EFFECTS OF MATERNAL PHYSICAL ACTIVITY ON METHYLATION PATTERNS IN OFFSPRING BLOOD SPOTS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Kinesiology – Doctor of Philosophy

2014

ABSTRACT

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A number of studies have suggested that lifestyle factors during pregnancy, including diet and cigarette smoking, may influence the long term health of offspring. Whether maternal leisure-time physical activity (LTPA) during pregnancy might have a similar effect is unknown. One mechanism by which LTPA might influence offspring health is DNA methylation, where methyl groups are added to cytosine bases in DNA particularly at areas in the genome rich in cytosine and guanine bases (CpG islands). The purpose of this dissertation was to determine the relationship between maternal LTPA during pregnancy and offspring DNA methylation, both globally and in metabolism-related candidate genes, in infant blood spots.

We used the Archive for Research on Child Health (ARCH) study data set, which has been ongoing at Michigan State University (MSU) since 2007. At enrollment, subjects completed questionnaires on demographic information as well as LTPA. We matched highly active subjects (averaged 637.5 minutes per week of moderate or vigorous physical activity (MVPA); n = 14) to low active subjects (averaged 60.4 minutes per week MVPA; n = 28) based on maternal age and race. Subjects were contacted via telephone to ask for consent to link physical activity data to their child's blood spot which was obtained at birth.

Following DNA isolation and bisulfite treatment, we used pyrosequencing to determine methylation levels of long interspersed nucleotide elements (LINE-1) (global methylation) and PPARγ, PGC1-α, IGF2, PDK4, and TCF7L2.

We found no differences between high active and low active groups for LINE-1 methylation, an indicator of global methylation (Aim 1). For Aim 2, the only differences in candidate gene methylation between the two groups was at two CpG sites in the P2 promoter of IGF2; in both instances the low active group had significantly higher DNA methylation than the high active group ($p = 0.020$ and 0.047). However, the meaning of these differences is not clear. Also, mean methylation over eight P2 promoter sites for IGF2 was significantly higher in the low active group ($p = 0.045$), but there were no other differences in mean methylation levels for any candidate gene.

Overall these results suggest no effect of maternal LTPA on global and candidate gene methylation profiles, with the exception of IGF2 methylation. This research is a unique pilot study of maternal LTPA in pregnancy and its effects on DNA methylation, in humans. Possible reasons for the mostly null findings include our choice of biologic sample (blood spots), lack of transfer of methylation profile from mother to offspring, measurement of methylation rather than gene expression, and inadequate LTPA stimulus. Future studies should include examination of the role of higher levels of maternal LTPA on both maternal and infant umbilical cord blood samples, and determination of the role of maternal LTPA on both DNA methylation and gene expression.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and encouragement of many. Most of all I would like to thank my PhD advisor Dr. Jim Pivarnik for his many hours of assistance with this project. I am extremely grateful for his dedication to both students and to research, and am fortunate to have had the opportunity to train with him at MSU. I would also like to thank my other committee members. Dr. John Gerlach permitted me to use his laboratory space for analyses and provided much feedback regarding the laboratory work portion of this dissertation. Dr. Lanay Mudd provided excellent feedback on study design and statistical analyses. Dr. Nigel Paneth oversees the ARCH study and has permitted me to use this data. The work would not have been possible without each of them.

Many others have assisted in making this work possible as well. Thank you to Lynette Biery, ARCH study coordinator, for her assistance working with this data. Carrie Langbo, BioTrust Coordinator for the State of Michigan, worked tirelessly with me to obtain consent for use of the infant blood spots. Nancy Christ at the Michigan Neonatal Biobank was also heavily involved in this process.

I am also grateful to my fellow PhD students at MSU who have made my four years here fun, memorable, and a great learning experience. I was fortunate to start the program with an incredible group of people: these include Chris Connolly, Alex Montoye, Larissa True, and Kimbo Yee. I am thankful to have

found a wonderful church family here in East Lansing and am indebted to many friends at University Reformed Church for the support and prayers provided over the last several years. To my daughter Addy, thank you for providing much distraction and many opportunities for breaks from studying and writing—these were needed! And finally, many thanks to my husband Dave who moved to Michigan, to a poor job market and cold winters, so I could pursue this degree. He is a great blessing to me and to many others as well.

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KEY TO ABBREVIATIONS

ACOG: American College of Obstetricians and Gynecologists

ACSM: American College of Sports Medicine

AGA: Average for gestational age

ANOVA: Analysis of variance

ARCH: Archive for Research on Child Health

BMI: Body Mass Index

CVD: Cardiovascular disease

eNOS: endothelial nitric oxide synthase

GDM: Gestational diabetes mellitus

GR: Glucocorticoid receptor

GWG: Gestational weight gain

HEI: Healthy Eating Index

IGF2: Insulin-like growth factor 2

IOM: Institute of Medicine

LBW: Low birth weight

LDL: Low-density lipoprotein

LGA: Large for gestational age

LINE-1: Long interspersed nucleotide elements

LTPA: Leisure-time physical activity

MC4r: Melanocortin 4 receptor

MET: Metabolic Equivalent

MSU: Michigan State University

MVPA: Moderate or vigorous physical activity

Ng: Nanograms

OPA: Occupational physical activity

PCR: Polymerase chain reaction

PDK4: Pyruvate dehydrogenase lipoamide kinase 4

PGC1-α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPARγ: Peroxisome proliferator-activated receptor gamma

RXRA: Retinoid-x receptor-α

SES: Socioeconomic status

SGA: Small for gestational age

SNP: Single nucleotide polymorphism

TACSTD2: Tumor-associated calcium-signal transducer 2

TCF7L2: Transcription factor-7 like 2

INTRODUCTION

The number of overweight and obese adults and children is increasing worldwide. In the United States, 16.9 percent of children and adolescents ages 2-19 were classified as obese and another 31.7 percent were classified as overweight based on national survey data from 2007-2008 (Ogden, Carroll, Curtin, Lamb, & Flegal, 2010), and two thirds of American adults were classified as either overweight or obese (Flegal, Carroll, Ogden, & Curtin, 2010). Because obese children are more likely to become obese adults compared to normal weight children (Dietz, 1998), identifying the causes of the rise in obesity and implementing preventative and treatment programs is important from a public health perspective. Traditionally the "big two"—poor diet and physical inactivity, have been blamed for the rising obesity epidemic in both children and adults. However, recent evidence suggests that numerous other factors, including the prenatal and maternal environment, may also play a role (Poston, Harthoorn, & Van Der Beek, 2011).

Cardiovascular disease (CVD) is responsible for approximately 34% of all deaths in the United States each year (Lloyd-Jones et al., 2009). Atherosclerosis begins early in life (Berenson et al., 1998; Mahoney et al., 1996), and there is also evidence that CVD risk factors track moderately well from childhood into adulthood and likely contribute to CVD morbidity (Katzmarzyk et al., 2001). The prevalence of CVD risk factors in children has increased significantly over the past three decades and thus, it is important to address this issue. However,

interventions designed to reduce CVD risk factors in children and adolescents have been only moderately successful at best (van Sluijs, McMinn, & Griffin, 2008). Investigators have begun to consider alternative strategies, such as determining whether a child's CVD risk factor profile is determined before birth, and if so, whether positive genetic alterations in offspring can occur through altered maternal behaviors.

That CVD and obesity risk factors can be traced to fetal origins is believed to be related to a phenomenon known as “programming” (Barker, 1992). Programming posits that critical developmental periods for the heart and blood vessels exist during embryonic and fetal life. If, during these periods, the maternal-fetal unit is unduly stressed, permanent developmental alterations can occur. This has been shown experimentally in animal models (Hahn, 1984; Langley & Jackson, 1994; Winick & Noble, 1966). Epidemiological evidence has shown that programming may occur in humans, primarily in low birth weight offspring and those at the lower end of the normal birth weight range (Law et al., 2002). Most studies on fetal programming have focused on birth weight and offspring health, but few have considered the relationships between maternal behaviors during pregnancy and offspring health.

Results from a number of animal studies of maternal nutrition during pregnancy and epigenetic programming effects in the offspring (reviewed in (Burdge & Lillycrop, 2010b; Heerwagen, Miller, Barbour, & Friedman, 2010), suggest that the maternal environment during development influences the offspring phenotype, particularly in terms of metabolic health. It is reasonable to

pursue the study of the effects of maternal physical activity in the same manner, yet this has not been done to date.

Numerous long-term benefits of maternal activity for the offspring have been suggested, but few well designed studies have been conducted. The majority of research in this area has been focused on risk of development of obesity and co-morbidities in the offspring of exercising pregnant mothers, and much of that work has been conducted in animal models due to the ease of controlling both diet and genetic predisposition to obesity. In one recent human study, Hopkins and colleagues published work demonstrating that a moderate-intensity cycling program during the second half of pregnancy resulted in offspring who weighed less and were less fat at birth (Hopkins, Baldi, Cutfield, McCowan, & Hofman, 2010).They also found that serum IGF-I and IGF-II were lower in the cord blood of the offspring of exercising mothers compared to controls, suggesting that hormonal stimulation of fetal growth was less in the exercise group.

A mechanism by which offspring phenotype might be affected by maternal exposures or behaviors is epigenetics, which are heritable chemical changes in DNA that may influence the expression of the gene. In terms of lifestyle and its influence on the epigenome, the most well-studied epigenetic change is DNA methylation (Alegría-Torres, Baccarelli, & Bollati, 2011), where methyl groups are added to cytosine bases in CpG islands. High levels of methylation are associated with low promoter activity of the particular gene (and lower gene expression) and low levels, then, are associated with high promoter activity and

high amounts of gene expression. Whether high or low methylation level is advantageous in terms of programming depends on the gene in question and may depend on the tissue being studied as well. In humans, only one study has shown varying levels of DNA methylation with a lifestyle exposure; individuals who were in utero during the Dutch Famine of 1944 had lower levels of DNA methylation genome-wide compared to their same-sex siblings who were not prenatally exposed to the famine (Heijmans et al., 2008). Other studies in animal models have shown DNA methylation differences in animals fed normal diets compared to protein restricted diets; the direction of difference depended on the gene being studied (Burdge & Lillycrop, 2010a).

Though the rationale for studying DNA methylation as a mechanism of how fetal programming might occur is strong, to date, no studies have measured methylation differences in human tissue from offspring of exercising or otherwise physically active mothers compared to low active controls. The purpose of this dissertation study is to evaluate the differences in global DNA methylation and methylation of candidate genes with known metabolic function in blood spots from infants born to physically active compared to low active mothers. Specifically, leisure time physical activity (LTPA), defined as activity performed during leisure time that resulted in a rise in energy expenditure above baseline levels, was assessed via questionnaire. Physical activity data have been collected on over 300 pregnant women as part of the Archive for Research on Child Health (ARCH) study. These data, as well as possible covariates, were used to select the most and least active subjects whose infants' blood spots were

then requested from the Michigan Neonatal Biobank. Using the ARCH data and DNA extracted from the requested blood spots, this dissertation addresses the following Research Aims.

Research Aims

Specific Aim 1: Investigate global DNA methylation of newborn dried blood spots of infants born to pregnant women reporting high levels of LTPA compared to low active controls, in order to determine overall methylation in repetitive DNA stretches in humans and how they may be affected by high levels (greater than three times minimum recommended levels) of maternal LTPA. The MethylFlash assay and long interspersed nucleotide element (LINE-1) methylation will be used to assess global DNA methylation.

Hypothesis 1.1: Global DNA methylation will be greater in blood spots of offspring of pregnant women reporting high LTPA compared to offspring of low active controls.

Specific Aim 2: Determine whether methylation levels of metabolism-related candidate genes are different in newborn dried blood spots of infants born to pregnant women reporting high levels of LTPA compared to offspring of low active controls. Candidate genes are peroxisome proliferator-activated receptor-γ (PPAR-γ), proliferator-activated receptor-γ co-activator α (PGC1-α), insulin-like growth factor 2 (IGF2), pyruvate dehydrogenase kinase 4 (PDK4), and transcription factor 7-like 2 (TCF7L2).

Hypothesis 2.1: Methylation levels will be different for each of the five candidate genes in blood spots of infants born to pregnant women

reporting high levels of LTPA compared to low active controls. Specifically, we hypothesize that, compared to low active controls, women with high LTPA will have lower methylation levels for PPAR-γ, PGC1-α,and PDK4, but higher methylation levels for IGF2 and TCF7L2.

Organization of Dissertation

Chapter one is the introduction and specific aims while the literature review is presented as Chapter two. The methods section of the dissertation is Chapter three and all results are presented in Chapter four. Lastly, Chapter five is a summary of the results and discussion of how these results should be interpreted, as well as future research directions. References and appendices of relevant supplemental materials are also included in the final dissertation.

REVIEW OF LITERATURE

Physical Activity and Pregnancy

Leisure time physical activity (LTPA) is activity that causes a rise in energy expenditure above baseline levels and occurs during an individual's leisure time rather than as part of their occupation or transportation (Dishman, Heath, & Washburn, 2004). Occupational physical activity (OPA) is energy expenditure above baseline that is used to perform vocational or work activities, often in an eight hour work day, and other types of activity that may, depending on the researcher, include transportation, care for children, pets, and other individuals, and household chores (Howley, 2001). Exercise is planned, purposeful physical activity, often done with the goal of improving physical fitness and health (Dishman, et al., 2004). The intensity, duration, and total volume of physical activity or exercise that a person engages in influences health and well-being. Therefore, it is important to consider physical activity when studying public health outcomes.

LTPA levels, as assessed by both accelerometer and self-report, decline over the course of pregnancy (Rousham, Clarke, & Gross, 2006). The reasons for the decline are many, but include physical limitations, response to medical advice to reduce or cease exercising, or perceived risks associated with activity, particularly late in pregnancy (Clarke & Gross, 2004). Similar reasons for pregnant women reducing LTPA or stopping completely were found by Duncombe et al., but their study participants added that feeling tired or unwell and lack of time kept them from maintaining LTPA levels throughout pregnancy

(Duncombe, Wertheim, Skouteris, Paxton, & Kelly, 2009). In all, the evidence is quite consistent that LTPA levels decrease as pregnancy progresses, and the reasons for this decline are numerous. More data supporting the safety and efficacy for improved maternal and birth outcomes of LTPA during pregnancy may encourage pregnant women to engage in LTPA and may inspire their physicians to spend more time discussing the benefits of LTPA for both mother and baby.

Benefits of Physical Activity During Pregnancy--Fetal

Historically there has been concern for pregnant women participating in physical activity, particularly in strenuous activities. Concerns include shunting of blood away from the placental perfusion toward exercising skeletal muscle, hyperthermia that might result from activity and how this might influence the fetus, and the impact of repetitive movements such as running or jumping(Hopkins & Cutfield, 2011). However, the relatively limited research to date have not substantiated these concerns.

