cr and Glutamate/Cr ratios in the prefrontal cortex of the brain (Simões *et al*., 2015). Similarly, preterm FGR infants have increased absolute NAA levels in various areas of the brain (Lazeyras *et al.*, 2003; Roelants-Van Rijn *et al.*, 2004). The latter are very different finding from that in the fetal brains, which suggests that there are post-natal developmental events that occur in human brains that must be investigated further (Simões *et al.*, 2015). There are a limited number of studies in the field of MRS and FGR; therefore, more research is required to explore the development of imaging biomarkers in the neonate for early diagnosis and monitoring of abnormal brain development following an adverse *in utero* environment exposure.

In this report we aimed to provide an MRS study of a reliable, robust animal model of FGR using guinea pigs exposed to maternal nutrient restriction (MNR). MNR produces FGR in precocious offspring after a lengthy gestation of 68-70 days. This model has been widely studied and is considered a moderate nutrient restriction model whereby the nutrient restricted sows eat 70% of the *ad libitum* diet on a daily basis three weeks prior to conception and continues on throughout pregnancy until mid-pregnancy, 35 days, when the restriction is lifted to 90% of an *ad libitum* diet (Sohlstrom *et al.*, 1998; Roberts

et al., 2001*a*, 2001*b*, Kind *et al.*, 2002, 2003, 2005). MNR in guinea pigs has been shown to mimic placental insufficiency through a decrease in placental weight and a decrease in the nutrient and oxygen exchange surface area; both contributing to a decrease in placental function and a decrease in fetal weight (Jansson & Persson, 1990; Dwyer *et al.*, 1992; Roberts *et al.*, 2001*a*, 2001*b*; Jansson & Powell, 2007).

Our goal was to study brain metabolite differences in the hippocampus of FGR-born neonates as a means to generate more accurate biomarkers for FGR aberrant neurodevelopment. This study will provide more evidence of post-natal brain metabolism in guinea pigs born growth-restricted and determine neuro-metabolic correlates in the neonate FGR guinea pig brain on a histological level. We tested the hypothesis that the guinea pig neonate model of FGR after MNR would display differences in hippocampus metabolite levels in the neonate, post natal day 21-27; specifically a decrease in the NAA/Cr ratio, which could be used as a marker for neuronal density and integrity, thus correlating to neuro-metabolic correlates in histological tissue and potential later neurodevelopmental disorders.

5.2 MATERIALS AND METHODS

5.2.1 Animal Model

A model, previously established by Sohlstrom and colleagues (1998), was used to produce moderate Maternal Nutrient Restriction (MNR) in nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) (Sohlstrom *et al.*, 1998). All experimental procedures were approved by the University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. The guinea pig sows were housed in individual cages in a dedicated small animal care facility with daily monitoring of the 12 hour light/dark cycles, humidity at 40-60%, and a constant temperature of 19 degrees Celsius. Animals were fed a guinea pig ration diet (Guinea Pig Diet 5025, Lab Diet, St. Louis, MO) and after a two-week period of acclimatization to the facility, daily food consumption, weight, and estrous cycles were tracked. The guinea pigs used in this study are the same that were used for both Chapter 3 and 4.

Thirty-two guinea pig sows were randomly assigned to either a Control group or a Maternal Nutrient Restriction (MNR) Group. The control group were fed ad libitum while the MNR group were fed 70% of the average food intake per kilogram of body weight of the ad libitum fed animals as described by Sohlstrom et al (1998). After two weeks of their respected diets, the animals began the breeding cycle. A female found to be in estrous, indicated by an opening of the vaginal membrane, was placed in a cage with a male guinea pig (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) for 48-72 hours or until the vaginal membrane was again closed. Day 0 of gestation was recorded at the day that fell in the middle of the estrous opening cycle. Animal pregnancies were confirmed by external abdominal ultrasound at gestational days 14-21. Animals that were not successful in the breeding attempt continued on their respected diets and were re-bred at their next estrous cycle opening. Sows that were successful and deemed pregnant continued on their diets until day 35 of gestation at which point the MNR animals food intake was increased to 90% average food intake of the control animals per kilogram body weight until spontaneous pupping.

Neonates were considered to be Appropriate for Gestational Age (AGA) if their birth weight was >95 g and born from a Control sow and Fetal Growth Restricted (FGR) if <85 g and born from a MNR sow. These criteria are extrapolations from past studies completed in fetuses (Elias *et al.*, 2016). These weights are representative of the 20^{th} percentile and 10^{th} percentile, respectively, from our Control Population of neonates (Chapter 3). Any pups that were born on or at an earlier gestational age than 66 days (average 67-68 days) received a corrected post-natal age to ensure and appropriate age corresponding to the number of days since conception at the time of testing. Control sows remained on an ad libitum diet throughout the nursing period. The MNR sows remained on a diet consisting of 90% of the average food intake of the ad libitum-fed Control animals per kilogram of body weight per day. MNR sows were switched to eating 100% of the average food intake of the ad libitum-fed animals per kg per day at post-natal day 15.

On corrected post-natal day 21-25, one female and one male neonate from each AGA-Control and FGR-MNR litter underwent MRI studies.

5.2.2 Animal Handling

Each animal was handled in the Animal Preparation Facility at the University of Western Ontario alongside the 9.4T magnet MRI machine in the Robarts Imaging Facility by an animal technician. The guinea pig was initially anesthetized with a nose cone using 4% isofluorane and 1.5L oxygen in a chamber, followed by continued sedation at 1.8% isofluorane for the duration of the testing period. The guinea pig was placed on a warm water pad to maintain core body temperature, which was measured with a rectal thermometer throughout the MRI scan. Heart rate and oxygen saturation were all monitored with a foot monitor. After the scan was complete, the guinea pig was taken out of the scanner, injected with 1cc of saline and placed under a heat lamp for recovery. Animals were allowed to recover for 48-72 hours before necropsy.

5.2.3 Imaging Protocol

Animals underwent the MRI study on a 9.4T VARIAN magnet in the Robarts Imaging Facility in the Centre for Metabolic Mapping. The MRS imaging took place immediately after the anatomical imaging on the same animal. A head surface coil was placed over the guinea pig head to ensure all brain anatomy was included. The 3-dimensional voxel of interest had dimensions of $3x8x4mm^3$ and was placed with careful precision for each animal over the superior hippocampus to include Cornu ammonis (CA) 1, CA2, and the Dentate Gyrus (DG) as shown in Figure 1. This voxel also included the corpus callosum as the voxel spanned from the left to the right brain hemisphere.

Localized voxel shimming was completed to obtain a relatively homogeneous magnet field. A VAPOUR pulse sequence was used remove the water signal, which was followed by a LASER single voxel pulse sequence, which was acquired over 128 averages, a repetition time (T_R) of 4000ms and an echo time (T_E) of 20ms (Kassem & Bartha, 2003).

