PLACENTAL 11β-HYDROXYSTEROID DEHYDROGENASE EXPRESSION AND BIRTH WEIGHT IN HAMILTON COUNTY, TN

By

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ABSTRACT

Infants born below 2,500 grams are classified as low birth weight. Reduced birth weight has been shown to increase the risk of infant mortality and chronic adulthood diseases. In 2007, Hamilton Country reported 12.0% of live births to be low birth weight, compared to the state average of 9.4%. An excess *in utero* exposure to cortisol has been linked to restricted fetal growth. Placental production of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol before passage into the fetus. This study tests the hypothesis that increased placental 11β-HSD2 expression has a positive correlation with an individualized birth weight centile. A Spearman's rank correlation reported a significant correlation between these two variables ($p = 0.024$). Additionally, birth weight was significantly different between underweight and obese mothers, married vs. single mothers and black vs. white mothers. These results reinforce the importance of proper 11β-HSD2 expression for optimal fetal growth.

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11β-HSD, 11beta-hydroxysteriod dehydrogenase

ACTB, beta actin

ACTH, adrenocorticotropic hormone

AGA, Average for Gestational Age

BLUES, Building Lasting Unshakeable Expectations into Successes

BMI, Body Mass Index

BWC, birth weight centile cDNA, complementary DNA

cDNA, complementary DNA

CRH, corticotropin-releasing hormone

C_T, cycle number

DBP, di-n-butyl phthalate

DBT, Dibutyltin

DPT, diphenyltin

DPrP, dipropyl phthalate

dsDNA, double stranded DNA

DTC, dithiocarbamate

GROW, Gestation Related Optimal Weight

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GR, glucocorticoid receptor

IHRFI, Infant Health Risk Factor Index

IL, interleukin

IUGR, Intrauterine Growth Restriction

LBW, low birth weight

LGA, Large for Gestational Age

LMP, last missed period

M, gene expression stability measure

NRFHR, non-reassuring fetal heart rate

PCBs, polychlorinated biphenyls

PE, Preeclampsia

PIH, Pregnancy Induced Hypertension

PTL, pre-term labor

qRT-PCR, quantitative (real time) reverse transcription polymerase chain reaction

SGA, Small for Gestational Age

TBT, tributyltin

TPT, triphenyltin

VLBW, very low birth weight

WHO, World Health Organization

CHAPTER 1

INTRODUCTION

Birth weight carries with it strong ties to infant mortality, and also adult health. Children born under 2500 grams places that child at a 20x greater risk for death within the first year than a child born above that weight (Wilcox and Skjaerven, 1992). Being born on the smaller end of the normal birth weight range carries an increased risk of adult chronic diseases, such as heart disease and type 2 diabetes (Barker et al., 1989; Yajnik et al., 1995). Maternal habits, such as smoking, are well known to be an influence on birth weight (Krentz et al., 2011). Others, such as marital status, maternal body mass index, maternal age of greater than 35, and race have shown to correlate with birth weight (David and Collins, 1997; Ehrenberg et al., 2003; Joseph et al., 2005; Shah et al., 2011). Hamilton County, TN has an elevated rate of low birth weight infants, when compared to other metro areas in the state, and to the national rate (Decosimo et al., 2010). Maternal habits and socioeconomic factors cannot fully explain this increased rate of low birth weight births, though certain community-based initiatives have been implemented to help alleviate some of those factors (Featherstone, 2010).

One of the most recent theories to explain the mechanism of low birth weight births is an over-exposure to cortisol *in utero*, which is the focus of the current study. Cortisol is a stress hormone and is regulated by the hypothalamus (Sherwood, 2006). Localized production of cortisol is required for proper fetal organ maturation (Speirs et al., 2004). However, an excess of maternally derived cortisol, via passage through the placenta, has been correlated with reduced birth weight and intrauterine growth restriction (McTernan et al., 2001).

The passage of maternally derived cortisol is modulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2, which is highly expressed in the syncytiotrophoblast layer, where the maternal-fetal interface is located (Yang, 1997). Active cortisol is inactivated to cortisone by this enzyme before passage into the fetal blood supply. Local amplification of cortisol, from cortisone, is modulated by 11β-HSD type 1 and is expressed in fetal tissues (Alfaidy et al., 2003). These isozymes (11β-HSD1 and 11β-HSD2) work in concert with each other to ensure proper fetal development. Stymied fetal growth has been linked in the reduction of the 11β-HSD2 placental barrier (Dy et al., 2008; McTernan et al., 2001; Shams et al., 1998).

However, other maternal characteristics have been shown to influence birth weight (i.e., maternal height, maternal weight at first clinical visit, parity, ethnic group, geographical region), as well as infant sex and gestational age (Gardosi et al., 1992). When those factors are controlled for, a more accurate representation of the growth potential of an infant can be made, allowing for a better detection of potentially pathogenic factors that affect fetal growth and development. Gardosi and Francis (2009) developed such software, which takes into account the conditions listed above, and returns a birth weight centile calculation, which is a more accurate assessment of intrauterine conditions.

The current study investigates whether the expression of 11β-HSD2 in placenta tissue correlates with birth weight in infants born in Hamilton County, TN. With the importance of protecting the fetus from an excess of maternal cortisol, through the workings of the 11β-HSD2 enzyme, the current study measured the expression of this enzyme in 242 placenta samples collected from Erlanger Hospital in Chattanooga, TN. Expression measurement was done via quantitative reverse transcription polymerase chain reaction assay. Additional comparison were

made based on maternal age (less than 35 vs. 35 and up), marital status (single vs. married), pre-pregnancy BMI (under vs. average vs. overweight vs. obese), maternal drug use in non-complicated pregnancies (use vs. non-use), race (Caucasian vs. African American vs. Hispanic) and maternal education (less than high school vs. high school/GED vs. some college) to see if these groupings had any influence on infant birth weight, 11β-HSD2 enzyme expression or the calculated birth weight centile.

A molecular examination of the placenta, via quantification of 11β-HSD2 expression, could be used as a means to easily assess the health and growth potential of an infant, which could alleviate the need for more invasive procedures, such as the collection of cord blood, to occur. The current study examines the usefulness of the placenta as a diagnostic tool in regards to fetal health as measured by birth weight or assessment of the fetal growth potential. No attempt was made to measure actual enzyme activity, which is the efficiency of which cortisol is converted to cortisone. Enzyme activity is more sensitive to environmental pollutants and would be a better end point to examine if indeed Hamilton County's (especially Chattanooga's southern neighborhoods) industrial past and long lived organic compounds and/or metals have a significant influence on the birth weight of these infants. However, since expression begets activity, 11β-HSD2 enzyme expression is an effective measurement of the cortisol protection that this enzyme provides.

CHAPTER 2

LITERATURE REVIEW

Hamilton County, TN and Low Birth Weight Infants

Hamilton County Tennessee has an elevated incidence of low birth weight (LBW) births, defined as infants born below 2,500 grams. In 2007, Hamilton Country reported 12.0% of live births as low birth weight, with the state average of 9.4% (Decosimo et al., 2010). This trend is disproportionate among races, with the black population reporting a rate of 18.6% and the white population at 9.7%, though both races have an elevated incidence compared to the national average of 8.2%. Even among other metropolitan areas in the state, the low birth weight rate is still elevated, with Shelby (Memphis), Davidson (Nashville) and Knox (Knoxville) counties following with 11.1%, 9.3% and 8.8%, respectively. Very low birth weight (VLBW) babies (defined as less than 1,500 g) were reported at 2.2% of live births in 2007. Again, when race is factored into these statistics, the black population is represented disproportionally, with a rate of almost double the white population (3.7% and 1.7%, respectively). Low birth weight is a significant factor in infant mortality (Wilcox and Skjaerven, 1992). Hamilton County reported 9.7 deaths/1000 births, which is greater than both the national and state rates (6.8 and 8.7 deaths/1000 births respectively).

A study done by the Ochs Center for Metropolitan Studies (2010) reported an Infant Health Risk Factor Index (IHRFI) for sub-regions of Hamilton County. This IHRFI score considered six pregnancy outcome indicators: percentage of low birth weight births, percentage of preterm births, percentage of delayed or no prenatal care pregnancies, percentage of births to

teenage mothers, percentage of births to single mothers, and percentage of births to mothers without a high school degree. Possible scores ranged from 6, representing the lowest risk, to 216, the highest risk. Areas of Hamilton County with the greatest IHRFI were Amnicola/East Chattanooga (zip code 37406) at 206, followed by Glenwood/Eastdale (37404/37406) at 200, South Chattanooga (37409/37410) at 195, Bushtown/Highland Park (37404) at 194 and Downtown (37408/37402) and Ridgedale/Oak Grove/Clifton Hills (37407) receiving the same score of 192. In contrast, Signal Mountain (37377) received a score of 12. The full Ochs Center for Metropolitan Studies report contains a full list of Hamilton County sub-regions and IHRFI and LBW listings, along with additional demographic characteristics.

Community based incentives in the state of TN have been developed in order to improve pregnancy outcomes. The BLUES (Building Lasting Unshakeable Expectations into Successes) project started in Shelby County in 2005, with the first cohort study ending in 2009 (Kimberly Lamar, 2010). Objectives of the program included increasing pregnancy health education and prenatal health services for low-income mothers, as well providing social support and community outreach. This holistic approach to improving pregnancy outcomes was designed to target minority populations, especially African Americans, where disparities in pregnancy outcomes are widely reported. Data from the 2005-2009 cohort showed significant improvement in low birth weight rates (9.81% in BLUES compared to 18.60% in control group, $p < 0.001$), as well as significant improvement in prematurity and infant deaths ($p = 0.0001$). Additional expansion of this program into Hamilton County targeted specific zip codes (37410, 37408, 37406, and 37403). The Hamilton County pilot study showed an 8% drop in prematurity and LBW, and an 11% drop in infant mortality in the BLUES subjects when compared to the county as a whole.