Maternal pregnancy physical activity does not appear to place the fetus at additional risk for adverse outcomes such as LBW or preterm birth. For example, Kardel and Kase showed that maternal exercise did not increase risk of LBW, nor did it affect Apgar scores or duration of labor (Kardel & Kase, 1998). In another study, Sternfeld et al. found that gestational age and birth weight were unrelated to exercise intensity during pregnancy (Sternfeld, Quesenberry, Eskenazi, & Newman, 1995b). There is limited evidence that LTPA is related to preterm birth, but these data are not strong enough or of sufficient quantity to make a

conclusive statement in favor of a protective effect of LTPA. Owe and colleagues found a protective effect of maternal exercise against preterm birth in a large cohort study (n = 61,098) (Owe, Nystad, Skjaerven, Stigum, & Bø, 2011) while a similar but smaller study in Brazil also showed a protective effect of LTPA on preterm birth risk (n = 4,147) (Domingues, Barros, & Matijasevich, 2008). It is important to keep in mind that there are a number of possible confounders to the relationship between preterm birth and LTPA (as well as any birth or maternal outcome and LTPA) including maternal age, smoking, race and so on, and different studies control for some but not all of them. This may explain, at least in part, differences in results among studies.

The risk for large for gestational age (LGA) birth appears to be reduced with maternal physical activity during gestation. Mothers who performed at least two hours per week of physical activity during pregnancy had a 70% reduced risk of giving birth to a LGA infant compared to those who were physically active less than two hours per week (Alderman, Zhao, Holt, Watts, & Beresford, 1998). In the same study, the infants born from active mothers were not at increased risk of small for gestational age (SGA) birth. Similarly, the authors also found a link between LGA odds and maternal size, and suggested that maternal glucose tolerance may play a role in predicting LGA risk. In a study utilizing the Danish National Birth Cohort data, exercising mothers had a slightly decreased risk of having either a SGA or LGA infant compared to non-exercisers (hazard ratios 0.87 and 0.93, respectively), even after controlling for gestational age and mother's pre-pregnancy body mass index (BMI) (Juhl, Olsen, Andersen, Nøhr, &

Andersen, 2010). Mudd et al. found that maternal LTPA during pregnancy was related to significantly lower odds of LGA (odds ratio = 0.3, 95% CI = 0.14 – 0.64) but not SGA (Mudd, 2009; Mudd et al., 2012). The study also showed a positive association between maternal BMI and LGA risk. Overall, the literature shows mixed results as to whether SGA risk is reduced or unaffected, but lower LGA risk is consistently related to maternal LTPA during pregnancy. It is important, however, to additionally study the effects of maternal size and glucose handling on offspring size, as these factors also appear to influence LGA risk. At worst, it appears that physical activity during pregnancy has no deleterious effect on birth weight. At best, maternal physical activity appears to reduce the risk of birth weight falling outside the normal range.

Benefits of Physical Activity During Pregnancy--Maternal

In addition to not causing harm, physical activity during pregnancy has been shown to have a number of beneficial effects for the mother. First, physical activity is associated with a reduced risk of preeclampsia, a condition characterized by high blood pressure and proteinuria that can develop in the second half of pregnancy. A case-control study that included women who had a prior preeclamptic pregnancy found that women who participated in light or moderate intensity activity prior to pregnancy had reduced risk of a subsequent preeclamptic pregnancy by 24 percent, while those who performed vigorous activity had a reduced risk of 54 percent (Sorensen et al., 2003). In another study, Saftlas et al. asked women to recall leisure time and work physical activity after their pregnancy and found that women who regularly participated in LTPA

or who had occupations requiring physical activity were at decreased risk of preeclampsia, yet the confidence intervals included one and thus the effect is not significant (Saftlas, Logsden-Sackett, Wang, Woolson, & Bracken, 2004). Though the study had a large number of controls (n = 2,422), there were many fewer cases (n = 44) which resulted in limited statistical power. LTPA may also reduce risk for development of gestational diabetes (GDM) during pregnancy. In a randomized control trial where pregnancy women were assigned to either a pregnancy-long physical activity program consisting of light resistance and toning exercises or a control group, the physically active women performed better on a glucose screen at 24 to 28 weeks gestation and had lower incidence of GDM (Barakat, Cordero, Coteron, Luaces, & Montejo, 2011), but there were design flaws in the study. Another group instigated a 12-week physical activity intervention in the second half of pregnancy and found no effect of the intervention on insulin resistance of GDM incidence (Stafne et al., 2012). Meta-analyses of studies of either dietary or physical activity intervention during pregnancy on GDM risk have shown that most interventions are ineffective in preventing this outcome (Oostdam, van Poppel, Wouters, & van Mechelen, 2011). Many studies on this topic are either small or poorly designed, but better designed randomized control trials are currently being conducted.

Few studies have addressed whether physical activity or exercise during pregnancy is related to a mother's risk for needing a caesarean section for delivery. The largest of these studies (n = 1,342), was conducted using the PRAMS database in North Carolina. The authors found no relationship between

maternal physical activity and delivery mode, though there was a trend toward a protective effect of physical activity against caesarean section (Bovbjerg & Siega-Riz, 2009). Bungum and colleagues showed that low active women had a 4.48 (95% CI = 1.2 – 16.2) times higher odds of having a caesarean delivery compared to women who reported LTPA during their pregnancy, even with a small sample size of 137women (Bungum, Peaslee, Jackson, & Perez, 2000). However, another group did not find that maternal LTPA predicted delivery mode (Sternfeld, Quesenberry, Eskenazi, & Newman, 1995a). More research needs to be conducted to determine whether physical activity during pregnancy might reduce need for medically indicated caesarean deliveries. All three studies included a cohort design, but Bovbjerg et al. used frequency of exercise (days per week) as the independent variable while the other two studies included total volume of physical activity. The differing results may also be explained by different timings of physical activity exposure. The Bovbjerg et al. study only evaluated exercise in the last three months of pregnancy while Bungum et al. looked at only the first two trimesters and Sternfeld et al. collected data over all trimesters.

The role of physical activity on miscarriage risk is not well studied, and resulting evidence is equivocal. Of six published studies on this topic, two found that physical activity during pregnancy was associated with increased risk of miscarriage (Hjollund et al., 2000; Madsen et al., 2007), two found no relationship (Clapp, 1989; Rose, Haddow, Palomaki, & Knight, 1991), and two found a protective effect of physical activity (Latka, Kline, & Hatch, 1999; X. Zhang et al.,

2011). The Madsen study was a large cohort study that found more activity to be associated with higher miscarriage risk. But, when the authors excluded participants with potential recall bias due to physical activity ascertainment after miscarriage, the relationship between physical activity and miscarriage was attenuated (Madsen, et al., 2007). Two other cohort studies on this topic were prospective while the Madsen cohort was primarily retrospective interviews with a subset of the cohort interviewed prior to miscarriage. The other three studies on this topic are case-control studies: two found maternal physical activity was associated with decreased miscarriage risk (Latka, et al., 1999; X. Zhang, et al., 2011) and the Clapp study results showed no difference in risk between cases and controls (Clapp, 1989). It appears that study design may be related to the study findings, with case-controls favoring a finding of a protective effect of LTPA and cohort studies favoring a finding of an elevated risk. Because of the small number of studies, though, it is difficult to know for certain. Once the Madsen et al. study is removed from the list of studies finding a positive relationship between LTPA and miscarriage (due to the recall bias issue), though, the effect of study type is less obvious. Another possible reason for differences is that two of the cohort studies (Rose et al. 1991 and Hjollund et al. 2000) assessed LTPA differently than did the case-control study authors. Rose and colleagues used total activity (including OPA) as their dependent variable and the Hjollund group assessed intensity rather than quantity of activity. The current consensus by the American College of Obstetricians and Gynecologists (ACOG) and American College of Sports Medicine (ACSM) is that maternal physical activity performed

by women with low-risk pregnancies does not increase risk of miscarriage (ACSM, 2006).

Physical Activity Quantification and Measurement

There are both objective and subjective methods for assessment of physical activity. Subjective measures include questionnaires, surveys, and diaries while objective techniques include pedometers, which count the number of steps an individual takes, and accelerometers, which quantify accelerations of the hip and are reported in 'counts' where a high number of counts indicates a high amount of activity (Trost, McIver, & Pate, 2005). Direct observation involves a researcher observing an individual participating in physical activity and using a designed system to quantify that activity (McKenzie, 2002). Other techniques for assessment of physical activity include indirect calorimetry which requires the collection of expired gases, but this technique is not feasible in a free-living environment or on large samples (Dishman, et al., 2004; McKenzie, 2002), and doubly-labeled water, which is useful for quantifying energy expenditure over a period of several days or weeks but can thus not separate physical activity from resting metabolic rate (Lifson, Gordon, & McClintock, 1955). It is important to point out that pedometers and accelerometers measure total daily physical activity rather than solely LTPA or OPA, so it is necessary to have another means, such as a questionnaire, to assess what percentage of an individual's total physical activity meets the definition of LTPA versus OPA.

Selection of physical activity assessment technique depends upon the research question being asked, the number of subjects to be assessed, and the

type of physical activity to be assessed. For example, doubly labeled water is useful for assessing total energy expenditure, rather than just physical activity, over a period of time, often a week or more. It is very costly and therefore is impractical for large samples, but can be very useful as a criterion method against which other physical activity assessment techniques are validated. Similarly, direct observation is not realistic for large sample sizes because of the time-consuming, and thus expensive, nature of the data collection process. Accelerometers produce large volumes of data and can be expensive in terms of up-front cost, but provide objective measurement that takes into account intensity of the activity, which pedometers are not able to do. Surveys and questionnaires vary widely in the types of questions that they ask; some are lengthy and have been validated for use in particular populations, and some may include a single question such as “are you physically active?” The type of data that obtained depends on the questions asked, and these surveys or questionnaires may be designed for a specific research question.

Physical activity can be quantified in terms of frequency (how many days per week or month), intensity (light, moderate, or vigorous), and time (minutes or hours per day). In order to obtain a total volume of physical activity that takes into account all of these variables, the units MET·min·week may be used. MET stands for metabolic equivalent and one MET is equal to the value, at rest, of the average individual’s energy expenditure. Three METs, then, is equal to three times resting energy expenditure, and so on. Generally moderate physical activities are considered those that raise metabolic rate to between three and six

METs and vigorous activities are those that have intensity greater than six METs. By taking into account the intensity, in METs, for any given activity (see the Compendium of Physical Activity for activities and their MET values (Ainsworth et al., 2011)) and multiplying by the number of minutes per week the activity was performed, a total physical activity volume can be calculated in MET·min·week.

One difficulty when performing physical activity research is being able to separate LTPA from other types of physical activity. For example, it may be that some investigators would classify child care and household chores as LTPA but others would call it occupational. Additionally, objective measurements of physical activity such as accelerometers and pedometers do not provide any information regarding the type or purpose of the activity; they simply provide a value for total physical activity over the time the device was worn. This may influence their ability to accurately measure certain types of activities. For example, in adult females ages 25 to 74, pedometer step counts were found to correlate well to LTPA but not OPA (Sequeira, Rickenbach, Wietlisbach, Tullen, & Schutz, 1995). Another issue with monitors is subject compliance, which may change throughout pregnancy. Rousham et al. measured physical activity throughout pregnancy by both accelerometry and interview, and found that the correlation between the two methods decreased significantly as pregnancy progressed (Rousham, et al., 2006). For these reasons, it may be beneficial to measure physical activity during pregnancy using both subjective and objective techniques when feasible. If the investigator's interest is in LTPA alone, it is most realistic to assess total physical activity with objective measures and use

questionnaires or diaries to determine what type of activities are being done or what percentage of total physical activity can be classified as LTPA.

It is important for many research questions to be able to differentiate between LTPA and OPA. Some studies have shown that strenuous physical work is associated with intrauterine growth restriction (OR 2.46 95% CI 1.36 to 4.21 for women who report moderate to vigorously strenuous versus light) (Spinillo et al., 1996) and premature delivery (OR 2.72 95% CI 1.24 to 5.95 for women whose jobs required prolonged standings compared to those whose did not) (Teitelman, Welch, Hellenbrand, & Bracken, 1990). One reason for the possible adverse effects of OPA is the psychological as well as physical stress that is associated with these working conditions (Clapp, 1996). Due to these physiologic differences in the effects of OPA and LTPA, it is often important to study them separately, and therefore methods to assess one or both separately should be developed.

Depending on the research question, it may be important to assess physical activity levels during pregnancy *and* determine if women are meeting the recommendations. Ideally, this assessment should be repeated across trimesters as the exposure may change over time as pregnancy progresses. In addition, accurate measurement helps us determine which types of physical activity, and how much, might result in the greatest benefit to the maternal-fetal unit. In the first trimester women tend to report feelings of fatigue and/or nausea as reasons for not participating in activity, and in the last trimester, change in body size and center of gravity is more likely to prevent them from meeting physical activity

recommendations (Leiferman, Swibas, Koiness, Marshall, & Dunn, 2011). Therefore, methods of physical activity assessment may need to be adapted for use in pregnant women. In addition, assessment of activity via devices such as pedometers or accelerometers may be influenced by pregnancy in terms of monitor tilt due to gestational weight gain. For example, DiNallo and colleagues found that during a controlled exercise bout on a treadmill, pedometer steps, Actigraph accelerometer counts per minute, and minutes of moderate or vigorous physical activity (MVPA) all were lower at 32 weeks gestation compared to 20 weeks (DiNallo, Downs, & Le Masurier, 2012). Therefore, considerations for maternal weight gain, monitor tilt, and gestational age must be taken into account when designing studies for assessing physical activity during pregnancy.

Physical Activity Recommendations for Pregnancy

The first generally accepted recommendations for pregnancy physical activity in the US were introduced in 1985 and were quite conservative; pregnant women were advised to keep their heart rates under 140 beats per minute at all times and avoid strenuous activity for more than 15 minutes at a time (ACOG, 1985). Since then, much research has been conducted on the topics of safety and benefit of physical activity or exercise throughout pregnancy, and the overwhelming consensus is that it is both safe and effective at improving birth outcomes in the majority of pregnant women (ACSM, 2006). Recommendations for physical activity during pregnancy have also been updated, and are now very similar to recommendations for the general population. In 2002, ACOG published their expert committee opinion that most women can benefit from physical activity

during pregnancy and that in the absence of obstetric complications, they should aim for 30 minutes of moderate intensity exercise on most if not all days of the week (ACOG, 2002). The ACOG guidelines stipulate, however, that activities potentially involving abdominal trauma, as well as scuba diving, which places the fetus at risk for decompression sickness, should be avoided. The 2008 Physical Activity Guidelines for Americans provide very similar recommendations; they suggest 150 minutes per week of moderate intensity physical activity and indicate that the 30 minutes per day can be accumulated in bouts over the course of the day ("Physical Activity Guidelines Advisory Committee report, 2008. To the Secretary of Health and Human Services. Part A: executive summary," 2009).

It is important to note that most pregnancy physical activity recommendations are for aerobic exercise, and that the safety of resistance training has been studied very little in pregnancy. To our knowledge, only one group has conducted a randomized control trial of resistance training in pregnancy. Pregnant women were randomly assigned to either a resistance training group, where they did light resistance and toning exercises three times a week for 35 minutes per session, or a control group. There were no differences between the groups in gestational age at delivery (Barakat, Stirling, & Lucia, 2008), mode of delivery (Barakat, Ruiz, Stirling, Zakynthinaki, & Lucia, 2009), or birth size (Barakat, Lucia, & Ruiz, 2009). These studies were conducted on relatively small samples (70 to 80 interventions and the same number of controls), so it is difficult to determine whether resistance training might have

positive effects on birth outcomes or offspring health. Another group recently examined the association between resistance training during pregnancy and birth outcomes using a cross-sectional study design; they found no difference in gestational age at delivery or delivery mode between women who participated in resistance training and those who did not (E. White, Pivarnik, & Pfeiffer, 2013). In addition to no effects on gestational age or delivery mode, the authors found a protective effect of resistance training on gestational diabetes and hypertensive disorders during pregnancy, suggesting that, at the least, resistance training during pregnancy is not detrimental to the health of the fetus. Taken together, these studies suggest that light resistance training is safe during pregnancy. The effects of more strenuous activity are still unknown.