5.2.4 Spectrum Processing and Analysis

Spectra were processed using fitMAN_SUITE Version 1.6 as developed by Bartha and colleagues (Bartha *et al.*, 1999). A Hankel singular value decomposition (HSVD) manual

subtraction (of resonances between 4.1 and 5.1 parts per million) was used to decrease noise in the metabolic spectrum (van den Boogart *et al.*, 1994). The area of the peak for each remaining metabolite was analyzed and calculated using a standard set of spectra as provided by fitMAN (Bartha *et al.*, 1999). The peaks that were measured include: N-acetylaspartate (NAA), Glutamate and Glutamine (Glu and Gln, respectively), Creatine (Cr), Choline-containing compounds (Cho), and Myo-Inositol (Myo), all with different parts per million (ppm) ranges as shown in Figure 1. To accurately measure the change in metabolites between groups, each metabolite was normalized to creatine by dividing each metabolite's area under the spectrum by creatine's area under the spectrum, due to its relative stability regardless of the metabolic state of the animal (Levine *et al.*, 1992; Li *et al.*, 2003; Jansen *et al.*, 2006; Story *et al.*, 2011; Simões *et al.*, 2015).

5.2.5 Neonate Brain Histology for Neuroanatomical Correlates

5.2.5.1 Tissue Processing and Embedding

After neonate MRI, guinea pigs were allowed to recover fully for 48-72 hours, after which they underwent a full necropsy. Prior to necropsy, animals were weighed and then euthanized with 0.3 cc intra-peritoneal injection of Euthanol (sodium pentobarbital, MTC Pharmaceuticals, Cambridge, ON, Canada). The brains were extracted and sectioned at the cerebral peduncles and the rostral two thirds of the brains were placed in 4% paraformaldehyde for immersion fixation for later histological study. The fixed brains then underwent three rinses of Phosphate Buffer Saline (PBS) over 72 hours, and were then stored in 70% Ethanol for 2-3 weeks. The rostral two thirds of the brains were then dissected at the optic chiasm to create the rostral brain section and middle or hippocampal section. These two blocks were embedded in paraffin wax for histological analysis.

5.2.5.2 Tissue Sectioning of Hippocampal Block

The hippocampal blocks were sectioned into five cuts at a 5um thickness. The sectioning occurred at level 880, a representative level of the Online Guinea Pig Atlas (Welker *et al.*, 2010) found at the more caudal face of the block where much of the hippocampus is present. A 200um section was trimmed off the cutting surface of the block until the

representative guinea pig atlas level 840 was visible (Welker *et al.*, 2010), and then 5 sections per animal were sectioned.

5.2.6 Tissue Staining and Histological Analysis

5.2.6.1 Nissl Stain of Guinea Pig Brain Tissues

To visualize neurons and examine the morphology of the coronal brain sections, Nissl staining was performed on paraffinized sections to stain the Nissl bodies using 0.1% cresyl violet (The British Drug Houses Ltd., Poole, England). All sections were first de-paraffinized in two changes of xylene, and sequentially hydrated in 100%, 95% and 70% alcohol, followed by rinsing twice in double distilled water. The sections were immediately stained in 0.1% cresyl violet for 5 minutes, after which they were rinsed quickly in double distilled water and differentiated in 95% ethyl alcohol for 15 minutes. Next, the sections were dehydrated in 100% alcohol, cleared in xylene, and then the slides were mounted with permanent mounting medium.

Each brain slide was scanned at 2.5x magnification on the Zeiss AxioImager Z1 Brightfield microscope and captured with the Zen Pro 2012 software. Measurements of the hippocampal layer thickness were completed using Image-Pro Premier, v9.1 made by MediaCybernetics.

Hippocampus neuron cell count was completed on both the pyramidal neuron layer and the granule cell layer of the dentate gyrus (DG). Four sequential images at a magnification of 20X were taken of the pyramidal cell layer of the CA1 in the hippocampus. Five sequential images at a magnification of 40X oil immersion were taken of the granule cell layer of the dentate gyrus. Each image was taken on the Zeiss AxioImaher Z1 Brightfield microscope and captured with the Zen Pro 2012 software.

5.2.6.2 Hippocampal Layer Thickness Measurements

Both the 840 level and the 880 level in the brains were analyzed for layer thickness differences. Ten AGA-Control and seven FGR-MNR brains were assessed at both levels for hippocampus layer thickness. The hippocampal proper layer thickness in the CA1 region was measured as the CA1 neuronal projections rarely change lengths throughout

coronal sections the hippocampus (Anderson *et al.*, 2007) and this is the area that would have been included in the MRS voxel placement. The stratum oriens, stratum pyramidal, stratum radiatum, and stratum lacunosum underwent width measurements, as shown in Figure 2. In the dentate gyrus, the moleculare layer widths were measured, as well as the granule layer widths, which all make up the 'C' or 'cup' shape of the dentate gyrus, as shown in Figure 2. In addition, the polymorphic layer, which is the brain tissue being encompassed by the dentate cup, was measured for width. Each layer on each side of the brain underwent 15 width measurements, which were then averaged together to make up the mean layer width for each guinea pig. No width measurements deviated further lateral than the CA1/CA3 border of the hippocampus proper.

5.2.6.3 Hippocampus Neuronal Cell Count

Both the 840 and 880 hippocampus layers were analyzed for the neuron cell count. Neuron cell counts were completed automatically on Image-Pro Premier, v9.1 made by MediaCybernetics, which identified neurons by the positively stained nucleolus with specific roundness and size parameters. Cell count analysis was displayed as number of neurons/mm^2. Percent area stained was also calculated in the same regions as the cell count through a set threshold based on the intensity of staining, and was scored as a percentage stained based on the area of mm^2. Total area in the location of interest (mm^2) was measured by manually tracing the relevant cell layers to create a region of interest (ROI) on the 20X and 40X high power field images. The blue channel was pulled out of the black and white image to build an even black outline of the ROI, and a threshold was set to include everything from intensity 1-255 and to fill holes. This produced an area expressed as um^2, which was then converted to mm^2.

