Maternal Energetics, Placental Epigenetics, and Developmental Programming

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THESIS

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This thesis is dedicated to my mother, Teresa, and my father, Kim. They each provided me with unwavering support and encouragement to accomplish this thesis and never failed to sprinkle this journey with humor.

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"Beyond the very extreme of fatigue and distress, we may find amounts of ease and power we never dreamed ourselves to own; sources of strength never taxed at all because we never push through the obstruction"

William James

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CpG site	Cytosine-guanine dinucleotide; Region of the DNA sequence in which a cytosine base is followed by a guanine base and separated only by one phosphate in the 5’ to 3’ direction.
DNAm	DNA methylation; Epigenetic modification in which a methyl group is added to a cytosine base at the fifth carbon.
DOHaD	Developmental Origins of Health and Disease
TSS	Transcription Start Site
SNPRC	Southwest National Primate Research Center

SUMMARY

The purpose of this dissertation was to integrate well-established aspects of the marmoset monkey with innovative DNA methylation (DNAm) methodologies (i.e. reduced representation bisulphite sequencing) to gain insight about the role of placental DNAm in a valuable model of developmental programming of obesity. The introduction lays the theoretical and conceptual groundwork and is followed by two independent, but complementary papers. The aim of the first paper, *The Common Marmoset Monkey: Avenues for Exploring Prenatal and Placental Mechanisms in Developmental Programming of Pediatric Obesity*, was to position the marmoset monkey as a sophisticated model for exploring the maternal and placental mechanisms involved in developmental programming of obesity. The two aims of the second paper, *DNA Methylation in the Marmoset Monkey: Maternal weight epigenetically affects genes involved in metabolic pathways,* were (1) to describe placental DNAm for the first time in this species; and (2) to explore the association between maternal metabolic health and DNAm of genes and gene pathways in the placenta.

To the best of our knowledge, this is the first study to report genome-wide placental DNAm in the common marmoset monkey. Importantly, these results were obtained in an animal model with a high degree of biological salience for translational research. The findings from the primary aim of the second paper establishing DNAm patterns in the marmoset placenta provide a valuable step in developing the marmoset monkey as model of investigating the role of placental epigenetics in developmental programming. The secondary aim of paper two was to begin to understand the impact of maternal metabolic status on placental DNAm at the epigenome-wide level by

identifying genes and gene pathways that are affected by maternal weight during gestation. One of the principal findings of this aim was that maternal weight is associated with DNAm in genes that are predominantly involved in energy metabolism and homeostasis such as the *regulation of glycolytic processes* (GO:0006110), and the *regulation of lipid metabolic processes pathways* (GO:0019216). Combined, the findings of this study establish the marmoset as a model worthy of exploring placental epigenetic contributions to developmental programming and reinforce the growing body of evidence that demonstrate the effects of maternal metabolic status on placental DNAm.

1. Introduction

Across the world overweight/obesity affects 42 million children and is a predisposing factor for all of the major health conditions that form the dominant health burden of societies worldwide [1, 2]. There is increasing evidence that obesity has prenatal origins, as fetal exposure to altered nutrient supply in utero is consistently linked to greater risk of obesity and metabolic disorders later in life [3-5]. Maternal metabolic health during pregnancy has major implications for developmental programming of obesity in offspring [5-7]. Yet, the intrauterine mechanisms at play in the maternal metabolic conditions that result in offspring obesity are largely unknown. As the physical interface between mother and fetus during pregnancy, the placenta is the key regulator of nutrient allocation between the mother and fetus [8]. Interrogating the placenta is thus essential for understanding the intrauterine mechanisms that link maternal metabolic condition to pediatric obesity.

1.1 Theoretical Basis: Developmental Origins of Health and Disease

The link between exposures of the intrauterine environment and disease later in life is captured by the developmental origins of health and disease (DOHaD) framework [3, 9-11]. DOHaD posits that "exposure to an unfavorable environment during development (either in utero or in the early postnatal period) programs changes in fetal or neonatal development such that the individual is then at greater risk of developing adulthood disease," [6]. Much of the developmental programming of obesity research focuses on fetal outcomes (i.e. birth weight, epigenetic changes in offspring metabolic tissues) which does not elucidate the mechanisms that lead to these outcomes and obesity later in life. Interrogating the placenta has the potential to do so. Recent

incorporation of the placenta into DOHaD research demonstrates that the placenta plays a central mechanistic role in developmental programming and that optimizing placental structure and function likely optimizes lifelong health of offspring [12-14]. Inclusion of the placenta in developmental programming has led to innovative findings that have started to unravel the role of the placenta in developmental programming of obesity, diabetes, hypertension, and osteoporosis [10, 15, 16]. Yet due to its recent entry into developmental programming research, much is still unknown about the precise placental mechanisms involved, especially as they pertain to maternal metabolic conditions that influence developmental programming of obesity.

1.2 Maternal metabolic health and offspring obesity

Maternal metabolic health (e.g. under-/over-nutrition, weight, weight gain, etc.) before and during pregnancy is a primary contributor to the conditions of the intrauterine environment, fetal development, and birth outcomes [7, 17]. Both extremes of maternal metabolic status lead to developmental programming of obesity in offspring [7]. The groundbreaking epidemiological study linking maternal undernutrition to obesity came from the Dutch famine during World War II [18]. Offspring of mothers who experienced the famine during the first half of pregnancy had lower birth weights but a significantly higher incidence of obesity at 19-years-old than those without prenatal exposure to famine. Animal studies consistently demonstrate that maternal nutrient restriction (e.g. caloric, protein) is linked to low birth weight and obesity later in life [3]. Postnatally, low birth weight infants typically follow a growth pattern that involves rapidly crossing from a lower weight centile to a higher weight centile ("centile crossing") [16, 19]. Centile

crossing is a classic high-risk phenotype in developmental programming that has been repeatedly linked to obesity and other metabolic disorders later in life [16, 19].

While maternal undernutrition has been the historical precept of developmental programming research, recent studies have linked maternal metabolic conditions of obesity, high-fat diet, and diabetes with offspring obesity later in life [5-7]. Commonly, these maternal conditions lead to high birth weight infants that follow a growth pattern towards obesity that is characterized by consistently high weight [7]. Recently, there has been a 25% increase in large for gestational age infants that is concurrent with the 25-36% increase in maternal obesity [6]. Studies of the Pima Indian population have elucidated much about the effects of diabetes on birth outcomes and obesity [20, 21]. Mothers with diabetes have increased maternal glucose levels which stimulates fetal production of insulin and insulin-like-growth factors. This typically leads to high birth weight infants with high insulin levels that develop obesity and insulin resistance later in life. However, normal birth weight infants born to diabetic mothers are also born with high insulin levels and develop obesity and insulin resistance later in life [21, 22]. Low birth weight is not limited to conditions of maternal undernutrition, and low birth weight infants resulting from pregnancies complicated by maternal overnutrition typically exhibit the centile crossing growth pattern toward obesity [7]. The available evidence implies that the intrauterine mechanisms leading to obesity are much different for low, normal and high birth weight infants and that these mechanisms are highly influenced by maternal metabolic status.

1.3 Incorporating the placenta into developmental programming of obesity

Historically, birth weight has been used as a proxy of fetal growth and the intrauterine environment [23]. Research continues to show that obesity manifests in low, normal, and high birth weight infants which suggests that birth weight may not be on the direct causal pathway leading to obesity later in life. Birth weight alone is a discrete output measure of synergistic inputs throughout gestation [14, 23]. While birth weight is important for a foundational understanding of developmental programming of obesity, use of birth weight alone is limited in its ability to reflect dynamic processes of the intrauterine environment and to elucidate developmental programming mechanisms. Across the spectrum of birth weights, interrogating the placenta has the potential to reveal the intrauterine mechanisms that are at play in developmental programming of obesity. Throughout pregnancy, the placenta functions as a nutrient sensor that is responsive to maternal nutrient supply and fetal nutrient demand [8, 17, 24]. The ability of the placenta to alter and mediate nutrient transport to the fetus makes it a key regulator of fetal growth and developmental programming [8]. This provides an active biological tissue that is plastic and responsive to the maternal conditions and fetal signals that contribute to developmental programming. On a molecular level, alterations in placental epigenetic profiles may provide a robust reflection of maternal metabolic health with the potential to predict of fetal developmental programming outcomes. For example, the epigenetic modification of DNA methylation has been examined in placental genes and related to pregnancy complications such as intrauterine growth restriction and preeclampsia; maternal conditions such as obesity, gestational diabetes,

and caloric restriction; and fetal outcomes such as birth weight, neurobehavior, and allergies [25-27].

1.4 Maternal metabolic health and epigenetic profiles

Epigenetic alterations can result from gene-environment interactions and play an important role in gene regulation [28]. Epigenetic changes in the placental genome occur in response to the intrauterine environment—which is shaped by maternal metabolic, nutritional, and hormonal status [25-27]. DNA methylation (DNAm) is a well-studied epigenetic modification which is responsive to the maternal metabolic condition and influences placental gene expression with consequences for fetal growth and obesity outcomes [29]. Examining DNAm status of placental genes that are involved in metabolic gene pathways can provide molecular level insight into the placental mechanisms mediating maternal metabolic conditions and birth outcomes linked to obesity. For instance, in a recent genome-wide DNAm study, 23 genes were identified whose DNAm levels explained 70-87% of the variance in birth weight suggesting that these genes are part of important gene networks of growth control and may be an important contributor to the underlying mechanisms of developmental programming outcomes that have previously been linked to birth weight [30]. In a comparative genome-wide study of obesity, gestational diabetes, and preeclampsia, a lower level of placental DNAm was observed in mothers with gestational diabetes and a higher level of placental DNAm in mothers with obesity [31]. Human placentas exposed to maternal obesity have also demonstrated higher DNAm levels in *LEP* which encodes for leptin and both high and low levels of leptin exposure in utero are associated with obesity later in life [32-34]. Examining placental DNAm can elucidate placental strategies at play in

the associations between maternal metabolic health and developmental programming outcomes.

1.5 The marmoset monkey as a model for developmental programming of obesity

The common marmoset monkey (*Callithrix jacchus)* is a biologically relevant nonhuman primate model for studying reproduction as well as maternal and offspring obesity. In particular, marmosets exhibit reproductive biology and hemochorial placental characteristics that are fundamentally similar to humans but ideal for research studies due to their small body size (283-500 grams), quick sexual maturity (~15 months), frequent reproduction (~2 times/year), short gestation (143 days), and a lifespan of 8-10 years in captivity [34]. At the Southwest National Primate Center, research has shown that marmosets in captivity spontaneously develop obesity and demonstrate similar alterations in metabolic markers that are evident in humans [10, 14, 23, 35, 36]. In consideration for maternal characteristics, the marmoset provides a continuum of maternal metabolic states for which to explore reproductive effects. In consideration for developmental programming obesity, birth outcomes and postnatal growth patterns leading towards obesity reflect those seen in human developmental programming studies and the obese phenotype emerges as early as 1 month of age. Additionally, low birth weight infants from triplet litters have demonstrated the classic high-risk phenotype of developmental programming: low birth weight followed by “centile crossing” into a higher weight in adolescence and adulthood. These aspects expose an intriguing and biologically salient model for developmental programming of obesity research [37, 38]. These characteristics permit efficient assessment of

developmental programming exposures and outcomes as they relate to short-term, long-term, and intergenerational contexts.

In the marmoset, placental structure and function has been explored in relation to the conditions of the intrauterine environment and provides evidence in support of the role of the placenta in developmental programming [39]. Yet, the placenta has yet to be examined in the context of maternal metabolic health nor has placental DNAm been explored in this species. Foundational studies in the marmoset have established it as a valuable model for developmental programming of obesity while other studies have established the association between placental characteristics and the intrauterine environment. There is a prime opportunity to merge these aspects of the marmoset monkey as a model to offer insight into the role of the placenta in linking maternal metabolic health to developmental programming of obesity. Additionally, with the recent mapping of the marmoset genome, it is now possible to advance our understanding to include the role of placental DNAm in developmental programming through the use of the marmoset monkey.

The purpose of this dissertation was to integrate well-established aspects of the marmoset monkey with innovative DNAm methodologies to gain insight about the role of placental DNAm in a valuable model of developmental programming of obesity. The two papers that follow address this purpose. The aim of the first paper, *The Common Marmoset Monkey: Avenues for Exploring Prenatal and Placental Mechanisms in Developmental Programming of Pediatric Obesity*, was to position the marmoset monkey as a sophisticated model for exploring the maternal and placental mechanisms involved in developmental programming of obesity. The aim of the second paper, *DNA*

Methylation in the Marmoset Monkey: Maternal weight epigenetically affects genes involved in metabolic pathways, was to characterize placental DNAm for the first time in this species and explore the association between maternal metabolic health and DNAm of genes and gene pathways in the placenta. Combined these two papers address the void of developmental programming research in nonhuman primate models that will offer critical time advantages and provide a biologically relevant model for translating findings to human populations. Additionally, this work lays the groundwork for future prospective studies to explore intergenerational and long-term developmental programming exposures and effects in the rich context of longitudinally gathered maternal and offspring data.

