INVESTIGATING THE RELATIONSHIP BETWEEN MATERNAL DIET AND INFANT ADIPOSITY

by

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ABSTRACT

The role of fetal programming in the early development of obesity is an important area of focus for chronic disease prevention. Maternal diet during pregnancy represents a specific in-utero exposure with the potential to impact fetal development. Little is known in human populations regarding the association between maternal diet and offspring adiposity. This thesis addresses this knowledge gap using data from the Healthy Start study, a diverse, pre-birth cohort of 1,410 mother-infant dyads. In addition, there is little information as to what specific nutrient exposures affect fetal fat accretion. Here, we propose a novel pathway linking maternal dietary niacin consumption during pregnancy with neonatal adiposity at birth. The micronutrient niacin, specifically its amide form, nicotinamide, has been linked to increased adipogenesis through inhibition of Sirtuin 1 (SIRT1) protein and activation of PPARγ in cell culture experiments. Within a translational framework, this project incorporates population-level analyses to explore the relationships between maternal diet quality, and the dietary composition of fat and niacin and fat mass at birth, as well as examine potential clinical markers. Further we employed cell culture experiments to elucidate the protein mediators of the hypothesized association. We analyzed the impact of overall diet quality, dietary niacin and high-fat diet on percent fat mass (%FM) of neonates in the full Healthy Start cohort and found that both a low-quality and high-fat diet, but not dietary niacin, are associated with higher %FM in the neonate without influencing the total mass or fat-free mass (FFM). Further,
we investigated the association between maternal dietary niacin and SIRT1 protein levels in umbilical cord tissue and the association between SIRT1 protein and neonatal %FM in a small subset of the parent Healthy Start cohort but found no significant relationships. Finally, in cell culture of fetal mesenchymal stem cells, nicotinamide exposure significantly increased protein markers of adipogenesis, PPARγ and fatty acid binding protein (FABP)-4 and decreased SIRT1 protein activity. In summary, our results suggest that maternal diet quality and dietary fat during pregnancy influence fat development in-utero, and that nicotinamide in an in-vitro cell model induces greater adipogenesis, possibly through a SIRT1-dependent mechanism.

The form and content of this abstract are approved. I recommend its publication.

Approved: Dana Dabelea
This thesis is dedicated to my parents, Gary Buti and Roberta Stafford, my brother,
Nathan Buti, my grandfather, Bruno Buti, and to my husband, Jason Shapiro.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>GWG</td>
<td>Gestational weight gain</td>
</tr>
<tr>
<td>FM</td>
<td>Fat mass</td>
</tr>
<tr>
<td>FFM</td>
<td>Fat-free mass</td>
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<tr>
<td>%FM</td>
<td>Percent fat mass</td>
</tr>
<tr>
<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>c/EBPα</td>
<td>CCAAT/enhancer binding protein alpha</td>
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<tr>
<td>FABP4</td>
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CHAPTER I

INTRODUCTION

Background

The role of fetal programming in the early development of obesity is an important area of focus for metabolic disease prevention. Maternal diet during pregnancy represents a specific and modifiable in-utero exposure with the potential to impact developmental pathways that influence future metabolic disease risk. In addition to excess calories, factors within the diet such as fat content and micronutrients may play an important role in fat tissue development and metabolic programming by mechanisms that remain poorly understood. Apart from glucose, little is known in human populations as to whether, what, and how specific nutrient exposures impact fetal programming, specifically fetal fat accretion. While fat in the diet is an obvious contributor of the lipid substrate for adipose tissue growth, micronutrient components of the diet may play a key role in direct modulation of cellular mechanisms responsible for adipogenesis. The overarching goal of this proposal is to explore the associations between the quality of maternal diet and specific dietary components and neonatal body composition. We first aimed to explore the relationship between maternal diet quality using the Health Eating Index 2010, and neonatal body composition, a topic with potential clinical and public health implications. Second, we aimed to explore a novel hypothesis that maternal dietary niacin intake during pregnancy affects infant adiposity through a cellular pathway involving inhibition of fetal SIRT1 and activation of PPARγ protein action. We further hypothesized that the dietary niacin effect would be apparent or enhanced in the presence of excess lipid substrate, as provided by a maternal high-fat diet or maternal obesity. Finally, we tested a
mechanistic pathway (Figure 1) using *in-vitro* approaches in umbilical cord tissue-derived mesenchymal stem cells. All these research aims were attempted and conducted within the unique infrastructure of the Healthy Start study, an ongoing pre-birth cohort of approximately 1,410 women and infants in Colorado.