5.2.7 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism. All data was presented as the mean ± the standard error of the mean (SEM). Metabolite ratios were statistically analyzed using a two-tailed Student t-test. Metabolite values with a coefficient of variation below 30% were accepted in the study. Hippocampus layer thickness and hippocampus neuron cell counts were all analyzed using a two-tailed student t-test for

comparison between AGA-Control and FGR-MNR neonates. $P<0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 Population Characteristics

Eighteen AGA-Control and eighteen FGR-MNR neonates were studied at the age of 21-25 days post-natal. FGR-MNR neonates were 28% smaller at birth compared to the AGA-Control neonates, while at the time of MRI were only 15% smaller compared to the AGA-Control neonates ($p<0.05$). At time of necropsy, 48-72 hours after MRI, the FGR-MNR neonates were still 15% smaller than the AGA-Control population ($p<0.05$). Brain weights at necropsy in the FGR-MNR group were 5% smaller than the AGA-Control neonates with values of 3.13 ± 0.04 g and 3.32 ± 0.04 g, respectively ($p<0.05$). This was accompanied by a 15% reduction in hippocampus volume and a 6% reduction in hippocampus volume/brain volume ratio in the FGR-MNR neonates compared to the AGA-Control neonates. There were no sex differences in the results shown.

5.3.2 Metabolite Analysis

After shimming the magnet to obtain a uniform magnet field, 15 metabolites were identified on each spectrum. After setting a coefficient of variation below 30% and running statistical analysis, no metabolite levels were statistically different from each other between the FGR-MNR and AGA-Control neonate populations; NAA/Cr was not different between groups, nor were the Myo/Cr, Glu/Cr, or Choline/Cr ratios, as shown in Table 1. There were no sex differences in the results shown.

5.3.3 Hippocampus Histology

5.3.3.1 Hippocampus Layer Thickness

At the 840 level of the brain of the hippocampus proper, as shown in Table 2, there was a 16% decrease in the stratum oriens layer thickness of the CA1 region of the hippocampus proper for the FGR-MNR neonates. In the dentate gyrus area of the hippocampus, the FGR-MNR neonates have a 19% in the infra-moleculare layer thickness and a 19% decrease in the polymorphic layer thickness.

At the level of 880 in the neonate brain tissues in the hippocampus proper, as shown in Table 2, there was a 16% decrease in stratum oriens width in the CA1 region of the hippocampus proper, and a 14% decrease in the stratum radiatum. In the dentate gyrus region, there is a significant decrease by 11% in the supra-moleculare layer, and a 16% decrease in the width of the polymorphic layer. There were no significant differences in widths of any of the densely populated cellular layers in either the hippocampus proper, composed of the pyramidal cell layers, nor in the dentate gyrus, composed of the granule cells layers, in either level of the hippocampus studied. There were no sex differences in the results shown.

5.3.3.2 Hippocampus Cell Count

At the level of 840 and 880 in the guinea pig brains, neurons were counted in both the CA1 and the DG of the hippocampus for 10 AGA-Control and 7 FGR-MNR neonates. At the level of 840 in the CA1, the FGR-MNR neonates had 3445 ± 227.6 cells/mm^2 while then AGA-Controls had 2937 ± 297.4 cells/mm^2, and there were no statistically significant differences between groups. As a percent area stained, the FGR-MNR neonate CA1 pyramidal cells stained 49 ± 2 % of the ROI while the AGA-Control neonates had a percent area stained of 47 ± 2% of the overall ROI. These values were not statistically different. In the DG at the level of 840, the FGR-MNR neonates had on average 5174 ± 222.7 cells/mm^2 versus the AGA-Control neonates, which had 4283 ± 319.6 cells/mm^2, again with no statistical differences between the two groups. No statistical differences were found in the DG percent area stained between the FGR-MNR neonates and the AGA-Control neonates with values of 59 +/- 4% area stained and 51 +/- 5% area stained, respectively. There were no sex differences in the results shown.

At the level of 880 in the brain in the CA1 region, there were no differences in the number of cells/mm^2 between the FGR-MNR and AGA-Control neonates with values of 3156 ± 93.4 cells/mm^2 and 3071 ± 168.1 cells/mm^2. Similarly, the percent area stained in the CA1 region between the FGR-MNR and AGA-Control neonates showed no statistical differences with values of 50 ± 4% area stained and 52 ± 3 % area stained, respectively.

In the dentate gyrus, there was a significant increase in the number of cells/mm^2 in the FGR-MNR neonates with values of 4920 ± 108.3 cell/mm^2 compared to the AGA-Control neonates that had on average 4030 ± 249 cells/mm^2. However, the percent area stained showed no significant differences between the neonates groups with the FGR-MNR neonates having 52 ± 2% area stained and the AGA-Control neonates have a DG with 50 ± 3% area stained of neurons. There were no sex differences in the results shown.

5.4 Discussion

In this study, 36 interpretable spectra were obtained that identified many metabolites in the brain. There were no significant differences in brain metabolite levels between the AGA-Control and FGR-MNR neonate groups, which does not confirm our tested hypothesis. However, there was a significant decrease in stratum oriens polymorphic layer widths in the FGR-MNR neonate brains, while no differences were found in the dense neuron cell layer thicknesses. Upon further investigation into the cell body dense layers of the hippocampus, the CA1 and DG, there were no differences in the number of cells per mm^2 or the % area stained at level 840 and 880 in the guinea pig brain, with the exception of the cells/mm^2 in the DG. This suggests that there are few differences in neuron numbers in these regions of the hippocampus, further supporting the neonate MRS findings and hippocampus layer thickness findings.

To our knowledge, this is the only FGR MNR neonate animal model used to study *in vivo* brain metabolite levels. The only other studies are in neonate humans, and the majority are in premature infants, and demonstrated a significant increase in NAA/Cr levels and absolute NAA levels (Lazeyras *et al.*, 2003; Roelants-Van Rijn *et al.*, 2004; Simões *et al.*, 2015). Human fetal FGR studies using MRS to measure brain metabolite levels showed a consistent decrease in NAA/Cr and NAA/Cho ratios in the FGR brains compared to appropriately grown Control fetuses (Azpurua *et al.*, 2008; Story *et al.*, 2011; Sanz-Cortes *et al.*, 2015). The present study demonstrates no differences between metabolite levels, specifically in NAA/Cr, suggesting that there is an increase in NAA/Cr levels from the age of the fetus to the neonate. This could be explained by an increase in growth and maturity from the growth-restricted fetal brain to the age of the neonate after a period

of post-natal growth. Kok *et al.* (2002) found that the NAA/Cr and NAA/Cho levels increased while the Cho/Cr ratio decreased with increasing gestational age in humans (Kok *et al.*, 2002). Furthermore, Kreiss and colleagues (2002) examined an increase in NAA/Cr with increasing gestational age in humans, thus suggesting that there is constant growth and maturation of the fetal brain, which may be stalled or altered in a FGR brain as displayed by FGR fetal MRS studies (Kreis *et al.*, 2002; Azpurua *et al.*, 2008; Story *et al.*, 2011).