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2. The Common Marmoset Monkey: Avenues for Exploring the Prenatal and Placental Mechanisms in Developmental Programming of Pediatric Obesity

The emergence of adult metabolic disorders in children is a worldwide health concern [1]. The pediatric obesity epidemic is incalculably problematic given that obese children are at risk for all of the major chronic health conditions that burden adults[1, 2]. Undoubtedly, the pediatric obesity epidemic is partially influenced by our external environments of caloric excess and sedentary behaviors [3]. However, there is increasing evidence that the prenatal experience and intrauterine environment are capable of exerting profound and permanent effects on metabolic health through developmental programming [4-7].

Birth weight is an outcome of particular interest in developmental programming research. Many studies have laid much of the groundwork in demonstrating that both low and high birth weight infants are at increased risk for obesity in childhood and as adults [8, 9]. The Dutch famine and Pima Indian studies, along with other epidemiological studies, have strongly demonstrated the association between low birth weight and obesity and metabolic syndrome in adulthood [10-14]. Low-birth weight followed by rapid catch-up growth during infancy is known as “centile crossing” and is a well-established and evocative phenotype in developmental programming of obesity [9, 15]. Human studies have demonstrated that centile crossing is significantly associated with childhood obesity and have also linked centile crossing to other metabolic disorders such as diabetes and hypertension [8, 15-19]. While low birth weight is typically the outcome of fetal “undernutrition” during the prenatal period, high birth weight is a common outcome of fetal “overnutrition,” particularly in pregnancies characterized by

maternal obesity and excessive gestational weight gain. High birth weight is also an established risk factor for the development of obesity later in life and the U-shaped association between birth weight and adulthood obesity is widely accepted [12, 17, 20].

While human studies have demonstrated the link between birth weight and obesity later in life, birth weight itself is an outcome of the complex and dynamic processes occurring in the intrauterine environment throughout gestation [21, 22]. As such, the discrete measure of birth weight is limited in its ability to reflect dynamic processes and focusing our exploration on the underlying intrauterine mechanisms that lead to the outcomes of birth weight will be essential in advancing our understanding of developmental programming of obesity. The organ that supports the developing fetus and has a prominent role in shaping the intrauterine environment throughout gestation is the placenta [21]. Recent inclusion of the placenta in developmental programming research has led to innovative findings that have started to unravel the role of the placenta in developmental programming of obesity, diabetes, hypertension, and osteoporosis [22-24]. Yet due to its recent entry into developmental programming research, much is still unknown about the placental mechanisms involved, especially as they pertain to childhood metabolic disorders.

Animal models have been critical to our understanding of the pathogenesis of obesity and the lifespan advantages of animal models offer significant opportunities in developmental programming research [25]. The short lifespans of animals permit efficient assessment of short-term and long-term health consequences, as well as intergenerational effects [26]. They also allow for control over environmental and

genetic factors, which are key components of developmental programming processes that cannot be controlled for in human population studies.

Rodents provide time and cost effective animal models that are a commonly used in obesity and developmental programming research [27]. They also provide a model that allows for a high degree of experimental control, and for these reasons, rats and mice are by far the most commonly used animal models of obesity [27]. However, rodent models lack the biological relevance to humans that is necessary for translating findings to the childhood obesity epidemic. The reproductive biology of rodents constrains the ability to fully elucidate placental mechanisms of developmental programming, while the differences in fat cell function and distribution, feeding behaviors, and adipokine function limit applicability of findings to the human population [25]. Additionally, many rodent models are experimentally manipulated to develop obesity through genetic engineering, selective breeding or extreme diets [28]. This is an additional limitation to rodent models, as the most common etiology of pediatric obesity is spontaneous and multifactorial [3].

In addition to offering phylogenetic advantages, nonhuman primate models of obesity exhibit reproductive processes similar to humans and the spontaneous development of obesity [29]. Across all primates the reproductive physiology is quite similar. Monkeys, apes, and humans exhibit placentas that are hemochorial with similar tissue composition that contributes to the biological relevance of exploring placental mechanisms of developmental programming in nonhuman primates [21]. In captive colonies, marmoset monkeys, macaques, baboons, and vervet monkeys spontaneously develop high body weight and high relative fat concentrations [29, 30]. Adding to the

value of these models is that overweight and obese nonhuman primates develop a suite of sequelae similar to that of obese humans including alterations in glucose, lipid and cholesterol levels, thus providing physiologically significant models for exploring the developmental programming mechanisms of obesity [30].

In particular, the common marmoset monkey (*Callithrix jacchus)* is a powerful model to explore developmental programming. Marmosets have been used as a biomedical model for well over fifty years and their use in research continues to increase [31]. The basic biology and development of the marmoset monkey offers many advantages for its use as an economical research model and as the first New World Monkey to have its genome sequenced there are rich opportunities for increased utility and novel research applications [31, 32]. When considering the placental and intrauterine contributions to developmental programming of pediatric obesity, a powerful animal model will offer reproductive physiology and placentation similar to humans. Additionally, to understand the underlying mechanisms of pediatric obesity, an animal model that closely resembles humans in the spontaneous, multifactorial and early life development of obesity is much needed. The purpose of this paper is to demonstrate that the presence of these aspects in the marmoset monkey make it a valuable research model for exploring the role of prenatal and placental mechanisms involved in developmental programming of pediatric obesity. First, a brief overview of the marmoset and obesity will be given, followed by a discussion marmoset reproduction and placental characteristics. I will then discuss the occurrence and utility of variable intrauterine environments in developmental programming in marmosets, evidence of developmental

programming of obesity will be given, and finally I will put forward future directions for including the placenta in developmental programming of obesity in marmosets.

2.1 The common marmoset monkey and obese phenotypes

The common marmoset monkey (*Callithrix jacchus)* is a New World Monkey that is part of the Cebidae family and the subfamily Callitrichinae, which also includes tamarins, lion tamarins, and Goeldi's monkeys (also called callimicos) [21, 32, 33]. The callitrichines are native to South and Central America, with marmosets living primarily in the coastal region of northeastern Brazil [21, 33].

Marmosets exhibit a fast life history that, "appears genetically engineered for 5-year NIH or MRC grants," [31]. At about 1 month old, infants begin weaning and are completely weaned by 2-3 months of age [29, 34]. The onset of puberty begins as early as 7-8 months and can last until about 15-16 months, although sexual maturity is typically reached between 11 and 12 months of age [31, 34]. When paired for mating in captive colonies, first conception can occur at about 14-15 months, and following an average gestation period of 143 days, first births can occur at about 19-20 months of age [31]. With an established mating pair, marmosets can produce offspring twice a year throughout their lifespan, which averages 6 years in captivity [21, 31]. In addition to their ability to frequently reproduce, marmosets regularly carry litters of multiples. Twins are the most common litter size in the wild but twins, triplets, quadruplets, and even, rarely, quintuplets are produced in captive colonies [33-35], and indeed, triplet litters have been observed in the wild. As discussed below, the natural variation in litter size creates unique opportunities for developmental programming research with larger litter sizes representing "undernourished" intrauterine environments.

A defining characteristic of the marmoset is its small body size, making it one of the least expensive nonhuman primate models to maintain in research laboratories [31]. In the wild, average adult weights range from 320-340 grams, and in captivity adult weights range from 283-500 grams [31, 36]. This is similar in size to the Sprague-Dawley rat that is commonly used in research, yet the marmoset monkey provides greater biological significance [21].

The spontaneous trends of increased weight in captivity have led to the characterization of an obese phenotype that is evident in early life with an etiology that is likely multifactorial, exposing an intriguing avenue for pediatric obesity research [25, 29]. Obesity in marmosets has been characterized based on body mass and body fat criteria similar to those used for classification of obesity in humans [25, 36]. A weight-based definition of obesity in marmosets is greater than the 90th percentile of body mass at 17 months of age and a percent body fat definition of obesity is greater than 14% relative body fat at 12 months of age. An important element of the marmoset obese phenotype that strengthens its use for obesity research with translational goals is that the increases in body mass reflect differences in metabolic markers that are similar to those seen in obese humans: higher fasting blood glucose, hemoglobin A1C, triglycerides and very low density lipoprotein [36]. Like humans and other primates, sex differences are evident in marmosets with females demonstrating a higher propensity to store fat [25].

The small size, fast life history, high fertility, natural variations in litter size, and biologically relevant characterization of obesity in the marmoset make it a prominent

and efficient model for research studies of primate reproduction and developmental programming.

2.2 Marmoset reproduction and placental characteristics

Marmosets demonstrate singular, cooperative breeding in which a single dominant male and female breed within an established breeding group [31]. Unlike most primates, marmosets experience a postpartum ovulation that usually occurs 10-20 days after delivery. Because postpartum ovulation typically results in conception and delivery, marmosets have a relatively short interbirth interval that averages 162 days and allows for marmosets to reproduce about 2 times per year [34].

Upon successful mating and conception, the 143-day gestation period begins [24, 31, 34]. Implantation typically occurs around day 11 to 13, and there is a quiescent period from this point until approximately day 50, a period that roughly coincides with weaning of the previous litter. The fetal stage of development typically does not begin until around day 80 [34]. Although this is a relatively late initiation of fetal development in primates, once fetal development begins the rate of organogenesis and other fetal developmental processes is quite similar to that of humans [34].

During days 19-30 of the quiescent period, investment is largely focused on the growth and development of the placenta. Similar to humans and all anthropoid primates, marmoset monkeys develop the most invasive type of placenta, the hemochorial placenta [37, 38]. Yet in an unusual deviation from other litter bearing animals, fusion of the chorionic membranes of marmoset embryos produces a unified bidiscoid placental mass with a chimeric layer of trophoblast that will be shared by all the fetuses of the litter [33, 37, 39]. The shared placental mass provides unique

opportunities for exploring the variable intrauterine environments that are related to litter size.

2.3 Litter size and natural variability in the intrauterine environment: Implications for developmental programming of obesity

The foundation of developmental programming is that an intrauterine environment characterized by alterations in fetal access to nutrients and resources leads to alterations in prenatal physiological development that may predispose the individual to obesity or other chronic illnesses [4, 25, 40]. For the intrauterine mechanisms and effects of developmental programming to be explored, a model must have the ability to exhibit alterations and variability in the intrauterine environment. Unlike other animal models of developmental programming that experimentally alter the intrauterine environment, due to the marmoset monkey's unique feature of gestating multiples with a shared placental mass, differences in litter size create variable intrauterine environments [21]. The natural variation in litter size creates intrauterine environments in which the developing fetuses compete for available nutrients and resources with outcomes that are in alignment with the tenets of developmental programming.

During the 143-day gestation period, the marmoset fetuses grow and develop in a complex intrauterine environment—an environment that is influenced by maternal and fetal factors. The marmoset placenta plays a key role in mediating these factors and when viewed as a nutrient sensor is responsive to both fetal demands and maternal supply [21, 24, 37, 41, 42]. The fetal demands can be altered by litter size with additional fetuses creating additional fetal demands [21].

The regular production of multiples is unique to the callitrichines, with the exception of the Goeldi's monkey [32, 34]. Gestating multiples is the result of the breeding female ovulating multiple ova during each cycle, therefore, marmoset litters are multizygotic, resulting from fertilization of multiple ova, rather than monozygotic [34, 35]. The determinants of litter size in marmosets are not entirely known although there are genetic underpinnings to the gestation of multiples with *GDF9, BMP15, BMP4,* and *WFIKKN* likely playing a role in regulation of ovulation number. Yet, litter size is highly variable within marmoset mothers and suggests that the heritability is quite low [21, 32, 33].

Beyond the genetic underpinnings, ovulation number and litter size is associated with maternal energetic investment [35, 43]. There is a positive association between maternal body weight (a reflection of maternal investment capacity), and the number of ovulations in a given cycle [35]. Across marmoset mothers, it has been consistently demonstrated that larger mothers tend to have larger litters [35, 43]. Within individual mothers the number of ova that she ovulates per cycle, and thus the potential litter size she carries increases when she weighs more [35]. Therefore, the litter sizes that she carries throughout her reproductive lifespan can vary depending on her weight. The within-subject association of maternal weight to number of ovulations and litter size suggests that there are pre-conceptual maternal energetic influences that can mediate litter size [35]. Additionally, there have been increases in the average birth weight of triplets in association with the trend of increased maternal prepregnancy weight which demonstrates the ability of the marmoset intrauterine environment to reflect the pre-

conceptual resources available for maternal investment in gestating multiple offspring [25].

Outside of the relationship between maternal energetic status influencing ovulation number, it has also been established that maternal energetic status influences pregnancy success and birth outcomes. In a study of maternal caloric restriction (i.e. 75% *ad libitum* intake), very different birth outcomes were observed in relation to the timing of restriction which is consistent with epidemiological studies such as those from the Dutch Famine [44, 45]. Mid-pregnancy restriction resulted in spontaneous abortion in all cases (N=8). Late-pregnancy restriction resulted in term deliveries with normal size neonates in 4/7 cases, and preterm delivery in the remaining 3/7 cases. Preterm delivery cases were related to mothers who had lower prepregnancy weights or who lost more weight during restriction.