Birth weight is a common study parameter due its abundant data sets (being a vital population statistic) and is strong predictor for infant mortality. However, raw statistics of LBW incidences often disregard gestational age, which is the main cause for low birth weight. Historically, low birth weight and prematurity were used interchangeably. Consequently, a new term, intrauterine growth restriction (IUGR), was introduced. IUGR infants are clinically full term (>37 weeks), but still have stunted growth (10^{th} centile for birth weigh), whereas premature infants have not had the proper gestational length in order to achieve a healthy weight (Peleg et al., 1998; Wilcox, 2001).

Birth Weight as a Measure of Adult Health

One of the pioneer researchers that proposed a link between birth weight and subsequent adult cardiovascular health was David J. P. Barker. His work on this subject is collectively known as the Barker Hypothesis (http://www.thebarkertheory.org), and has since broadened to be known as the Developmental Origins of Health and Disease. He first proposed a link in a 1989 manuscript analyzing areas with high rates of infant mortality (which is strongly correlated with LBW) and rates of death by ischemic heart disease (Barker et al., 1989). The study followed over 5,000 English men and found that those at the lowest end of the normal birth weight scale, as well as those who were small at one year of age, had the greatest heart disease death rates. Weighing 18 pounds or less at one year of age had doubled the mortality ratio from ischemic heart disease compared to those who weighed 27 pounds or more at one year of age. In addition to those findings, another parameter of cardiovascular health, blood pressure, was measured (Barker et al., 1990). This was also found to correlate with placental mass and birth weight; the smallest babies with the largest placentas had the highest blood pressure at age 46 to 54, independent of elevated Body Mass Index (BMI) and alcohol consumption. The ratio of fetal

size to the mass/area of the placenta was the key measurement in Dr. Barker's findings. An enlarged placenta is thought to be an attempt to compensate for poor fetal nutrition. By expanding the surface area of the placenta, the availably of whatever nutrients are carried in the maternal blood supply increases (as reviewed by Robinson et al., (1995)). These early findings supported Barker's theory that a healthy intrauterine environment was a greater factor in preventing adulthood diseases than childhood upbringing (Barker, 1990). Continued longitudinal studies have confirmed these findings in Scandinavia cohorts (Finland and Sweden) (Barker et al., 2002; Lithell et al., 1996).

Research into the fetal origin of disease has broadened into other chronic diseases, including insulin resistance and diabetes, leading to the phrase, "the thrifty phenotype". This phrase emphasizes the need for survival of the fetus under sub-optimal growth conditions (as reviewed by Hales and Barker, 1992). Hales and Barker (1992) explain that development of brain neurons, renal glomerui and Beta cells are accelerated compared to other organ systems, with Beta cell formation at about half of adult capacity at one year of age. If there is a nutritional deficiency, a thrifty phenotype would allocate available resources to neural and heart development, resulting in an underdevelopment of the other early developing visceral organs (kidneys and pancreas) and skin (Thornburg and Morton, 1994). Indeed, amino acid deficiencies are cited as the largest cause for stymied fetal growth. Not only are amino acids the building blocks of the tissues themselves, but also the main substrate in energy production early in fetal life. The main repercussion of this amino acid deficiency is a reduction of Beta cells numbers, but also causes an abnormal islet structure and vascularization. In an undernourished fetal state, the need for insulin is low and the consequences of an underdeveloped pancreas in the fetus would be nil. However, this reduced number and function of the pancreatic cells begets the onset

of Type 2 diabetes when over-nutrition in adult life occurs, as the pancreas cannot produce enough insulin to control blood-glucose levels. In addition to impaired glucose tolerance, an under-development of renal glomerui *in utero* leads to renal failure (Hales and Ozanne, 2003). Studies with Indian children support that reduced fetal weight impaired glucose tolerance and increased the risk of adult onset of Type 2 diabetes (Yajnik et al., 1995). Although resources are allocated to the heart and brain differentially than the rest of the developing fetus, heart conditions can still arise when cardiac structures (specifically the left ventricle and aortic root) are reduced in size. Adverse remodeling and growth catch-up later in life can lead to hypertrophy, which is a risk factor for heart disease (as reviewed by Geelhoed and Joddoe, (2010)). In concert with heart disease, vascular structure can also be compromised with abnormal fetal growth, contributing to endothelial dysfunction and loss of normal elasticity of the blood vessels, leading to hypertension and atherosclerosis (as reviewed by Geelhoed and Joddoe (2010)).

Though the emphasis on maternal nutrition is merited, and easy to measure and manipulate experimentally, a review by Harding (2001) emphasizes that what truly affects fetal growth is *fetal* nutrition. Fetal nutrition is dependent on not only maternal nutrient intake, but maternal metabolism, hormone interplay, blood flow through the uterus and placenta, placenta metabolism, and nutrient transfer as well. Therefore, compromising the blood flow through the placenta, as with pregnancy-induced hypertension (PIH) (preeclampsia (PE), toxemia), could be equally as detrimental to fetal health as low protein intake on the resulting fetal phenotype. These periods of nutrient deficiency may affect different organ systems by reduced cellular division, or fluctuations in proper growth factors or hormone production during a specific system's critical window of development (Barker, 1995).

Cortisol's Double Edge: Cortisol, Fetal Development and the Role of Placental 11β-Hydroxysteroid Dehydrogenase Type 2

During critical time points in fetal development, events *in utero* induce irrevocable physiological changes to the fetus, also known as fetal programming (Lucas, 1991; Meaney et al., 2007). While the link between birth weight and adult disease has been supported by Dr. Barker's research, further investigation into the mechanism of fetal programming, birth weight, and disease leads to the complex dynamic of glucocorticoid (mainly cortisol) exposure during pregnancy (Charmandari et al., 2004).

In adults, cortisol is released in response to a stressor and is the main regulator that maintains proper blood glucose levels by stimulating gluconeogenesis. Gluconeogenesis is the conversion of non-carbohydrate substrates (lipids, amino acids) to glucose in the liver. Proper brain function is dependent on sufficient blood glucose levels because of its inability to use any other substrate as a fuel source. High concentrations of synthetic glucocorticoids are used to control inflammation by immune system suppression. Cortisol release is regulated ultimately by the hypothalamus which releases corticotropin-releasing hormone (CRH). The release of CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which is what actually acts to release cortisol (Sherwood, 2006)

This stress hormone is also vital to fetal organ maturation, and is involved in additional pregnancy related events. Cortisol is one potential ligand for the glucocorticoid receptor (GR), which functions as a ligand-dependent transcription factor. The α-isoform is expressed throughout most tissue types and modulates target genes by promoter region binding or interaction with additional transcription factors (as reviewed by Charmandari et al., (2004)). Once activated, it functions to remodel chromatin, target initiation sites, and stabilize the RNA-polymerase II machinery in order for multiple transcription of target genes (Nicolaides et al.,

2010). Describing the role of the receptor in regards to specific gene regulation is beyond the scope of this review (Oakley and Cidlowski, 2011). Cellular responses to GR activation is tissue and ligand type dependent. There are also eight receptor variants (A, B, C1, C2, C3, D1, D2 and D3) that influence gene targets, as well as cofactor surroundings and promoter regions (Karolien, 2010).

Cortisol and the GR play a large role in cellular regulation and fetal development (described below). However, when a fetus is exposed *in utero* to an excess of maternally produced cortisol (via the placenta), a reduction in fetal growth and reduced birth weight can occur (McTernan et al., 2001). The moderator of this cortisol equilibrium is the enzyme, 11β-hydroxysteroid dehydrogenase (11β-HSD) and where considerable focus has been placed in trying to elucidate the mechanism of low birth weight and fetal programming. 11β-HSD is an intracellular membrane bound enzyme, found in two isoforms, type 1 and 2 (Lakshmi et al., 1993). Type 1 (11β-HSD) plays a role in local production of cortisol and is found mainly in the chorion and amnion (fetal membranes) and fetal tissues. Type 1 converts cortisone, the biologically inactive ketone, back to the active cortisol via reduction reactions (Sun et al., 1997). Type 2 performs the reverse reaction, facilitating the unidirectional oxidation of cortisol to cortisone, with NAD+ as a cofactor (Murphy, 1981). The 11β-HSD2 enzyme is highly expressed in at-term placental tissue (37-42 weeks), specifically in the syncytiotrophoblast layer, where the maternal-fetal interface is located (Pepe et al., 1999; Staud et al., 2006).

Speirs (2004) mapped the GR and 11β-HSD1 expression throughout development in rats. Increased expression for both was seen in central nervous system, liver, gastrointestinal tract and lung tissues, indicating that the maturation of these tissues was dependent on the local production of cortisol. The increase in expression was organ system dependent and varied across

developmental time points. The lungs were among the last tissue types to see 11β-HSD1 promotion. For pregnancies at risk for pre-term delivery, cortisol can be used as a therapy by accelerating the structural development of the lungs, thereby increasing pre-term infant survival rates (Ballard, 2000). Further evidence of the importance of cortisol in lung maturation was discovered using GR knock-out mice (Cole et al., 1995). Neonatal death, due to lung immaturity, resulted when expression of this critical receptor was repressed.

An additional role of glucocorticoids is labor induction, through prostaglandin production. When the expression of 11β-HSD1 was increased by the addition of dexamethasone (a synthetic glucocorticoid) in culture, increased cortisol production in turn stimulated prostaglandin production in the chorionic trophoblast. Prostaglandins are the main driver of the positive feedback loop which induces labor (Sun et al., 2002). Alfaidy et al. (2003) found an increase of 11β-HSD1 expression in the intrauterine (fetal) membranes during late gestation. This increase in 11β-HSD1 expression accounts for the increase in cortisol measured in fetal circulation and amniotic fluid. These findings support the need for additional cortisol production to induce parturition, with cortisol originating from the placenta, rather than fetal (adrenal) or maternal origins.