The present study did not find any changes in the NAA/Cr ratio between the AGA-Control and the FGR-MNR neonates in the hippocampus, which suggests that there are no differences in neuronal density and integrity between groups. However, there is a reduction in the hippocampus volume as seen in the T2 weighted MRI imaging study in Chapter 4. Therefore, the organization of the hippocampus was investigated in the present study to determine the cause of volumetric differences. A decrease in the stratum oriens layer width at both levels of the hippocampus studied and a decrease in the infra-moleculare layer and polymorphic layer widths in the dentate gyrus was found in the FGR-MNR brain compared to the AGA-Control neonate brains. Interestingly, there were no differences in the pyramidal and granule cell layer widths of the hippocampus. This suggests that there are reductions in neuronal processes in the FGR-MNR neonate with no differences in the neuronal number as the stratum oriens is composed of the apical dendrites and axons of the CA1 and CA3 pyramidal neurons (Anderson *et al.*, 2007). This reduction in the stratum oriens width has also been shown in guinea pig FGR studies induced by uterine artery ligation/ablation studies, therefore this region seems to be vulnerable to FGR (Mallard *et al.*, 1999, 2000). Fetal sheep have also shown a reduction in stratum oriens width after a reduction in placental blood flow for 7 days, suggesting this is a pathological change occurring in FGR (Rees *et al.*, 1997).

FGR studies report findings of decreased neuronal cell numbers in various areas of the brain, however the present study demonstrates no statistical differences in CA1 pyramidal cell number and DG granule cell number in FGR-MNR neonates. Such studies include those that focus on fetal sheep after *in utero* exposure to hypoxemia, and an intrauterine

artery ligation model of FGR in guinea pigs in post-natal brains (Rees *et al.*, 1997; Mallard *et al.*, 2000). Each of these studies showed a decrease in hippocampus cell number in the pyramidal neurons regardless of the age or FGR-model used (Rees *et al.*, 1997; Mallard *et al.*, 2000). The post-natal guinea pig brain, as investigated by Mallard *et al* (2000), utilized a different model of FGR, compared to the present study, which could be accounting for the cell number differences. The uterine artery ligation/ablation model is much more invasive as it is a surgical intervention, and an abrupt change in oxygenated blood flow, compared to the chronic MNR model, which could acutely increase levels of hypoxemia in the FGR brain during development leading to an increase in hippocampal neuron apoptosis, and a decrease is neuron number (Mallard *et al.*, 2000; Turner & Trudinger, 2009). Although there are differences between the present study and other studies of FGR, this is the only study that utilizes MRS, hippocampus layer thicknesses, and cell counts in an FGR-MNR neonate model to further understand the physiological and pathological outcomes in the brain to assist in the determination and use of biomarkers for detection of later neurodevelopmental disorders.

5.5 Conclusion

Although there are no differences in the metabolite levels or cell numbers between the FGR-MNR and AGA-Control neonates, inconsistent with our hypothesis, there are hippocampus layer width differences, which hold the dendrites and axonal projections of hippocampal neurons. These findings suggest dendrite growth disparities in the FGR-MNR neonates and propose that the level of NAA in the neonate hippocampus is a good indication of neuronal number, growth, and integrity. The hippocampus organization and dendritic projection length may be restricted in regards to the neuron projections in FGR after MNR and can be a potential indicator of poor neurodevelopment and have implications to later neurodevelopmental disorders and cognitive ability regardless of the neuronal cell number present. More research is required to study neuron connections and synapses in the FGR-MNR neonate in order to identify and determine the presence and extent of hindered dendritic tree growth. It is also important to develop a longitudinal MRS study to assist in the understanding of metabolite levels over the intrauterine and

post-natal developmental periods to assist in the development of potential therapeutic targets that could decrease the risk of developing neurodevelopmental disorders.

5.6 References

Anderson P, Morris R, Amaral D, Bliss T & O'Keefe J (2007). *The hippocampus book.*

Azpurua H, Alvarado A, Mayobre F, Salom T, Copel J & Guevara-Zuloaga F (2008). Metabolis Assessment of the brain using proton magnetic resonance spectroscopy in a growth restricted human fetus: case report. *Am J Perinatol* **25,** 305–309.

Bartha R, Drost D & Williamson P (1999). Factors affecting the quantification of short echo in vivo 1H MR spectra:prior knowledge, peak elimation, and filtering. *NMR Biomed* **12,** 205–216.

Bejjani A, O'Neill J, Kim JA, Frew AJ, Yee VW, Ly R, Kitchen C, Salamon N, McCracken JT, Toga AW, Alger JR & Levitt JG (2012). Elevated glutamatergic compounds in pregenual anterior cingulate in pediatric autism spectrum disorder demonstrated by1H MRS and 1H MRSI. *PLoS One*; DOI: 10.1371/journal.pone.0038786.

van den Boogart A, van Ormondt D, Pijnappel W, de Beer R & Ala-Korpela M (1994). *In mathematics in Signal Processing III, JG McWhirter Ed.*

Dwyer C, Madgwick A, Crook A & Strickland N (1992). The effect of maternal undernutrition on the growth and development of the guinea pig placenta. *J Dev Physiol* **18,** 295–302.

Eichenbaum H, Yonelinas AP & Ranganath C (2007). The medial temporal lobe and recognition memory. *Annu Rev Neurosci* **30,** 123–152.

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Fattal-Valevski A, Toledano-Alhadef H, Leitner Y, Geva R, Eshel R & Harel S (2009). Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *J Child Neurol* **24,** 846–851.

Geva R, Eshel R, Leitner Y, Fattal-Valevski A & Harel S (2006*a*). Memory functions of children born with asymmetric intrauterine growth restriction. *Brain Res* **1117,** 186–194.

Geva R, Eshel R, Leitner Y, Valevski AF & Harel S (2006*b*). Neuropsychological

outcome of children with intrauterine growth restriction: a 9-year prospective study. *Pediatrics* **118,** 91–100.

Haglund NGS & Källén KBM (2011). Risk factors for autism and Asperger syndrome. Perinatal factors and migration. *Autism* **15,** 163–183.

Jansen JFA, Backes WH, Nicolay K & Kooi ME (2006). 1H MR Spectroscopy of the Brain: Absolute Quantification of Metabolites1. *Radiology* **240,** 318–332.

Jansson T & Persson E (1990). Placental transfer of glucose and amino acids in intrauterine growth retardation: studies with substrate analogs in the awake guinea pig. *Pediatr Res* **28,** 203–208.

Jansson T & Powell TL (2007). Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)* **113,** 1–13.

Kassem MNE & Bartha R (2003). Quantitative proton short-echo-time LASER spectroscopy of normal human white matter and hippocampus at 4 tesla incorporating macromolecule subtraction. *Magn Reson Med* **49,** 918–927.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS & Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Roberts CT, Sohlstrom AI, Katsman A, Clifton PM, Robinson JS & Owens J a (2005). Chronic maternal feed restriction impairs growth but increases adiposity of the fetal guinea pig. *Am J Physiol Regul Integr Comp Physiol* **288,** R119–R126.