In non-experimental contexts, it's been demonstrated at SNPRC and other colonies that when marmoset mothers are pregnant, they do not increase their energy intake in comparison to when they are not pregnant [46]. While there are genetic and evolutionary adaptations that allow marmosets to gestate multiples, the amount of energy and nutrients that the mother is able to invest in a pregnancy does not increase in relation to the number of offspring she is carrying. In comparing twin to triplet pregnancies, this results in a maternal mass to fetal mass ratio that is reduced by 24.9% in triplet pregnancies [47]. Additional fetuses create an intrauterine environment of high fetal demand with limited maternal energetic resources—an environment with major implications for developmental programming. The low maternal energetic supply and high fetal demand of triplet litters represents a model of an "undernourished" or

restricted intrauterine environment, while twin pregnancies represent a non-restricted intrauterine environment.

In alignment with developmental programming outcomes, birth weights of twin and triplet marmosets reflect their very different intrauterine environments: triplets are born at significantly lower weights than twins. In a captive colony of marmosets, this inverse relationship between birth weight and litter size was initially reported in 2004 [43]. Among male and female newborns, the average birth weight for twins was 30.24 grams, and triplets demonstrated a lower average birth weight of 27.73 grams [45]. In a more recent study, the mean birth weight of twin females was significantly higher than the mean birth weight of triplet females, 31.17 grams and 28.72 grams, respectively (P=0.002) [26]. It is suggested that regular demonstration of an inverse relationship between litter size and birth weight is likely a “reflection of intrauterine competition for resources accessed through the shared, bidiscoid placenta, and maternal resources (as reflected by maternal size) apparently cannot compensate for this higher demand,” [45]. The weight differences between individual fetuses of triplet and twin litters tends to be evident by day 120 of the 143-day gestation period (Figure 1) [47]. This suggests that the associated intrauterine alterations may also be detectable prior to birth. With the associations between maternal energetic status, litter size, and birth outcomes, marmosets present a natural experiment for exploring the intrauterine mechanisms involved in developmental programming [25].

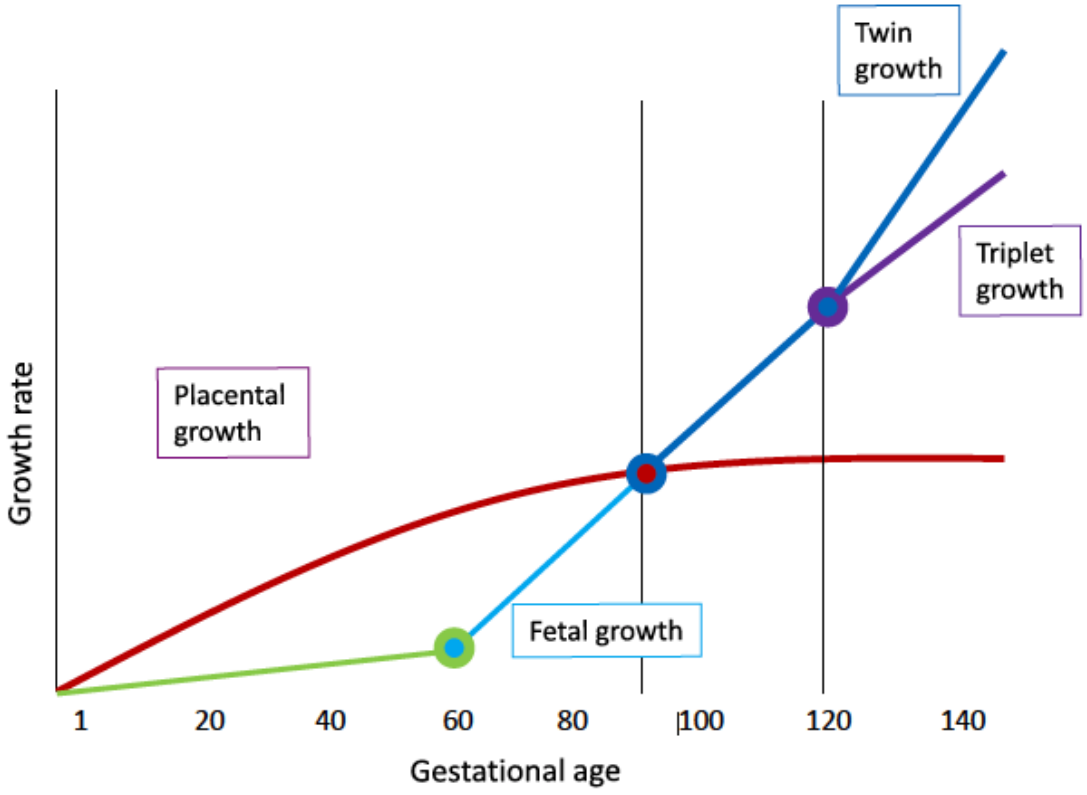

Figure 1: Placental and fetal growth rates across gestation (143 days)

2.4 Placental phenotypes reflect conditions of the intrauterine environment

Placental phenotypes, which are comprised of placental structural and functional measures, are robust indicators of the intrauterine environment that can reflect placental strategies taken to mediate maternal and fetal signals [24, 42]. In a study of 26 full-term pregnancies, Rutherford et al. demonstrated that placentas of triplets are relatively smaller than placentas of twins, which means that throughout triplet pregnancies a smaller placental mass is supporting a larger total fetal mass [28]. A reduction in the allocation of placental mass from which to access maternal resources in

triplet pregnancies may be the limiter of fetal growth that results in lower birth weights and reinforces the model of a restricted intrauterine environment in larger litter sizes [41]. Stereological analyses of the placental microstructure has shown that the surface area of the maternal-fetal interface is 40% larger in the triplet placenta than in the twin placenta. This increase demonstrates a placental compensatory strategy to maintain adequate surface area for nutrient transport to occur [48]. Although the total surface area is increased, the surface area is decreased by 25% per fetus in triplet compared to twin pregnancies. The placenta compensates through increasing surface area of the maternal-fetal interface, but only to an extent and the continued increase in surface area that would fully compensate for the increased litter size is not attained. In addition to significant differences in absolute and relative maternal-fetal surface area, significant differences have also been shown in placental efficiency and insulin-like growth factor concentrations per fetus [21].

These differences in placental phenotypes between twin and triplet pregnancies not only reflect the very different intrauterine environments, but also suggest potential mechanisms by which the placenta negotiates maternal supply and fetal demand with varying litter sizes. The underlying mechanisms which allow and/or limit the structural compensatory changes in the marmoset placenta are still unknown but likely have implications for birth outcomes and developmental programming in marmosets. Currently, placental phenotypes have not been explored in the longitudinal context that would allow for the associations between placental phenotypes and developmental programming outcomes of obesity to be determined.

2.5 Early life emergence of the obese phenotype and evidence of development programming in the marmoset monkey

A primary advantage of the marmoset as a model for developmental programming of obesity is the early life emergence of the obese phenotype. Using a definition of obesity as relative body fat greater than 14% at one year of age, researchers found that body mass differences between individuals who became obese and those who did not were present at 6 months of age [25, 29]. Significant differences in percent body fat between obese and non-obese individuals were present by 1 month of age and those individuals destined to become obese showed a distinctly different pattern of fat growth. Percent body fat increased between 1 and 12 months in those who became obese and decreased in those who did not become obese.

Using a weight-based definition of obesity (>90th percentile at 17 months of age), Tardif and colleagues detected significantly different weights as early as 1 month of age with differences that persisted at 2, 4, 6, and 24 months [25, 30]. The subjects classified as obese in the weight-based study weighed an average of 514 grams as adults while subjects classified as obese in the body fat percent study weighed an average of 461 grams. This comparison combined with the results of the weight-based study suggest that there may be a “super-obese” subset that demonstrate stronger associations between early life body mass and the development of obesity in adulthood.

Tardif & Bales examined patterns of early and late postnatal growth rates in relation to birth weight and maternal conditions [43]. During the first five months of life, marmosets demonstrated a linear early growth rate that averaged 1.15 grams/day. The growth rate during this time appears to relatively inflexible, and the small variation that

did exist was related to birth weight and maternal size. Early growth rate was related to birth weight in that lower birth weight was associated with a slower initial weight gain. Yet, this trend was less obvious when low birth weight infants were born to mothers with low body weight. The effect of maternal size is not surprising given that the early growth period occurs while infants are nursing and their growth is dependent on maternal resources. Indeed, studies in marmoset lactation found that smaller mothers rearing twins had less milk fat and less milk output than larger mothers [49].

One of the most lucrative findings for developmental programming of pediatric obesity in marmosets comes from observations in later growth rates. In the study by Tardif & Bales, the average later growth rate, between 5 and 24 months, was 0.83 grams/day [43]. During this later growth period, there was an interactive effect of birth weight and litter size. In twins, there was a positive association such that low birth weight twins exhibit slower later growth rates. Hence, the slower early growth rates of low birth weight twins extend into the later growth period, such that these individuals go on to be lower weight adults. Yet, low birth weight triplets exhibit a faster later growth rate. Overall, low birth weight triplets exhibit the highest rates of body mass gain during this period. This is a demonstration of “centile crossing,” which is characterized as low birth weight followed by rapid catch-up growth in infancy [9, 50]. As mentioned, centile crossing is a highly evocative phenotype in developmental programming with human and animal studies demonstrating that centile crossing is significantly associated with pediatric obesity [16-19, 21]. Epidemiological studies have also linked centile crossing to other metabolic disorders such as diabetes and hypertension, offering potential expansion of the use of the marmoset monkey as a model for developmental

programming [8, 15, 16]. These studies demonstrate that similarly to humans, the U-shaped association between birth weight and obesity is present in marmoset monkeys with high birth weight marmosets more likely to be obese and low birth weight triplets demonstrating centile-crossing and obesity development later in life.

Factors to consider in the postnatal growth patterns that lead to obesity in marmosets are diet and maternal size. In a study by Power et al, these two factors demonstrated independent effects [29]. In early life, high-fat diet exposure led to an increased relative rate of fat accumulation and at 6 months, 58% of subjects exposed to the high fat diet were classified as obese (>14% body fat). Yet, by 12 months four subjects had crossed over the normal-weight group to the obese group and the effects of diet were no longer evident. The subjects that crossed over from the normal weight group to the obese group were all the offspring of relatively large mothers (i.e. 12.9%-17% body fat). High-fat diet exposure is not necessary for marmosets to become obese. With standard diet intake, individuals can develop obesity over time, and the risk of obesity is associated with being the offspring of a relatively large mother. Maternal size is of particular interest given the discussed relationship between maternal weight and litter size and suggests that developmental programming mechanisms contribute to an individual's response to diet and propensity toward obesity. Combined, the birth outcomes that reflect the intrauterine environment, demonstration of centile-crossing, and the early life occurrence of obesity that can be altered by maternal condition and high-fat diet exposure position the marmoset for further expansion as a model for developmental programming of pediatric obesity.

2.6 Conclusion and future directions

Attending to the alarming rates of childhood obesity will require innovative and novel research strategies that offer avenues for preventative and therapeutic interventions. Applying our increasing knowledge of developmental programming to the pediatric obesity epidemic is likely to produce mechanistic insights about the intrauterine processes at play with the potential to initiate preventative measures at the earliest stages of life. Inclusion of the placenta in developmental programming studies offers a biological tissue to garner insights about the intrauterine mechanisms and a target tissue for implementing interventions [24].

The foundation for a powerful model of developmental programming of obesity has been laid in the common marmoset monkey presenting a worthy model for investigating the intrauterine processes at play. The natural occurrence of variable litter size in marmosets presents the unique opportunity for exploring prenatal and placental mechanisms of obesity development. The marmoset placenta has been extensively characterized in relation to litter size, and the demonstrated associations between litter size and obesity outcomes suggests that the placenta plays a critical role in developmental programming of obesity. Elucidation of placental phenotypes that are related to obesity development has the potential to reveal the structural and functional strategies the placenta takes in negotiating maternal supply and fetal demands that may have consequences for developmental programming and obesity later in life. Given the well-established association between litter size and postnatal growth patterns that lead to obesity, the marmoset is poised for longitudinal examination of the maternal and placental contributions to these outcomes.

Beyond placental phenotypes, the recent mapping of the marmoset genome now provides the opportunity to explore the genomic and epigenomic underpinnings of these phenotypes. A robust opportunity that our group is currently exploring in the marmoset is how placental epigenetic profiles relate to maternal, placental and fetal phenotypes. Human and rodent studies have demonstrated alterations in the epigenetic profiles of several gene loci including, *LEP ABCA1, Igf2r and Dlk1*, in relation to maternal energetic status (e.g. maternal obesity, gestational weight gain, diabetes, high-fat diet, etc) [51-54]. It is likely that placental epigenetic profiles differ in marmosets depending on maternal energetic status and that placental epigenetics influence the obesity outcomes that are seen in relation to litter size and maternal condition. Programming of metabolic syndrome in response to antenatal glucocorticoid excess was recently explored in the marmoset by Nyirenda and colleagues [55]. This study demonstrated that antenatal glucocorticoid exposure caused persistent increases in 11β-HSD1 gene expression in adipose tissue of offspring, and suggests a novel mechanism for developmental programming in response to antenatal glucocorticoid exposure. Further exploration of omics in the marmoset is a promising path towards elucidating gene pathways that can be targeted for intervention and treatment strategies. The early life emergence of obesity combined with the fast life history of the marmoset provides an efficient and economical model for implementing intervention and treatment strategies in a model that will allow for the immediate, short-term, long-term and intergenerational effects to be explored in a feasible timeframe.