**Specific Aims and Hypotheses**

*Specific Aim 1*

To investigate the associations between maternal diet quality during pregnancy and neonatal adiposity in a pre-birth cohort of mother-offspring pairs. We hypothesized that infants born to mothers with lower diet quality during pregnancy will have higher adiposity compared to infants born to women with higher diet quality.

*Specific Aim 2*

To investigate the associations between maternal dietary niacin, SIRT1 protein in the umbilical cord, and neonatal adiposity and the potential modifying effect of a high-fat diet or maternal obesity on these relationships.

**Hypothesis 1**

Increased consumption of niacin during pregnancy is associated with increased neonatal adiposity and this association is increased or apparent only in the presence of excess lipid substrate as provided by a maternal high-fat diet or maternal obesity.

**Hypothesis 2**

Increased maternal dietary niacin intake is associated with decreased SIRT1 protein concentration in umbilical cord tissue.
Hypothesis 3
Decreased SIRT1 protein concentration in umbilical cord tissue is associated with increased neonatal FM. This association is further increased in the presence of excess lipid substrate as provided by a maternal high-fat diet or maternal obesity.

Specific Aim 3
To measure the adipogenic differentiating response of umbilical cord tissue-derived mesenchymal stem cells (MSC) when treated with nicotinamide and lipid in-vitro.

Hypothesis 1
MSCs cultured from neonates born to non-obese mothers in the Healthy Start study will have significantly greater adipogenic response when treated with nicotinamide, lipid, or a combination of nicotinamide and lipid compared to MSCs treated with vehicle-control.

Hypothesis 2
In MSCs from neonates born to non-obese mothers in the Healthy Start study, SIRT1 protein activity will be significantly reduced upon treatment with nicotinamide.

Hypothesis 3
The adipogenic response of MSCs treated with nicotinamide will be significantly associated with adiposity at birth of the neonates from which the cells were derived.

Significance and Innovation
This study aims to contribute evidence regarding the association between maternal diet (overall diet quality, niacin and fat intake) and adiposity at birth using epidemiologic data provided by the Healthy Start cohort. We also intend to demonstrate, in-vitro, using umbilical cord tissue samples, the presence of a molecular pathway, by which maternal niacin intake, especially in the presence of a high fat diet, inhibits fetal
SIRT1, leading to PPARγ activation and increased adipogenesis. Our conceptual framework and pathways to be explored are shown in Figure 1.

Figure 1: Conceptual model of proposed pathway between a poor quality maternal diet, high fat, and niacin/nicotinamide (NAM) intake during pregnancy and neonatal adiposity.

The results of this study could significantly impact clinical treatment and dietary recommendations for the pregnant population. The diet quality measure used here was developed to reflect dietary adherence to the United States (US) recommendations for consumption of meats, dairy, fruits and vegetables, and other major contributors to the diet (e.g. added sugar, sodium). Our results could be used to develop short clinical screening tools for diet quality among pregnant women and help clinical dieticians counsel patients on improving their diet choices.

Further, SIRT1 protein activators (e.g. resveratrol, leucine) are being explored in pregnant and non-pregnant animal models as potential treatments for obesity and metabolic syndrome [1-3]. Nicotinamide competitive inhibitors (e.g. isonicotinamide), while not widely explored outside of cell culture, could also provide treatment options [4]. Further, while niacin-rich foods do not necessarily group into distinctly unhealthy dietary patterns (e.g. “Western” diet, high-fat diet), data linking this micronutrient to
excess fetal fat accretion may contribute to revised dietary recommendations for consumption of niacin-rich foods, especially when coupled with a high fat diet. Public health practice and policy may also benefit from this research. Nicotinamide is the form of niacin used in both grain enrichment and dietary supplements [5, 6] presenting a potential point for population-level intervention to decrease excess consumption of nicotinamide.

This project is innovative in that it is the first to investigate the joint effect of maternal dietary intake of niacin and fat on neonatal adiposity using a large epidemiology cohort. Moreover, we will explore a novel mechanistic pathway, suggested by in-vitro studies but not previously tested in animal models or humans, specifically investigating the role of SIRT1 and PPARγ as potential mediators of the above associations. To investigate the proposed mechanism, we will use methods that draw from both epidemiology and laboratory sciences through assessment of maternal diet and neonatal adiposity at the cohort level and analysis of protein action at the tissue and cellular levels. This is a novel, multidisciplinary approach to the fetal programming hypothesis. Furthermore, as a result of this study, we will have built a moderately sized biological cell bank of human umbilical cord-derived mesenchymal stem cells from a large and well-characterized mother-infant cohort. This provides our team and collaborating scientists with a powerful tool for future studies of the molecular and epigenetic basis for children who go on to develop a variety of metabolic phenotypes.
CHAPTER II

LITERATURE REVIEW

The Obesity Epidemic and Its Consequences

Currently in the United States, more than half (63%) of the adult population is overweight or obese [7]. While obesity in adulthood is recognized as an important target for chronic disease prevention, of significant concern is the increasing prevalence of overweight and obese children. The CDC reports that 27% of children in the United States are overweight or obese [8]. For these children, the influence of obesity on the development of adverse metabolic consequences, including heart disease and diabetes [9-12] is exerted earlier, increasing their risk of obesity-related chronic disease in young adulthood and throughout their lives. The premature onset of metabolic and cardiovascular outcomes associated with childhood obesity warrants investigation into the environmental and physiological influences on adipose tissue accretion in early life given the limited effects of treatment after metabolic disease has been identified.