Kind KL, Simonetta G, Clifton PM, Robinson JS & Owens J a (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Exp Physiol* **87,** 469–477.

Kok RD, van den Berg PP, van den Bergh AJ, Nijland R & Heerschap A (2002). Maturation of the human fetal brain as observed by 1H MR spectroscopy. *Magn Reson Med* **48,** 611–616.

Kreis R, Hofmann L, Kuhlmann B, Boesch C, Bossi E & Hüppi PS (2002). Brain metabolite composition during early human brain development as measured by quantitative in vivo 1H magnetic resonance spectroscopy. *Magn Reson Med* **48,** 949–958.

Lazeyras F, Chen A, Henry F, Borradori-Tolsa C, Magistretti P & Huppi P (2003).

Quantitative 1H-MRS shows prominent increase of lactate in cerebral white matter in premature infants with intrauterine growth restriction (IUGR). *Proc Intl Soc Mag Reson Med*.

Levine S, Helpern J, Welch K, Vande Linde A, Sawaya K, Brown E, Ramadan N, Deveshwar R & Ordidge R (1992). Human focal cerebral ischemia: evaluation of brain pH and energy metabolism with P-31 NMR spectroscopy. *Radiology* **185,** 537–544.

Li BSY, Wang H & Gonen O (2003). Metabolite ratios to assumed stable creatine level may confound the quantification of proton brain MR spectroscopy. *Magn Reson Imaging* **21,** 923–928.

Lodygensky G a., Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F & Hüppi PS (2008). Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr Res* **63,** 438–443.

Løhaugen GCC, Østgård HF, Andreassen S, Jacobsen GW, Vik T, Brubakk A-M, Skranes J & Martinussen M (2013). Small for gestational age and intrauterine growth restriction decreases cognitive function in young adults. *J Pediatr* **163,** 447–453.

Lui, Jing, Wang, Xiao-Feng, Yan W (2014). Long-Term Adverse Outcomes of Fetal Growth Restriction. **93,** 1–5.

Mallard C, Loeliger M, Copolov D & Rees S (2000). Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* **100,** 327–333.

Mallard EC, Rehn A, Rees S, Tolcos M & Copolov D (1999). Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: Implications for the aetiology of schizophrenia. *Schizophr Res* **40,** 11–21.

Morsing E, Åsard M, Ley D, Stjernqvist K & Maršál K (2011). Cognitive Function After Intrauterine Growth Restriction and Very Preterm Birth. *Pediatrics* **127,** e874–e882.

Moxon-Lester L, Sinclair K, Burke C, Cowin GJ, Rose SE & Colditz P (2007). Increased cerebral lactate during hypoxia may be neuroprotective in newborn piglets with intrauterine growth restriction. *Brain Res* **1179,** 79–88.

O'Keeffe MJ, O'Callaghan M, Williams GM, Najman JM & Bor W (2003). Learning,

cognitive, and attentional problems in adolescents born small for gestational age. *Pediatrics* **112,** 301–307.

Parkinson CE, Scrivener R, Graves L, Bunton J & Harvey D (1986). Behavioural differences of school-age children who were small-for-dates babies. *Dev Med Child Neurol* **28,** 498–505.

Pryor J, Silva PA & Brooke M (1995). Growth, development and behaviour in adolescents born small-for-gestational-age. *J Paediatr Child Health* **31,** 403–407.

Rees S, Stringer M, Just Y, Hooper SB & Harding R (1997). The vulnerability of the fetal sheep brain to hypoxemia at mid-gestation. *Dev Brain Res* **103,** 103–118.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001*a*). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA (2001*b*). Altered placental structure induced by maternal food restriction in guinea pigs: A role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22,** 77–82.

Roelants-Van Rijn AM, Van Der Grond J, Stigter RH, De Vries LS & Groenendaal F (2004). Cerebral structure and metabolism and long-term outcome in small-for-gestational-age preterm neonates. *Pediatr Res* **56,** 285–290.

Sanz-Cortes M, Simoes R V., Bargallo N, Masoller N, Figueras F & Gratacos E (2015). Proton magnetic resonance spectroscopy assessment of fetal brain metabolism in late-onset "small for gestational age" versus "intrauterine growth restriction" fetuses. *Fetal Diagn Ther* **37,** 108–116.

Simões R V., Cruz-Lemini M, Bargalló N, Gratacós E & Sanz-Cortes M (2015). Brain Metabolite Differences in One-Year-Old Infants Born Small at Term and its Association with Neurodevelopmental Outcome. *Am J Obstet Gynecol*1–11.

Sohlstrom A, Katsman A, Kind KL, Roberts CT, Owens PC, Robinson JS & Owens JA (1998). Food restriction alters pregnancy-associated changes in IGF and IGFBP in the guinea pig. *Am J Physiol* **274,** E410-6.

Story L, Damodaram MS, Allsop JM, Dcr R, Mcguinness A, Appsc BA, Patel A,

Wylezinska M, Hagberg H, Kumar S & Rutherford MA (2011). Brain metabolism in fetal intrauterine growth restriction : a proton magnetic resonance spectroscopy study. *YMOB* **205,** 483.e1-483.e8.

StrangKarlsson S, Raikkonen K, Pesonen a K, Kajantie E, Paavonen EJ, Lahti J, Hovi P, Heinonen K, Jaervenpaeae a L, Eriksson JG & Andersson S (2008). Very low birth weight and behavioral symptoms of attention deficit hyperactivity disorder in young adulthood: The Helsinki study of very- low-birth-weight adults. *AmerJ Psychiat* **165,** 1345–1353.

Turner a. J & Trudinger BJ (2009). A Modification of the Uterine Artery Restriction Technique in the Guinea Pig Fetus Produces Asymmetrical Ultrasound Growth. *Placenta* **30,** 236–240.

Welker W, Johnson J & Noe A (2010). Cell Stain Brain Atlas of the Domesticated Guinea Pig. Available at: http://neurosciencelibrary.org/specimens/rodentia/guineapig/sections/thumbnail.html .

Table 1: Hippocampus metabolite levels

Data presented as means ± SEM; n values were 18 and 18 for neonate AGA-Control and FGR- MNR metabolite levels, respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted, NAA = N-acetyl aspartate, Cr = Creatine, Cho = Choline.

Metabolite	AGA-Control N=18	FGR-MNR N=18
NAA/Cr	0.44 ± 0.01	0.44 ± 0.03
Cho/Cr	0.41 ± 0.02	0.46 ± 0.03
Glutamate/Cr	0.69 ± 0.03	0.74 ± 0.03
Myo-inositol/Cr	1.99 ± 0.08	1.96 ± 0.06

Table 2: Hippocampus Layer Widths

Data presented as means (um) ± SEM; * $p < 0.05$, ** $p < 0.01$, vs corresponding AGA-Control group value; n values were 10 and 7 for neonate AGA-Control and FGR- MNR hippocampus layer widths, respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted.