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3. DNA Methylation in the Marmoset Monkey: Maternal weight affects genes involved in metabolic pathways

As the physical interface between mother and fetus during pregnancy, the placenta is vital for the coexistence of these two individuals. For the mother, the metabolic and endocrine actions of the placenta enable her to maintain pregnancy and prepare her for post-pregnancy events such as milk production and caretaking [1]. For the fetus, the placenta orchestrates all the events of fetal development through mediating nutrient uptake, waste elimination and gas exchange [2]. As the biological bridge between mother and fetus, the placenta is not only important for the health of the mother and fetus during pregnancy, but also impacts the lifelong health of the fetus through developmental programming. Indeed, robust findings from foundational and contemporary studies that support the role of the placenta in the developmental origins of health and disease, have led to our understanding that there are placental origins of health and disease [3, 4].

Maternal metabolic health during pregnancy has been linked to developmental programming of obesity, diabetes, insulin resistance, and metabolic syndrome [5-9]. Although the mechanisms underlying developmental programming of metabolic disorders by altered maternal metabolic health are not entirely elucidated, accumulating evidence suggests that placental epigenetic dysregulation is involved [10, 11]. For example, maternal obesity is associated with globally higher levels of DNA methylation (DNAm) in the placenta than in placentas from non-obese pregnancies [12], and maternal glucose concentrations are associated with alterations in placental DNAm of *LEP* [10, 13-15]*, ADIPOQ [16],* and *ABCA1[17]* genes. As the placenta and fetus are

derived from the same genetic material, epigenetic dysregulation in the placental likely influences epigenetic dysregulation and developmental programming in the fetus.

While studying human populations is ideal, animal models offer many advantages in exploring the role of placental DNAm in developmental programming research. In addition to providing the longitudinal context necessary for studying long-term and intergenerational outcomes of developmental programming due to their short lifespans, animal models also provide a high degree of genetic and environmental control. To date, rodents are the most prevalent animal models in placental and developmental programming research [18].While rodents are time and cost efficient models that undoubtedly contribute to advancing science, they lack the biological relevance necessary for translating findings to human populations.

An emerging and biologically relevant nonhuman primate model in placental and developmental programming research is the common marmoset monkey (*Callithrix jacchus).* Marmosets exhibit reproductive biology and hemochorial placental characteristics that are fundamentally similar to humans but ideal for research studies due to their small body size (283-500 grams on average), quick sexual maturity (~15 months), frequent reproduction (~2 times/year), short gestation (143 days), and a lifespan of 8-10 years in captivity [19, 20]. At the Southwest National Primate Center, research has shown that marmosets exhibit metabolic and reproductive developmental programming outcomes [21-24]. Additionally, the marmoset provides a naturally occurring continuum of maternal metabolic states for which to explore placental and developmental programming effects. An attractive aspect of the marmoset is that, much like humans, maternal metabolic health is related to developmental programming

outcomes. Typically, heavier marmoset mothers carry larger litters and infants of larger litters are more likely to exhibit developmental programming outcomes such as obesity and decreased reproductive success [21-23]. Extensive characterization of placental phenotypes in marmosets by Rutherford and colleagues [22, 25-30] has provided convergent evidence for the role of the placenta in developmental programming in the marmoset. As the first New World Monkey to have its genome sequenced [31], there are extensive opportunities for genetic and epigenetic mechanism within the marmoset placenta to be explored. Yet, placental DNAm has yet to be explored in this highly valuable model of primate reproduction and developmental programming.

As a critical step towards understanding the utility of the marmoset as model for exploring placental DNAm in developmental programming, the initial aim of this study was to characterize genome-wide DNAm in the marmoset placenta for the first time. Given the known associations between maternal metabolic status and developmental programming outcomes in humans and the marmoset monkey, this study also aimed to identify genes and gene pathways that are affected by maternal weight during gestation.

3.1 Materials and methods

3.1.1 Subjects

This study was conducted using data and placental tissue from 10 adult female marmoset monkeys (five from each colony) that were enrolled in an ongoing project assessing pregnancy and pregnancy outcomes. The animals originated from two breeding colonies: The Southwest National Primate Research Center (SNPRC) in San Antonio, TX and the Barshop Institute for Longevity and Aging Studies at University of Texas Health Science Center at San Antonio. Between 2012 and 2015, each marmoset

female reproduced 1-2 times per year resulting in data and placental tissue available from a total of 17 pregnancies (10 pregnancies from mothers at The Barshop Institute, and 7 pregnancies from the mothers at SNPRC). All animals were housed in typical marmoset family groups consisting of one breeding female, one breeding male and their offspring up to 2-4 years of age. Prior, during and following pregnancy, dams were fed a commercial marmoset diet in ad lib quantities. All animal procedures, husbandry, and housing were reviewed and approved by Institutional Animal Care and Use Committees at the Southwest National Primate Research Center and the University of Texas Health Science Center at San Antonio.

3.1.2 Maternal weight

Upon detection of pregnancy via ultrasound, estimated gestational age was determined using previously established growth curves [32]. Body weight was collected from the female marmosets within one week of estimated gestational days 60, 90 and 120 (gestation=143 days). Weights were taken in the females' home cage by introducing a digital scale for her to stand on. All scales provided weights to the nearest gram and were calibrated every 6 months.

3.1.3 Infant birth outcomes

Infant birth outcome data was collected as described in Tardif et al. [33]. Briefly, at birth, litter size and sex of the littermates were recorded. Birth weights of each littermate were taken within 24 hours of birth using digital scales that provided weight in grams to the nearest hundredth with. The sum of individual littermate weights was used as the total litter weight. Average infant weight was calculated as the total litter weight divided by the litter size.

3.1.4 Placental collection and processing

Delivery was not induced in this sample and gestational age and delivery dates were estimated based on growth curves as done in previous work [21, 22, 28, 29, 32]. This combined with nocturnal delivery makes opportunistic placental collection a challenge, However, due to the stable nature of DNA methylation, both fresh (<1 hour after delivery) and found (1-12 hours after delivery) placentas were included in the study if the placenta was completely intact. The time of collection was recorded and gross appearance of maternal and fetal surfaces, membranes, and cords was described. Placentas were cleaned by gently dabbing away blood with gauze and then untrimmed and trimmed weights and volumes were taken. Using a 6mm biopsy punch one central and one peripheral tissue sample was taken from each placenta yielding 2 samples per placenta (N=34). The tissue samples were placed in RNA-free tubes with 5mL of RNAlater and stored at -80 degrees until further processing.

3.1.5 Placental DNA purification and quantification

After rinsing the RNAlater preserved samples with sterile water, 7-25mg of placental tissue was obtained for DNA purification. Total DNA purification was performed using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). All reagents and equipment in the manufacturer's protocol were followed with the addition of RNase A digestion to yield RNA-free genomic DNA. Genomic DNA was quantified using two dsDNA assay methods: Quant-iT Picogreen (Invitrogen, Carlsbad, CA) and Qubit System (Thermo Fischer Scientific, Waltham, MA). Quality of the DNA was assessed through gel electrophoresis.

3.1.6 DNA methylation libraries, sequencing and alignment

Using 100ng of genomic DNA input, reduced representation bisulfite sequencing (RRBS) was performed by the University of Illinois Genomic Core using Ovation RRBS Methyl-Seq System 1-16 (NuGEN Technologies, San Carlos, CA). The 34 samples were randomly assigned to 5 lanes and the samples were run in 5 pools, 4 lanes with 7 samples and 1 lane with 6 samples. RRBS uses MspI which is a methylation insensitive restriction enzyme that recognizes CCGG sites. As MspI is insensitive to methylation status and CCGG sites are more common in CpG islands and promoter regions, all CCGG sites were cut between the two C's resulting in small fragments with a high frequency of potential CpG methylation sites. Following MspI digestion, the fragments underwent adaptor ligation, final repair, bisulfite conversion, and PCR amplification to produce the final libraries for sequencing. The 5 pools were sequenced twice on 5 lanes in the HiSeq 4000 (Illumina, San Diego, CA) with paired-end reads 100nt in length. Before alignment to the marmoset genome, trimming was done to remove low-quality bases and adaptor sequences, and to remove the sequence diversity provided by the NuGEN RRBS adaptor. The trimmed bisulfite sequence was then aligned to the marmoset genome, PCR duplication artifacts were removed, and methylation status was determined using the pileometh package in R.

3.1.7 Statistical Analyses

All analyses were conducted using R (version 3.3.2) and RStudio (version 1.0.136) with packages available through CRAN and Bioconductor.

Each placenta was treated independently given that previous work has demonstrated that within subject variability in placental phenotypic characteristics from pregnancy to pregnancy.

The RRBS data was aligned to the marmoset genome (C_jacchus 3.2.1, version 85) obtained from Ensembl. Although methylation can occur and be detected at non-CpG sites (i.e. CpHpG and CpHpH) through RRBS, only CpG sites were evaluated in this study. Using the count data, beta-values and M-values at each CpG site were calculated using the following equations: β = methylated counts / (methylated counts + unmethylated counts); M = $\beta/(1-\beta)$ [34]. For statistical analyses, the M-values were used to promote normality and reduce heteroscedasticity of the data and allow for better alignment with statistical assumptions [34, 35]. Correlations were assessed using Pearson's pairwise correlation tests. Figure 1 demonstrates the analysis work flow. In CpG sites with variance >2, The association between methylation and maternal weight was assessed through three simple regression models, one for maternal weight at each time point (gestational day 60, 90, and 120) with methylation as the outcome and maternal weight as the single predictor. Following regression, a false discovery rate (FDR) < 0.05 was applied using the Benjamini-Hochberg method [36] and CpG sites that were significantly related to maternal weight at any time point were used to create a gene list for gene ontology analysis. Gene ontology analysis helps to add structure to the large amount of data that was obtained by RRBS and allows for determining whether genes significantly associated with maternal weight belong to functionally related biological networks more than by chance alone. Semi-automated enrichment analysis for gene ontology categories was performed using the topGO package in R

[37]. This method compares a predefined list of genes (in this case, genes significantly associated with maternal weight) to a reference list of genes (i.e. all the genes that were assessed in the regression models). A *P* value based on Fisher's exact test is provided to identify gene ontology categories that are over-represented in the predefined list of genes. Again, adjustment for multiple comparisons was done using the Benjamini-Hochberg procedure with a FDR < 0.05.

3.2 Results

3.2.1 Sample descriptive statistics

The maternal, placental, and infant characteristics are described in Table 1. The seventeen pregnancies resulted in litters ranging in size from two to five, with triplets being the most frequent (n=8). Total litter weight increased with respect to litter size and average infant weight for the sample was 29.64 grams. The sample demonstrated a wide range in maternal weight at each time point and as expected, mean maternal weight increased throughout gestation. Of note, when the sample was analyzed as a whole, maternal weights were not significantly correlated with infant birth outcomes (i.e. litter size, total litter weight, individual infant weight). However, quintuplet pregnancies are a rare occurrence and when the one quintuplet pregnancy that occurred in this sample was removed from analyses, maternal weight significantly correlated with litter weight at each gestational time point (R=0.44 at 60 days, R=0.58 at 90 days, R=0.64 at 120 days, $p<0.05$), as well as with litter size at gestational day 120 (R=0.39, $p<0.05$). There was a strong positive correlation between maternal birth weight and the average birth weight of her offspring (R=0.69, $p<0.01$). There were also strong positive correlations between maternal weight at each time point and placental weight (R=0.804

at 60 days, R=0.897 at 90 days, R=0.858 at 120 days, p <0.01) as well as placental volume (R=0.844 at 60 days, R=0.893 at 90 days, R=0.828 at 120 days, p <0.01).

TABLE I

MATERNAL, PLACENTAL AND INFANT CHARACTERISTICS

	N	Mean ± SD	Range
Maternal weight (g)			
60-day weight	17	440.47 ± 89.09	318.2 - 614.0
90-day weight	17	464.56 ± 93.93	360.2 - 656.0
120-day weight	17	507.61 ± 88.90	412.0 - 702.0
Placental outcomes			
Placental weight (g)	17	8.66 ± 2.56	6.2 - 14.9
Placental volume (mL)	17	8.82 ± 3.38	5.0 - 17.0
Infant outcomes (g)			
Total litter weight	17	83.04 ± 26.81	47.4 - 134.4
Average infant weight	17	29.64 ± 6.25	15.8 44.8
Infant weight (g) by litter size			
Twin	4	32.02 ± 2.15	28.95 – 34.00
Triplet	8	26.69 ± 5.93	15. 80 – 36.13
Quadruplet	4	29.41 ± 2.59	26.59 – 32.50
Quintuplet	1	44.80	-

3.2.2 Placental DNA methylation analyses

DNAm was assessed through RRBS on two placental samples per pregnancy, yielding a total of 34 placental samples. An advantage of RRBS and methylation sequencing is that it directly counts the number of methylated and unmethylated cytosines which offers more precision and accuracy than signal intensity of array based methods [38]. Figure 2 provides an overview of the analysis workflow.