However, an excess of *in utero* exposure to cortisol has been linked to intrauterine growth restriction (IUGR) and further health implications later in life such as hypertension (White, 2001). The presence of 11β-HSD2 in the syncytiotrophoblast, the tissue of maternal/infant exchange, suggest that this barrier is designed to protect the fetus from excess maternal cortisol (Yang, 1997). The 11β-HSD2 enzyme oxidizes (inactivates) 80-90% of the maternal cortisol, leaving 10-20% to pass into the fetal blood supply without adverse affects on fetal growth (Waddell et al., 1998). With an increase in cortisol levels later in pregnancy, there is

concurrent increase in the differentiation of placental cells. Cell differentiation, as a measure of human chorionic gonadotropin (hCG) secretion, was increased when an 11β-HSD2 inhibitor (carbenoxolone) was added to cultured trophoblast cells. This decrease in 11β-HSD2 activity lead to an increase in cortisol concentration (because of reduced cortisol oxidation). The increase in cortisol induced the shift from cytotrophoblasts, placental stem cells, into syncytiotrophoblast cells, the functional cell layer between maternal and fetal blood (Nacharaju et al., 2004). Syncytiotrophoblast cells express 11β-HSD2 and with this increased cell differentiation, the result would be an increase in the placental cortisol barrier, effectively shielding the fetus and reducing the cortisol levels *in utero* (Nacharaju et al., 2004)

The inhibition of placental 11β-HSD2 has been shown to alter glucose metabolism in offspring. Administration of carbenoxolone (inhibitor of 11β-HSD2) to pregnant rats reduced average fetal weight by 20% (Lindsay et al., 1996). Neonates of the carbenoxolone exposed pregnancies made up the weight reduction by six months of age, but had significantly higher fasting plasma glucose and exhibited significantly greater plasma glucose and insulin responses (10 and 38% higher respectively) to an oral glucose load, increasing the risk of hyperglycemia in adult life. A study by Welberg et al. (2005), described similar findings in rats when carbenoxolone was administered during pregnancy. That study also found that inhibited 11β-HSD2 activity was associated with reduced birth and body weight at adulthood. Administration of a single course of antenatal dexamethasone (a potent synthetic cortisol) reduced 11β-HSD2 expression and fetal weight compared to the placebo group in sheep (Kerzner et al., 2002). Fetal weight was further reduced with repeated administration of dexamethasone (once a week for five weeks), though this continued exposure did not significantly change the expression of 11β-HSD2 (anymore than the initial dose did). The positive and adverse consequences of cortisol exposure

in pregnancy are well documented. Without it, organ maturation and perturbation progression is hindered, but an over-exposure has been linked to adverse pregnancy outcomes.

The placental expression of 11β-HSD2 and the oxidation of cortisol becomes more pronounced as pregnancy progresses (Shams et al., 1998). McTernan et al. (2001) reported a significant increase (56 fold) in the enzyme's expression in placentas at term (37-42 weeks) with normal weight infants compared to first and second trimester measurements. The significant increase in the enzyme emphasizes its importance in protecting the fetus from excess glucocorticoid exposure to ensure that the final weeks of growth, which are influential on birth weight, are uninhibited by the steroid. Unabated fetal exposure, due to mutations in the 11β-HSD2 gene are linked to apparent mineralocorticoid excess, a disorder characterized by reduced birth weight, significant organ damage, and early death (Dave-Sharma et al., 1998). With the weight of evidence supporting the adverse effects of hypercortisolemia, understanding the potential influences on the modulation of the expression or the enzyme activity is key to preventing these adverse effects on infant health.

Maternal Health Influences on 11β-HSD2 Activity and Expression

Studies investigating the relationship between 11β-HSD2 and fetal cortisol exposure have used three main means of quantifying this enzyme's presence and efficiency. Enzyme activity is the measure of how well that enzyme converts its substrate, cortisol, into the product, cortisone. A decrease in activity could occur through two mechanisms: enzyme inactivation or substrate competition (Hardy et al., 2001), or due to a decrease in enzyme mRNA transcripts, a measure of enzyme transcriptional promotion (Sampath-Kumar et al., 1998). A third way of quantifying this process is to measure the cortisol:cortisone ratio in fetal plasma (Campbell and Murphy, 1977). Blood is not mixed in the placenta, rather nutrients and wastes are transported to

and from maternal and fetal blood lines via the syncytiotrophoblast layer of the placenta. This maternal/fetal interface not only handles nutrient and waste transfer, but also the passage of maternal cortisol. This interface is where the 11β-HSD2 enzyme is localized, and if properly functioning and in sufficient supply, 11β-HSD2 converts the majority of the maternal cortisol to cortisone before encountering the fetal blood supply. Thus, fluctuations in the cortisol:cortisone ratio in fetal plasma can reflect the efficiency of the enzyme.

A wide range of maternal health conditions have been shown to play a significant part in the activity/expression of 11β-HSD2. One that parallels Barker's findings is maternal nutrition. McMullen et al. (2004) tested 11β-HSD2 activity and the ratio of cortisol:cortisone in fetal plasma in pregnant ewes fed a 70% maintenance diet. While maintaining the ewes and fetuses, this diet was nutritionally deficient. Sustained from gestational day 26 onward, this diet decreased 11β-HSD2 enzyme activity by 52% on day 90 of gestation; fetal growth was also restricted as a result. This pattern of nutritional restriction, however, did not alter the fetal cortisol:cortisone plasma ratio. Lesage et al. (2001) placed rats on a 50% nutritional restriction during the last week of gestation. Maternal corticosterone (the murine cortisol equivalent) levels increased, along with maternal adrenal weight, while placental 11β-HSD2 expression decreased. This rise in maternal cortisol levels, coupled with a decrease in 11β-HSD2 expression, resulted in excess fetal cortisol exposure, and a decrease in fetal weight resulted. To deduce whether the increased cortisol exposure via increased maternal production, or the nutritional deficiencies was the cause of the 11β-HSD2 expression decrease and reduced fetal growth, a second group of mothers were placed on the same nutritional restriction pattern. In the second group however, a basal level of cortisol was maintained by artificial means following the removal of their adrenal gland. This was done to test the effects of the diet restriction and null maternal cortisol

production. The same reduction of 11β-HSD2 expression and fetal weight resulted, mirroring the adrenal intact mothers, which showed that the nutritional state of the doe, and not maternal cortisol levels, decreased 11β-HSD2 expression and lead to the reduced fetal weight. Langley-Evans et al. (1996) also showed a similar reduction of 11β-HSD2 enzyme activity in rats when mothers were placed on a mildly restricted protein diet. Pups of the protein-restricted mothers had an increased adult systolic blood pressure compared to controls. Additional placental enzymes were tested for activity (MDase activity, PKase activity), as well as total placental protein. These measurements were not significantly different from controls, indicating that overall placental function was maintained; with the protein deficiency specifically affecting the 11β-HSD2 enzyme.

Chronic maternal stress is another important maternal factor for maintaining the proper 11β-HSD2 placental cortisol barrier. At gestational day 20, a 160% increase in 11β-HSD2 activity was reported by Welberg et al., (2005) with the administration of acute maternal stress (via a restraint test) in rats. However, this ability to up-regulate the enzyme was inhibited by 90% with sustained chronic maternal stress on days 14-19. So although acute stress can induce enzyme activity, when stress is sustained, that ability is diminished. The effects of other stress hormones, catecholamines (norepinephrine, and epinephrine) and proinflammatory cytokines (interleukin (IL)-1, IL-6, and tumor necrosis factor-α) have all been shown to down regulate expression and activity of 11β-HSD2 in placenta tissue cultures (Kossintseva et al., 2006; Sarkar et al., 2001). This 11β-HSD2 reduction is also seen in reduced oxygen environments, such as pregnancies complicated with preeclampsia (PE). Hardy and Yang (2002) demonstrated that cytotrophoblast cells, when cultured under hypoxic conditions, do not differentiate into syncytiotrophoblast cells, the cells that predominantly express 11β-HSD2, which could lead to a

reduction of the enzyme. Placentas from normal and PE pregnancies were compared by Alfaidy et al., (2002); 11β-HSD2 expression was reduced with PE pregnancies when compared to gestationally matched controls. In the same study, villous explants from first trimester (5–8 week gestation) and at-term placentas were cultured under 20% O_2 or 3% O_2 atmospheres. At both time points, the increased O_2 atmosphere contributed to an increased in 11β-HSD2 expression. In the at-term placentas, an 140% increase in activity was observed.

Although not a maternal illness, intrauterine growth restriction (IUGR), babies born small even at full term (Adam, 2008; Wilcox, 2001), has been associated with a decrease in placental 11β-HSD2 activity and expression (Dy et al., 2008; McTernan et al., 2001; Shams et al., 1998). However, there is refuting research about this association (Rogerson et al., 1997).

With the importance of 11β-HSD2 in the prevention of excessive cortisol exposure to the fetus, and the possibility of its inhibition (activity and expression) further merits investigation as to what other compounds impact this important placental barrier.

Environmental Influences of 11β-HSD2 Activity and Expression

With the increasing understanding of the important physiological role of cortisol, research into potential environmental contaminates that disrupt the 11β-HSD2 axis need to be examined (Odermatt and Gumy, 2008; Odermatt et al., 2006). Metals have been shown to alter the activity and expression of this enzyme. Yang et al. (2006) reported that cadmium, a metal classified as a human carcinogen by the World Health Organization's (WHO) International Agency for Research on Cancer (World Health Organization, 1993) also is an inhibitor to 11β-HSD2 activity in cultured trophoblast cells. A 24-hour exposure to 1 μM $CdCl_2$ reduced the cortisol conversion activity of 11β-HSD2 by 80%. Along with enzyme activity, 11β-HSD2 gene

transcription was decreased with cadmium exposure, suggesting that the decrease in activity was due to a reduction of gene transcription. Ronco et al. (2009) reported a decrease in the birth weight (8.4%) in rats with a placental concentration of 50 ppm of Cd^{+2}, which was linked to an increase in corticosterone, the glucocorticoid equivalent in rats. There was no reduction in 11β-HSD2 activity, however. Hardy et al. (2001) demonstrated, after culturing placenta tissue *in vitro*, that similar to cadmium, calcium, another divalent element, inhibited 11β-HSD2 activity through an alteration in the catalytic efficiency by a disruption of the enzyme itself. Activity inhibition of up to 50% was observed at calcium concentrations from 22 to 268 nM, but the activity inhibition was reversible with the addition of a chelator. Zinc (also divalent) was identified by Niu and Yang (2002) as a 11β-HSD2 activity inhibitor, with an IC_{50} of 2.5 μM. The inactivation of metals, through the use of a metal chelator (e.g., EDTA, EGTA or TPEN) has been shown to significantly increase 11β-HSD2 activity in the placental microsomal fraction or tissue culture (Niu and Yang, 2002; Yang et al., 2002). It is clear through both the decrease in activity and expression of 11β-HSD2 by metals, and by the increase in activity with the chelation of metals, that 11β-HSD2 is sensitive to these trace elements. Organotins (Di- and tributyltin (DBT, TBT) as well as di- and triphenyltin (DPT, TPT)) were tested for 11β-HSD2 inhibition (Atanasov et al., 2005). The IC_{50} in intact cells ranged from 0.99 (± 0.24) to 2.89 (± 0.59) μM with TPT and DPT, respectfully.