	Hippocampus Proper				Dentate Gyrus		
	Stratum Oriens	Stratum Pyramidal	Stratum Radiatum	Stratum Lacunosum	Moleculare Layer	Granule Layer	Polymorphic Layer
Level 880							
AGA-Control N=10	244.6 ± 9.4	58.3 ± 1.9	336.9 ± 12.5	139.3 ± 6.1	184.8 ± 6.1	91.2 ± 2.8	806.1 ± 41.1
FGR-MNR N=7	205.1 ± 8.2	56.6 ± 3.3	291.2 ± 14.5	130.0 ± 3.9	172.2 ± 5.7	84.9 ± 3.1	674.3 ± 34.9
Students t test	**		*				*
Level 840							
AGA-Control N=10	235.0 ± 11.1	57.3 ± 2.2	334.1 ± 13.7	134.5 ± 5.9	220.9 ± 4.9	90.6 ± 2.3	849.2 ± 27.2
FGR-MNR N=7	197.6 ± 10.3	54.7 ± 3.9	290.1 ± 15.2	122.0 ± 4.4	187.4 ± 9.9	83.9 ± 4.7	682.3 ± 33.5
Students t test	*				**		**

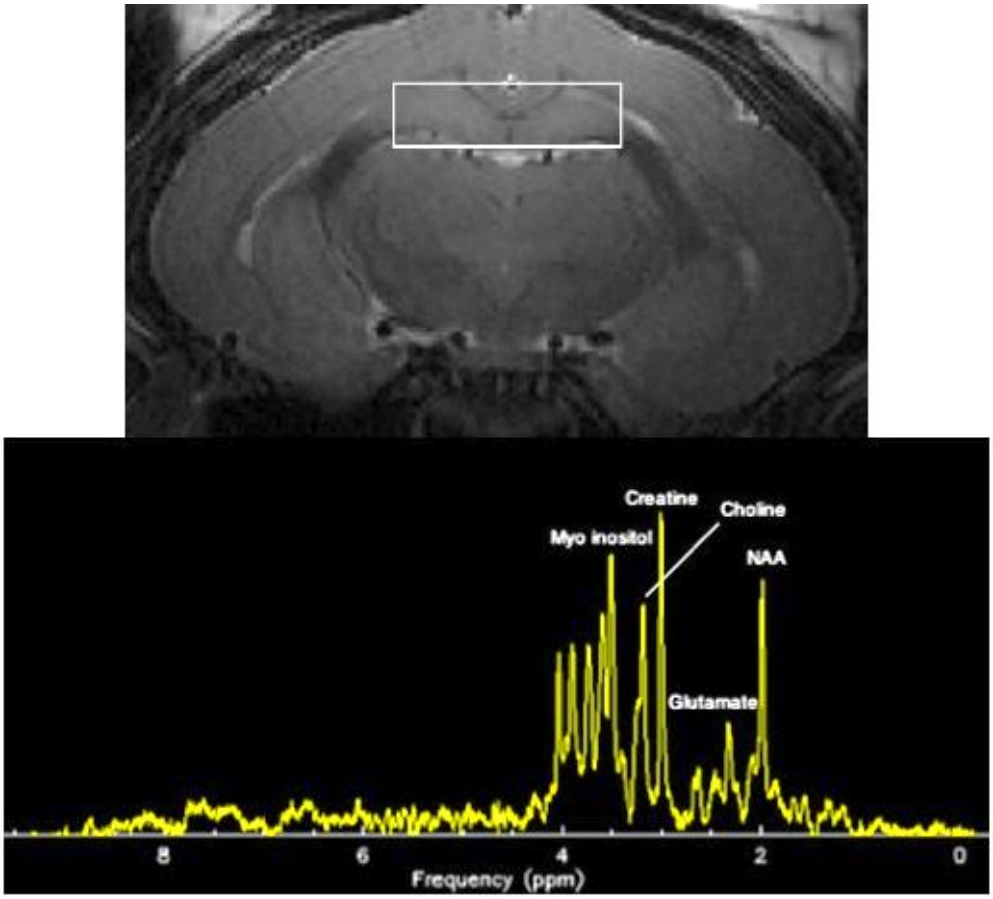


Figure 1: *In vivo* proton nuclear magnetic resonance spectroscopy.
(Above) T2 weighted image of GP brain with a highlighted hippocampal ROI for spectroscopy. Voxel dimensions are $3x8x4mm^3$ covering the majority of the hippocampus and corpus callosum. (Below) Spectrum acquired on the 9.4 Tesla magnet. Note the concise separations of the various high resolution peaks consisting of Myo, Myo-inositol; Cho, choline; Cr, creatine; Glt, glutamate; and NAA, *N*- acetyl aspartate.