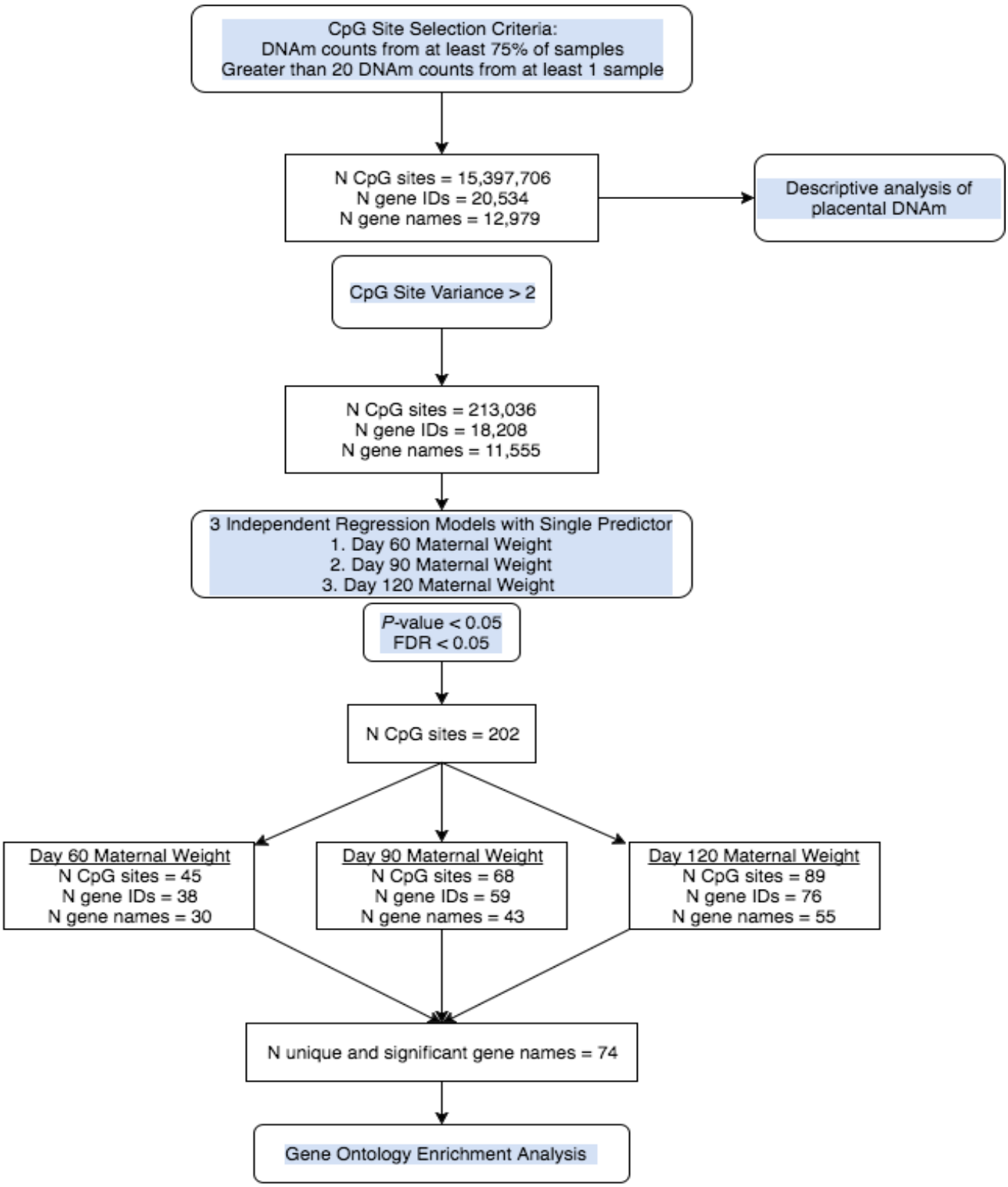

Figure 2. Workflow of the analytical strategy. This strategy was used to describe DNAm in the marmoset placenta and to identify genes and gene pathways that are associated with maternal weight during gestation.

For inclusion in analyses, a CpG site had to have counts from at least 75% of the samples and at least one sample had to have greater than 20 counts at that site. A total of 15,397,706 CpG sites met these criteria which corresponded to 18,208 marmoset gene IDs and 11,555 unique gene names. Table II provides summary statistics for the sequencing reads obtained per CpG site and per sample. The distribution of DNAm across all CpG sites is shown in Figure 3.A. An M-value of -7 represents completely unmethylated CpG sites, an M-value of 7 represents completely methylated CpG sites, and an M-value of zero represents hemi-methylated CpG sites (i.e. CpG sites with an equal number of sequencing reads that were methylated and unmethylated). The sequencing read data and distribution of DNAm demonstrate a slight negative skew with a higher proportion demonstrating M-values greater than zero. As demonstrated in Figure 3.B, 65% of CpG sites had an M-value greater than zero with 12% of CpG sites demonstrating complete methylation.

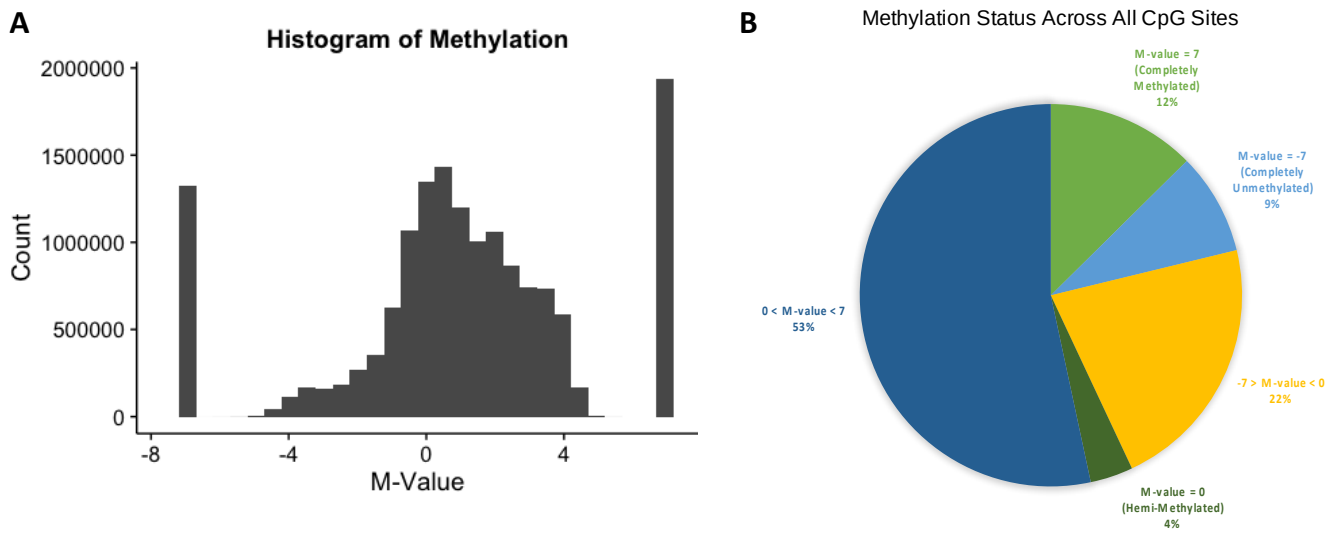

Figure 3. Distribution of methylation across all CpG sites. A) Histogram of M-values. M=-7 indicates completely unmethylated sites, M=0 indicates hemi-methylated sites, and M=7 indicates completely methylated sites. B) Frequency of methylation subdivided by M-values.

TABLE II

SEQUENCING READS OBTAINED BY WHOLE-GENOME RRBS

	N	Mean	Range
Per CpG site (N=15,397,706)			
Total reads	229,080,095	14.9	1-484
Methylated reads	141,710,362	9.2	0-433
Unmethylated reads	87,369,733	5.7	0-363
Per sample (N=34)			
Total reads	-	6,737,649.9	6,272,810-7,333,074
Methylated reads	-	4,167,951.8	3,941,390-4,444,733
Unmethylated reads	-	2,569,698.0	2,303,802-2,889,340

DNAm within gene regions was calculated using the reads that were aligned within ± 3 kb region of the transcription start site (TSS) (i.e. the promoter region). The general pattern observed was that DNAm decreased in the upstream region as it approached the TSS, DNAm was lowest at the TSS, and increased as the downstream distance from the TSS increased. In this study, 0.06% of CpG sites captured were located at a TSS and 32.97% were within the promoter region.

There are established patterns of completely methylated and completely unmethylated CpG sites in the promoter region of somatic and germ-cell tissues in vertebrate species and the global hypomethylation that occurs in the human placenta is not uniform across the genome [39, 40]. Therefore, completely methylated and completely unmethylated CpGs were compared within (< 3 kb) and outside (≥ 3 kb) the promoter region to explore any patterns in the occurrence of completely methylated and completely unmethylated CpG sites in the marmoset placenta. Figure 4 demonstrates a stark difference in the location of completely methylated and completely unmethylated CpGs with a high occurrence of completely unmethylated CpGs within the promoter region and a high occurrence of completely methylated CpGs outside the promoter region.

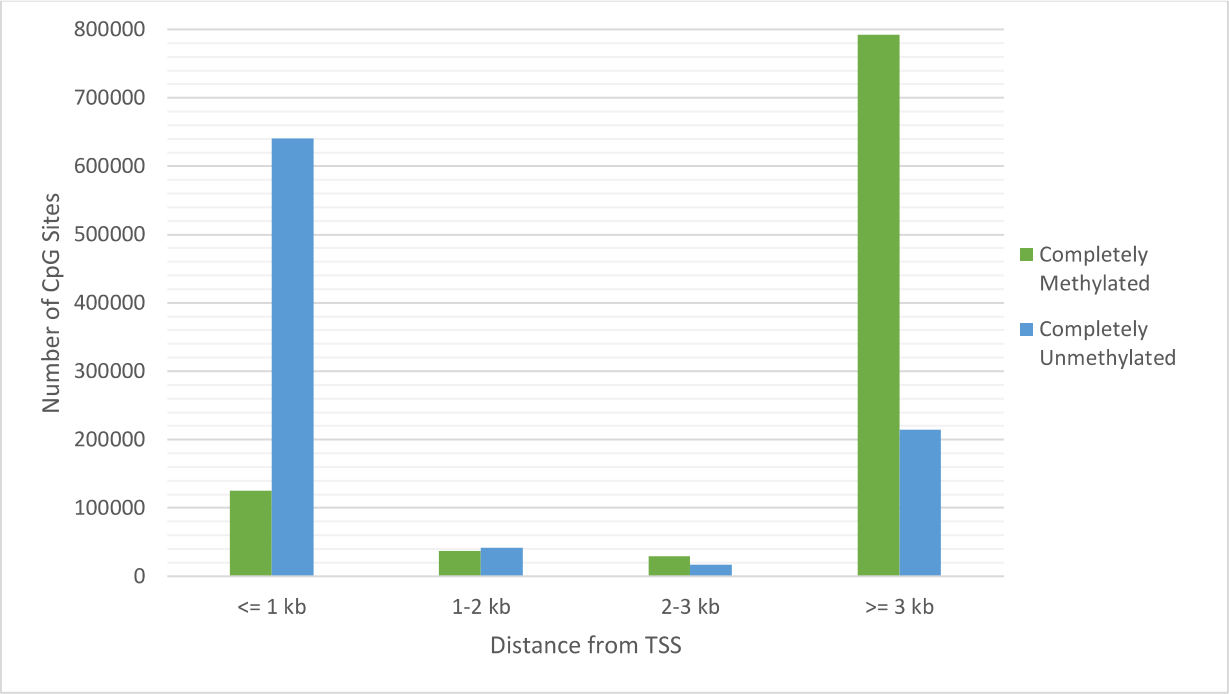

Figure 4. Comparison of completely methylated and completely unmethylated CpG sites at specific distances from the transcription start site. Distance from the TSS of <3kb is within the promoter region, while distances >3kb from the TSS are outside the promoter.

3.2.3 Placental DNAm and maternal weight

Figure 2 displays the workflow of these analyses. The association between maternal weight and placental DNAm, was assessed by three separate regression models with single predictors: maternal weight at (1) gestational day 60; (2) gestational day 90; and (3) gestational day 120. CpG sites with variance less than two were excluded from regression analyses, resulting in 213,036 CpG sites that were evaluated in each of the regression models. P-values were adjusted to account for multiple comparisons by using the Benjamini-Hochberg method [36] with a false discovery rate

(FDR) of 0.05. The number of significant CpG sites ($p<0.05$, FDR 0.05), unique gene identifiers, and unique gene names for each predictor are shown in Figure 2 and Tables III-V provides the gene list for each predictor. The unique gene names that were significantly associated with DNAm for each predictor were combined and de-duplicated to create a gene list for gene ontology analysis Table VI. The predefined gene list contained 74 genes and was compared to the reference gene list of 11,555 genes. Fisher's exact test was used for comparison, however after controlling for FDR <0.05 none of the gene ontology categories remained significant. Table VII lists the top 20 gene ontology categories that were enriched in relation to maternal weight. The top four categories were *regulation of glycolytic processes* (GO:0006110, 26 annotated genes), *carnitine shuttle* (GO:0006853, 6 annotated genes), *regulation of carbohydrate catabolic processes* (GO:0043470, 31 annotated genes), and *regulation of lipid metabolic processes* (GO:0019216, 188 annotated genes).