In addition to metals, other organic pollutants, phthalates (dipropyl phthalate (DPrP) and di-n-butyl phthalate (DBP), were shown to disrupt 11β-HSD2 activity when tested on rat kidney tissue (IC_{50} of 85.59 μM and 13.69 μM, respectfully) (Zhao et al., 2010). The extent of activity inhibition was structurally dependent, specifically on the number and arrangement of carbons in the alcohol moiety. One, two, and 5-10 carbons were ineffective in enzyme

disruption, except when cyclized (6-carbons). Another class of organic compounds, dithiocarbamate (DTC), showed similar inhibition (Atanasov et al., 2003). Six DTC congers were tested (Thiram, Disulfiram, DEDTC, PDTC, Maneb Zineb) and resulted in a range of IC_{50} from Disulfiram at 128 (±21) nM to Zineb 31 (±18) μM. Additional screening has been performed to find other possible 11β-HDS inhibitors (Schweizer et al., 2003). It is interesting to see the inhibition of type 2, and not type 1 in with organotins and dithiocarbamates, both through the proposed disruption at the Cys^{90} (Atanasov et al., 2005; Atanasov et al., 2003), suggesting sensitivity to outside xenobiotics and compounding factors for the disruption of proper placental cortisol barrier function.

Birth Weight as an Individual Measurement and the GROW Curve

A 2000 study done by Savitz et al. (2000) analyzed births in New York City, NY, US and found that while the majority of low birth weight infants were premature (69.2%), just more than half (50.2%) premature infants were low birth weight. So while gestational period is a confounder for overall birth weight, there are other mechanisms that induce low birth weight other than length of development.

A simple assessment based on fetal weight alone ignores other important variables that influence birth weight, including maternal height and weight, parity, ethnicity and infant sex (Gardosi et al., 1992). With the incorporation of these factors, infants can then be grouped as small for gestational age (< 10^{th} centile), average, or large for gestational age (>90^{th} centile) (i.e., SGA, AGA, LGA). With these physiological characteristics incorporated into an adjusted growth curve, a more accurate assessment of whether or not the infant's weight was influenced by pathological factors is gained. After assessing 4,179 births in the United Kingdom, Gardosi and Chang (1992) found that 28% of SGA babies were within the normal range when the

adjusted charts were used, emphasizing the importance of incorporating more than just gestational age into proper birth weight.

Software developed by Gardosi and Francis (2009) can be used to calculate an individual growth centile, using the physiological parameters (i.e., maternal height, maternal weight at first clinical visit, parity and ethnic group), which are customized to different geographical regions. These parameters generate a Term Optimal Weight, which is then extended proportionally to give a Gestation Related Optimal Weight (GROW) curve. Finally, the actual birth weight of the infant and the gestational age at delivery is factored in to produce the birth weight centile (BWC) ranking.

Expression Quantification and New Standards for qRT-PCR

Northern blots can be used as to compare mRNA production between experimental treatments. RNA is first separated by size via gel electrophoresis, transferred to a membrane and labeled probes are then used to target genes of interest (Glick and Pasternak, 2003). However, a more sensitive technique for quantification of gene expression is quantitative (real time) reverse transcription polymerase chain reaction (qRT-PCR). Total RNA is first isolated from the sample of interest at a specific time point or under a certain condition, and is then reverse transcribed to complementary DNA (cDNA). This cDNA sample is then placed into a thermocycler with target-gene specific primers, and a fluorescent dye that only fluoresces when bound to double stranded (ds) DNA, referred to as an intercalation dyes. As the thermocyler completes a cycle (denature, anneal, polymerize, repeat), the cDNA sample (PCR product), which originated from the RNA transcripts, is amplified, increasing the fluorescence proportionally. A threshold level for the fluorescence is set automatically by the cycler. When the fluorescence of a sample surpasses the threshold, the cycle number (C_t) is recorded. Fewer cycles are required to generate

fluorescence sufficient to surpass the threshold when increased gene-of-interest transcripts are present in the initial sample. In Figure 1, Position A represents the sample with the most RNA transcripts of interest (also known as copy number). Position B is the sample with the lowest copy number. The green horizontal line is the fluorescence threshold where the C_t value for each sample will be taken.

The specificity of the amplification process is assessed through a melt curve (Figure 2). After the polymerization step for qRT-PCR, the temperature is incrementally increased, with corresponding fluorescence detection. Increased temperature denatures the dsDNA strands (PCR product), decreasing the fluorescence produced by the intercalation dye, which only fluoresces in the presence of dsDNA. A desired melt curve has only one peak, indicating no primer dimer interaction and no additional amplification of contaminating DNA.

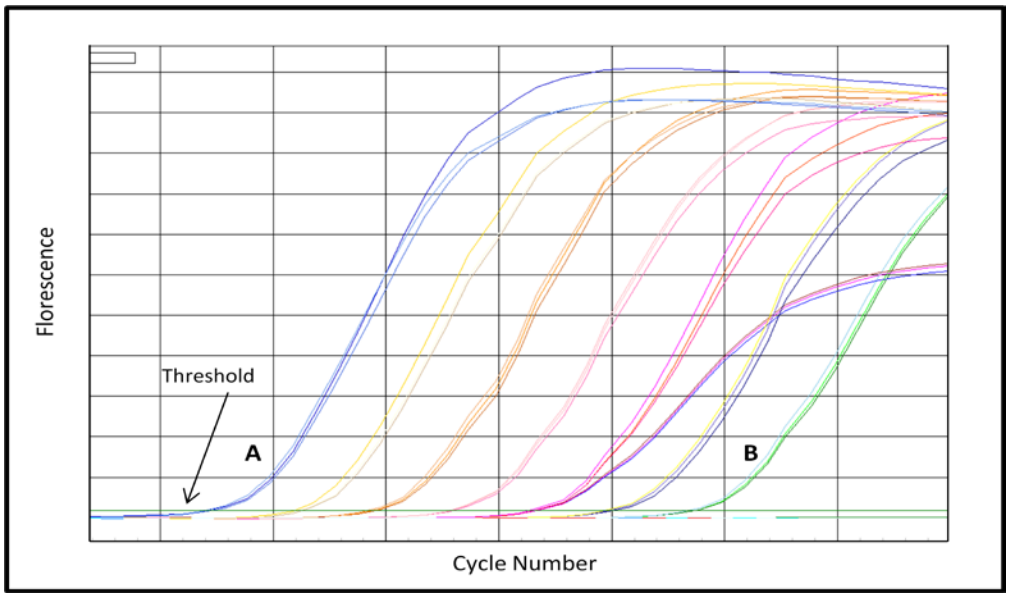

Figure 1

qRT-PCR reaction plot of fluorescence of intercalation dye and cycle number. Sample A has the most RNA transcripts of interest (copy number). B is the sample with the lowest copy number. The green line is the fluorescence threshold where the Ct value for each sample will be taken.

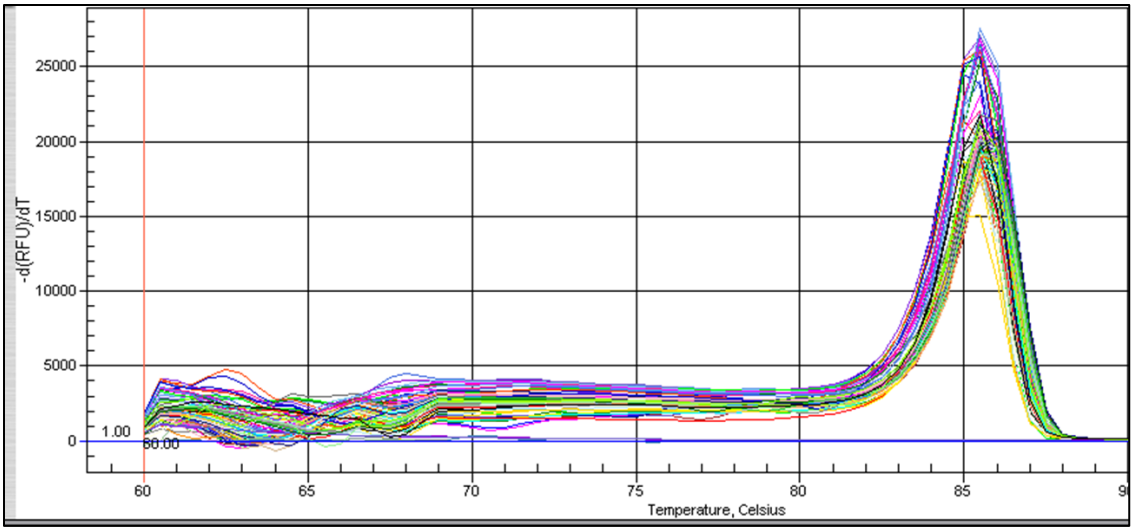

Figure 2

Melt curve plot of a single gene using qRT-PCR. Increasing the temperature incrementally denatures the dsDNA strands (PCR product), decreasing the fluorescence produced by the intercalation dye. One peak indicates no primer dimer interactions or DNA contamination.