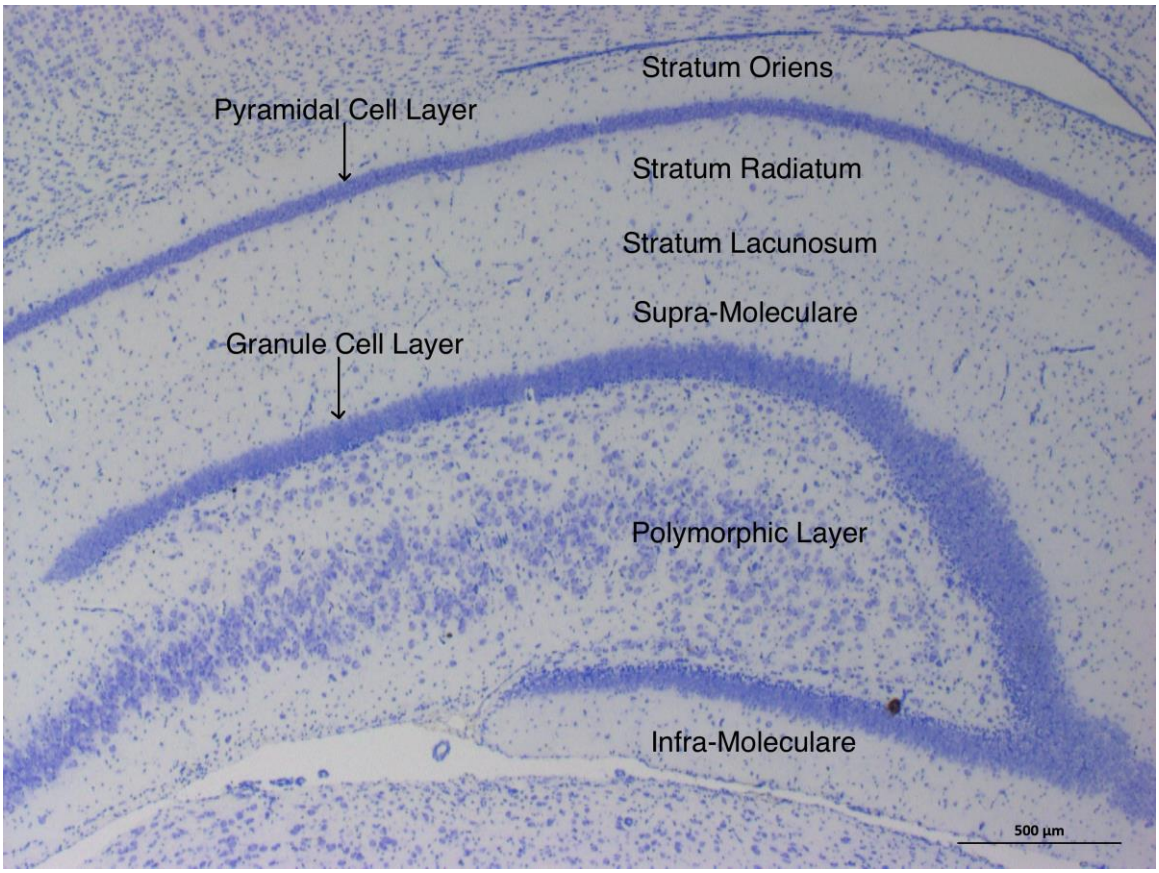


Figure 2: Representative bright-field microscope image (2.5X Objective lens) of Nissl stained guinea pig hippocampus at level 840 in brain.

Chapter 6
GENERAL DISCUSSION

6.1 General Discussion

The importance of a healthy intrauterine environment is a growing topic of interest as this milieu affects the growth and development of the fetus and can determine later life outcomes and potential diseases such as neurological disorders, cardiovascular disease, glucose intolerance, type II diabetes, and obesity due to fetal programming. Specific to neurodevelopmental disorders, FGR increases the risk of developing autism, attention deficit hyperactive disorder and schizophrenia. Although many factors contribute to fetal growth restriction (FGR), improper placental growth and therefore impaired nutritional transport to the fetus plays a major role in many human cases of FGR and continues to affect the neonate after birth. Although many animal models use techniques to induce placental insufficiency, this study was designed to further characterize how a nutrient restriction model would act as a more representative model of human FGR, and determine if there were any changes in brain structural and metabolic development in the neonate.

Past studies of FGR in guinea pigs have provided a large body of evidence regarding the fetal outcomes after uterine artery ligation/ablation, however this type of placental insufficiency model is abrupt and invasive, and causes FGR by an acute arrest of blood flow to the placenta, which can be quite severe (Turner & Trudinger, 2009; Elias *et al.*, 2016). The MNR model is analogous to the human situation as maternal under nutrition is one of the most common maternal causes of FGR. Maternal nutrient restriction (MNR) in guinea pigs has been used in the past to study maternal, placental, and the fetal characteristics of FGR, and the associated later life changes in the young adult, post-natal day 90, although limited studies have demonstrated the neonate organ outcomes and post-natal growth (Roberts *et al.*, 2001*a*, 2001*b*, Kind *et al.*, 2002, 2003). The present study further characterizes the neonate model of growth restriction after MNR, and is able to mimic post-natal growth as seen in the majority of human situations of FGR and catch up growth, as shown in Chapter 3. Additionally, this study provided justification for using a neonate weight threshold of >95 g for categorizing AGA-Control and <85 g for characterizing FGR-MNR cohorts. The organs affected in our FGR-MNR model are

related to those that are associated with FGR later life diseases so this model can be used to help elucidate the underpinnings of FGR disease processes associated with accelerated post-natal growth and fetal programming.

The present study utilized MRI and MRS in the brain of a growth restricted neonate guinea pig to determine adverse brain development or injury biomarkers after being born growth restricted. Structural differences were found in the brains of the growth restricted neonates, with statistical decreases in overall brain volume, hippocampus volume, and lateral ventricle volume on MRI, which were also found in in the histological study. Although the lateral ventricles volumes were different from what was hypothesized, this information provides additional knowledge on the growth and development of the post-natal neonate guinea pig. Additionally, the present study found a statistical decrease in the hippocampus volume to brain volume ratio suggesting that the hippocampus is a vulnerable brain structure to an adverse *in utero* environment such as MNR. Similar findings have been shown on MRI in the FGR preterm human neonate, whereby researchers have found a decrease in hippocampus volume and intracranial volume (Lodygensky *et al.*, 2008; Tolsa *et al*., 2004). Although the present study does not focus on pre-term birth and FGR, it provides good evidence of a hippocampus biomarker that is present across different forms of growth restriction and can assist in the determination of essential biomarkers for FGR neuro-developmental disorders. Histologically, the hippocampi cross sectional was significantly reduced, similar to that investigated in FGR induced by uterine artery ligation in the fetus and the neonate (Mallard *et al*., 1999, 2000). As the findings in the present study were correlated with the neuro-anatomical correlates, MRI was able to identify brain developmental abnormalities in the growth-restricted neonate in comparison to the AGA-Controls.

Metabolite levels in the hippocampus, specifically NAA, did not change between the FGR-MNR and AGA-Control and thus did not support our hypothesis, suggesting there are no differences in neuronal density and mitochondria function, even though there is a volumetric decrease in the hippocampus. As described in Chapter 5, the present study found no differences in hippocampus layer thickness in the cell soma layers, including

the pyramidal cell layer and the granule cell layers histologically, which supports the MRS findings. Although there were decreases in the non-cell dense layers of the stratum oriens, and stratum radiatum of the hippocampus proper, as well as decreases in the moleculare layer and polymorphic layer, which make up the dentate gyrus. A decrease in stratum oriens layer thickness has been shown in the guinea pig fetus after uterine artery ligation and in growth restricted sheep (Rees *et al.*, 1997; Mallard *et al.*, 1999), suggesting that there are pathological changes in this region, specific to FGR, that may be causing neurodevelopmental disorders.

Together, the past and preset studies of moderate MNR as a model in guinea pigs support the utility of this model for inducing FGR in the neonate with many similarities to that of human FGR with regards to the growth patterns and organ weights displayed in Chapter 3. This was the first time to date that the neonate FGR-MNR model has been used to study neonate post-natal and organ growth, and non-invasive magnetic resonance imaging and magnetic resonance spectroscopy techniques. The growth results are similar to that seen in the human situation and the MRI and MRS results display hippocampus findings in accordance with MRI studies of neurodevelopmental disorders such as autism and schizophrenia and provided further insight into the structural and metabolic changes seen after fetal programming and fetal growth restriction.