TABLE III

45 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIONAL DAY 60[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
10	16507933	NA	ENSCJAG00000032743	8.21E-09	0.00075
5	135692805	5S_rRNA	ENSCJAG00000024555	2.04E-08	0.00163
9	13850661	CACNA1C	ENSCJAG00000000732	6.67E-08	0.00251
1	177324707	NA	ENSCJAG00000017966	1.01E-07	0.00287
1	207684919	NA	ENSCJAG00000018966	1.03E-07	0.00287
4	1639040	NA	ENSCJAG00000022438	1.26E-07	0.00310
1	177313750	NA	ENSCJAG00000017966	2.16E-07	0.00446
3	183761230	CRMP1	ENSCJAG00000010648	2.37E-07	0.00470
3	183761811	CRMP1	ENSCJAG00000010648	3.69E-07	0.00553
18	2316759	PRKAB2	ENSCJAG00000002530	5.04E-07	0.00685
2	3048585	WBSCR17	ENSCJAG00000005178	5.70E-07	0.00739
22	12418791	CACNA1A	ENSCJAG00000004458	9.00E-07	0.01031
22	21451357	NA	ENSCJAG00000032315	1.03E-06	0.01112
1	195085254	FBXO7	ENSCJAG00000006238	1.17E-06	0.01200
6	155933814	NA	ENSCJAG00000034894	1.39E-06	0.01289
22	21451391	NA	ENSCJAG00000032315	1.71E-06	0.01542
1	141811277	ANKS6	ENSCJAG00000011142	1.75E-06	0.01557
16	71400669	WDYHV1	ENSCJAG00000000865	2.10E-06	0.01742
18	36565866	SCYL3	ENSCJAG00000010050	2.35E-06	0.01925
7	36083382	TMEM240	ENSCJAG00000037689	2.80E-06	0.02047
7	49305869	KAZN	ENSCJAG00000008920	2.82E-06	0.02047
18	2316773	PRKAB2	ENSCJAG00000002530	2.90E-06	0.02073
ACFV01190020.1	1340	NA	NA	3.11E-06	0.02162
7	37009749	TNFRSF14	ENSCJAG00000002555	3.48E-06	0.02304
1	177313680	NA	ENSCJAG00000017966	3.64E-06	0.02346
16	91386366	BAALC	ENSCJAG00000016141	4.31E-06	0.02462
1	177956403	VAV2	ENSCJAG00000010024	4.51E-06	0.02504
12	84494722	PGAM1	ENSCJAG00000016575	4.76E-06	0.02577
9	88988805	ANKS1B	ENSCJAG00000002273	5.60E-06	0.03003
6	157494660	CROCC2	ENSCJAG00000006365	5.64E-06	0.03003
20	11687025	ADGRG1	ENSCJAG00000011920	6.36E-06	0.03025
5	126698006	SEPT9	ENSCJAG00000013203	6.47E-06	0.03025
2	192181103	CTNND2	ENSCJAG00000009238	6.55E-06	0.03025
15	5509601	NA	ENSCJAG00000031315	7.06E-06	0.03155
3	67058583	U6	ENSCJAG00000027016	7.47E-06	0.03182
GL285864.1	261	NA	NA	8.05E-06	0.03317
2	202460667	Y_RNA	ENSCJAG00000028941	8.22E-06	0.03329
3	183762034	CRMP1	ENSCJAG00000010648	8.45E-06	0.03384
22	7374258	TRAPPC5	ENSCJAG00000036638	8.47E-06	0.03384
2	200569162	NA	ENSCJAG00000036280	9.41E-06	0.03577

TABLE III (continued)

45 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIONAL DAY 60[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
6	12987155	MCTP2	ENSCJAG00000005975	9.94E-06	0.03705
11	109603018	DGKZ	ENSCJAG00000011773	1.11E-05	0.03992
4	34401203	BAK1	ENSCJAG00000016047	1.15E-05	0.04090
13	10797937	EPHX2	ENSCJAG00000019677	1.39E-05	0.04601
GL285558.1	1187	snoU13	ENSCJAG00000036742	1.52E-05	0.04799

[a]Shown in descending order of P value with FDR <0.05. Alphanumeric chromosomes represent unassembled contigs.

TABLE IV

68 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIONAL DAY 90[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
4	1639040	NA	ENSCJAG00000022438	6.00E-10	0.00019
10	16507933	NA	ENSCJAG00000032743	1.88E-09	0.00024
1	207684919	NA	ENSCJAG00000018966	2.75E-08	0.00177
2	3048585	WBSCR17	ENSCJAG00000005178	3.04E-08	0.00177
1	177324707	NA	ENSCJAG00000017966	3.69E-08	0.00196
9	13850661	CACNA1C	ENSCJAG00000000732	4.12E-08	0.00202
1	177956403	VAV2	ENSCJAG00000010024	1.01E-07	0.00287
3	183761230	CRMP1	ENSCJAG00000010648	1.03E-07	0.00287
6	155933814	NA	ENSCJAG00000034894	1.20E-07	0.00310
6	155718341	HDAC4	ENSCJAG00000009423	1.45E-07	0.00330
1	177313750	NA	ENSCJAG00000017966	1.73E-07	0.00375
7	36083382	TMEM240	ENSCJAG00000037689	2.43E-07	0.00470
22	12418791	CACNA1A	ENSCJAG00000004458	2.69E-07	0.00479
1	177313680	NA	ENSCJAG00000017966	3.39E-07	0.00548
18	36565866	SCYL3	ENSCJAG00000010050	3.58E-07	0.00553
3	186729794	NA	ENSCJAG00000031790	4.93E-07	0.00685
13	10797937	EPHX2	ENSCJAG00000019677	5.78E-07	0.00739
5	135692805	5S_rRNA	ENSCJAG00000024555	6.00E-07	0.00752
2	192181103	CTNND2	ENSCJAG00000009238	9.04E-07	0.01031
6	157494660	CROCC2	ENSCJAG00000006365	1.02E-06	0.01112
2	3048584	WBSCR17	ENSCJAG00000005178	1.18E-06	0.01200
12	118225906	NA	ENSCJAG00000014400	1.20E-06	0.01200
1	181715957	EXD3	ENSCJAG00000013170	1.28E-06	0.01223
20	11687025	ADGRG1	ENSCJAG00000011920	1.99E-06	0.01713
22	988671	EFNA2	ENSCJAG00000005546	2.06E-06	0.01732
20	27662976	VAC14	ENSCJAG00000014847	2.52E-06	0.02015
16	5683907	Metazoa_SRP	ENSCJAG00000037430	2.57E-06	0.02018
GL285864.1	261	NA	NA	2.59E-06	0.02018
16	71400669	WDYHV1	ENSCJAG00000000865	2.92E-06	0.02073
6	152377322	NA	ENSCJAG00000004382	3.04E-06	0.02137
1	141811277	ANKS6	ENSCJAG00000011142	3.47E-06	0.02304
GL286251.1	47558	NA	ENSCJAG00000031964	3.50E-06	0.02304
13	15635804	NA	ENSCJAG00000020226	3.67E-06	0.02346
1	195085254	FBXO7	ENSCJAG00000006238	3.67E-06	0.02346
1	189497727	TPST2	ENSCJAG00000008612	3.97E-06	0.02453
4	159937586	NA	ENSCJAG00000022894	4.11E-06	0.02462
3	183762034	CRMP1	ENSCJAG00000010648	4.28E-06	0.02462
18	2316759	PRKAB2	ENSCJAG00000002530	4.46E-06	0.02504

TABLE IV (continued)

68 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIONAL DAY 90[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
12	15186696	TNFRSF17	ENSCJAG00000016037	4.63E-06	0.02553
22	5416191	HSD11B1L	ENSCJAG00000016948	6.24E-06	0.03025
19	24318939	SYT2	ENSCJAG00000006030	6.36E-06	0.03025
3	183761811	CRMP1	ENSCJAG00000010648	6.40E-06	0.03025
7	152246015	MAB21L3	ENSCJAG00000006194	6.51E-06	0.03025
22	1104834	DAZAP1	ENSCJAG00000005609	6.55E-06	0.03025
15	5271984	KLHL24	ENSCJAG00000021216	6.58E-06	0.03025
20	44359746	DEF8	ENSCJAG00000010490	7.12E-06	0.03162
8	95080471	PRKAG2	ENSCJAG00000020422	7.99E-06	0.03317
22	21451357	NA	ENSCJAG00000032315	8.54E-06	0.03392
1	198828190	NA	ENSCJAG00000022385	8.72E-06	0.03442
10	125596501	EML1	ENSCJAG00000006842	8.82E-06	0.03449
3	67058583	U6	ENSCJAG00000027016	9.00E-06	0.03486
9	21381896	NA	ENSCJAG00000025904	9.46E-06	0.03577
12	84494722	PGAM1	ENSCJAG00000016575	9.97E-06	0.03705
5	126698006	SEPT9	ENSCJAG00000013203	1.08E-05	0.03968
5	114123373	PTRF	ENSCJAG00000000406	1.10E-05	0.03988
Y	1988813	SCAPER	ENSCJAG00000010868	1.10E-05	0.03988
10	132055046	NA	ENSCJAG00000000003	1.11E-05	0.03992
16	91386366	BAALC	ENSCJAG00000016141	1.13E-05	0.04025
GL284703.1	79888	NA	NA	1.21E-05	0.04237
5	157776512	TSC22D1	ENSCJAG00000019808	1.24E-05	0.04319
1	177313674	NA	ENSCJAG00000017966	1.25E-05	0.04319
4	34401203	BAK1	ENSCJAG00000016047	1.25E-05	0.04319
ACFV01190020.1	1340	NA	NA	1.26E-05	0.04319
8	22818148	U6	ENSCJAG00000034393	1.39E-05	0.04601
2	57730252	PDGFA	ENSCJAG00000004948	1.40E-05	0.04601
17	61597034	GADL1	ENSCJAG00000004395	1.40E-05	0.04602
6	155718429	HDAC4	ENSCJAG00000009423	1.45E-05	0.04676
20	44133661	VPS9D1	ENSCJAG00000010650	1.50E-05	0.04799

[a]Shown in descending order of P value with FDR <0.05. Alphanumeric chromosomes represent unassembled contigs.

TABLE V

89 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIOINAL DAY 120[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
1	207684919	NA	ENSCJAG00000018966	2.57E-10	0.00016
7	36083382	TMEM240	ENSCJAG00000037689	9.78E-10	0.00020
4	1639040	NA	ENSCJAG00000022438	1.23E-09	0.00020
1	177324707	NA	ENSCJAG00000017966	7.87E-09	0.00075
10	16507933	NA	ENSCJAG00000032743	2.84E-08	0.00177
2	3048585	WBSCR17	ENSCJAG00000005178	4.57E-08	0.00202
1	177956403	VAV2	ENSCJAG00000010024	4.74E-08	0.00202
12	118225906	NA	ENSCJAG00000014400	6.52E-08	0.00251
22	12418791	CACNA1A	ENSCJAG00000004458	8.45E-08	0.00287
6	155718341	HDAC4	ENSCJAG00000009423	8.63E-08	0.00287
5	140947022	URAD	ENSCJAG00000019404	1.23E-07	0.00310
GL284914.1	52816	NA	NA	1.32E-07	0.00312
6	155933814	NA	ENSCJAG00000034894	1.76E-07	0.00375
9	13850661	CACNA1C	ENSCJAG00000000732	2.63E-07	0.00479
12	108358469	PPAPDC1A	ENSCJAG00000006402	2.70E-07	0.00479
16	5683907	Metazoa_SRP	ENSCJAG00000037430	2.78E-07	0.00481
4	34401203	BAK1	ENSCJAG00000016047	3.34E-07	0.00548
5	135692805	5S_rRNA	ENSCJAG00000024555	3.43E-07	0.00548
22	48099867	ZNF211	ENSCJAG00000000037	3.78E-07	0.00553
2	192181103	CTNND2	ENSCJAG00000009238	3.80E-07	0.00553
1	181715957	EXD3	ENSCJAG00000013170	4.03E-07	0.00572
13	10797937	EPHX2	ENSCJAG00000019677	5.40E-07	0.00720
7	49305869	KAZN	ENSCJAG00000008920	7.94E-07	0.00976
2	3048584	WBSCR17	ENSCJAG00000005178	8.34E-07	0.01005
3	186729794	NA	ENSCJAG00000031790	8.97E-07	0.01031
12	404895	DECR2	ENSCJAG00000011883	9.20E-07	0.01031
21	49728719	COL6A2	ENSCJAG00000001863	1.06E-06	0.01133
18	36565866	SCYL3	ENSCJAG00000010050	1.16E-06	0.01200
20	27662976	VAC14	ENSCJAG00000014847	1.25E-06	0.01223
19	24318939	SYT2	ENSCJAG00000006030	1.28E-06	0.01223
1	177313750	NA	ENSCJAG00000017966	1.37E-06	0.01289
1	177313680	NA	ENSCJAG00000017966	1.48E-06	0.01347
4	133634568	EYA4	ENSCJAG00000002817	1.97E-06	0.01713
1	177313674	NA	ENSCJAG00000017966	2.01E-06	0.01713
6	152377322	NA	ENSCJAG00000004382	2.40E-06	0.01941
22	988671	EFNA2	ENSCJAG00000005546	2.68E-06	0.02047
20	44359746	DEF8	ENSCJAG00000010490	2.70E-06	0.02047

TABLE V (continued)