With the use of qRT-PCR increasing over the past decade, more stringent and consistent requirements for the publication of qRT-PCR are needed (Bustin et al., 2009). One important area is in the selection and number of reference genes (Bustin et al., 2005). Reference genes are used as an internal standard to normalize the gene of interest, which controls for variations in several steps: RNA extraction, reverse-transcription and amplification. These controls allow for the comparisons of RNA concentrations across different samples (Bustin et al., 2009). Reference genes are selected based on their consistent expression over developmental time points and cellular modulations. An abundance of reference gene possibilities have been archived in the RTPrimer Data Base, with the goal of uniformity and standardization in primer design (Pattyn et al., 2003). Currently, the database holds 8,310 primer sets for twenty-four organisms, bacteria and viruses. For this study, three genes were evaluated as possible reference

genes, ribosomal 18S RNA, beta actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

With the mounting evidence that over-exposure to cortisol *in utero* influences birth weight, the current study examined the correlation between birth weight and a calculated birth weight centile with placental expression of 11β-HSD2. Additionally, other maternal factors that affect birth weight, the birth weight centile and 11β-HSD2 expression were examined.

CHAPTER 3

MATERIALS AND METHODS

Subjects

This study received approval from and annual renewal by two IRBs in the University of Tennessee system: Chattanooga Unit of the College of Medicine Institutional Review Board and University of Tennessee Graduate School of Medicine Institutional Review Board. Collection was done at Erlanger Hospital, Chattanooga, TN and was sustained between June of 2007 to July of 2010. Sample selection was based on personal arrival time and time of placental delivery. From placentas collected one hour after the delivery (n=242), and which would have otherwise been discarded, a thin, five gram sample was removed from an area adjacent to the umbilical cord, preserved with 25 ml of RNAlater (an RNA Stabilization reagent, Qiagen, Valencia, CA) and stored at -20°C. Additional maternal and infant data were collected from medical records. Placentas were collected from singleton births from HIV and hepatitis negative mothers over 18 years of age. Consent was obtained when possible.

Assessment of 11β-HSD2 Expression: qRT-PCR

To determine whether the expression of 11β-HSD2 correlated with birth weight, the relative abundance of *11β-HSD2* mRNA in preserved placenta samples was assessed by a two-step qRT-PCR, as described previously (Sharma et al., 2009). Briefly, total RNA was extracted from an approximately 30 mg RNALater preserved sample using an RNase Mini Kit Plus (Qiagen Hercules, CA) according to manufacturer's instructions. Briefly, tissues were

mechanically homogenized in lysis buffer containing 1% β-mercaptoethanol. Genomic DNA removal from homogenized lysate was carried out by supplied gDNA Eliminator spin columns. Aliquots were stored at - 80° until time of use. Agarose gel electrophoresis was used to evaluate RNA quality through detection of 18S and 28S rRNA bands on a 1% RNase-free agarose gel, as well as assessment of RNA purity, ensuring the absence of DNA contamination. RNA was quantified by spectrophotometry (NanoDrop 2000c, Thermo Fisher Scientific) measuring the A260/A280. One microgram of total RNA was reverse transcribed in a total volume of 20 ul using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Reactions were carried out in supplied reaction buffer according to the following incubation parameters: 5 min equilibration at 25°C, 30 min reverse transcription at 42°C, 5 min enzyme inactivation at 85°C. The diluted RNA was retroactively assayed to confirm absence of genomic DNA contamination. Gene transcript levels of ribosomal *18S, ACTB, GAPDH* and *11β-HSD2*, using primers listed in Table 1, were quantified in triplicate with the Ssofast Eva Green assay using the Ssofast Eva Green master mix (Biorad) under universal thermal cycling parameters (2 min at 50 C and 10 min at 95 C, followed by 40 cycles of 15 sec at 95 C and 1 min at 60 C) on the Biorad iQ5 Real-Time PCR Detection System. The specificity of the Ssofast Eva Green assays was verified by performing a melting curve analyses, confirming only amplification of the primer/gene product with the generation of a single peak (Figure 2). Levels of *18S, ACTB, GAPDH* and *11β-HSD2* mRNA in each RNA sample were quantified using the relative standard curve method, which involves the generation of a standard curve from serial dilutions of untreated control cDNA, ranging from 2×10^1 to 2×10^8 copies. For each RNA sample, the relative amounts of *ACTB, GAPDH* and *11β-HSD2* were determined and the ratio of *11β-HSD2* to both *ACTB* and *GAPDH* was calculated using the $\Delta\Delta C_T$ method (Livak and

Schmittgen, 2001), resulting in the final normalized *11β-HSD2* expression level (Sharma et al., 2009) (see Determination of Reference Gene Expression Stability below). An identical cDNA sample was used as an inter-run control across all plates of the same gene to assess the consistency between qRT-PCR runs of the same gene (Hellemans et al., 2007). Plate efficiencies for each gene exceeded 92.5% (14 plates/gene).

Table 1

Sequences for 11β-HSD2 and all potential reference genes primers, forward (FP) and reverse (RP).

Gene Name	Primer sequence (5'-3')	Product size (base pairs)	Reference
18S-FP	GTAACCCGTTGAACCCCATT	153	(Zhang et al., 2011)
18S-RP	CCATCCAATCGGTAGTAGCG		
ACTB- FP	GGCCGCGGTGTACGCCAACACAGTGCTG	228	(Murphy, 2002)
ACTB- RP	CCCGGGGCCGTCATACTCCTGCTTGCTG		
GAPDH-FP	CCTGTTCGACAGTCAGCCG	101	(Sturla et al., 2009)
GAPDH- RP	CGACCAAATCCGTTGACTCC		
11β-HSD2 -FP	TCAAGACAGAGTCAGTGAGAAACG	129	(Murphy, 2002)
11β-HSD2 - RP	GGAACTGCCCATGCAAGTG		

Determination of Reference Gene Expression Stability

From the three possible reference genes (*18S*, *ACTB* and *GAPDH*), sixty samples were analyzed for consistency of expression using geNorm (Vandesompele et al., 2002). The geNorm software ranks potential reference genes according to variances in their expression profile. Those with the lowest gene expression stability measure (M) are selected as the best possible reference genes. NormFinder (Andersen et al., 2004) was also used to calculate the stability of the reference gene expression. The initial analysis resulted in *18S* receiving the greatest M value of all three potential genes, and was subsequently eliminated. The final M value for *ACTB* and *GAPDH* was 0.879, indicating an inherently stable expression.

Birth weight centile calculation

GROW software was obtained for the United States coefficients (Gardosi and Francis, 2009). Data for the gestational age adjustment (parity, maternal height/weight, ethnicity, infant sex and weight) was obtained from medical charts. Gestational age was based on best obstetric estimate. This incorporated the use of first trimester ultrasound that was within +/- 3 days of last missed period (LMP) or a second trimester (< 20 weeks gestation) that was +/- 7 days of LMP.

Statistical Analysis

Study parameters included normalized 11β-HSD2 expression, birth weight centiles and unadjusted birth weight. These three endpoints were analyzed based on maternal age, marital status, pre-pregnancy BMI, maternal smoking and drug use, race and education. Additional comparisons were done between infant sex, and complicated vs. non-complicated pregnancies. Data were complete for the three study parameters (birth weight centiles, 11β-HSD2 expression, and birth weight) for a total of 242 samples. Of those 242, 51 were classified as complicated, which included IUGR (n=5), oligohydramnios (n=5), PIH/PE (n =9), Non-reassuring fetal heart rate (NRFHR) (n=15), and pre-term labor (PTL) (n=13). One hundred and ninety one were considered uncomplicated. Self-reported smoking, drug use (n =5) and alcohol consumption (n =2) were lumped together (n = 44) and compared against uncomplicated pregnancies with no drug/tobacco use (n = 147). Education and marital status data were missing from some births (n = 199 and 234 respectfully). The top three races (Hispanic, Caucasian, African American) were used for comparison of 11β-HSD2 expression, birth weight centile and unadjusted birth weight (n = 240). Calculation of maternal Body Mass Index (BMI) was based on weight at first booking and maternal height followed the standard calculation from Keys (Keys et al., 1972). Individual BMI was calculated for all subjects (n = 242) and delineated according to standard guidelines,

released by the World Health Organization (2011). A BMI score of less than 18.5 is considered underweight, 18.5-24.9 is considered normal, 25-29.9 is classified as overweight, and a BMI score of greater than 30 is considered obese.

Comparisons based on maternal age (less than 35 vs. 35 and up), marital status (single vs. married), pre-pregnancy BMI (under vs. average vs. overweight vs. obese), maternal drug use in non-complicated pregnancies (use vs. non-use), race (Caucasian vs. African American vs. Hispanic) and maternal education (less than high school vs. high school/GED vs. some college), infant sex and pregnancy type (complicated vs. non-complicated; complications are listed above) were done with either a Mann-Whitney Rank Sum Test or a Kruskal-Wallis One Way Analysis of Variance on Ranks, with accompanying Dunn post hoc test. Correlations between normalized 11β-HSD2 expression, birth weight centiles, gestational age, and unadjusted birth weight were analyzed with a Kruskal-Wallis One Way Analysis of Variance on Ranks. Statistical analysis was done with SigmaPlot 12.0.

CHAPTER 4

RESULTS

Analysis of Study Parameters: Birth Weight Centiles, 11β-HSD2 Expression, and Birth Weight

All data were subjected to a Shapiro-Wilk Normality Test and were non-normal ($p < 0.001$). Log and inverse transformations were attempted, but did not improve data distribution. Non-parametric tests, or tests of ranks, (Kruskal-Wallis One Way Analysis of Variance on Ranks, Mann-Whitney Rank Sum Test, Spearman Rank Order Correlation) were continued throughout the analysis, and medians reported (Table 2).

Table 2

Distribution of study parameters reported as 25th, 50th and 75th quartiles as well as mean and standard deviation (Std Dev).