Fetal Origins

Maternal Weight and Weight Gain

The Fetal Origins of Adult Disease hypothesis, also termed the Barker hypothesis, was developed based upon an ecological model that showed correlations between geographic regions with high infant mortality rates and high death rates, in adults, from cardiovascular and respiratory disease(Barker & Osmond, 1986). Studies revealed that both placental size and infant birth weight were related to systolic and diastolic blood pressure in adulthood (Barker, Bull, Osmond, & Simmonds, 1990), and growth patterns in early life were related to incidence of coronary events and insulin resistance in adulthood (Barker, Osmond, Forsen, Kajantie, & Eriksson, 2005). However, there is controversy surrounding some of the statistical analyses that Barker used, particularly in the

study of the relationship between birth weight and blood pressure. For example, Paneth and colleagues argue that controlling for adult weight in such an analysis is inappropriate (Paneth, Ahmed, & Stein, 1996), and Kuzawa demonstrates by investigating a supply-and-demand hypothesis that prenatal nutrition is related to low-density lipoproteins (LDL) and systolic blood pressure—not diastolic blood pressure or other cholesterol measures (Kuzawa, 2004). Still, other outcomes, including hypertension and endothelial dysfunction, do have stronger evidence supporting an association with prenatal nutrition (Burdge & Lillycrop, 2010a; Langley-Evans & McMullen, 2010), and certainly there are ample data suggesting that various events during fetal development, not limited to nutrition, influence the offspring phenotype. For instance, maternal smoking is strongly related, in a dose-response manner, to low birth weight risk (reviewed in (Walsh, 1994)) and heavy alcohol consumption during pregnancy has a robust association with central nervous system impairment and diminished fetal growth in offspring (reviewed in (Little & Wendt, 1991)).

The prenatal environment may predispose the fetus to chronic disease later in life. Maternal pre-pregnancy weight status and gestational weight gain (GWG) are two relatively easy-to-assess maternal characteristics that may alter the prenatal environment and may predict future poor maternal health and elevated chronic disease risk (Linné, Dye, Barkeling, & Rössner, 2004; Whiteman et al., 2011). Pre-pregnancy obesity and excessive or inadequate GWG may reflect poor diet, physical activity, socioeconomic status, or other stressors in the mother and these environmental factors potentially contribute to

a less than optimal environment in utero (Viswanathan et al., 2008). Numerous studies have identified a relationship between pre-pregnancy weight status and offspring weight status at both birth and throughout childhood and adolescence (Gale et al., 2007; He, Ding, Fong, & Karlberg, 2000; Koupil & Toivanen, 2008; Laitinen, Power, & Jarvelin, 2001; Reynolds, Osmond, Phillips, & Godfrey, 2010). Rooney et al. found that in their cohort, maternal obesity was the strongest predictor of offspring obesity in childhood and adolescence (Rooney, Mathiason, & Schauberger, 2010), and another longitudinal study demonstrated that maternal obesity was associated with weight and length at birth but not at ages 1 and 3 months (Regnault et al., 2010). There are a large number of studies on the topic of maternal obesity and offspring weight status (at various time points) with mixed results; a meta-analysis of 45 medium or high-quality studies on the topic showed an odds ratio of 1.53 (95% CI 1.44 to 1.63) for LGA and 1.95 (95% CI 1.77 to 2.13) for subsequent offspring overweight/obesity in mothers who were overweight or obese prior to pregnancy (Yu et al., 2013). Thus, it appears that in general there is a positive association between maternal and offspring obesity, but study findings might be affected by the timing of offspring measurements, and maternal weight status may be more influential at some time points than others. Taken together, these results suggest that environmental factors influencing the offspring after birth are more strongly at play than is the prenatal environment, but this is not yet clear from the literature.

Additional studies have found independent effects of maternal BMI prior to pregnancy on other adverse health outcomes in offspring. Obese but not

overweight women are more likely to give birth to an infant with a congenital heart defect, with the most obese women having the greatest risk (Mills, Troendle, Conley, Carter, & Druschel, 2010). Another study showed that maternal BMI is negatively associated with cardiorespiratory fitness in 9-year old offspring, as measured by maximal cycle ergometer test, even after controlling for child body fatness. However, there was no relationship between maternal BMI and other risk factors for cardiovascular disease, including blood pressure, total cholesterol, glucose and insulin levels, and triglycerides (Labayen et al., 2010). In another analysis, researchers showed a higher odds (1.87) of development of severe asthma in the first seven years of life in children whose mothers were obese (BMI > 35) during pregnancy (Harpsøe et al., 2013). A review article on the topic of maternal overweight/obesity during pregnancy and offspring health articulated increased risk of congenital anomalies, small for gestational age, LGA, preterm birth, neonate mortality, reduced breastfeeding success, long-term obesity risk, and metabolic and cardiovascular abnormalities later in life (Ruager-Martin, Hyde, & Modi, 2010). Overall, there is significant evidence that a mother's weight status prior to pregnancy affects the health of her child in a variety of ways throughout his or her life.

Despite a fairly large amount of published data on maternal weight status in pregnancy and effects on offspring, it is not well known whether or how maternal pre-pregnancy obesity or weight gain during pregnancy contributes toward a more risky prenatal environment. In 2009, the Institute of Medicine (IOM) updated their guidelines for weight gain during pregnancy based on

evidence that inadequate or excess gestational weight gain may be related to adverse health outcomes for the offspring (IOM, 2009). Recommended weight gain is now based upon the mother's BMI prior to becoming pregnant. Underweight women (BMI less than 18.5) should gain 28 to 40 pounds over the course of the pregnancy, normal weight women (BMI 18.5 to 24.9) should gain 25 to 35 pounds, overweight women (BMI 25 to 29.9) should gain 15 to 25 pounds, and obese women (BMI greater than or equal to 30) should gain 11 to 20 pounds (IOM, 2009).

Since the IOM update, research has indicated that weight gains outside of the recommended ranges may affect the offspring adversely. In a study from the Copenhagen Perinatal Cohort, investigators examined the relationship between gestational weight gain (GWG) and child obesity in a group of 4,234 mother-offspring dyads. The authors found that GWG was related to offspring BMI at all ages, including childhood, adolescence, and adulthood (Schack-Nielsen, Michaelsen, Gamborg, Mortensen, & Sorensen, 2010). They also demonstrated a linear relationship between GWG and odds of offspring adult obesity. This suggests that inadequate weight gain based on IOM cut points might actually be beneficial in regards to offspring weight status because lower GWG, even if less than recommended by the IOM, is associated with low risk of becoming an obese adult. Another study showed that the offspring of women gaining less than recommended weight based on pre-pregnancy BMI had lower levels of adiposity compared to normal and excess gain offspring, but the cardiovascular risk profiles were similar among groups (Fraser et al., 2010). In the same study,

women who gained greater than recommended weight, additionally, had offspring who had higher adiposity and greater cardiovascular disease risk at age nine compared to normal and inadequate gain offspring. Together, these data indicate that offspring health may be protected by weight gain within or below IOM recommendations.

Several investigators have used gestational weight gain as a continuous independent variable to investigate offspring health outcomes. In infants, maternal weight gain was a significant predictor of weight at birth and at age 3 months (Regnault, et al., 2010). In another study, subjects (offspring) were followed up at several time points; GWG was a significant predictor of offspring BMI at adolescence and early adulthood but not childhood (Rooney, et al., 2010), though the reason is unclear. In 21-year old offspring, mother's GWG was significantly and directly related to BMI (Mamun et al., 2009). Mamun and colleagues also found that systolic blood pressure was greater in offspring whose mothers had higher GWG, though the relationship was not significant. Another study showed that GWG was independently associated with offspring percent fat, measured via skinfolds, at age 30 years (Reynolds, et al., 2010). These studies all followed individuals to varying ages, and therefore it is difficult to compare the results. Both the Regnault et al. and Mamun et al. study were large cohorts (n = 1,418 and n = 2,432, respectively) and the other two studies were considerably smaller. Also, a wide possible number of covariates, including mother's pre-pregnancy BMI, breastfeeding or formula feeding, offspring dietary behaviors and physical activity, and so on, potentially affect the results. Each study used these

and other covariates in different combinations, making results difficult to compare.

Diet

Like maternal BMI and GWG, maternal diet too may affect offspring health. Natural experiments of famine, either by food shortage (Japan) or war (Dutch Hunger Winter, Seige of Leningrad), have provided evidence that food restriction during pregnancy influences offspring health. Data from the Dutch Hunger Winter show that those offspring prenatally exposed to famine have diminished glucose tolerance as adults compared to controls who were not exposed (de Rooij et al., 2006). Also, offspring prenatally exposed to famine in early gestation had higher risk of coronary heart disease and had adverse lipid profiles compared to those unexposed (Roseboom, de Rooij, & Painter, 2006). Effects of famine exposure even affected the children of those individuals who were prenatally exposed to the Dutch Famine. As infants, the offspring of those who were in utero during the famine had higher adiposity and as adults, had poorer overall health compared to controls whose parents were not exposed (Painter et al., 2008).

Infant hormone levels are affected by mothers' diet even when the mother does not have GDM (Butte, 2000). Mothers with lower Healthy Eating Index (HEI) (i.e., poor diet) scores delivered infants with higher cord blood insulin levels (Gesteiro, Bastida, & Sánchez Muniz, 2011).The offspring also had a relative risk of 7.6 for glycemia (cutoff for high glucose was 81.25 mg/dl for males and 82.5 mg/dl for females) and 6.7 insulinemia (cutoff was 4.8 mUI/l for males and 6.4

mUI/l for females) (95% CI not given; p = 0.008 and 0.011, respectively) compared to infants of high-HEI moms (Gesteiro, Rodríguez Bernal, Bastida, & Sánchez-Muniz, 2012).These hormone levels that influence in utero conditions may set the stage for infants to develop chronic disease later in their life, regardless of their own dietary behaviors because their cells are programmed to deal with the perpetually high levels glucose experienced during fetal development . It is not known whether some type of intervention, at the maternal level during pregnancy, might attenuate or even prevent this cellular programming that places the fetus at risk for disease.

Maternal metabolic health may also contribute to the health of offspring. Gestational diabetes is a condition that affects 3 to 7 percent of pregnancies and is associated with maternal obesity (Ferrara, Kahn, Quesenberry, Riley, & Hedderson, 2004). Mothers with high plasma glucose are more likely to have a baby born with hypoglycemia (OR = 2.61, 95% CI = 0.99 – 6.92) or macrosomia (OR = 3.4, 95% CI = 1.55 – 7.43) than pregnant women who have normal plasma glucose levels (Ferrara et al., 2007). In children, a combination of maternal GDM and LGA birth weight resulted in a 10.4 times greater odds of insulin resistance at age 11 compared to children whose mothers did not have GDM and were not LGA (Boney, Verma, Tucker, & Vohr, 2005).There appears to be a dose-response relationship between maternal glucose and offspring disease risk, as the higher the glucose levels the fetus is exposed to in utero, the higher the risk of metabolic disease in adulthood (Silverman, Metzger, Cho, & Loeb, 1995).

Interventions to consider during pregnancy include maternal weight loss (or behavioral interventions that promote weight loss or body composition change, such as physical activity). It is possible, but not clear whether maternal weight loss (in overweight or obese prospective mothers) might improve birth outcomes and ultimately offspring health later in life. There are limited data on this topic, particularly on the offspring at any follow-up period after birth. Marceau and colleagues studied women who underwent biliopancreatic diversion surgery, a form of bariatric weight loss surgery, and evaluated subsequent pregnancies and live births (Marceau et al., 2004). The authors found that incidence of macrosomia in the offspring decreased from 34.8 to 7.7 percent and coincided with an increase in normal-weight offspring from 62.1 to 82.7 percent. However, women who had surgery had a higher than normal miscarriage rate (26.0%) which did not decline with weight loss. The same research group studied children of women who had bariatric surgery at a time point between two pregnancies. The authors compared children born prior to and after maternal surgery. They found that children born after surgery had lower weight at birth (but still in normal range), and at follow up at ages 2.5 to 26 years. Children born after maternal surgery also had higher insulin sensitivity, better lipid profiles, and lower C-reactive protein (Smith et al., 2009). In another study utilizing the same design, prevalence of offspring obesity was 52 percent less in children born after surgery compared to those born before, though the authors did not present data on ages of the children when height and weight measurements were taken (Kral et al., 2006).

Taken together, these data suggest that maternal weight loss somehow influences offspring disease risk, presumably independent of child lifestyle behaviors. It is possible that the mother's lifestyle changed considerably following bariatric surgery. The result, theoretically, is that her child born after surgery was exposed to different dietary and physical activity behaviors, which might partially explain this phenomenon. Paternal factors and offspring diet, physical activity, and screen time were not accounted for in either bariatric surgery study. The authors proposed that the in utero exposures to the fetus after maternal weight loss somehow program better metabolic health that persists for many years after birth. Though no studies have shown what mechanism might be driving these findings, epigenetic changes are suspected (Smith, et al., 2009). Epigenetic modifications are chemical changes in the DNA that then are passed to the offspring and may alter gene expression but without changing DNA sequence (Tost, 2009). In the bariatric surgery studies, the theory is that the maternal weight loss results in epigenetic changes in the maternal genome that are passed on to her offspring whose disease risk is lower, compared to a sibling born before the mother's weight loss, due to the epigenome alterations.

Other lifestyle behaviors related to metabolic health, including physical activity, might also drive epigenetic change that predisposes toward or protects from disease. The role of physical activity in epigenetic effects on DNA has not been well-studied, especially in humans. There are a number of animal studies of maternal nutrition during pregnancy and epigenetic programming effects in the offspring (reviewed in (Burdge & Lillycrop, 2010b; Heerwagen, et al., 2010)), with

results suggesting that the maternal environment during development does indeed influence the offspring phenotype. It thus seems reasonable to pursue the study of the effects of maternal physical activity in the same manner. However, we found no studies evaluating the role of physical activity during pregnancy on epigenetic processes.

DNA Methylation

The basis of the Fetal Origins Hypothesis is that nutrient supply during fetal development and early infancy influences later health, and epigenetics is a mechanism by which this may occur. Epigenetics is a heritable change in a gene that does not result from a change in the DNA sequence (Tost, 2009). The most commonly studied form of epigenetic variation is DNA methylation, in which a methyl group binds to cytosine bases, often in the regulatory region of the gene, altering the expression of that gene (Weber et al., 2007). The idea of 'developmental programming' is based upon the concept of epigenetics, where environmental influences, including diet and physical activity, might alter the epigenome and then be passed to subsequent generations, predisposing offspring to particular phenotypes or even chronic diseases. Much methylation occurs in CpG islands, which are regions of the genome rich in cytosine and guanine bases (Jones & Takai, 2001). Promoter region of genes that are mostly methylated are less transcriptionally active and promoters with low methylation levels are more transcriptionally active (Weber, et al., 2007).