89 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIOINAL DAY 120[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
11	130031608	RNH1	ENSCJAG00000012214	2.78E-06	0.02047
22	47093771	ZNF471	ENSCJAG00000020296	2.80E-06	0.02047
22	48494355	NA	ENSCJAG00000000381	3.16E-06	0.02169
1	195085254	FBXO7	ENSCJAG00000006238	3.37E-06	0.02290
4	159937586	NA	ENSCJAG00000022894	3.73E-06	0.02363
1	184797700	NA	ENSCJAG00000004879	3.93E-06	0.02453
15	5271984	KLHL24	ENSCJAG00000021216	3.99E-06	0.02453
5	135692806	5S_rRNA	ENSCJAG00000024555	4.15E-06	0.02462
1	141811277	ANKS6	ENSCJAG00000011142	4.23E-06	0.02462
13	15635804	NA	ENSCJAG00000020226	4.26E-06	0.02462
19	43294549	7SK	ENSCJAG00000032509	4.30E-06	0.02462
11	55289953	FAM181B	ENSCJAG00000014709	4.31E-06	0.02462
3	152412117	U6	ENSCJAG00000025583	4.48E-06	0.02504
16	71400669	WDYHV1	ENSCJAG00000000865	4.72E-06	0.02577
11	125324021	FGF3	ENSCJAG00000009730	5.79E-06	0.03025
4	163744391	NA	ENSCJAG00000012365	5.98E-06	0.03025
4	106452640	NA	ENSCJAG00000006178	6.05E-06	0.03025
1	177965978	VAV2	ENSCJAG00000010024	6.14E-06	0.03025
20	44133661	VPS9D1	ENSCJAG00000010650	6.17E-06	0.03025
16	12801023	CA8	ENSCJAG00000008976	6.26E-06	0.03025
22	21451357	NA	ENSCJAG00000032315	6.42E-06	0.03025
12	40118839	NA	ENSCJAG00000015397	6.43E-06	0.03025
15	65610998	FBLN2	ENSCJAG00000016873	6.48E-06	0.03025
5	114123373	PTRF	ENSCJAG00000000406	6.57E-06	0.03025
6	157494660	CROCC2	ENSCJAG00000006365	6.73E-06	0.03061
GL284914.1	50043	NA	NA	6.75E-06	0.03061
4	19855367	SLC35B3	ENSCJAG00000021320	7.06E-06	0.03155
5	157155159	NA	ENSCJAG00000017549	7.30E-06	0.03182
1	189497727	TPST2	ENSCJAG00000008612	7.40E-06	0.03182
7	54370823	VWA5B1	ENSCJAG00000006551	7.41E-06	0.03182
Y	1988813	SCAPER	ENSCJAG00000010868	7.49E-06	0.03182
GL285864.1	261	NA	NA	7.51E-06	0.03182
22	30047764	ZNF146	ENSCJAG00000009524	7.57E-06	0.03182
1	177788729	SARDH	ENSCJAG00000009864	7.57E-06	0.03182
22	5416191	HSD11B1L	ENSCJAG00000016948	7.78E-06	0.03249
ACFV01192733.1	1291	NA	NA	8.18E-06	0.03329
6	155718429	HDAC4	ENSCJAG00000009423	8.23E-06	0.03329
ACFV01192733.1	1207	NA	NA	8.85E-06	0.03449
4	30674268	NA	ENSCJAG00000020676	9.13E-06	0.03515

TABLE V (continued)

89 CpG SITES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT GESTATIOINAL DAY 120[a]

Chromosome	Start Position	Gene Name	Gene ID	*P* value	FDR
GL284900.1	12752	NA	NA	9.22E-06	0.03527
GL286232.1	66374	NA	ENSCJAG00000003723	9.95E-06	0.03705
6	46430125	DLX2	ENSCJAG00000007389	1.04E-05	0.03834
1	177385764	SURF6	ENSCJAG00000017951	1.16E-05	0.04109
12	15186696	TNFRSF17	ENSCJAG00000016037	1.27E-05	0.04327
11	113515486	CLP1	ENSCJAG00000008748	1.27E-05	0.04327
4	169752716	NA	ENSCJAG00000003511	1.29E-05	0.04366
11	123170411	GRK2	ENSCJAG00000001006	1.38E-05	0.04601
2	188068188	FBXL7	ENSCJAG00000010823	1.38E-05	0.04601
1	76580306	BARX1	ENSCJAG00000017399	1.42E-05	0.04634
6	158394771	PDCD1	ENSCJAG00000019795	1.44E-05	0.04668
ACFV01192733.1	1135	NA	NA	1.47E-05	0.04735
5	30031004	NA	ENSCJAG00000032266	1.52E-05	0.04799

[a]Shown in descending order of P value with FDR <0.05. Alphanumeric chromosomes represent unassembled contigs.

TABLE VI

SEVENTY-FOUR GENES SIGNIFICANTLY ASSOCIATED WITH MATERNAL WEIGHT AT ANY TIME POINT[a,b]

U6	FBXO7	KLHL24	ADGRG1	CRMP1	TMEM240
Y_RNA	EML1	FBLN2	VAC14	SLC35B3	TNFRSF14
7SK	FAM181B	CA8	VPS9D1	BAK1	KAZN
snoU13	DGKZ	WDYHV1	DEF8	EYA4	VWA5B1
5S_rRNA	CLP1	BAALC	COL6A2	PTRF	MAB21L3
Metazoa_SRP	GRK2	GADL1	EFNA2	SEPT9	PRKAG2
BARX1	FGF3	PRKAB2	DAZAP1	URAD	CACNA1C
ANKS6	RNH1	SCYL3	HSD11B1L	TSC22D1	ANKS1B
SURF6	DECR2	SYT2	TRAPPC5	MCTP2	SCAPER
SARDH	TNFRSF17	WBSCR17	CACNA1A	DLX2	
VAV2	PGAM1	PDGFA	ZNF146	HDAC4	
EXD3	PPAPDC1A	FBXL7	ZNF471	CROCC2	
TPST2	EPHX2	CTNND2	ZNF211	PDCD1	

[a]p<0.05 and FDR<0.05.
[b]Duplicates of genes that were significantly associated with maternal weight at more than one time point were removed and unassembled contigs were omitted.

TABLE VII

TOP 20 GENE ONTOLOGY THAT DEMONSTRATE ENRICHMENT BASED ON MATERNAL WEIGHT

GO.ID	GO Term	Annotated Genes	*P* value	FDR
GO:0006110	regulation of glycolytic process	26	0.00047	1
GO:0006853	carnitine shuttle	6	0.00051	1
GO:0043470	regulation of carbohydrate catabolic pro...	31	0.00079	1
GO:0019216	regulation of lipid metabolic process	188	0.00082	1
GO:0009118	regulation of nucleoside metabolic proce...	37	0.00133	1
GO:1903578	regulation of ATP metabolic process	37	0.00133	1
GO:0051193	regulation of cofactor metabolic process	43	0.00206	1
GO:0051196	regulation of coenzyme metabolic process	43	0.00206	1
GO:0006096	glycolytic process	46	0.00251	1
GO:0006757	ATP generation from ADP	46	0.00251	1
GO:0042451	purine nucleoside biosynthetic process	47	0.00267	1
GO:0046129	purine ribonucleoside biosynthetic proce...	47	0.00267	1
GO:0046031	ADP metabolic process	51	0.00337	1
GO:0010882	regulation of cardiac muscle contraction...	15	0.00345	1
GO:0046128	purine ribonucleoside metabolic process	180	0.00416	1
GO:0030258	lipid modification	181	0.00426	1
GO:0042278	purine nucleoside metabolic process	182	0.00436	1
GO:0006165	nucleoside diphosphate phosphorylation	56	0.00439	1
GO:0046034	ATP metabolic process	114	0.00458	1
GO:0046939	nucleotide phosphorylation	58	0.00484	1

3.3 Discussion

To the best of our knowledge, this is the first study to report genome-wide placental DNAm in the common marmoset monkey. The primary goal was to characterize DNAm in the marmoset placenta. Importantly, these results were obtained in an animal model with a high degree of biological salience for translational research. The marmoset is emerging as a prime model for exploring the developmental origins of health and disease, specifically obesity [23, 24, 41] and reproductive health outcomes [21, 26]. Placental phenotypes have been extensively characterized in the marmoset [21, 22, 25, 26, 28, 30] which demonstrates the role of the placenta as a key contributor to developmental programming outcomes and suggests that underlying epigenetic mechanisms may be at play. As such, the findings from the primary aim of this study establishing DNAm patterns in the marmoset placenta provide a valuable step in developing the marmoset monkey as model of investigating the role of placental epigenetics in developmental programming. Much like humans, developmental programming outcomes in the marmoset are associated with maternal metabolic status. Generally, marmoset mothers with higher pre-pregnancy weight carry larger litters, and offspring of larger litters are more likely to develop obesity and reproductive health complications later in life [21, 24, 28]. Based on this, the secondary aim of this study was to begin to understand the impact of maternal metabolic status on placental DNAm at the epigenome-wide level by identifying genes and gene pathways that are affected by maternal weight during gestation. One of the principal findings of this aim was that maternal weight is associated with DNAm in genes that are predominantly involved in

energy metabolism and homeostasis such as the *regulation of glycolytic processes* (GO:0006110), and the *regulation of lipid metabolic processes pathways* (GO:0019216). Combined, the findings of this study establish the marmoset as a model worthy of exploring placental epigenetic contributions to developmental programming and reinforce the growing body of evidence that demonstrate the effects of maternal metabolic status on placental DNAm.

The previously established relationship of heavier mothers carrying larger litters was not observed in this sample. Due to the rare occurrence of quintuplets, the relationship between maternal weight during gestation and infant birth outcomes was assessed in the sample after omitting the one quintuplet pregnancy that occurred in this sample. With removal of this pregnancy, there was a moderate-low significant correlation between maternal weight at gestational day 120 and litter size. It is important to note that the previously established relationship used maternal prepregnancy weight due to the positive association between prepregnancy weight and ovulation number, and in turn, litter size [42]. The focus of this study was on maternal weight during gestation and therefore the preconceptual effects of maternal weight could not be directly assessed.

Additionally, initial studies of developmental programming in marmosets were restricted to twin and triplet litters and utilized twins as model of a “non-restricted intrauterine environment” and triplets as a model of a “restricted intrauterine environment.” In that framework, twins typically had higher birth weights than triplets and low birth weight triplets were the most likely to demonstrate centile crossing and developmental programming of obesity and reproductive health outcomes ([21, 43].

Yet, there is a secular trend of increasing weight in breeding female marmosets in the colonies used in this study [23, 24]. As maternal weights have increased, there has also been a trend for increasing birth weights of their offspring. This study and others show a more extensive overlap of birth weights between twin and triplet litters [23]. While studies comparing twin and triplet litters were critical for establishing the marmoset as a model of developmental programming, our group has studies underway to examine developmental programming effects in the current context of increasing maternal weight combined with increasing litter sizes and increasing birth weight of infants from larger litters. The relationship between maternal weight (both before and during pregnancy) and infant outcomes associated with developmental programming is likely more nuanced such that developmental programming effects are not limited to low birth weight triplets, but rather developmental programming acts across the spectrum of litter sizes and birth weight. This study demonstrates a shift in the relationship between maternal weight and infant outcomes and limits the ability to infer how the findings of this study apply to developmental programming outcomes that have been based on litter size and birth weight. As the primate placenta is an agent of developmental programming, understanding the associations of maternal weight and placental DNAm will be significant step in advancing the marmoset as model of primate reproduction and developmental programming.

In humans and other mammals, the placenta is a globally hypomethylated organ [reviewed in 44]. Interestingly, analysis of marmoset placental DNAm through RRBS captured a higher level of DNAm in the marmoset placenta than expected with 65% of the covered CpG sites demonstrating M-values greater than zero, suggesting that the

marmoset placenta may be more globally methylated than the human placenta. The function of DNAm is context specific and the relationship between DNAm and transcription differs depending on genomic location (e.g. TSS, promoter, gene body, etc) [45]. As an initial assessment of where DNAm occurred in the marmoset placental genome, gene regions around the TSS were examined. The TSS is within the promoter region of the gene. Methylation in the promoter region of a gene inhibits the recognition of the gene by transcription factors and RNA polymerase which leads to gene silencing [46]. Conversely, unmethylated promoters are actively transcribed [45]. The effect of DNAm status on gene transcription in the promoter impacts gene expression, and in human embryonic stem cells 20% of the most highly expressed genes demonstrated the lowest levels of methylation within the ± 1 kb from the TSS in gene promoters [47]. While gene expression was not analyzed in this study, we did analyze the pattern of DNAm within the promoter region (± 3 kb region of TSS). In general, DNAm decreased as it approached the TSS, was lowest at the TSS and increased as the downstream distance from the TSS increased. The lower level of DNAm at TSS of promoter regions, suggests that increased transcription is occurring.