Study Parameter [a]	**25% quartile**	**Median**	**75% quartile**	**Mean**	**Std Dev**
Birth weight centile (%)[b]	18.3	44.0	72.1	46.1	31.5
11β-HSD2 Expression[c]	0.159	0.291	0.488	0.365	0.279
Birth weight (g)	2776.0	3184.0	3539.5	3121.6	664.4

[a] For all parameters, n = 242.

[b] Birth weight centile was calculated using GROW Software (Gardosi and Francis, 2009).

[c] Expression of 11β-HSD2 was normalized using the $\Delta\Delta C_T$ method with reference genes, *ACTB* and *GAPDH*.

Birth weight ranged from 968 g to 4733 g and the birth weight distribution was negatively skewed (Figure 3). Fourteen percent of the sample population was LBW. According to the birth weight centile calculation, 17% were of births were SGA, 70% were AGA and 13% were LGA.

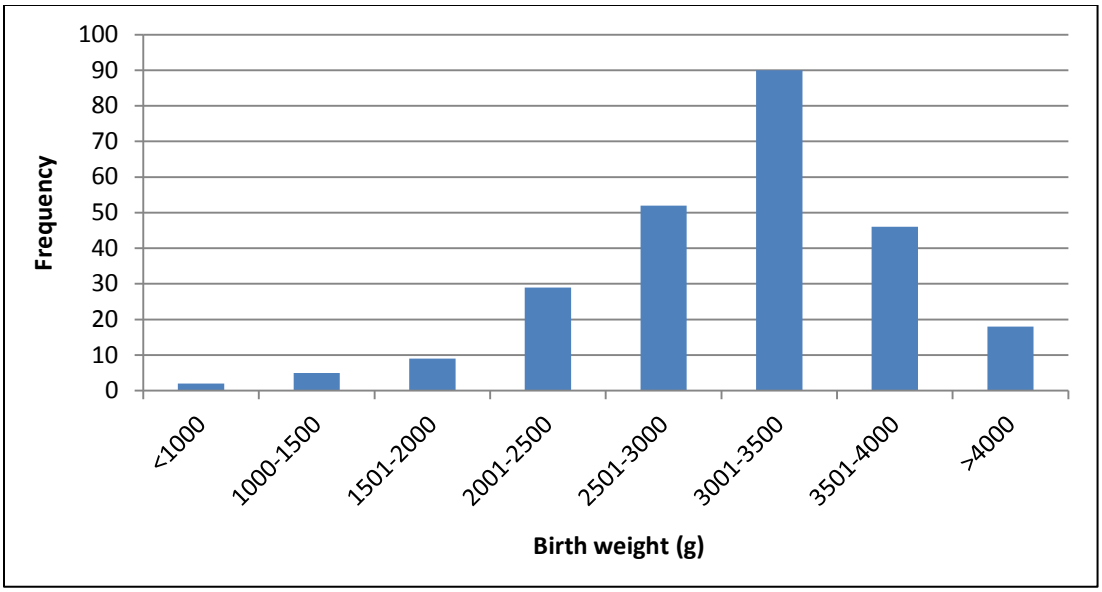

Figure 3

Distribution of birth weights, n = 242.

Birth weight centile, 11β-HSD2 expression, and birth weight were analyzed based on maternal age, marital status, pre-pregnancy BMI, maternal smoking and drug use, race and maternal education. Additional comparisons were done between infant sex and complicated vs. non-complicated pregnancies. Birth weight was significantly different between married and single women ($p < 0.001$) (Figure 4), Caucasian and African American mothers ($p = 0.023$) (Figure 5), and obese and underweight women ($p = 0.029$) (Figure 6) and complicated vs. non-complicated pregnancies ($p = <0.001$). Significant differences in infant birth weight centile were found between married and single women ($p = 0.011$) and complicated vs. non-complicated pregnancies ($p = 0.027$). Between those eight categories, there was no difference in 11β-HSD2 expression (Table 3). Thus, race, education, age, smoking, marital status, maternal BMI, nor pregnancy complications were associated with changes in 11β-HSD2 expression. Additionally,

there was no significant difference in Type 2 expression between low birth weight (<2,500 g) and normal birth weight infants, or when comparing SGA, AGA and LGA infants.

There was a significant, positive correlation between birth weight and maternal BMI (n = 242, p=0.0471) and gestational age (p < 0.001) using a Spearman Rank Order Correlation, as well as with 11β-HSD2 expression and birth weight centile in the uncomplicated pregnancies (n = 191, p=0.024.

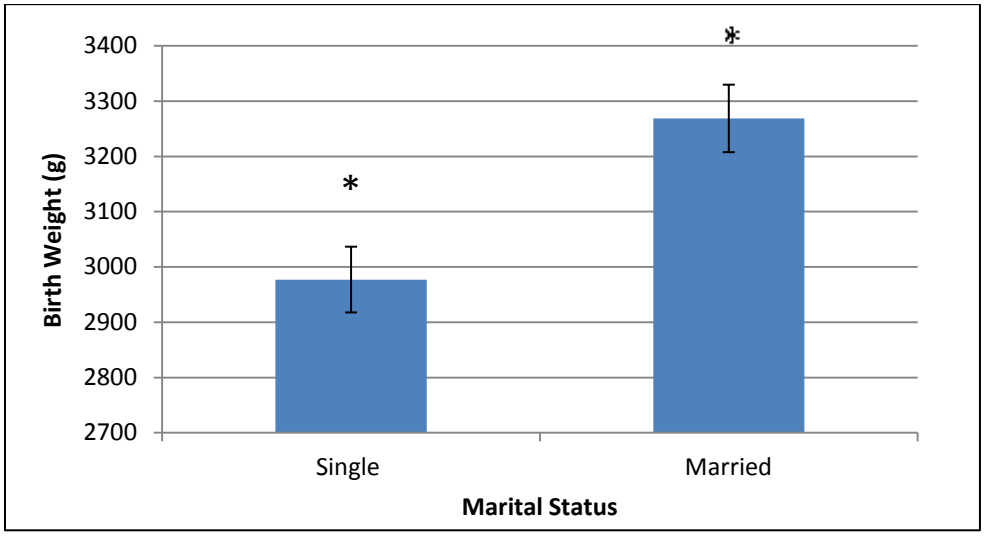

Figure 4

Effects of Marital Status on Average Birth Weight (Single Mothers vs. Married Mothers). Significant differences (p < 0.05) are denoted with an asterisk (*). Error bars signify standard error. n = 126 for single mothers, 108 for married mothers. Average (± standard deviation) birth weight for single mothers was 2977.1 g (±618.1) and 3268.6 g (±685.3) for married mothers.

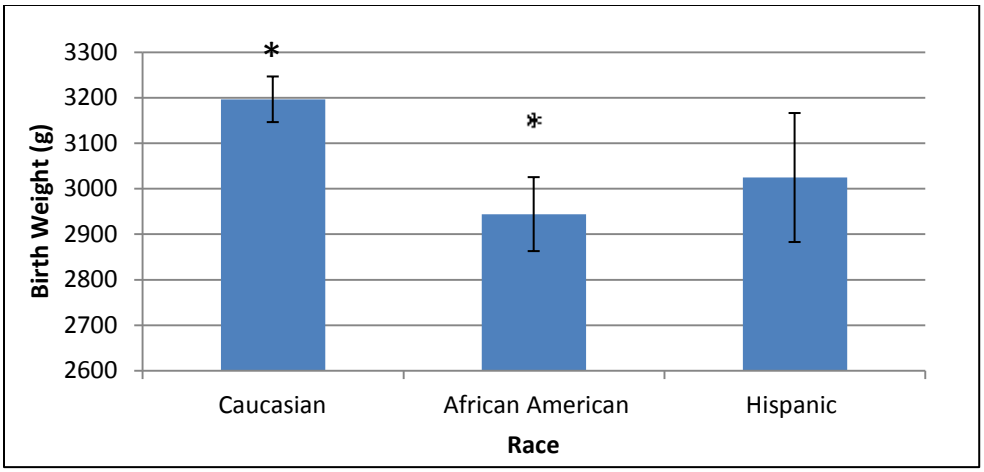

Figure 5

Effects of Race on Average Birth Weight (Caucasian vs. African American vs. Hispanic). Significant differences ($p < 0.05$) are denoted with an asterisk (*). Error bars signify standard error. n = 155 for Caucasian mothers, 46 for African American mothers and 39 for Hispanic mothers. Average (± standard deviation) birth weight for Caucasian mothers was 3196.7 g (±624.5), 2944.2 g (±551.4) for African American mothers and 3024.7 g (±885.6) for Hispanic mothers.

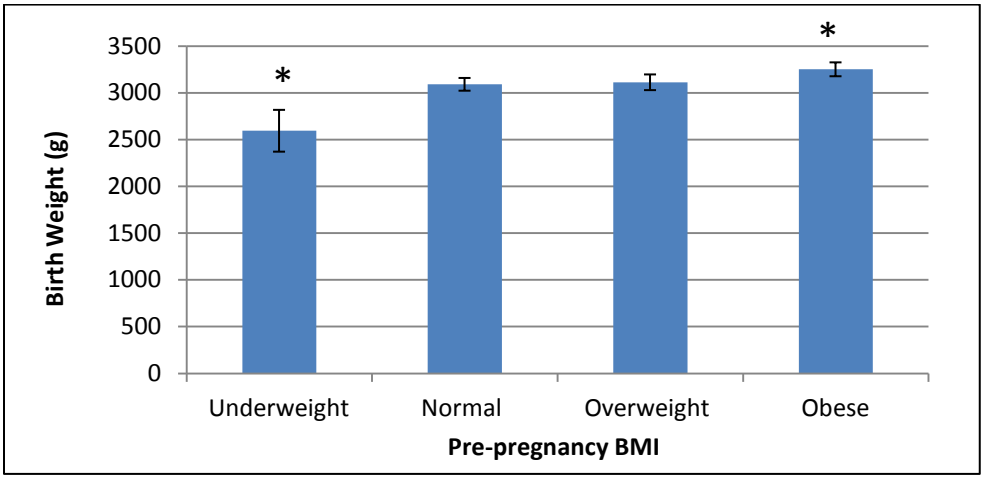

Figure 6

Effects of Pre-Pregnancy BMI on Average Birth Weight (under vs. average vs. overweight vs. obese). Significant differences ($p < 0.05$) are denoted with an asterisk (*). Error bars signify standard error. n = 9 for underweight mothers, 96 for average weight mothers, 70 for overweight mothers and 67 for obese mothers. Average (± standard deviation) birth weight for underweight mothers was 2594.3 g (±670.2), 3090.8 g (±663.7) for normal weight mothers, 3112.8 g (±701.6) for overweight mothers and 3251.5 g (±607.8) for obese mothers.