It would be expected, then, that DNA methylation levels correspond to gene expression, but this is not always the case. For instance, Turan and

colleagues identified 23 candidate genes whose methylation levels were able to explain considerable (70 to 87 percent) variance in birth weight in humans (Turan et al., 2012). The gene expression levels for these same genes, however, predicted far less of the variance in birth weight, and correlations between methylation levels and gene expression was low and for the most part, not statistically significant. Thus, it is not safe to assume that methylation levels correlate with gene expression levels of the same gene. Gene expression measurements are also known to be specific to the timing of the exposure of interest and thus this outcome can be difficult to study. Methylation levels are more stable than gene expression and are less affected by measurement and exposure timing (Tost, 2009). Another issue in the study of DNA methylation is tissue specificity; some genes have similar methylation levels across a number of tissues (brain, gonads, heart, intestine, liver, lung, muscle, cord blood, and so on) while other genes have methylation profiles that vary considerably among the tissues (Murphy, Huang, & Hoyo, 2012). It is important to be aware of this issue when reading and interpreting literature on this topic.

Environmental Factors Influence DNA Methylation

Because DNA methylation is mitotically stable, it follows that environmental factors do not play a large, if any, role in determining DNA methylation patterns. However, recent evidence suggests otherwise (Lim & Song, 2012). Schar and Fritsch have described DNA methylation as possessing ‘dynamic stability’ because some methylation patterns are modeled after a template and other patterns are developed de novo (Schär & Fritsch, 2011). Twin

studies have shown that older twin pairs have greater divergence of methylation patterns compared to younger twins, suggesting that environmental factors affect these patterns (Fraga et al., 2005). Most evidence for developmental programming comes from animal studies, due to ethical limitations regarding the manipulation of the human environment. Several animal studies have shown that environmental changes in pregnant animals affect their offspring despite no direct exposure of the offspring to the particular environment.

In mice fed either a standard or obesogenic (high fat, high sugar) diet for six weeks prior to breeding, offspring of the obese dams, who were all fed standard chow, ate more, had lower locomotor activity, and higher abdominal fat pad mass compared to the offspring of the control dams(Samuelsson et al., 2008). Additionally, offspring of the obese dams demonstrated adipocyte hypertrophy as well as altered mRNA expression of several genes expressed in adipocytes. In another mouse study, Kavanagh et al. examined the effects of neonatal and fetal exposure to trans-fatty acids by developing a complex study design that exposed offspring to trans-fat, either in utero, through breast milk, or both. Experimental animals were compared to controls exposed to an identical diet except for the replacement of trans-fats with cis-fats (Kavanagh et al., 2010). Exposure to trans-fatty acids via breast milk resulted in slower growth, elevated fasting glucose, and higher plasma levels of IGF-1, though insulin sensitivity did not differ. Mice exposed to trans-fatty acids in utero but fed breast milk free of trans-fatty acids experienced rapid early neonatal growth and had impaired insulin sensitivity as well as higher abdominal fat pad mass. In another animal

study, George et al. examined the effects of overfeeding and maternal weight gain on offspring growth and development in sheep (George et al., 2010). The authors found that at mid-gestation, fetal crown to rump length, thoracic and abdominal girths, and perinatal fat mass were all higher in the offspring of overweight and obese ewes compared to offspring of controls who gained normal weight. Additionally, fetal heart, pancreas, and liver weights were greater in the offspring of obese compared to overweight and control animals. Taken together, these data show that a variety of environmental exposures during pregnancy, in animals, can influence offspring characteristics related to metabolic health.

In addition to animal studies, a few human studies have focused on dietary alterations during pregnancy and influences on offspring. Khulan et al. (2012) studied umbilical cord blood of offspring born to Gambian mothers who were given micronutrient supplements during pregnancy (compared to controls given placebos). The authors found different methylation patterns in the groups in a large number of genes (108 in males, 106 in females), some of which are imprinted genes (Khulan et al., 2012). The imprinted genes, which are genes in which epigenetic mechanisms 'silence' either maternal or paternal expression of the gene, tended to be different not at birth but at 9 months of age, suggesting that these genes are not directly influenced by pregnancy micronutrient supplementation. Instead, the authors suggested that a small number of genes that are affected by supplementation might in turn affect a larger number of genes later in postnatal development. In another study, individuals prenatally exposed to famine during the Dutch Famine had less DNA methylation of the

IGF2 gene compared to siblings of the same sex, who were not exposed to the famine (Heijmans, et al., 2008). Low methylation of this gene, which is related to growth, should correlate to higher gene expression and thus more growth than in the unexposed sibling. These studies demonstrate that human offspring are affected by the maternal environment and that environmental stimuli such as diet may impact the newborn throughout his or her lifetime.

Though few studies have directly measured methylation levels in the DNA of experimental animals, evidence for the developmental programming of these animals is strong. When all else is held constant, maternal nutrition appears to alter fetal environment in such a way that the offspring experiences altered cellular growth. Further research is required to understand how this might occur in humans and what role interventions (i.e., physical activity, weight loss) might have in preventing the passage of adverse epigenetic modifications to the next generation. Not surprisingly, data supporting developmental programming suggests that the obesity epidemic is a complex one that will not be solved in one or two generations.

DNA Methylation is Heritable

Burdge and colleagues conducted the first study that specifically measured DNA methylation differences as a result of intergenerational transmission (2007).In this study, the grand-offspring of male rats who were fed a protein-restricted diet had lower methylation of glucocorticoid receptor (GR) and PPAR-α gene promoter regions, even though individual animals measured were all fed normal chow diets their entire lives (Burdge et al., 2007). In another

experiment, rodents were overexposed to glucocorticoids which affected both fetal and placental weights in two subsequent generations (Drake, Liu, Kerrigan, Meehan, & Seckl, 2011).

Gervin et al. studied heritability of DNA methylation in humans by comparing the similarities of monozygotic versus dizygotic twins. The authors found low heritability (2 to 16 percent), suggesting that the environment influences DNA methylation to a greater extent than do genetic factors (Gervin et al., 2012). In contrast, Dunn and Bale studied mice and demonstrated that body length and insulin sensitivity phenotypes were heritable across two generations (i.e., the "grandchildren" were affected by the diets of two generations previous) (Dunn & Bale, 2009). Though specific methylation patterns were not measured, studies of women who were pregnant or gave birth during or shortly after the Dutch Famine of 1944-1945 showed that some phenotypes were altered in offspring. The offspring (F1; the first generation of offspring of those directly exposed to a stimulus) of those exposed to the famine in utero (F0) had greater adiposity as infants, and poorer health later in life, compared to F1 generation whose mothers were not exposed to famine in utero (Painter, et al., 2008). However, prevalence of Metabolic Syndrome and cardiovascular disease was not affected, nor was birthweight. Timing of the exposure may also be important; birthweight was lower in the F1 generation if the mother was exposed to caloric deficit in the first or second trimester, but there was no difference in birthweight if the exposure came in the 3rd trimester (Lumey & Stein, 1997). Overall, DNA

methylation appears to be heritable in both humans and rodents, though the type and timing of exposure influence the outcome in terms of phenotype.

Factors that Influence DNA Methylation

Diet

Diet-induced cellular changes (in the mother or child) may also influence phenotypic variance among infants and children. Methyl groups come mainly through consumption in the diet, and these methyl groups are needed for DNA methylation, a heritable change in the chemical composition but not sequence of a gene (Choi, Corrocher, & Friso, 2009). It is plausible, then, that diet affects DNA methylation, which in turn affects gene expression and potentially alters the phenotype that is expressed. Evidence of this in humans in scarce, but animal models suggest it likely occurs (Dominguez-Salas, Cox, Prentice, Hennig, & Moore, 2012). In one of the few studies of epigenetic modifications in healthy human children, newborn methylation patterns were found to explain 25% of the variance in adiposity amongst 9 year olds. Some of these methylation pattern differences were related to lower maternal carbohydrate intake in early pregnancy (Godfrey et al., 2011). This is significant because the lower maternal carbohydrate metabolism was also associated with elevated adiposity in childhood. In a very recent study, Zhang and colleagues distributed self-administered questionnaires on diet and physical activity to 165 healthy subjects, ages 18-78 years. White blood cell global methylation patterns were not associated with any diet or physical activity variables except for dietary folate intake from fortified foods (Zhang et al., 2012).

Because there are so few data available, it is difficult to achieve consensus on the role of diet on human DNA methylation. Still, it appears that early pregnancy diet, particularly carbohydrate and folate intake, affects DNA methylation patterns in offspring. In animal models, maternal protein restriction during gestation has been associated with hypermethylation of total DNA in fetal liver (Rees, Hay, Brown, Antipatis, & Palmer, 2000) and also in PPAR-α promoter of the offspring's liver (Lillycrop et al., 2008) and X-receptor promoter (van Straten et al., 2010). A high fat diet resulted in altered methylation of the melanocortin-r receptor (Mc4r) in the brain tissue of two lines of mice (Widiker, Karst, Wagener, & Brockmann, 2010). This receptor has been implicated in weight regulation. Animal model studies have shown that maternal diet influences methylation of several different genes in a variety of tissues, and the alteration appears to be very specific to the stimulus. Further study is required to better understand the mother's diet in pregnancy and how it may affect her offspring not only at birth, but beyond.

Sex

Little is known about methylation pattern differences between the sexes, but one recent study found that, with the exception of imprinted genes, CpG sites studied were significantly more methylated in males compared to females (El-Maarri et al., 2007). The DNA for this study was isolated from blood samples of 96 healthy males and 96 healthy females. The authors selected another sample of 48 individuals from each gender to confirm their findings; they found very similar results. In another study (which was not designed to examine differences

between sexes, but the results stood out to the investigators), white blood cells were extracted from whole blood of 134 male and 157 female subjects (Sarter et al., 2005). They investigated methylation patterns on four autosomal genes, and males had higher levels of methylation compared to females in three of those genes (no difference in methylation in the fourth gene). It appears that males tend to have higher methylation levels than females and therefore, depending on the study design, it may be important to use subject sex as a covariate or to stratify by sex for analysis.

Race

Racial differences in methylation patterns also exist. African-American and Caucasian newborns have different methylation patterns in their cord blood DNA; 13.7% of CpG sites investigated (out of 26,000+ sites) were found differentially methylated according to race, while only 2% were differentially methylated according to gender (Adkins, Krushkal, Tylavsky, & Thomas, 2011). Another study found that global DNA methylation, measured as LINE-1 methylation in peripheral blood in adults, is lower in non-Hispanic blacks compared to non-Hispanic whites (F. F. Zhang, R. Cardarelli, J. Carroll, K. G. Fulda, et al., 2011). Like sex, race might be an important potential confounder to consider during design and analysis in DNA methylation studies.

Maternal Age

Another factor that has been found to be associated with different DNA methylation patterns at birth is maternal age. Methylation levels of umbilical cord blood samples at 144 CpG sites on 142 genes were significantly correlated to the

mother's age at birth, and a weaker correlation was found with paternal age; the direction of the correlation varied by gene but the majority of correlations were inverse (i.e., greater age was associated with lower methylation of the gene) (Adkins, Thomas, Tylavsky, & Krushkal, 2011). However, parity was not used as a covariate in the analysis and therefore it is not clear if the effect is the result of age or of accumulation of subsequent pregnancies. Assuming it is indeed an age effect, the clinical significance of this finding is not yet clear, however, and to our knowledge, no additional studies have investigated this relationship. Adkins et al. speculated that their findings might have something to do with the fact that greater maternal age is related to offspring risk of type 1 diabetes (Cardwell, Carson, & Patterson, 2005) and childhood cancers such as leukemia (Johnson et al., 2009; Maule et al., 2007; Podvin, Kuehn, Mueller, & Williams, 2006). Possibly, increased parental age results in methylation changes that are passed on to offspring and then contribute to disease predisposition, though the degree of speculation on this mechanism is great.

Body Mass Index (BMI)

Zhang and colleagues found no difference in peripheral blood DNA methylation in adults by BMI, waist circumference, fat deposition location, or percent body fat (F. F. Zhang, R. Cardarelli, J. Carroll, K. G. Fulda, et al., 2011). In another study of children, DNA methylation in peripheral blood (studied on a panel of 24 genes, with one to three sites per gene) was not associated with BMI, fat mass, or lean mass at average age 12.35 years, after controlling for multiple comparisons (Relton et al., 2012). In contrast, Godfrey et al. found that umbilical

cord blood DNA methylation at two gene promoters, retinoid-X receptor-α (RXRA) and endothelial nitric oxide synthase (eNOS), is associated with that child's adiposity at age nine years (Godfrey, et al., 2011). Most recently, Groom et al. found that tumor-associated calcium signal transducer 2 (TACSTD2) gene expression and methylation was associated with fat mass at age nine to 15 years, but they were not able to replicate the finding in a second cohort (Groom et al., 2012).

Variability in the findings of the relationships between BMI/body weight and DNA methylation speaks to the complexity of this topic. Some data are from children but the ages are varied, and some data are from adults. Two of these four studies found a relationship between DNA methylation at birth and later body composition or BMI, but the role of the mother's BMI and adiposity on her child's epigenetic profile was not assessed. To our knowledge, no data exists to address the effects of a mother's BMI or body fatness on the epigenetic profile of her offspring. Maternal physical activity is known to influence body weight and GWG, but how a woman's LTPA during pregnancy might alter the offspring epigenome has also not been studied. More research is needed to clarify these mixed results and provide further understanding of how maternal weight and body composition may contribute to the programming of offspring phenotypes.

Physical Activity

The beneficial effects of physical activity for pregnant women are fairly clear, but the effects on the developing fetus are less clear and not as well-studied. Hopkins and colleagues have recently published work demonstrating

that a moderate-intensity cycling program during the second half of pregnancy resulted in offspring who weighed less and were less fat at birth, though there was no effect on maternal insulin sensitivity (Hopkins, et al., 2010). The authors also found that serum IGF-I and IGF-II (both growth factors) were lower in the cord blood of the offspring of exercising mothers compared to controls, suggesting that hormonal stimulation of fetal growth was less in the exercise group. Mothers in this study were normal weight and healthy, but Hopkins et al. speculated that effects of maternal exercise on the offspring of overweight or obese women might be even more profound. To our knowledge, no other studies have examined the effects of maternal PA on offspring blood markers that may predict future offspring health. More specifically, no studies have been designed to examine the effects of maternal physical activity on epigenetic changes in humans. Though they did not assess gene expression or DNA methylation in their blood samples, the Hopkins et al. study findings, along with others, suggest that gestational physical activity influences fetal growth, and it is worth exploring the mechanism driving this occurrence (Hopkins, et al., 2010).

Metabolism-related Genes Subject to DNA Methylation

There is evidence that metabolic function in adulthood may, at least in some cases, be ‘programmed’ by exposures in utero (Gabory, Attig, & Junien, 2011). Because physical activity is a known regulator of metabolic function, it follows that physical activity might alter genes with known metabolic functions that have the potential to be regulated epigenetically. The following genes have

some function in metabolism and the rationale for studying each is expressed in the following paragraphs.