DNAm in promoter regions was further explored by assessing the pattern of completely methylated and completely unmethylated CpG sites within the ± 3 kb region of TSS. CpG sites that are completely methylated or completely unmethylated are CpG sites that demonstrate less variability. Examining these sites provides insight into DNAm patterns that may be more stable with likely implications for gene expression and provides a baseline understanding of DNAm in the marmoset placenta. Figure 4 demonstrates a clear pattern of a higher occurrence of completely unmethylated CpG

sites within the ± 1 kb region of the TSS. The opposite was true for CpG sites outside of the promoter region which demonstrated a higher occurrence of completely methylated CpG sites. While this study captured a higher extent of methylation than expected, it appears that higher levels of methylation are occurring outside of the gene promoter, and lower levels of methylation occur in the gene promoter regions potentially leading to increased transcription and expression. In somatic and germ-line tissues there is a global pattern complete methylation of CpG sites across the genome, with the exception of CpG sites within the promoter region which demonstrate high variability based on tissue-type [39]. The results of this study suggest that similar to the human, hypomethylation is not uniform across the entire placenta [40]. The high occurrence of completely unmethylated CpG sites within the promoter suggest that there is a stable amount of hypomethylation occurring within the promoter, likely leading to similar increases in gene expression that are observed in the human placenta [48].

Three regression models were used to identify CpG sites and genes that had associations between DNAm and maternal weight at any time point. A deduplicated gene list for gene ontology analysis was created using the CpG sites that had significant associations between DNAm and maternal weight at any time point. Gene ontology pathway analysis revealed that genes with significant associations between DNAm and maternal weight were enriched in major metabolic pathways including *regulation of glycolytic processes* (GO:0006110), *carnitine shuttle* (GO:0006853), *regulation of carbohydrate catabolic processes* (GO:0043470), and *regulation of lipid metabolic processes* (GO:0019216). An important and biologically significant aspect of obtaining these results in the marmoset is that heavier marmosets exhibit metabolic alterations

such as increased fasting glucose, hemoglobin A1C, triglycerides and very low density lipoproteins [49]. This suggests that the maternal metabolic milieu associated with increased weight influences DNAm of genes that regulate energy and metabolism within the placenta.

Placental DNAm of genes within the pathways that were enriched in this study of the marmoset placenta have been explored in association with various indicators of maternal metabolic status (including maternal weight and glucose levels) in human and animal models. In human pregnancies characterized by obesity, placentas demonstrate globally higher levels of DNAm than those from non-obese pregnancies [12]. On a gene-specific level, genes within the *regulation of lipid metabolic processes* pathway (GO:0019216) have been of particular interest. Leptin, which is regularly produced by adipose tissue and is also produced by the placenta during pregnancy, is an adipokine central for energy homeostasis and plays a role in regulating nutrient exchange as well as placental and fetal growth [10, 50, 51]. Investigations of DNAm of the leptin gene (*LEP*) have contrasting results. Lesseur et al., [10] found no significant associations between maternal pre-pregnancy obesity and placental DNAm at 23 CpG sites within *LEP*, but a trend of higher DNAm of placental *LEP* in pregnancies affected by pre-pregnancy obesity was observed. In a follow-up study that also included pregnancies affected by gestational diabetes mellitus, placental DNAm of *LEP* was significantly higher in pregnancies exposed to pre-pregnancy obesity, however this was due to a partial mediation effect of gestational diabetes [51]. In another study exploring gestational diabetes mellitus, Bouchard et al., [52] found decreased DNAm with increasing blood glucose levels. The reason for the discrepancy in the results is

unknown, but suggests that there are dynamic interactive effects of the maternal metabolic milieu and that placental DNAm of *LEP* is sensitive to maternal metabolic dynamics.

Other genes within the *regulation of lipid metabolism* pathway that warrant further investigation in the marmoset are the genes of the peroxisome proliferator-activated receptor (PPAR) transcription factor family. The PPAR family of genes play a major role in cellular and systemic lipid metabolism as well as in placental development and function [reviewed in 53]. Placental DNAm of *PPARGC1A* is associated with maternal glucose levels [54, 55] and placental DNAm of *PPARA* is significantly different between pregnancies with and without gestational diabetes [56]. *PPARA* encodes for a transcriptional regulator that is involved in nutrient exchange between mother and fetus and is highly expressed in human and rodent placentas [53, 57]. Although little is known about placental DNAm of *PPARA* in relation to maternal metabolic status, maternal prenatal nutrition has been shown to influence the DNAm of *PPARA* in offspring with alterations that persist in adulthood [58]. Another PPAR gene of interest in the *regulation of lipid metabolism* pathway is *PPARG. PPARG* is a key regulator of trophoblast differentiation and metabolism. As a transcription factor, *PPARG* regulates the expression of other target genes. Not all of its target genes are known, but increased activation of *PPARG* in trophoblasts does target genes that regulate lipid uptake and transport including upregulation of *FATP1*, *FATP4* which emphasizes the role of *PPARG* in trophoblast lipid metabolism [59, 60].

In addition to the potential impacts on growth and development of the fetus, alterations in DNAm of *LEP, PPARs*, and other genes within the enriched pathways of

this study is also likely to influence the growth and development of the placenta itself. The placenta is a metabolically active tissue with a significant amount of nutrient uptake from the maternal circulation. The maternally-sourced nutrients are metabolized by the placenta and used for generating cellular energy and synthesizing components essential for cellular growth within the placenta such as DNA, RNA, proteins, membranes and other biological building blocks [57]. Genes that are a part of the *regulation of glycolytic processes* and *regulation of carbohydrate catabolic processes* pathway will be of particular interest given that glucose is the primary substrate that the placenta uses to generate energy for growth and development of the fetoplacental unit. The placenta exhibits a very high rate of glucose consumption to meet its own metabolic demands as well as that of the fetus [57]. In the human placenta, 45% of the glucose taken up is transported to the fetal circulation, while 50% is metabolized via glycolysis to serve the metabolic needs of the placenta [57]. In human pregnancy, glucose is not synthesized by the fetus, and is only minimally synthesized within the placenta if at all, and therefore the maternal circulation is the predominant source of glucose for the fetus and placenta. Currently, the consumption and disposition of glucose in the marmoset placenta is unknown. Exploring this in a multilevel context along with placental DNAm is likely to contribute to a better understanding of metabolic dynamics in the marmoset placenta.

The results of this study provide support for the marmoset as a model for exploring the impact of maternal metabolic status on placental DNAm. There are many strengths of the marmoset model including the natural occurrence of a continuum of maternal metabolic states. In this study, maternal weight in early pregnancy ranged

from 318-614 grams, and provides a broad spectrum of maternal metabolic states to be explored. It is likely that increasing maternal weight is accompanied by similar metabolic alterations as seen with increasing weight in non-pregnant marmosets such as increased glucose, hemoglobin A1C, triglycerides and very low density lipoprotein [49]. This provides the opportunity to explore the association of placental DNAm with specific indicators of maternal metabolic status in a model with a high degree of biological salience for translation to human research. Another major advantage of further investing placental DNAm in the marmoset, is that there is evidence of developmental programming of obesity and reproductive health outcomes. The short life course of the marmoset provides a model in which placental DNAm can be examined in the longitudinal context necessary for making associations with developmental programming outcomes in a time efficient manner.

Yet there are many steps that must first be taken to understand placental DNAm in the marmoset on a fundamental level. This study assumed that the marmoset genome acts similarly to the human genome in the orchestration and execution of biological processes. It was also assumed that DNAm has the same impacts on gene transcription and expression as is known in other species. Considering the phylogenetic similarities and shared sequence homology among primates it is likely that these similarities do exist, however further studies will be necessary to rule out evolutionary differences and variants that may impact the dynamics of the marmoset genome and DNAm. An important next step will be assessing the association between gene expression and DNAm in the marmoset placenta.

Another necessary step will be accounting for cellular heterogeneity. The placenta is comprised of many different cell types and DNAm is known to differ among cell types within the placenta [11, 61]. Variations of cell types within samples can be a major contributor of sample-to-sample variation in DNAm [11, 62]. This is an important consideration for interpreting studies of DNAm in the placenta that was not accounted for in this initial assessment of placental DNAm in the marmoset. Future studies should employ cell-sorting approaches to gain a more robust understanding of cell-specific DNAm patterns in the marmoset. In addition to being cell-specific, DNAm is also gestational age specific [11, 62]. In human placentas, there are dramatic changes in placental DNAm throughout gestation which may be due to the changes in cellular composition that occur throughout gestation or may be epigenetic changes that are a part of normal placental development [11, 62, 63]. Deciphering this in the marmoset will require obtaining placentas at various gestational ages and focusing studies on cell-specific DNAm. A biological confounder that will be difficult to account for in the marmoset due to chimerism and the high frequency of mixed-sex litters is fetal sex. Studies have demonstrated that sex-specific differences occur not only in placental DNAm, but also in gene expression, protein expression and immune function [reviewed in 64]. As of now, this remains a limitation of all studies exploring placental function in the marmoset.

3.4 Conclusion

The results of this study contribute to an increasing body of evidence that demonstrate associations between maternal metabolic status and placental DNAm that likely have consequences for placental function, fetal growth, and developmental

programming. This study also positions the marmoset as a model for examining placental DNAm. This is the first study to report genome-wide placental DNAm in the marmoset monkey. As such, establishing DNAm patterns in the marmoset monkey is a valuable step for elucidating the placental epigenetic mechanisms that contribute to placental function and lifelong health trajectories of offspring in a model that provides high biological salience for translating findings to human studies.

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PEER-REVIEWED PUBLICATIONS

Fritschi, C., Park, H., Richardson, A., Park, C., Collins, E. G., Mermelstein, R., **Riesche, L.,** and Quinn, L. (2015). Association between daily time spent in sedentary behavior and duration of hyperglycemia in type 2 diabetes. *Biological Research for Nursing.* doi: 10.1177/1099800415600065

Lee, Y., Florez, E., Tariman, J., McCarter, S., and **Riesche, L.** (2015). Factors related to sexual behaviors and sexual education programs for Asian-American adolescents. *Journal of Applied Nursing Research, 28*(3), 222-228. doi: 10.1016/j.apnr.2015.04.015

PRESENTATIONS (Presenting author underlined, *professional meeting)

***Riesche, L.,** Ziegler, T., Tardif, S., Ross, C., and Rutherford, J. (2016). Maternal insulin and placental growth in the common marmoset monkey.

International Federation of Placenta Associations Annual Meeting. Portland, OR (poster).

*Rutherford, J., Tardif, S., Ross, C., **Riesche, L.**, deMartelly, V., Sills, A., Layne Colon, D., and Ziegler, T. (2016). Metabolic hormone dynamics of the marmoset monkey pregnancy. International Federation of Placenta Associations Annual Meeting. Portland, OR (poster).

*Rutherford, J.N., Tardif, S.D., Ross, C., Layne Colon, D., Sills, A., **Riesche, L.**, and Ziegler, T. (2016). Metabolic hormone dynamics across marmoset monkey pregnancy: sources of variation and implications for birth outcomes. American Association of Physical Anthropologists Annual Meeting. Atlanta, GA (podium).

***Riesche, L.,** & Rutherford, J. (2016). Maternal energetics and placental epigenetics: Implications for offspring outcomes. Midwest Nursing Research Society (MNRS) Annual Meeting. Milwaukee, WI (poster).

***Riesche, L.,** & Rutherford, J. (2015). Placental epigenetics and developmental programming. International Society of Nurses in Genetics (ISONG) World Congress. Pittsburgh, PA. (poster).

KEYNOTE: Riesche, L. (2015) The role of every nurse in the collective commitment to compassionate care. 2015. White coat ceremony, College of Nursing, University of Illinois, Chicago, IL (podium).

***Riesche, S. L.,** & Apatira, O. (2012) An exploration of first-time mother's childbirth experience and factors influencing the decision of childbirth delivery method. Grace Peterson research colloquium, Chicago, Illinois (poster).

MEDIA

Riesche, L. (2015, January 26). Like a koala bear hanging out in a tree: Anxiety and excitement as a nurse starts her PhD journey. *RWJF Culture of Health.* Available at http://www.rwjf.org/en/culture-of-health/2015/01/like_a_koala_bearha.html

Wisby, G. (2014, August 18). New program helps nurses move forward, faster, with PhDs. *UIC News*. Available at http://news.uic.edu/new-program-helps-nurses-move-forward-faster-with-phds

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2014-2016 College of Nursing Curriculum Committee, University of Illinois Chicago, Doctoral Student Member

2012-2015 Bridges to the Doctorate Program, University of Illinois Chicago, Peer Mentor

2011-2014 Robert Wood Johnson Foundation, New Careers in Nursing, DePaul University, Peer Mentor

PROFESSIONAL NURSING MEMBERSHIPS

2015-Present International Society of Nurses in Genetics (ISONG), Communications Committee member

2015-Present Midwest Nursing Research Society (MNRS), member

2014-Present National Association of Hispanic Nurses (NAHN), member

2014-Present Illinois Hispanic Nurses Association (IHNA), member

2012-Present Sigma Theta Tau International Honor Society (STTI), Zeta Sigma Chapter member

2011-2014 Student Nursing Association of Illinois (SNAI), member

INTERDISCIPLINARY MEMBERSHIPS

2016 International Federation of Placenta Associations, member

2015-Present Society for Advancement of Chicano and Native Americans in Science (SACNAS), member

2014-Present Graduate Women in Science, Sigma Delta Epsilon, member

CURRENT PROFESSIONAL LICENSE & CERTIFICATION

2016 Basic Cardiac Life Support Certification

2013 Registered Nurse, Professional License

2013 Neonatal Resuscitation Program Certification