Table 3

Relationship of maternal and infant parameters relative to birth weight centile, normalized 11β-HSD2 expression, and unadjusted birth weight. Total number (n) in each category are reported along with median values. p-values comparing study parameters within maternal/infant characteristics are in gray.

Statistical Grouping	Birth Weight Centile	Normalized 11β-HSD2 Expression[a]	Birth Weight (g)	n	% of Sample Population
Maternal Age	0.352	0.617	0.933		
<35	41.53	0.297	3195	225	93
≥35	60.53	0.254	3155	17	7
Marital Status	0.011*	0.595	<0.001*		
Single	38.19	0.301	2972	126	54
Married	50.41	0.289	3269	108	46
Pre-preg. (BMI)[c]	0.128	0.480	0.029*		
Underweight (<18.5)	18.55	0.446	2578 (A)[b]	9	4
Average (18.5-24.9)	51.69	0.275	3155 (AB)	96	39
Overweight (25-29.9)	37.02	0.298	3228 (AB)	70	29
Obese (>30)	47.50	0.284	3354 (B)	67	28
Drug use[d]	0.618	0.732	0.090		
No drugs consumed	50.38	0.266	3333	147	77
Drugs used	46.53	0.248	3282	44	23
Race	0.167	0.771	0.023*		
Caucasian	44.56	0.303	3248 (A)[b]	155	64
African American	36.23	0.293	2984 (B)	46	19
Hispanic	53.82	0.266	3127(AB)	39	17
Education	0.307	0.639	0.114		
< high school	49.88	0.253	3126	67	34
HS/GED	51.91	0.284	3334	56	28
any college	36.76	0.306	3073	76	38
Pregnancy type	0.027*	0.507	<0.001*		
Non-complicated	45.525	0.282	2565	191	79
Complicated	27.671	0.332	3301	51	21
Infant Sex	0.863	0.753	0.886		
Female	39.17	0.304	3163	132	54
Male	44.25	0.286	3207	110	46

[a] *11β-HSD2* was normalized using the $\Delta\Delta C_T$ method with *ACTB* and *GAPDH*.

[b] Significant differences were accompanied by a Dunn's post hoc test. Statistically similar median values are indicated by using identical letters (e.g., A is significantly different than B).

[c] Body Mass Index

[d] In un-complicated pregnancies

[*] Significant differences among groups (p-value < 0.05)

CHAPTER 5

DISCUSSION

The present study was designed to investigate the expression of 11β-HSD type 2 and its possible correlation with raw birth weight and (or) a maternal physiology/gestational age adjusted birth weight centile. To our knowledge, no research has used such a large sampling to assess the relationships between the expression of this enzyme using qRT-PCR over a range of birth weights of non-complicated pregnancies and various maternal parameters and fetal parameters. The 11β-HSD2 expression data, in addition to birth weight and the adjusted birth weight centile, were examined in a broader scale with such maternal factors as age, BMI, marriage status, education, race and smoking.

Significant Findings: Race, Maternal BMI and Marital Status Are Correlated With Birth Weight

One significant finding that is well supported in the literature is the disparity between white and black infant birth weight. The present study shows a significantly lower birth weight between Caucasian and African American women, which is mirrored by the state as a whole (TN Department of Health). In addition, data from the State shows that black Tennessee infants are still at greater risk for infant mortality than white infants across all maternal ages, among mothers who hold a college degree, and mothers without maternal care (Corniola et al., 2006).

The pattern of reduced birth weight infants born to American born black mothers is a nationwide trend. A study by David and Collins (1997) sought to determine whether this trend was due to a genetic predisposition to smallness by compiling data from infants born to African-

born women in the United States. The researchers looked at mothers who immigrated from West Africa, where it is thought that American born blacks can trace back ancestry due to the slave trade of the 17th and 18th century. Three quarters of American black genetic heritage originates from this area; the last quarter coming from European descent, so if there was a genetic predisposition to reduced birth weight, these two black populations should share that. However, birth weight distribution between U.S.-born white women and African-born black women were the same, and both populations gave rise to larger infants than infants born to U.S. born black women. The same parallel was seen in very low birth weight births. Without a genetic link to smaller infant size, continued investigation into community and psychological factors are merited (Geronimus, 1992).

The current study showed a significant difference in birth weight between under weight and obese mothers, as well as between married versus single mothers. Ehrenberg et al. (2003) reported that mothers that were underweight prior to conception had an increased risk of preterm labor, intrauterine growth restriction and low birth weight. Increased maternal BMI has been shown to increase birth weight due to an increase in placental nutrient transport (Jansson et al., 2008) and incidences of macrosomia (excessive birth weight) increase with maternal BMI (Frederick et al., 2008; Hull et al., 2008). Previous research shows that LBW, pre-term labor and small for gestational age births were seen more often with single versus married women (Shah et al., 2011). A large scale study done by the Centers for Disease Control in the U.S. found an increased risk of stillbirth, total infant deaths, and Sudden Infant Death Syndrome (SIDS) among single woman (Balayla et al., 2011). The significant differences in birth weight reported in the current study between difference races, marital status and maternal BMI are all supported in the literature. An accompanying decrease in 11β-HSD2 expression was not reported in this study,

thus the difference between these categories was not due to an insufficient placental cortisol barrier.

There was no difference in birth weight, birth weight centile or 11β-HSD2 expression in mothers over the age of 35. Maternal age of 35 or more years is considered high risk and correlated with adverse pregnancy outcomes compared to mothers between the ages of 20-24 (Joseph et al., 2005). Mothers between 35-39 years of age had an increased risk of maternal complications (hypertension, diabetes mellitus, placental abruption, or placenta previa) as well as pre-term and SGA births. Although these effects are seen with increasing age, these data support that aging mothers in the present study are able to maintain normal 11β-HSD2 expression, and birth weight, as well as mothers under the age of 35.

An additional variable that has been shown to influence fetal growth is the use of tobacco, drugs or alcohol during pregnancy (Lieberman et al., 1994; Wright et al., 1983; Zuckerman et al., 1989). The current study lumped any use of drugs (tobacco, alcohol or marijuana) together, though tobacco use was most commonly consumed (85%). The use of these substances during pregnancy was compared among uncomplicated pregnancies. It is well documented that smoking has a significant negative effect on birth weight. A study by Krentz et al., (2011) showed that birth weight was affected in a dose-dependent manner, ranging from 0-20 cigarettes smoked per day. Chen et al., (2007) tested the effects of nicotine on fetal growth through subcutaneous administration of nicotine in rats. Placenta 11β-HSD2 expression decreased at mid to late gestation, resulting in IUGR neonates. Despite these studies, there was no significant difference in birth weight or 11β-HSD2 expression between mothers whom used drugs, alcohol or tobacco and those that did not.

11β-HSD2 expression was compared among LBW infants and normal weight infants. Additionally, 11β-HSD2 expression was compared among SGA infants, as well as AGA and LGA infants. Several studies have shown a concurrent decrease in expression and birth weight (McTernan et al., 2001; Murphy et al., 2002), but this study did not. Rogerson et al. (1997) quantified 11β-HSD2 expression through a Northern blot analysis and compared expression with raw birth weight, as well as placental weight, in 110 placentas across various gestational ages, races, and maternal ages. Rogerson et al. found no difference in expression between maternal age, gestational age or race, which mirror results from the present study. However, no correlation was found between expression and infant birth weight. Although a similar design, the current study uses a more precise technique, a larger sample size, and adjusts for confounding birth weight variables not done by Rogerson et al., thus, a different study outcome.

Importance of Birth Weight Adjustment

Many factors contribute to an infant's final birth weight. Although it has been used to make predictions of adult adverse health effects of smaller infants (as with Dr. Barker's studies discussed above), the use of raw birth weight alone may not be a sensitive enough endpoint for subtle correlations with variables such as environmental contaminates, and as the data showed in this study, 11β-HSD2 expression. Therefore, confounding variables that have been shown to significantly influence birth weight need to be controlled for in order to see these extraneous variables. This is a well-established practice. For example, Fei et al., (2007) found a negative correlation with perfluorinated compounds and birth weight after adjusting for maternal age, parity, socio-occupational status, pre-pregnancy BMI, smoking during pregnancy, infant sex, and gestational age, with the most substantial confounding variables being parity and pre-pregnancy BMI. Fei's study also excluded births from the Danish cohort with congenital malformations,

supporting the need for some selection before a final correlation between the BWC and enzyme expression was found. Similar elimination of pregnancy complications was done by Washino et al., (2009) and a significant inverse correlation between the perfluorinated compounds and birth weight was found only after the above parameters were adjusted for (in addition to alcohol, caffeine consumption, annual house hold income and education level). The lumping of non-reassuring fetal heart rate (NRFHR) in with complicated pregnancies, and being subsequently discarded from the final expression/BWC correlation could be disputed. Clinically, NRFHR is assigned for a variety of conditions, which may or may not indicate, in and by itself, abnormal fetal conditions or affect birth weight (Adair, 2011). However, because of its high incidence in the sample population, it was lumped into pregnancy complications.