Proliferator-activated receptor-γ co-activator α (PGC1-α) and Peroxisome proliferator-activated receptor-γ (PPAR-γ)

PGC1-α is involved in glucose and fat oxidation and gluconeogenesis (in liver) and PPAR-γ is involved in adipogenesis and insulin signaling. Gemma and colleagues found a positive correlation between mother's BMI prior to pregnancy and methylation level of PGC1-α promoter, though they found no relationship between maternal BMI and PPAR-γ (Gemma et al., 2009). In another study, PGC1-α promoter was hypomethylated in a dose-dependent manner in adults after acute exercise (Barrès et al., 2012). No exercise component was included in the Gemma et al. study, yet physical activity is known to affect insulin signaling. PPAR-γ, then, may be epigenetically altered by physical activity. In a comparable study, subjects were monozygotic twins discordant for type 2 diabetes. The authors assessed methylation levels of a large number of candidate genes; there was a significantly higher methylation level in the skeletal muscle of the diabetic twin compared to the non-diabetic (Ribel-Madsen et al., 2012). Because physical activity is a well-known treatment for type 2 diabetes, methylation levels of this gene might be influenced by physical activity behavior. Whether this effect is passed on to offspring is unknown.

Insulin-like growth factor 2 (IGF2)

IGF2 is a maternally imprinted gene, meaning that the maternal copy of the gene is silenced during embryonic development (via DNA methylation), and

only the paternal copy is expressed in the normal human phenotype. Beckwith-Wiedemann Syndrome is a condition in which silencing of the maternal IGF2 gene copy fails, and the primary phenotypic outcome is overgrowth. Because IGF2 is related to human growth and known to be epigenetically regulated, this gene has been well-studied in the DNA methylation literature.

Some maternal attributes have been linked to IGF2 methylation in offspring. Folate supplementation after twelve weeks of pregnancy was found to be associated with higher IGF2 methylation in both maternal red blood cells and offspring cord blood at delivery (Haggarty et al., 2013). Circulating level of IGF2 in maternal circulation during the third trimester of pregnancy was associated with placental IGF2 methylation, and placental methylation status was also associated with birth weight (St-Pierre et al., 2012). In another study, cord blood IGF2 methylation was associated with IGF2 protein concentration, particularly in the offspring of obese women (Hoyo et al., 2012). Together, these data suggest that maternal characteristics such as weight status and diet may influence methylation of the IGF2 gene and in turn, offspring growth. It is plausible then, that maternal physical activity might influence IGF2 methylation as well.

Pyruvate dehydrogenase kinase 4 (PDK4)

Another candidate gene is pyruvate dehydrogenase kinase 4 (PDK4).Methylation levels of this gene promoter were lower in Type 2 Diabetes patients compared to non-diabetic controls, and physical activity intervention (increase of 5 hours per week of activity) resulted in elevated mRNA expression of PDK4 in individuals with normal glucose tolerance (Kulkarni et al., 2012).

Acute exercise also resulted in decreased PDK4 methylation in non-pregnant adults (Barrès, et al., 2012), but this gene has not been studied in terms of maternal transmission of methylation to the offspring. There may be differences, then, in methylation levels in non-pregnant individuals who exercise (chronically) compared to those who don't, and this effect may extend to the offspring of exercising pregnant women.

Transcription factor 7-like 2 (TCF7L2)

Six CpG sites in this gene were found to have altered methylation levels in adipose tissue, in humans, following a six month exercise intervention (Rönn et al., 2013). Five of the six genes had increased methylation in response to the intervention while the other had decreased methylation. This gene has been associated with Type 2 Diabetes; in a recent study TCF7L2 methylation of pancreatic islet cells was associated with the TCF7L2 single nucleotide polymorphism (SNP) genotype, suggesting that the gene regulates islet cell function to some extent (Dayeh et al., 2013). The combined role of this gene in etiology of diabetes and its exercise-induced alteration in the Ronn study suggest it is a reasonable candidate gene for this study.

Summary

In summary, physical activity during pregnancy has known benefits to both mother and offspring, though the effects on the offspring in particular are less well understood. Fetal programming occurs when an exposure in the mother is passed along to the fetus and influences the offspring phenotype; this programming can be either advantageous or predisposing for the offspring. This

dissertation will a) seek to identify if and how the specific exposure of maternal physical activity programs the fetus and b) determine if metabolism-related candidate genes or global DNA methylation is affected.

METHODS

Study Design

We used a nested exposure-control design for this study, and selected our participants from an ongoing cohort study described in detail in the Study Population section below. Three groups of women were selected: one group was highly active, one was considerably less active and was matched to the high active group on maternal age, race, and BMI, and one was also low active but was matched to the high active group on maternal age and race only. We matched infant blood spots taken at birth to the study subjects and used the spots to compare total global methylation as well as candidate gene methylation among the groups.

Study Population

The Michigan State University Department of Epidemiology & Biostatistics has, in collaboration with the College of Human Medicine, been operating the ARCH Study since 2007. Questionnaire data were collected on pregnant women at a mean gestational age of 12.8 weeks, and a copy of the questionnaire is included in Appendix I. Basic demographic data were collected at enrollment: name, date of birth, social security number, race, ethnicity, education level, marital status, household income, home and car ownership, ownership of stocks/bonds, height, pre-pregnancy weight, and whether pregnancy was planned.

ARCH participants are recruited from one of three mid-Michigan clinics that provide prenatal health care to primarily low-income pregnant women. After

enrollment, the women and their offspring are followed longitudinally. We opted to use only subjects enrolled since 2010 due to the need for infant blood spots (described in more detail later) as our biologic sample. Blood spots maintained by the State of Michigan have been stored frozen since 2010. In addition to demographic information, we have collected prospective physical activity data on more than 300 women. Study participants were queried regarding their participation in moderate and vigorous LTPA. Those who responded in the affirmative provided additional information including days per week and minutes per day. The LTPA questionnaire completed by participants is included in the Appendix I. Specifically, moderate physical activity was defined as an activity that caused a small increase in breathing and heart rate, and vigorous activity was any that caused a large increase in breathing and resulted in sweating.

On average, the most active subjects in the ARCH data set, excluding extreme outliers, reported 1,056 minutes of moderate or vigorous physical activity per week (~150 minutes per day). This represents roughly seven times the minimum amount of LTPA recommended in the 2008 Physical Activity Guidelines for Americans (Physical Activity Guidelines for Americans, 2008), and suggests that these women are very dissimilar to low active participants, with respect to physical activity behaviors. Women who reported participating in more than 2,500 minutes per week (approximately 6 hours per day) of moderate or vigorous LTPA were not included in this analysis due to our concerns about reporting accuracy. We were unable to use all of the most active subjects (for

whom the average LTPA was greater than 1,000 minutes per week in the analysis) due to consent issues and the need for subject matching.

Subject Matching

We contacted prospective subjects by phone to request verbal consent to utilize infant blood spots for our study. The script for consent phone calls is included in the Appendix II. Phone numbers were available for approximately 150 subjects who had completed the physical activity portion of the questionnaire. We called all subjects and after excluding those who did not answer despite multiple attempts, had disconnected phone lines, or who refused, we obtained consent from a total of 59 subjects. From these, we selected the most active subjects as the 'active' group and matched on maternal BMI (for BMI group only), age, and race as closely as possible to each individual 'active' woman. We did not use pre-defined criteria for matching due to the limited (n = 59) number of subjects from whom we could choose. In order to ensure the largest matched sample possible, we matched as closely as we could for each variable. For instance, we matched a high active woman of age 30 and BMI of 25 to a control with as similar an age as we could find who had a BMI within a range we deemed reasonably close. The average difference for age between the high active and combined low active group was 6.0 years (range was 0.1 to 14.4 years) and the average BMI difference for the BMI matched low active was 5.4 (range was 0.0 to 17.3). The average BMI difference for the unmatched low active group compared to the high active group was 10.8 (range was 3.5 to 21.0). Some high active women were matched to women with a higher BMI and some were matched to women with

lower BMI, and the same was true for age. In total, 42 subjects were included in the study.

We selected the 14 most active subjects in the database for whom we were able to identify a similar control and from whom we received consent to participate in the study. Another consideration in choosing the 14 most active women was how recently they participated in the ARCH study; we sought to select women who participated in the study within the last three years in hopes of being more successful at contacting them for verbal consent to release their infant's blood spot for analysis.

The 14 most physically active women (termed 'high active') were individually matched on maternal age and race/ethnicity to 14 low active controls (i.e. each active woman was matched to a single control similar in age and race/ethnicity). Matching criteria were selected because these factors are known to alter DNA methylation (Adkins, Krushkal, et al., 2011; Adkins, Thomas, et al., 2011; Borghol et al., 2012; El-Maarri, et al., 2007; Gemma, et al., 2009; Michels, Harris, & Barault, 2011). Other research indicates that maternal smoking is a strong predictor of DNA methylation (Knopik, Maccani, Francazio, & McGeary, 2012) so we attempted to exclude women who indicated that they smoked during pregnancy. Due to the difficulty of matching subjects, we did include a total of five smokers, but the number of smokers is not different across the three comparison groups. Previous research also suggests that maternal BMI influences DNA methylation of candidate genes (Gemma, et al., 2009; Liu et al., 2014) but not global methylation (Michels, et al., 2011) so we opted to select a third group of 14

subjects who were individually matched to the 14 high active women for age, race/ethnicity, and BMI. Thus, our total sample included 42 subjects (3 groups x 14 subjects per group). The purpose of the BMI match group was to distinguish the effects of physical activity, which has a known role in weight management, from BMI itself. Once we calculated group means for BMI, however, we found that the three groups had very similar BMI values (26.5 ± 6.0 for high active, 26.3 ± 5.5 for low active BMI match, 26.9 ± 9.4 for low active), and therefore we opted to analyze the data as high active (n = 14) compared to low active (n = 28), which was the combination of the BMI matched and unmatched low active groups. In addition, we know that other potential confounders are related to DNA methylation. Due to limitations with the total sample size, we were unable to match for baby's birth weight, and due to known difficulty with dietary recall, particularly several years after the time frame of interest, we did not assess or match on mother's diet during pregnancy despite evidence that these covariates might influence DNA methylation (Haggarty, Hoad, Campbell, et al., 2013; Lan et al., 2013; Michels, et al., 2011). DNA methylation also varies according to gender (El-Maarri, et al., 2007), but because there is no evidence to suggest that maternal physical activity influences the baby's gender, we did not match on this variable. Finally, we opted not to match on socioeconomic status (SES) despite its relationship with DNA methylation (Borghol, et al., 2012) because the ARCH cohort is fairly homogeneous in this variable, as subjects are recruited from clinics that serve primarily low-income women. We did, however, analyze demographic information between the two groups and found no statistically

significant differences in any of these variable including baby's gender, mother's household income, mother's education (trend toward statistical significance was observed), prevalence of maternal smoking during pregnancy, mother's marital status, or birthweight (see Table 3).

Sample Size

While sample size is small, a power analysis for this proposal was not computed because no human data of a similar nature have been published to date. However, others have performed similar studies using small samples. For example, Aagaard-Tillery et al. (Aagaard-Tillery et al., 2008) found that in age and weight matched adult female Japanese macaques, a high fat diet resulted in an alteration of the fetal chromatin structure. The authors compared 10 experimental primates with 9 controls, similar to our proposed sample size. Thus, we believed that an exploratory analysis of 14 active and 28 controls would provide pilot data so that future studies can use our resulting effect sizes to design studies to answer more specific research questions.

Identification numbers of the 42 selected subjects (14 high active matched with 28 low active controls) were matched to subject names by the study investigator so that blood spots for their infants could be requested from the Michigan Neonatal Biobank. The Biobank was able to retrieve all 42 of our requested spots; some were stored at the Detroit location and others at the Lansing location. Though samples that have been in storage since 2010 are frozen, we received four, three mm punches from each blood spot that had been thawed. Samples were mailed overnight from the Biobank. DNA was extracted

using the QIAamp DNA Micro Kit (Qiagen) according to manufacturer recommendations. Upon extraction, DNA was stored at -20 degrees Celsius.

Laboratory Analysis

Aim 1

Our first aim was to determine whether there are differences in global DNA methylation in the offspring of physically active compared to low active pregnant women. After extraction, DNA was quantified via spectrophotometry (NanoDrop). Next, we examined total 5-methyl cytosine content by using a colorimetric ELISA-like assay called MethylFlash (Epigentek, Farmingdale, NY),which assesses total content of 5-methyl cytosine in the genome using a standard curve metric. The assay includes positive controls for dilution into a standard curve as well as a negative control.

In addition to the MethylFlash assay, we sent isolated genomic DNA to a commercial laboratory (EpigenDX, Worcester, MA) where they conducted pyrosequencing analysis for LINE-1 methylation. We assayed four regions of repetitive elements throughout the genome; methylation of these long regions of repetitive DNA stretches are commonly used as indicators of methylation of the entire genome (Haggarty, Hoad, Campbell, et al., 2013; Michels, et al., 2011; A. J. White et al., 2013). Methylation of these repetitive elements is similar between mother (peripheral blood) and her newborn (umbilical cord blood); thus we believe that differences observed between offspring of physically active women versus low active can be attributed to maternal exposures (Kile et al., 2010).

Aim 2

Our secondary aim was to evaluate gene-specific DNA methylation differences in newborn dried blood spot samples of infants whose mothers reported high LTPA levels during pregnancy compared to infants of low active mothers, either matched or unmatched for BMI. We selected five candidate genes that have been shown to be altered epigenetically by exercise/physical activity or that, because of their function in metabolism, might likely be affected. None had been studied previously in terms of maternal transmission of the methylation pattern to the offspring.

PGC1-α and PPAR-γ are genes involved in metabolism of glucose and fat. PDK4 gene expression is affected by physical activity, and IGF2 is an imprinted gene that has been frequently associated with fetal growth. TCF7L2 is a gene with a known role in etiology of Type 2 Diabetes. Table 1 shows the descriptions of each candidate gene and the regions within these genes that were assessed. These genes are described in greater detail in the literature review portion of this dissertation (Chapter 2).

To assess methylation levels of selected candidate genes, we sent isolated genomic DNA to EpigenDX (Worchester, MA), a commercial laboratory specializing in methylation analyses. Upon receipt of DNA samples, EpigenDX treated samples with bisulfite and used polymerase chain reaction (PCR) to amplify the bisulfite treated DNA. Primers used for the PCR reactions are considered proprietary information by EpigenDX and thus are unavailable for publication. Cycling conditions are included in the Appendix III. Pyrosequencing

was then used to sequence the regions of candidate genes shown in Table 1; these conditions are also presented in Appendix III. Each pyrosequencing assay has been validated by EpigenDX by use of low, medium, and highly methylated DNA as well as a no template control. Results from each analysis are a percentage of methylated DNA at the region of interest.

It is important to note that to our knowledge, no studies have examined how a mother's LTPA might influence methylation of offspring cells in any tissue. Thus, this dissertation research should be considered a pilot project designed to generate hypotheses for future studies in the area of pregnancy physical activity and influences on the offspring's metabolic health.

Statistical Analysis

Descriptive characteristics of the sample are reported for the high active and both low active groups in Table 2. These data are presented as means ± standard deviations. Methylation data results are provided as % methylation of the genome (for global assays in Aim 1) or a given gene (Aim 2). No covariates were included in the analysis due to the matching design.

We checked for normality of LINE-1 data using the Kolmogorov-Smirnoff test. The LINE-1 data were normally distributed and therefore analysis of variance (ANOVA) was used to assess methylation differences between groups. Finally, we calculated a Spearman correlation coefficient to assess agreement between the LINE-1 data and minutes per week MVPA within the total sample (n=42).

We checked all candidate gene data for normality using Kolmogorov-Smirnoff tests and found that none of the data met criteria for normal distributions ($p < 0.05$). All data analyses, then, were performed using non-parametric tests. We compared the differences in percent methylation between groups, for each gene, using Mann Whitney U tests. Additionally we evaluated Spearman correlations for each mean methylation value for each gene region to number of minutes of MVPA per week within the total sample (n=42).