6.2 Future Studies

Based on the present findings, continued studies on the use of biomarkers in FGR are imperative in order to better understand the underlying factors associated with later life diseases, specifically neurodevelopmental disorders.

Future studies should utilize the non-invasive imaging tools of MRI and MRS starting in the fetus and continue on to the age of the neonate in order to gather longitudinal data. This could provide a longitudinal aspect of brain structure morphology, and brain metabolites throughout the changing intrauterine and post-natal environments to further understand the brain developmental stages occurring in FGR.

In conjunction with non-invasive MRI, a more in depth histological study would be beneficial to determine microstructural changes in the FGR neonate guinea pig brains. Brain development, myelination, and synapse formation is a continuous process in the age of the fetus and neonate and require a high degree of energy and oxygen, which can be disrupted in a MNR model of FGR. All of these processes can be measured indirectly through antibody stains including, myelin basic protein, synaptophysin and synaptopoetin in both the fetus and the neonate. Glial cells in the brain are also a vital component to essential brain structure and function, therefore investigation into glial cells and morphology might be useful to complete the story of growth restricted brains, specifically in the hippocampus.

Additionally, a follow up neurobehavioral would be of interest to understand how the findings from the present study affect the actual function, cognitive ability, and behavioural aspects of FGR after MNR in the animal model. Such studies can involve functional MRI studies, a Morris Water Maze test, an open field test, or a T-arm test, and have all been used for a variety of animal models to display neurobehavioural outcomes of the animals. The timing of neurobehavioural studies would be most beneficial in the age groups when neurodevelopmental disorder symptoms, such as those for autism, attention deficit hyperactive disorder and schizophrenia all start to present themselves (StrangKarlsson *et al.*, 2008; Haglund & Källén, 2011; Lui, Jing, Wang, Xiao-Feng, Yan, 2014).

6.3 Conclusions

In conclusion, this thesis was focused on determining the extent to which a MNR diet *in utero* would produce FGR offspring with specific interest in the brain to further investigate non-invasive brain imaging biomarkers and neuroanatomical correlates. The major findings of this study were:

1. MNR leads to FGR offspring that display accelerated post-natal growth that has differential effects of specific organs, indicated by a high brain to body weight ratio, no changes in liver to body weight, and decreased heart to body weight ratio compared to AGA-Control neonates.

2. FGR-MNR neonates have smaller brains, hippocampi, and lateral ventricles as seen with T2-weighted MRI and confirmed with neuroanatomical correlates. In addition, the FGR-MNR neonates have a reduced hippocampus/whole brain volume ratio suggesting that the hippocampus is a vulnerable brain structure to MNR and FGR.
3. FGR-MNR neonates do not show alterations in hippocampus metabolite levels through MRS, however, histologically, there are decreases in the layers of the stratum oriens and polymorphic layer of the hippocampus, as well as no changes in neuron cell number in the hippocampus which supports our MRS findings and begin to explain the decrease in hippocampus volume seen in objective 2 that may be associated with neurodevelopmental disorders.

These studies support MNR as a way to induce FGR in the newborn that mimics many neonatal growth events in the human situation. Furthermore, these studies provide insight into the use of MRI and MRS together as tools to build and obtain structural and metabolic biomarkers of FGR-born neonate guinea pigs for the use of potential biomarker identification in humans

6.4 References

Elias AA, Ghaly A, Matushewski B, Regnault TRH & Richardson BS (2016). Maternal Nutrient Restriction in Guinea Pigs as an Animal Model for Inducing Fetal Growth Restriction. *Reprod Sci* **23,** 219–227.

Haglund NGS & Källén KBM (2011). Risk factors for autism and Asperger syndrome. Perinatal factors and migration. *Autism* **15,** 163–183.

Kind KL, Clifton PM, Grant P a, Owens PC, Sohlstrom A, Roberts CT, Robinson JS & Owens J a (2003). Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. *Am J Physiol Regul Integr Comp Physiol* **284,** R140-52.

Kind KL, Simonetta G, Clifton PM, Robinson JS & Owens J a (2002). Effect of maternal feed restriction on blood pressure in the adult guinea pig. *Exp Physiol* **87,** 469–477.

Lodygensky G a., Seghier ML, Warfield SK, Tolsa CB, Sizonenko S, Lazeyras F & Hüppi PS (2008). Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr Res* **63,** 438–443.

Lui, Jing, Wang, Xiao-Feng, Yan W (2014). Long-Term Adverse Outcomes of Fetal Growth Restriction. **93,** 1–5.

Mallard EC, Rehn A, Rees S, Tolcos M & Copolov D (1999). Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: Implications for the aetiology of schizophrenia. *Schizophr Res* **40,** 11–21.

Rees S, Stringer M, Just Y, Hooper SB & Harding R (1997). The vulnerability of the fetal sheep brain to hypoxemia at mid-gestation. *Dev Brain Res* **103,** 103–118.

Roberts CT, Sohlstrom a., Kind KL, Earl R a., Khong TY, Robinson JS, Owens PC & Owens J a. (2001*a*). Maternal food restriction reduces the exchange surface area and increases the barrier thickness of the placenta in the guinea-pig. *Placenta* **22,** 177–185.

Roberts CT, Sohlstrom A, Kind KL, Grant PA, Earl RA, Robinson JS, Khong TY, Owens PC & Owens JA (2001*b*). Altered placental structure induced by maternal food restriction in guinea pigs: A role for circulating IGF-II and IGFBP-2 in the mother? *Placenta* **22,** 77–82.

StrangKarlsson S, Raikkonen K, Pesonen a K, Kajantie E, Paavonen EJ, Lahti J, Hovi P, Heinonen K, Jaervenpaeae a L, Eriksson JG & Andersson S (2008). Very low birth weight and behavioral symptoms of attention deficit hyperactivity disorder in young adulthood: The Helsinki study of very- low-birth-weight adults. *AmerJ Psychiat* **165,** 1345–1353.

Turner a. J & Trudinger BJ (2009). A Modification of the Uterine Artery Restriction Technique in the Guinea Pig Fetus Produces Asymmetrical Ultrasound Growth. *Placenta* **30,** 236–240.

CURRICULUM VITAE

Name:	Catherine Nevin
Post-Secondary Education and Degrees:	University of Guelph Guelph, Ontario, Canada 2010-2014 B.Sc.
	The University of Western Ontario London, Ontario, Canada 2014-2016 M.Sc. Candidate
	The University of Ottawa Ottawa, Ontario, Canada 2016-Present, B.Sc.N
Honours and Awards:	Obstetrics and Gynaecology Graduate Research Scholarship 2014-2015
	Province of Ontario Graduate Scholarship 2015-2016, declined
	Ontario Women's Health Scholars Award 2015-2016
Related Work Experience:	Teaching Assistant The University of Western Ontario 2014-2016