Although the present study lacked the resources to incorporate some of the variables mentioned above, the GROW software adjusted for parity, pre-pregnancy BMI, infant sex, and gestational age. Only after these variables were accounted for was there a correlation with 11β-HSD2 expression. More extensive analysis to control for these confounding factors not used in GROW would presumably produce a more accurate account of fetal growth. Additional maternal parameters that have been shown to affect fetal health, and 11β-HSD2 expression or activity (e.g., maternal nutrition, stress) should be incorporated into to future studies.

Data Gaps and Uncertainties

The present study examined the expression of 11β-HSD2 in placental tissue over a variety of infant and maternal parameters. However, this measurement of fetal health is incomplete without accompanying 11β-HSD2 activity data. Enzyme activity is affected by both reduced expression (enzyme production), but also by the physical disruption of the enzyme. If environmental contaminants are indeed a factor in increased *in utero* cortisol exposure, and beget

restricted fetal growth in Hamilton County, a physical disruption of the enzyme seems a more plausible mechanism over reduced expression. In addition to activity, the ratio of cortisol:cortisone in fetal plasma is a measure of actual cortisol exposure. With these two additional measurements, a more complete picture of cortisol, the placental 11β-HSD2 barrier and fetal growth could be constructed.

The current study made no attempt to evaluate any additional parameters that are important in maintaining an healthy pregnancy. Numerous studies have emphasized the importance of maternal and/or cord blood levels of adipocytokines (leptin, adiponectin, ect.) and Insulin-Like Growth Factors (Briana and Malamitsi-Puchner, 2009; Christou et al., 2001; Kyriakakou et al., 2008; Street et al., 2006; Tsai et al., 2004) on proper fetal growth. Proper fetal nutrition, in particular amino acid transport via the placenta, is another potential focus for research pertaining to proper fetal health and birth weight (Bajoria et al., 2002; Keating et al., 2009; Roos et al., 2004; Sibley, 2009). Begetting sufficient fetal nutrition is proper placental function, especially in the area of uterine perfusion and its effect on fetal weight and subsequent adult cardiovascular health (Alexander, 2003). Langley-Evans' study (1996) quantified three other placental enzymes, as well as protein to ensure that placental function was not a confounding variable on the growth of the fetus.

The birth weight centile calculation did take into consideration gestation age, which is strongly correlated with birth weight. However, studies have shown that clinical estimates of gestational age can be inaccurate. Dietz et al. (2007) reported that African American and Hispanic women, younger and less-educated women, and those who entered prenatal care after the second month of pregnancy have the most inconsistent gestational age estimates. The 'gold standard' of the gestational age estimate is based off of ultrasounds before the 20^{th} week of

pregnancy, but even then there have been reported inaccuracies (Henriksen et al., 1995). However, gestational age estimates based on ultrasound data have been shown to be much more accurate than recalling the mothers last missed period (Gjessing et al., 1999; Kramer et al., 1988). Adjustments to birth weight are dependent on high quality gestational age data, however, even the best measurements of gestational age can be a source of uncertainly.

Finally, the definition of low birth weight and its importance in infant health (infant mortality) is under scrutiny. A review by Wilcox (2001), rebukes that the proportion of births under 2,500 gram in a population automatically reflects the same risk of infant mortality. His alternative is based on the fact that some populations (for example, infants born at a higher elevation) have an overall reduction in birth weight, though the risk of infant mortality does not increase. Setting the same weight-cutoff which defines increased risk to the infant to one population at sea-level and one at 6,000 feet, would assign a unnecessarily greater risk to those infants born at elevation. It is the increased elevation that has shifted the entire birth weight distribution to the lower end, but this is done without a change in the infant mortality rate of the population. His proposal for assessing a population at risk is the not the percent of infants born under 2,500 grams; instead an examination of the 'residual' portion of a population's birth weight distribution. On a large scale, birth weight is normally distributed, with an extended tail on the left, which encompasses the IUGR and LBW/pre-term births. These infants outside the predominate distribution (defined by mean and standard deviation) are the residual population. It the percent of births in the residual portion of the birth weight distribution that should be the index for infant risk. He explains that defining a birth weight with increased risk within a population is plausible. However, between populations, that same weight may not be an accurate measure of infant mortality risk.

In regards to Hamilton County, the coinciding LBW incidence and infant mortality supports that there is an increased risk of infant mortality below the 2,500 gram weight. However, the analysis that Wilcox proposed would no doubt be interesting to investigate. In the end, he does stress the importance and strong predictive measurements the birth weight implies, however, the definition of the LBW classification is in need of re-evaluation.

A continued investigation into the dynamic relationship between fetal programming, the developmental origin of adult disease, and the 11β-HSD mediated balance of glucocorticoid production during gestation is highly warranted. If indeed a reduced placental expression of 11β-HSD2 affects the growth potential of infants, longitudinal studies linking expression and adult disease should be initiated and additional studies on the characterization of common animal models should be continued.

11β-HSD2 as a Screening Tool for Infants at Risk

The role of 11β-HSD2 in a healthy pregnancy is scientifically well supported. There is evidence to support that the molecular mechanism of reduced fetal growth is connected to the balance of cortisol exposure *in utero*; there must be enough to ensure organ maturation, but not enough to abate fetal growth. This makes 11β-HSD type 2 an important endpoint when investigating high incidences of LBW births. Since in the majority of cases the placenta is discarded after delivery, molecular examination of this organ in assessing fetal health would eliminate the need for more invasive collection procedures, such as collection of cord blood.

The utility of 11β-HSD2 activity measurement in a clinical setting has yet to be determined. These data presented here supports a correlation between expression and fetal growth, but the association is far from causational. The factors that control its expression are complex and greatly beyond the scope of this study, but if indeed this enzyme were to be used as

a screening tool, those factors may have to be controlled for in order to fully assess the risk associated with a decrease of expression. Although expression begets activity, activity reduction can also occur independent of expression. Thus, it would have to be determined whether expression or activity would make a better endpoint for assessment. Several of the studies concerning the regulation of this enzyme considered both in order to determine whether a decrease in activity was due to reduced expression, or due to a physical disruption of the enzyme itself. With the advent of qRT-PCR, expression evaluation is easier and faster than activity would be, especially when activity is concentrated in the microsomal portion. But enzyme activity is directly responsible for the *in utero* exposure to the fetus, this would be a more relevant, albeit more labor intensive, end point for infant risk.

Environmental contaminants and pregnancy outcomes

Additional environmental factors should be considered when studying fetal or child health. Ambient air pollution has been shown to affect birth weight (Bell et al., 2007; Bobak, 2000). Chemicals detected in cord blood, such as polychlorinated biphenyls (PCBs) (Murphy et al., 2009) and Perfluorinated Chemicals (Apelberg et al., 2007) have also been correlated with reduced birth weight. Other pregnancy complications (preterm labor) have been linked to the predominate DDT metabolite, DDE (Longnecker et al., 2001).

Domestically, the environmental effects of growth, development, and health of children will be examined through The National Children's Study, which was created through the Children's Health Act of 2000 (http://www.nationalchildrensstudy.gov/Pages/default.aspx). Not only will the study report on numerous environmental variables (air and water quality, sound pollution), but it will also examine what potential effects community, culture and family structure have on the health of U.S. children through their first 21 years. Increased global

concern for environmental effects on children brought about the creation of the International Society for Children's Health and the Environment, which aims to bring together the many disciplines that study and play a part in children's health, from policy makers to analytical chemists to public health practitioners (Bellinger et al., 2011).

The present study shows that in uncomplicated pregnancies, the growth potential of an infant positively correlates with placental expression of the 11β-HSD2. This finding is consistent with the hypothesis that a reduction in placental 11β-HSD2, leads to a reduced oxidation of active cortisol to inactive cortisone before passage into the fetus. Without this cortisol inactivation, an elevated cortisol level in the fetus leads to deleterious programming before birth, which could result in reduced growth and increased risk of adult diseases, such as Type-2 diabetes and hypertension.

CHAPTER 6

CONCLUSION

The current study expands research into the hypothesis that an over-exposure to cortisol *in utero* can negatively affect proper fetal growth. The passage of maternal cortisol into the fetus, via the placenta, is modulated by the enzyme, 11β-hydroxysteroid dehydrogenase type 2. To date, this is the largest study of its kind to look at this enzyme's expression over a large population and it's relation to the growth potential of a fetus. A significant positive correlation between 11β-HSD2 and a calculated birth weight centile for non-complicated pregnancies was found ($p = 0.024$). Only after confounding maternal and infant factors were controlled for did a correlation of fetal growth potential and placental expression of 11β-HSD2 occur. Continued molecular characterization of the placenta, and increased understanding of the critical functions the placenta performs, is warranted. Deciphering how deleterious pregnancy outcomes are linked to improper placental function could result in diagnostic tests designed to determine potential disease risk factors for childhood or adult life. Molecular assessments of the placenta, coupled with longitudinal studies of specific endpoints of adult health, would help define to what extent the intrauterine environment plays into the lifespan of an individual.

Significant differences in birth weight were found between Caucasians and African American mothers, single and married mothers and underweight vs. obese mothers. Without an accompanying difference of 11β-HSD2 expression suggests that this enzyme did not influence these differences in birth weight. Further studies could be conducted to deduce whether there is

an accompanying molecular cause to this difference in BW, in addition to potential socioeconomic factors.

Despite the potential flaw of the LBW definition, evidence supports that birth weight can be a predictor of adult health. However, further research into additional molecular endpoints hold promise to understanding, and ultimately preventing, this pathological reduction of fetal growth in Hamilton County. Successful suppression of Hamilton County's high rate of pathologically small infants requires further study. Certainly interdisciplinary, community-focused programs like BLUES, Centering Pregnancy (https://www.centeringhealthcare.org) and Infant Mortality Reduction Initiative (Featherstone, 2010) are aiding to curb this LBW trend and reduced infant mortality. However, when considering the industrial past of this area, and the areas of Hamilton County of greatest LBW incidence, it is plausible to consider these long lived contaminates in the soil as a possible confounding variable in the LBW equation. These contaminates, particularly metals, are of concern to those interested in deciphering the mechanism of restricted fetal growth in Hamilton County, TN.

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