Table 1: Candidate Gene Descriptions. Description of each candidate gene region assessed for percent methylation in this dissertation.

Gene	Description	PCR Size	# CpGs
PPARγ	Promoter	132 bp	14
PPARγ	Intron 2	281 bp	3
PGC1α	Proximal promoter	238 bp	3
PGC1α	Exon 1 to intron 1	174 bp	4
IGF2	P2 promoter	271 bp	8
IGF2	P4 promoter	130 bp	5
IGF2	Distal promoter	270 bp	3
PDK4	Promoter	149 bp	7
TCF7L2	5'UTR	150 bp	2
TCF7L2	5'UTR to promoter	150 bp	5

RESULTS

Sample

A total of 42 subjects were included in the analyses. Of these, 14 were classified as 'high active' and reported 360 to 1,020 minutes per week of MVPA, with mean participation in MVPA of 637.5 minutes per week. We planned originally to use a three group design with 14 low active subjects matched for BMI, race, and maternal age and another 14 low active subjects matched for race and age only. However, there were no differences in BMI among the low active groups matched and unmatched for BMI (see Table 2), so we opted to group these 28 subjects into one group to increase statistical power. The expanded low active group reported an average of 59.46 minutes per week MVPA.

Table 3 shows the sample characteristics for the two groups. As expected, MVPA was significantly higher in the high active group compared to either low active group ($p = 0.001$). There were no differences in maternal BMI, maternal age, or infant birth weight between groups. Also, we found no difference in the percentage of white participants, male offspring, smoking prevalence, income, marital status, or planned pregnancies between groups. While not statistically significant ($p = 0.060$), the active group was more highly educated than the low active group.

Table 2: Mean MVPA and BMI by the Three Originally Planned Groups.
Mean ± standard deviation plus ranges are presented for MVPA min/week and means ± standard deviation are presented for Maternal BMI.

	High Active (n=14)	**Low Active – BMI Match (n=14)**	**Low Active (n=14)**
MVPA/week	637.5 ± 220.7** (360 – 1020)	80.7 ± 93.9 (0 – 285)	38.2 ± 60.6 (0 – 180)
Maternal BMI	26.5 ± 6.0	25.8.3 ± 5.1	27.4 ± 9.5

Table 3: Descriptive Characteristics for Two Groups. Means ± standard deviations for MVPA, age, BMI, and birth weight were compared between two groups using analysis of variance (ANOVA). Mean percentages smoking were compared using chi-square tests. For categorical variables, data are presented as % (n). *indicates significant difference between high active group and low active group

	High Active (n=14)	**Low Active (n=28)**	**P-value**
MVPA per week	637.5 ± 220.7	59.5 ± 79.1	*0.000
Maternal age (years)	26.4 ± 8.2	27.4 ± 5.2	0.562
Maternal BMI	26.5 ± 6.0	26.6 ± 7.4	0.971
Infant Birth Weight (grams)	3247 ± 419	3409 ± 470	0.280
Maternal race (% white)	92.9 (13)	71.4 (20)	0.111
Sex (% male)	57.1 (8)	57.1 (16)	1.000
Smoking (% reported)	14.3 (2)	10.7 (3)	0.736
Income (% <$25,000)	57.1 (8)	39.3 (11)	0.273
Marital Status (% unmarried)	24.1 (3)	21.4 (6)	1.000
Marital Status (% married/living with baby's father)	35.7 (5)	39.3 (11)	0.822
Education (% attended at least some college)	85.7 (12)	64.3 (18)	0.060
Planned Pregnancy (% unplanned)	50.0 (7)	53.6 (15)	0.827

Aim 1

We used the MethylFlash assay to assess percent global methylation. We successfully ran a practice assay with a standard curve equation of y = 17.03x – 0.958. The R-squared value for the line of best fit was 0.99. While this initial standard curve was acceptable, we were unable to replicate a useful standard curve on subsequent assays when samples were analyzed. Two attempts were made to use the kit but were unsuccessful; we did not have enough isolated DNA to attempt a third assay. Therefore, no data from the MethylFlash assay are shown.

In addition to the conducting the MethylFlash assay, we also had pyrosequencing for LINE-1 performed, in order to estimate global DNA methylation. We used a Kolmogorov-Smirnoff test to determine that the LINE-1 data was normally distributed. Results from this assay are presented in Table 4. There were no differences between groups for LINE-1 percent methylation. We also assessed the relationship between MVPA minutes per week and LINE-1 percent methylation, but found no significant relationship based on Spearman correlation coefficients (data not shown).

Table 4: LINE-1 Methylation. Percent methylation for LINE-1 at four different CpG sites plus the overall mean methylation of LINE-1 for two groups. Data are presented as mean ± standard deviation. Significance was set at an alpha level of P<0.05.

LINE-1	CpG	High Active	Low Active	P-value
	CpG 1	84.57 ± 2.97	85.06 ± 2.85	0.602
	CpG 2	77.86 ± 1.23	78.21 ± 1.62	0.482
	CpG 3	75.09 ± 2.24	75.66 ± 2.05	0.415
	Cpg 4	71.46 ± 2.36	71.23 ± 1.96	0.738
	Mean	77.25 ± 1.44	77.54 ± 1.58	0.558

Aim 2

Five candidate genes were analyzed for percent methylation at various CpG sites. Table 5 shows data for PPARγ, a gene located on chromosome 3. Two gene regions were analyzed; the first region, in the promoter, contains 14 CpG sites and the second region, in intron 2, contains three. We found no differences in percent methylation at any site within the gene. Data for PGC1-α, on chromosome 4, is presented in Table 6; two regions were examined via pyrosequencing. One region, in the proximal promoter, has three CpG sites and the other, covering an area ranging from exon one to intron one has four. We found no differences in percent methylation in this gene.

Results for IGF2, on chromosome 11, are presented in Table 7. We analyzed three regions of interest within the gene: the first region we examined is in the P2 promoter and has eight CpGs, another is in the P4 promoter with five CpGs, and the last is in the distal promoter with three CpGs studied. There were two sites within the P2 promoter that were significantly different between the two groups; for CpG 3 and 4, percent methylation was higher in the low active group compared to high active. Although not significantly different, it is also worth noting that we found the same directional effect (low active group had higher methylation than high active group) for CpGs 1 and 4 through 6 for the P2 promoter for IGF2. We found no differences between physical activity groups for PDK4, a gene located on chromosome seven (see Table 8). The analysis for this gene was conducted on a region of the promoter that included seven CpG sites.

Table 9 shows methylation for TCF7L2. Seven total CpGs were studied and there were no differences between groups at any site.

We calculated total percent methylation for each gene region (two regions for PPARγ, three for IGF2, two for PGCA-α, one for PDK4, two for TCF7L2) in order to compare average methylation between groups. Data are presented in Table 10. The only statistically significant difference was in the P2 promoter for IGF2. We found that the low active group had a significantly higher percent methylation than the high active group ($p = 0.045$).

Correlations with Physical Activity

We calculated Spearman correlation coefficients to assess the relationship between total MVPA per week and percent methylation of each gene region. These data are shown in Table 11. No significant correlations were found.

Table 5: PPARγ Methylation. Percent methylation for PPARγ at each CpG site (column 2) within each studied gene region (column 1). Data are presented in mean ± standard deviation for each group. P-values are presented (alpha = 0.05).

PPARγ	CpG	High Active	Low Active	P-value
Promoter	CpG 1	0.72 ± 0.58	0.58 ± 0.57	0.320
Promoter	CpG 2	1.11 ± 0.60	0.87 ± 0.60	0.468
Promoter	CpG 3	0.79 ± 0.53	0.60 ± 0.63	0.143
Promoter	CpG 4	0.57 ± 0.65	0.54 ± 0.58	0.966
Promoter	CpG 5	0.53 ± 0.87	0.60 ± 0.70	0.506
Promoter	CpG 6	0.73 ± 0.85	0.82 ± 0.71	0.581
Promoter	CpG 7	1.02 ± 0.71	1.13 ± 0.70	0.809
Promoter	CpG 8	1.55 ± 0.24	1.41 ± 0.71	0.739
Promoter	CpG 9	0.64 ± 0.89	0.54 ± 0.68	0.976
Promoter	CpG 10	0.44 ± 0.47	0.30 ± 0.47	0.265
Promoter	CpG 11	0.33 ± 0.46	0.44 ± 0.58	0.561
Promoter	CpG 12	0.71 ± 0.77	0.74 ± 0.78	0.732
Promoter	CpG 13	0.46 ± 0.64	0.18 ± 0.46	0.118
Promoter	CpG 14	0.64 ± 0.96	0.25 ± 0.63	0.137
Intron 2	Cpg 1	75.47 ± 8.80	76.03 ± 7.77	0.749

Table 5 (cont'd).

Intron 2	Cpg 2	62.13 ± 6.92	62.87 ± 4.79	0.423
Intron 2	Cpg 3	57.84 ± 12.66	59.49 ± 3.60	0.423

Table 6: PGC1-α Methylation. Percent methylation for PGC-1α at each CpG site (column 2) within each studied gene region (column 1). Data are presented in mean ± standard deviation for each of the three groups. P-values are presented (alpha = 0.05).

PGC1-α	CpG	High Active	Low Active	P-value
Proximal promoter	CpG 1	0.69 ± 1.04	0.72 ± 1.20	0.861
Proximal promoter	Cpg 2	0.59 ± 0.84	0.17 ± 0.44	0.069
Proximal promoter	CpG 3	1.81 ± 1.77	2.09 ± 1.83	0.619
Exon 1 to intron 1	CpG 1	3.22 ± 1.65	2.79 ± 1.58	0.416
Exon 1 to intron 1	Cpg 2	4.85 ± 1.52	5.26 ± 2.14	0.531
Exon 1 to intron 1	CpG 3	7.26 ± 2.85	7.45 ± 2.71	0.622
Exon 1 to intron 1	Cpg 4	7.50 ± 1.92	6.81 ± 1.84	0.364

Table 7: IGF2 Methylation. Percent methylation for IGF2 at each CpG site (column 2) within each studied gene region (column 1). Data are presented in mean ± standard deviation for each group. P-values are presented (alpha = 0.05). *indicates significant difference between the two groups

IGF2	CpG	High Active	Low Active	P-value
P2 promoter	CpG 1	73.30 ± 4.84	74.66 ± 4.32	0.431
P2 promoter	CpG 2	87.22 ± 5.24	86.29 ± 4.35	0.393
P2 promoter	CpG 3	52.98 ±6.67	57.95 ± 5.61	*0.020
P2 promoter	CpG 4	69.86 ± 3.95	72.35± 2.89	*0.047
P2 promoter	CpG 5	80.78 ± 3.82	82.23 ± 2.63	0.182
P2 promoter	CpG 6	39.87 ± 3.76	41.68 ± 3.71	0.165
P2 promoter	CpG 7	98.19 ± 2.41	98.00 ± 2.23	0.581
P2 promoter	CpG 8	60.05 ± 2.23	60.77 ± 1.75	0.423
P4 promoter	Cpg1	1.42 ± 1.49	2.10 ± 4.97	0.540
P4 promoter	CpG 2	1.91 ± 5.24	1.86 ± 3.91	0.604
P4 promoter	CpG 3	0.30 ± 1.11	1.39 ± 3.67	0.137
P4 promoter	CpG 4	1.45 ± 2.47	1.43 ± 3.64	0.370
P4 promoter	CpG 5	0.21 ± 0.78	0.86 ± 2.61	0.477

Table 8: PDK4 Methylation. Percent methylation for PDK4 at each CpG site (column 2) within each studied gene region (column 1). Data are presented in mean ± standard deviation for each group. P-values are presented (alpha = 0.05).

PDK4	CpG	High Active	Low Active	P-value
Promoter	CpG 1	1.94 ± 1.51	1.09 ± 1.12	0.086
Promoter	CpG 3	1.16 ± 1.24	1.02 ±1.37	0.668
Promoter	CpG 4	0.14 ± 0.54	0.16 ± 0.60	0.952
Promoter	CpG 5	1.02 ± 1.40	1.07 ± 1.32	0.906
Promoter	CpG 6	0.41 ± 0.82	0.85 ± 1.20	0.234
Promoter	CpG 7	1.16 ± 1.26	0.62 ± 1.10	0.181

Table 9: TCF7L2 Methylation. Percent methylation for TCF7L2 at each CpG site (column 2) within each studied gene region (column 1). Data are presented as mean ± standard deviation for each group. P-values are presented (alpha = 0.05).

TCF7L2	CpG	High Active	Low Active	P-value
5'UTR	CpG 1	0.00 ± 0.00	0.00 ± 0.00	1.000
5'UTR	CpG 2	0.08 ± 0.31	0.06 ± 0.31	0.638
5'UTR to Promoter	CpG 1	0.09 ± 0.34	0.00 ± 0.00	0.157
5'UTR to Promoter	CpG 2	0.35 ± 0.70	0.08 ± 0.44	0.079
5'UTR to Promoter	Cpg 3	0.00 ± 0.00	0.00 ± 0.00	1.000
5'UTR to Promoter	CpG 4	0.12 ± 0.44	0.00 ± 0.00	0.157
5'UTR to Promoter	CpG 5	0.00 ± 0.00	0.00 ± 0.00	1.000

Table 10: Candidate Gene Mean Methylation. Percent methylation across all gene regions within 5 candidate genes. Data are presented in mean ± standard deviation for each region of the genes. P-values are presented (alpha = 0.05). *indicates significantly difference between the two groups.

Gene	Gene Region	High Active	Low Active	P-value
PPARγ	Promoter	0.73 ± 0.35	0.64 ± 0.31	0.463
	Intron 2	65.15 ± 7.29	66.13 ± 3.94	0.729
IGF2	P2 Promoter	72.83 ± 2.85	74.70 ± 2.25	*0.045

Table 10 (cont'd).

	P4 Promoter	1.06 ± 1.29	1.53 ± 2.43	0.724
	Distal Promoter	55.31 ± 9.12	52.48 ± 6.14	0.483
PGC1α	Proximal Promoter	1.03 ± 0.96	1.16 ± 0.94	0.947
	Exon 1 to Intron 1	5.71 ± 1.12	5.61 ± 1.38	0.683
PDK4	Promoter	0.85 ± 0.58	0.69 ± 0.61	0.445
TCF7L2	5'UTR	0.04 ± 0.16	0.03 ± 0.15	0.638
	5'UTR to Promoter	0.11 ± 0.24	0.02 ± 0.09	0.063

Table 11: Correlation Coefficients for LTPA and Candidate Genes.
Spearman correlation coefficients between gene regions and MVPA per week. P-values are presented (alpha = 0.05).

Gene	Gene Region	Correlation Coefficient	P-value
PPARγ	Promoter	0.089	0.574
	Intron 2	-0.008	0.958
IGF2	P2 Promoter	-0.176	0.266
	P4 Promoter	-0.057	0.720
	Distal Promoter	0.165	0.302
PGC1α	Proximal Promoter	-0.060	0.706
	Exon 1 to Intron 1	0.005	0.973
PDK4	Promoter	0.110	0.489
TCF7L2	5'UTR	0.058	0.713
	5'UTR to Promoter	0.155	0.327

DISCUSSION

The Developmental Origins of Adult Disease Hypothesis, sometimes termed the Barker Hypothesis, says that events and exposures that occur during fetal development affect health later in life. Though the exact mechanisms are not understood, there is ample evidence that this phenomenon does occur, at least to some extent, in humans (Burdge & Lillycrop, 2010a). The stimuli that elicit these changes that later affect disease risk are not clear. For instance, the Dutch Famine Study provides strong evidence that maternal diet during pregnancy, particularly caloric restriction, impacts offspring metabolic health (de Rooij, et al., 2006; Heijmans, et al., 2008; Painter, et al., 2008). Yet the effect of physical activity during pregnancy has been studied sparsely with respect to the Barker Hypothesis. Therefore, we designed our study to assess the effects of maternal LTPA during pregnancy on one known mechanism of fetal programming, called DNA methylation. We examined methylation throughout the genome (global methylation) as well as methylation of five candidate genes known to either be impacted (though not necessarily in a pregnant population) by physical activity, or with a known role in metabolism. This work is novel in that it is the first to examine the effects of pregnancy physical activity on DNA methylation and its potential transfer of methylation patterns to offspring in humans.

Our results suggest that there is no effect of physical activity during pregnancy on offspring global DNA methylation. To our knowledge, this is the first study to examine this topic in humans. Others have examined the role of

chronic physical activity on global methylation in non-pregnant populations. White and colleagues found that in adult women, higher physical activity levels were associated with higher levels of LINE-1 methylation in peripheral blood samples (A. J. White, et al., 2013). Sample size was n = 647 and the median physical activity level in the study was 12.5 hours per week. The authors adjusted for age, smoking, alcohol consumption, and BMI but none of the variables affected the statistical model. Another group found the same positive relationship between DNA methylation and physical activity in 165 subjects, but the relationship was attenuated after adjustment for confounders including race, gender, and age (F. F. Zhang, R. Cardarelli, J. Carroll, S. Zhang, et al., 2011). However, physical activity levels even in the most active group were low, with the most active group participating in no more than 30 minutes per day of physical activity. In Swedish older adults (n = 1,016, all age 70 years), however, physical activity was associated with lower levels of methylation (Luttropp, Nordfors, Ekström, & Lind, 2013). There was no adjustment for confounders. All three of these studies used blood samples from the individuals actually participating in the physical activity, whereas we examined the role of a mother's LTPA on her *offspring's* global methylation. We found no other studies of global methylation being transferred to offspring as a result of maternal LTPA, though the effects of adverse pregnancy conditions including gestational diabetes and preeclampsia have been shown to be related to lower placenta DNA methylation in offspring (Nomura et al., 2014). It seems plausible that some stimuli may certainly impact offspring DNA methylation but it is not clear from our one study, given the lack of other data on

the topic, whether this might occur with LTPA during pregnancy. Further research is needed to clarify this question.

For our candidate gene analysis, we hypothesized that the active group would have lower DNA methylation levels for PPARγ, PGC1-α, and PDK4while the active group would have the highest DNA methylation levels for IGF2 and TCF7L2. PPARγ and PGC1-α both have roles in metabolism and there is a known relationship between high BMI and higher PGC1-α methylation (Gemma, et al., 2009). PPARγ methylation is higher in diabetic versus non-diabetic twin pairs (Ribel-Madsen, et al., 2012), suggesting that high PPARγ methylation is not a 'good' thing. Methylation of PDK4 is also lower in diabetics compared to non-diabetics (Kulkarni, et al., 2012) and acute exercise resulted in an decrease in PDK4 and PGC1-α methylation (Barrès, et al., 2012). For all three of these genes, data suggest that high methylation represents a 'bad' phenotype. However, for IGF2 and TCF7L2, the opposite appears to be true. Higher IGF2 methylation is associated with gene silencing, which would turn off production of IGF2 protein, associated with growth. We found two studies examining the relationship between birth weight and IGF2 methylation; one found no relationship (Burris et al., 2013) and the other found that higher methylation was related to higher weight, within the normal birth weight range (St-Pierre, et al., 2012). Finally, TCF7L2 is a gene with a known relationship to Type 2 Diabetes. A six-month exercise intervention resulted in an increase in methylation in five of six studied sites within the gene (Rönn, et al., 2013). Thus it seems that for these two genes, high methylation levels represent a 'good' phenotype.

Despite previous results that helped formulate our hypotheses, we found few differences in DNA methylation among groups, and those we did find were opposite of what was expected. We found no influence of physical activity for methylation of PPARγ, PGC1-α, PDK4, or TCF7L2. There were two CpG sites in the P2 promoter region of IGF2 that did have different methylation levels. However, it was the low active group that had the higher DNA methylation levels, which was the opposite of what we expected to find.

Similar to our findings, Gemma and colleagues found no differences in PPARγ methylation among SGA, LGA and average for gestational age (AGA) infants; in fact, the promoter was nearly completely methylated in all of their samples (Gemma, et al., 2009). However, the investigators used umbilical cord blood as their biologic sample rather than peripheral blood. In the same study, infant DNA methylation of PGC1-α was also studied and was significantly correlated to maternal BMI. We were not able to replicate this finding in the present study.

Several exercise studies have been designed to examine PGC1-α methylation in lab animals, but data were collected using maternal, not offspring samples. In mice, maternal exercise during pregnancy prevented increased PGC1-α methylation associated with high-fat diet (Laker et al., 2014) and promoter DNA methylation decreased with a bout of acute exercise in a mouse study (Barrès, et al., 2012). In humans, an exercise intervention resulted in decreased PGC1-α methylation in skeletal muscle (Nitert et al., 2012) but not in adipose tissue (Rönn, et al., 2013). In another skeletal muscle biopsy study, bed

rest resulted in increased PGC1-α methylation and decreased gene expression (Alibegovic et al., 2010). Taken together, these data suggest that low levels of PGC1-α methylation represent a 'good' phenotype with possible better metabolic health. We did not see this effect in offspring of high active versus low active women, which could be due to our choice of biologic sample (infant peripheral blood taken at birth) or the lack of transfer of the methylation profile, at least for this gene, from mother to child.

Unlike PPARγ and PGC1-α, we expected the offspring of the active group to have the highest DNA methylation for IGF2. This is because IGF2 is involved in growth regulation, and we know that active women tend to have smaller babies (albeit within a healthy range) compared to sedentary women. High IGF2 methylation should, at least theoretically, result in less gene expression and therefore less growth. A study of folate supplementation in pregnancy resulted in higher methylation levels of IGF2 in offspring cord blood, suggesting that nutrition and perhaps other maternal lifestyle factors are influencing this gene's methylation status in offspring (Haggarty, Hoad, Campbell, et al., 2013). St-Pierre and colleagues found that placental DNA methylation was significantly correlated with infant birth size, but there was no association with mother's BMI (St-Pierre, et al., 2012). In another study, IGF2 methylation was shown to be inversely related to birth weight ($p = 0.052$) (Haggarty, Hoad, Horgan, & Campbell, 2013) and therefore we suspected that there might be birth weight differences between our groups. However, we found no significant difference in birth weights between high active and low active women (see Table 3).

IGF2 is a maternally imprinted gene, meaning that it is regulated by a process called imprinting, which involves silencing one parent copy of the gene via methylation. Because of the known regulation of this gene via methylation and its role in growth, we were surprised to find few effects of maternal physical activity on IGF2 methylation in offspring. But, we know of no studies that have examined the relationship between IGF2 methylation and exercise/physical activity, so it is not possible to directly compare our results to those of others. However, Hopkins et al. found that exercise training in pregnancy was not related to maternal serum IGF1 or IGF2 concentrations, which may mean that exercise is an insufficient stimulus to alter this particular gene's expression (Hopkins, Baldi, Cutfield, McCowan, & Hofman, 2011). Also, we found an effect of LTPA at only one of the promoters we studied (P2 promoter). Therefore, it may be that there is not a large enough effect for a net change in IGF2 hormone to be measured identified in serum. This may explain why the Hopkins et al. study, which assessed serum levels rather than gene expression or methylation found no effect of the physical activity intervention. Because the authors did not directly assess methylation or gene expression, it is difficult to know whether their findings should be compared directly to ours.

For PDK4, we expected the high active group to have lower DNA methylation compared to the low active group. Other data have shown lower levels of PDK4 methylation in Type 2 Diabetes patients, and higher levels of PDK4 gene expression in individuals with higher compared to lower BMI (Kulkarni, et al., 2012). Acute exercise, in mice and in humans (skeletal muscle),

resulted in decreased PDK4 methylation (Barrès, et al., 2012), so we expected chronic LTPA to do the same, even in offspring blood. Still, we found no relationship between physical activity and PDK4 methylation. We also found no effect of physical activity on TCF7L2 methylation. This was unexpected because a six month exercise intervention study found increased DNA methylation in TCF7L2 in adipose tissue in humans (Rönn, et al., 2013), but like for the other candidate genes, it may be that tissue specificity, maternal transmission, or amount of exercise stimulus impacted our findings.

The general lack of differences among the activity groups may be due to one or a combination of several reasons. First, we chose to study methylation rather than gene expression, which is, at least in theory, the effect of the methylation profile. There is not always direct correlation between methylation and gene expression, however. The location of the methylation within the genome likely influences the impact that the methylation change has on gene expression (Jones, 1999). For instance, methylation within certain gene areas including promoters of imprinted genes and DNA repair genes does seem to permanently silence promoter activity (resulting in a positive relationship between methylation level and expression) while methylation at other gene regions does not necessarily equate to less gene expression (Jones, 1999). It is plausible that our results might have been different had we measured mRNA levels (indicative of gene transcription) rather than the methylation at particular CpG sites. To our knowledge, few others have examined both gene expression and DNA methylation simultaneously. Barres and colleagues examined DNA methylation

of PGC1-α, PDK4, and PPAR-δ, in skeletal muscle samples, while simultaneously examining gene expression, and they found an inverse relationship between methylation and expression for each of these three genes (Barrès, et al., 2012). However, in the same study, the authors examined the effects of a 3-week exercise training intervention on PGC1-α and TFAM methylation and found no effect on DNA methylation but increased gene expression for both genes. This suggests that there is a timing effect in terms of the physical activity (i.e. acute versus chronic). Possibly we would have found different results for gene expression than we did for methylation since we examined habitual LTPA rather than an acute bout of exercise.

Another possible explanation for lack of differences among groups is that the methylation profile may not transfer from mother to child, at least in regard to physical activity. We found no studies showing heritability of methylation patterns related to maternal physical activity, even in animal models. Based upon the Fetal Origins of Adult Disease Hypothesis, however, it would be expected that such a stimulus might impact offspring health risk by transfer of methylation patterns. Other lifestyle-related stimuli have been shown to induce methylation changes that are passed to offspring. These include smoking (after 18 weeks gestation) (Joubert et al., 2014), mother's BMI (Liu, et al., 2014), and folate intake (Haggarty, Hoad, Campbell, et al., 2013; Steegers-Theunissen et al., 2009). Future research should involve a direct comparison of mother to offspring methylation patterns to assess similarity due to environment and/or transferred

epigenetic profile. If high active mothers are more comparable to offspring than are low active mothers, transfer of epigenetic markings can be assumed.

Yet another possibility is that the physical activity stimulus in this study was insufficient to elicit change in DNA methylation in the mother. However, the average woman in our high active group reported over 600 minutes per week of MVPA, and of those subjects, eight of 14 reported significant amounts of vigorous physical activity (i.e. more than one day per week).The total exercise stimulus in our study is considerably more than that used in Zhang and colleagues study; after controlling for confounders they found no relationship between global methylation and physical activity study when comparing individuals who exercised 26 to 30 minutes per day to those who were active less than ten minutes per day (F. F. Zhang, R. Cardarelli, J. Carroll, S. Zhang, et al., 2011). In the global methylation and physical activity study that did find a relationship between higher activity and more global methylation, the physical activity stimulus used in our study would be equivalent to their second lowest quartile of activity (A. J. White, et al., 2013); the highest active group reported more than 18 hours per week of activity. It may be, then that the total amount of activity was inadequate, or more likely, that the intensity was not great enough to elicit changes in methylation profiles. Barres and colleagues found that higher intensity (80% VO_{2peak}) acute exercise resulted in greater methylation changes than did lower intensity (40% VO_{2peak}) exercise (Barrès, et al., 2012).

It is also worth noting that the low active groups in our study reported an average of approximately 60 minutes MVPA per week. While neither group met

the recommendations for physical activity from the Physical Activity Guidelines for Americans (health.gov, 2008), they did report doing some activity. Ideally we would have compared the highly active group to two completely sedentary groups, who reported no physical activity whatsoever. Due to the need to match on other variables, however, this was not possible.

Another issue inherent to this type of study is recall bias. Our questions regarding physical activity asked about activity in the past month. It is possible that subjects did not properly recall the amount of LTPA they participated in, and such problems would affect study findings. In a study using the same ARCH sample, Schlaff and colleagues found no relationship between pregnancy LTPA and pre-pregnancy BMI or between LTPA and risk for gaining excess weight during pregnancy. Rather, BMI prior to pregnancy predicted gestational weight gain (Schlaff, Holzman, Maier, Pfeiffer, & Pivarnik, 2014). This suggests a possible combination of a recall bias issue and insufficient LTPA stimulus that may be leading toward our mostly null findings.

Another possible reason for our findings is that we have used a different biologic sample (blood spots) for analysis compared to most of the similar literature. Most research on methylation changes passed from one generation to the next has been performed on cord blood (Gemma, et al., 2009; Haggarty, Hoad, Campbell, et al., 2013; Joubert, et al., 2014) while another study used placenta (St-Pierre, et al., 2012). The majority of literature relating exercise or physical activity to DNA methylation has used either fat (Rönn, et al., 2013) or muscle biopsy (Barrès, et al., 2012; Kulkarni, et al., 2012; Nitert, et al., 2012;

Ribel-Madsen, et al., 2012; Wang, Psilander, Tonkonogi, Ding, & Sahlin, 2009) as the biologic sample. One research group examined methylation changes in a large number of genes following a six month exercise intervention. About 75 percent of the genes affected by exercise in skeletal muscle had decreased DNA methylation while most of the genes that were affected in adipose tissue had increased methylation (Nitert, et al., 2012; Rönn, et al., 2013), suggesting that different tissues are affected in unique ways by physical activity. It is less clear whether use of peripheral blood (blood spots) from birth compared to umbilical cord blood could influence results, and to what extent. We found no studies directly comparing methylation patterns of these two types of blood samples, though gene expression was highly correlated ($r = 0.941$) for cord blood and blood spots for three sets of genes (Slaughter et al., 2013). Due to known variation in DNA methylation patterns by tissue type, it is reasonable to suspect that cell type selection might lead to at least some differential findings across studies (more so for muscle, fat, and placenta rather than cord blood), and thus it is difficult to determine whether it is our sample selection or other factors that explain our mostly null findings.

Future Directions

There are several limitations to this study that should be addressed in future research. First, sample size was small, as we considered this to be pilot work. It is also important to compare methylation levels of genes in mother and her offspring in order to address the issue of transferability of these methylation profiles from one generation to another. Simply taking a maternal blood sample

at delivery (or shortly after, around the time of the infant's heel stick) would provide for this opportunity. It would be interesting to compare the DNA methylation levels of cord blood to blood spots to determine whether or not the biologic sample being used for the infant is influencing results. It is not likely that skeletal muscle biopsies would be plausible in infants or children due to ethical reasons.

Other future directions could include comparisons of high active, moderate active, and sedentary groups. It is possible that we see no differences in high active and low active groups because both ends of the activity spectrum result in a 'negative' epigenetic profile while the true effect lies with those who are moderately active. However, White et al. used physical activity quartiles and did not find this effect (A. J. White, et al., 2013). Still, similar u-shaped relationships are seen with exercise and immunity (Gleeson, 2007) and with exercise and telomere length (Ludlow et al., 2008), where moderate activity/exercise results in the 'good' phenotype and the low active and high activity groups have the 'bad' outcome. Finally, it would be useful to examine a greater selection of genes, possibly by use of a microarray or similar technology, to determine whether these candidate genes we selected might actually be the wrong place to look.

While there are important limitations to the study, there are also several strengths that should be acknowledged. This study is novel and is the first to examine the effects of pregnancy physical activity on offspring DNA methylation in humans. Though we found no differences in global methylation levels in offspring of active compared to sedentary women, we have been able to highlight

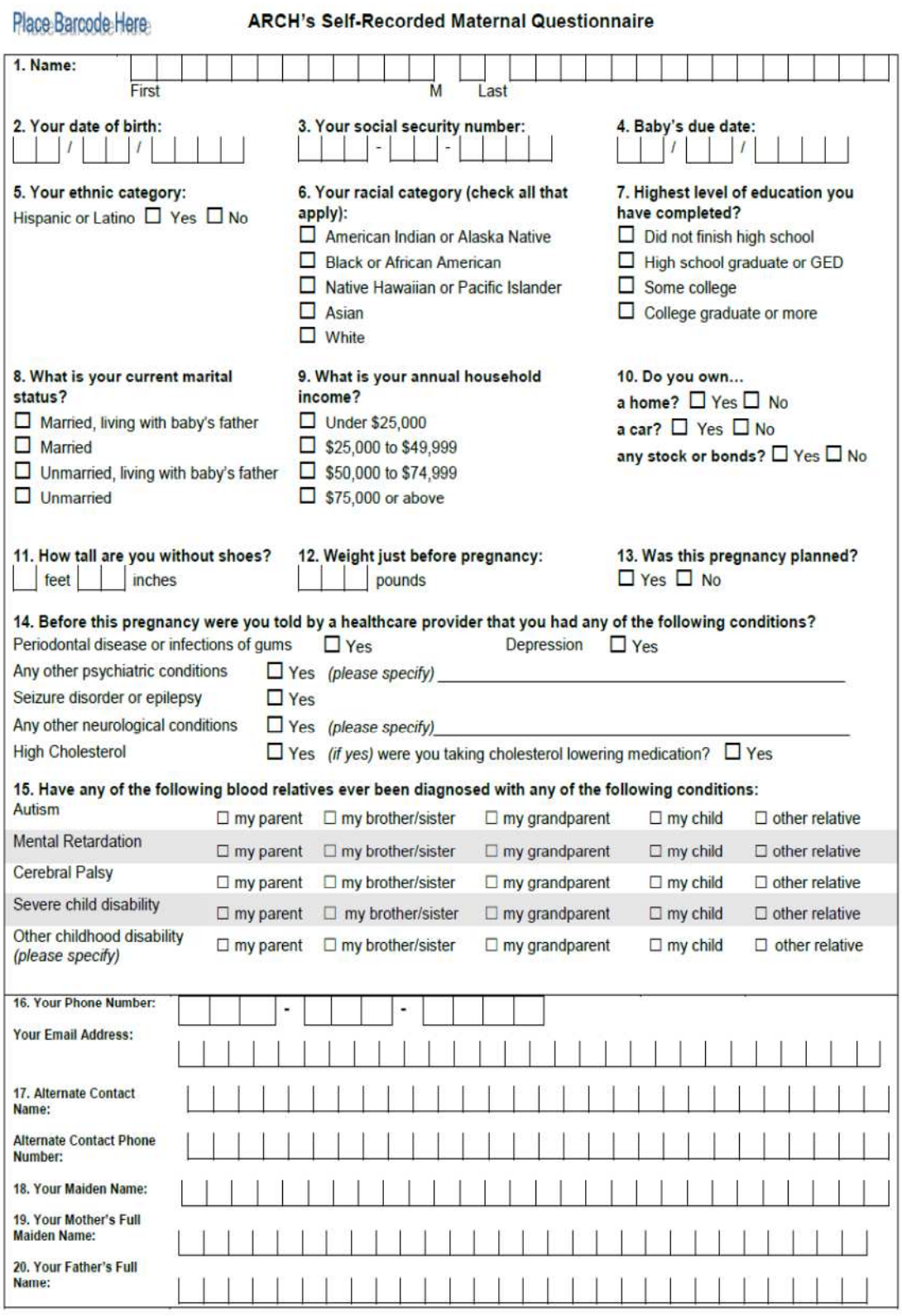
important issues to address in future research in this area. The groups were relatively well matched for known confounders, so future work can emphasize selection of appropriate tissue type for analysis, avoidance of recall bias, and adequate physical activity stimulus to further understand this issue and whether/how pregnancy LTPA might influence offspring susceptibility to non-communicable disease.

APPENDICES

APPENDIX A

Questionnaires

Figure 1: ARCH Questionnaire. Questionnaire is completed by subjects upon enrollment.



Place Barcode Here

ARCH's Self-Recorded Maternal Questionnaire

1. Name: First M Last

2. Your date of birth: / /

3. Your social security number: - -

4. Baby's due date: / /

5. Your ethnic category:
Hispanic or Latino ☐ Yes ☐ No

6. Your racial category (check all that apply):
☐ American Indian or Alaska Native
☐ Black or African American
☐ Native Hawaiian or Pacific Islander
☐ Asian
☐ White

7. Highest level of education you have completed?
☐ Did not finish high school
☐ High school graduate or GED
☐ Some college
☐ College graduate or more

8. What is your current marital status?
☐ Married, living with baby's father
☐ Married
☐ Unmarried, living with baby's father
☐ Unmarried

9. What is your annual household income?
☐ Under $25,000
☐ $25,000 to $49,999
☐ $50,000 to $74,999
☐ $75,000 or above

10. Do you own...
a home? ☐ Yes ☐ No
a car? ☐ Yes ☐ No
any stock or bonds? ☐ Yes ☐ No

11. How tall are you without shoes? feet inches

12. Weight just before pregnancy: pounds

13. Was this pregnancy planned? ☐ Yes ☐ No

14. Before this pregnancy were you told by a healthcare provider that you had any of the following conditions?
Periodontal disease or infections of gums ☐ Yes
Depression ☐ Yes
Any other psychiatric conditions ☐ Yes *(please specify)* _____
Seizure disorder or epilepsy ☐ Yes
Any other neurological conditions ☐ Yes *(please specify)* _____
High Cholesterol ☐ Yes *(if yes)* were you taking cholesterol lowering medication? ☐ Yes

15. Have any of the following blood relatives ever been diagnosed with any of the following conditions:

Autism	☐ my parent	☐ my brother/sister	☐ my grandparent	☐ my child	☐ other relative
Mental Retardation	☐ my parent	☐ my brother/sister	☐ my grandparent	☐ my child	☐ other relative
Cerebral Palsy	☐ my parent	☐ my brother/sister	☐ my grandparent	☐ my child	☐ other relative
Severe child disability	☐ my parent	☐ my brother/sister	☐ my grandparent	☐ my child	☐ other relative
Other childhood disability *(please specify)*	☐ my parent	☐ my brother/sister	☐ my grandparent	☐ my child	☐ other relative

16. Your Phone Number: - -

Your Email Address:

17. Alternate Contact Name:

Alternate Contact Phone Number:

18. Your Maiden Name:

19. Your Mother's Full Maiden Name:

20. Your Father's Full Name:

Figure 1 (cont'd).

These next sets of questions are about physical activity, exercise, and sports that you take part in during your free time. If you have any questions or if you are not sure if the activity you partake in is moderate or vigorous, please ask and we can help you.

We will first ask you about **moderate activities**. A moderate activity is one that causes a small increase in your breathing or heart rate. Some examples of moderate activities are brisk walking, bicycling, dancing and yoga.

21- During the past month, did you do any moderate activities for more than 10 minutes that caused a small increase in your breathing and heart rate?

☐ Yes ☐ No ☐ Don't Know/Refuse

21a- If yes, how many days a week do you usually do these moderate activities?

_____ Days per week ☐ Do not exercise at least 10min a week ☐ Don't know/Not sure

21b How much time do you usually spend doing these moderate activities in one day?

(Please fill in the blanks) _____ Hours and _____ Minutes

Now we will ask you about **vigorous activities**. A vigorous activity is one that causes an increase in your breathing or heart rate. or heart rate. Some examples of vigorous activities are running, jogging and aerobics.

22- During the past month, did you do any vigorous activities for more than 10 minutes that caused a large increase in your breathing and caused you to sweat?

☐ Yes ☐ No ☐ Don't Know/Refuse

21a- If yes, how many days a week do you usually do these vigorous activities?

_____ Days per week ☐ Do not exercise at least 10min a week ☐ Don't know/Not sure

21b How much time do you usually spend doing these vigorous activities in one day?

(Please fill in the blanks) _____ Hours and _____ Minutes

These next sets of questions are about physical activity, exercise at work

23- Do you currently work at least 30 hours a week?

☐ Yes ☐ No ☐ Don't Know/Refuse

23a- If yes, how do you spend most of your time at work on a typical day? Are you mostly sitting, standing, walking, or doing physical labor?

Please rank these activities from 1 to 4 in order of most (1) to least (4) time spent during a normal work day below. If there are any activities you do not normally do at work, please put a 0 next to it.

Please rank the following work activities using this scale:

1	2	3	4	0
I do the most			I do the least	I never do

Sitting: _____ Standing: _____ Walking: _____

Physical Labor (like lifting/moving things): _____

APPENDIX B

Oral Consent Script

ARCH Sub-Study: Effects of Maternal Physical Activity on Methylation Patterns in Offspring Blood Spots
PI: Jim Pivarnik and Mallory Marshall
Date:
Investigator (Caller) Name:
Subject Name:
Oral Consent Script:

Hello, my name is _____, a student from MSU; may I speak with (subject's first name) please?

If she answers:

Hello, my name is _____ and I am calling to follow up with you because you participated in the research studyArchive for Research on Child Health at MSU beginning in ____ (year) when you were pregnant with _____ (child's first name). May I ask you a question relating to your participation in the study?

If no: Thank you for your time. Goodbye.

If yes:
Researchers at MSU would like to use health information that you have already provided to us and request the Michigan Department of Community Health link it with your baby's blood spot sample that was taken from your baby's heel for newborn screening when ___ (he/she) was born at Sparrow. This is for a research study at MSU. The Department of Community Health oversees storage of remaining blood spots from newborn screening for potential future personal use or use in approved research. If you agree, we will request a small sample of your baby's blood spot, and we will use it for some genetic testing. The genetic testing will not tell us about any diseases or conditions your child might have. Very simply, the genetic testing will help us see whether certain genes which are related to the way the body uses energy are active or inactive (on or off). Your child's identity will be kept private; we will not put your child's name on any blood samples or other materials, and we will store the samples in a locked box in a laboratory where only study investigators can access the samples. Because no subjects will be identified, research results cannot be returned but a summary of the findings will be posted on-line at www.michigan.gov/biotrust when available. Would you agree to allow us to use your child's blood spot sample for this research? Please note that participation is voluntary and you can withdraw your consent at any time up until the research is completed, which we expect will be approximately April 2014. If you do agree, we will document that you consented over the phone, and you don't need to complete any other paperwork. However, if you would like a written copy of any materials including the original consent form you signed when you enrolled in the study or the information just provided to you via phone, I would be happy to mail that to you. If you have any questions

you may ask me or contact the study investigator, Mallory Marshall, at (517) 355-7696 or email mrm@msu.edu.

_____ Yes, my child's blood spot can be used.
_____ No, my child's blood spot may not be used.

Thank you for your time! Goodbye.

APPENDIX C

PCR and Pyrosequencing Conditions

PCR Cycling Conditions

PPARγ

Table 12: PPARγ Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
5X Q-Solution	3 μl (0.5x)
25 mM $MgCl_2$	1.8 μl (3.0 mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS2016FPre)	0.6 μl (6 pmol)
10 uM Rev primer (ADS2016RPB)	0.6 ul (6 pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1 μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 53ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

Table 13: PPARγ Intron 2 PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8 μl (3.0 mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS2020FPB)	0.6 μl (6 pmol)
10 uM Rev primer (ADS2020RP)	0.6 ul (6 pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1 μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 57ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

PGC1α

Table 14: PGC1α Proximal Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8μl (3mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer	0.6μl (6pmol)
10μM Rev primer	0.6 ul (6pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 53ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

Table 15: PGC1α Exon 1 to Intron 1 PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8μl (3mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer	0.6μl (6pmol)
10μM Rev primer	0.6 ul (6pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 56ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

IGF2

Table 16: IGF2 P2 Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8 μl (3.0 mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS3602FP)	0.6 μl (6 pmol)
10 uM Rev primer (ADS3602RPB)	0.6 ul (6 pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1 μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 65ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

Table 17: IGF2 P4 Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	0 μl (1.5 mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS173FP)	0.3 μl (3 pmol)
2.5 μM Rev primer (ADS173RPT)	0.12 ul (0.3 pmol)
10 uMUniversal RPB	0.27 ul (2.7pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 56ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

Table 18: IGF2 Distal Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8 μl (3.0 mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS1051FPB)	0.6 μl (6 pmol)
10 uM Rev primer (ADS1051RP)	0.6 ul (6 pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1 μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 60ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

PDK4

Table 19: PDK4 Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8μl (3mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer	0.6μl (6pmol)
2.5 μM Rev primer	0.6 ul (6pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 53ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

TCF7L2

Table 20: TCF7L2 5'UTR and 5'UTR-Promoter PCR Conditions

Component	Per 30μl reaction
10X PCR buffer (Contains 15mM $MgCl_2$)	3 μl (1x)
25 mM $MgCl_2$	1.8μl (3mM final conc.)
10 mMdNTPs	0.6 μl (200 μM of each)
10 μM Fwd primer (ADS173FP)	0.6μl (6pmol)
2.5 μM Rev primer (ADS173RPT)	0.6 ul (6pmol)
HotStar Taq Polymerase (5 U/μl)	0.15 μl (0.75 U)
DNA	1μl of bisulfite treated DNA
Water	Adjust to 30 μl

Cycling Conditions: 95ºC 15 min; 45 x (95ºC 30sec; 53ºC 30sec; 72ºC 30sec); 72ºC 5 min; 4ºC ∞

Pyrosequencing Protocol

Pyrosequencing is performed using PSQ 96HS system or PSQ 96HSA system.

1. Prepare master Binding Solution: Components for 1 reaction of binding solution: 2.2 uL streptavidin, 40 uL 2X Binding buffer, 25 uLMilli-Q-water

2. Add 65 uL binding solution to each 15 uL PCR sample.

3. Capture the PCR product as manufactory's protocol

4. Release the sepharose beads into annealing buffer containing 0.5 uM of a sequencing primer

5. Anneal the sequencing primer to the template by heating the plate to 85 ºC for 2 minutes

6. Turn off the heating block and leave the Pyro plate on the heating block for 10 minutes

7. Remove the Pyro plate from the heating block and allow the plate continuing to cool for 5 minutes

8. Run the Pyrosequencing as the manufactory’s instruction.

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