Fetal Programming and the Role of Maternal Diet

Increasingly, the effects of maternal exposures during pregnancy on fetal development and infant outcomes have become a significant area of focus among researchers investigating childhood obesity and its risk factors. Maternal phenotypes such as obesity and gestational diabetes (GDM) have been shown to significantly contribute to adverse infant outcomes, in particular, an increased risk of obesity and metabolic dysfunction in childhood and later in life [13]. Fetal exposures such as maternal diet during pregnancy may also play a significant role in fetal adipose accretion and the adverse “programming” of infants to be at higher risk of obesity and metabolic disease.
Diet quality in particular reflects the total composition of the diet, accounting for interactions between nutrients, and has been linked to fetal growth and development [14-16]. However, within the overall diet, the components that contribute to its quality such as fat content and micronutrients may play an important role in fat tissue development and metabolic programming by mechanisms that remain poorly understood. To date, animal models have shed important light on the role of diet composition during pregnancy and offspring development of adiposity [17-19]. For example, in non-human primates, exposure to maternal high-fat diet was associated with larger body size and liver pathology consistent with non-alcoholic fatty liver disease in the offspring [20]. In humans, however, evidence linking maternal dietary patterns and macro- and micronutrient intake to neonatal fat mass is limited. It is fair to say that diet is poorly, if ever, measured in most studies of human pregnancy, leaving the important contributions beyond obesity left to discover.

**Potential Mechanisms for Excess Fetal Fat Accretion**

One hypothesized molecular explanation for excessive adipose tissue accretion is the activation of PPAR-gamma (PPARγ) expression. PPARγ is a critical transcriptional regulator of adipogenesis [21, 22]. The natural ligand for PPARγ is a lipid-derived intermediated, PGJ2, which binds to the nuclear form of the receptor. In addition, its expression is controlled in part by Sirtuin 1 (SIRT1), a member of the Sirtuin family of NAD-dependent histone deacetylase proteins [23, 24]. SIRT1 has been shown to target the expression of PPARγ directly where it plays a key role in the deacetylation of chromatin necessary for suppressing PPARγ transcription and the adipogenic cascade [23, 25]. Importantly, with chronic maternal high-fat diet (HFD)-feeding and obesity,
hepatic SIRT1 protein content is decreased and PPARγ mRNA is increased in the fetal livers of non-human primates [20]. Furthermore, mice lacking SIRT1 display accelerated weight gain and hepatic metabolic dysfunction with chronic HFD-feeding [26], suggesting that attenuated SIRT1 activity is relevant for the progression of adiposity in response to caloric excess. While fat in the diet may contribute the lipid substrate for adipose tissue growth, micronutrient components of the diet may play a key role in direct modulation of SIRT1 activity and therefore significant control over PPARγ expression. Specifically, we hypothesize that excess nicotinamide, a potent SIRT1 inhibitor and the predominant form of dietary niacin absorbed through the human gut, may contribute to adipose tissue accretion through suppression of fetal SIRT1 protein activity and increased PPARγ expression, thereby promoting stem cell adipogenic fate during development.

Niacin and the Control of Sirtuin Function

Nicotinamide (NAM), the precursor for the synthesis of NAD(+) is an inhibitor of SIRT1. However, little is known about the effects of dietary NAM on human development of adipose tissue. Nicotinamide (NAM), also known as niacinamide is the amide form of niacin. The largest dietary sources of NAM include animal foods, some nut varieties, and enriched grains (e.g. cereal, wheat flour, etc.) [5, 27]. NAM is also the form of niacin added to dietary supplements [6]. Vegetables and fruits can also provide nicotinic acid (NA), the acid form of niacin. While a balanced diet will provide both niacin derivatives, during digestion the NA in the gut is reduced to several metabolites including NAM, which is the most readily absorbed metabolite [28]. Once absorbed, NAM is distributed to the body’s tissues where it is taken up by cells and converted to nicotinamide adenenine dinucleotide (NAD) by the NAM salvage pathway [29]. NAD is
a central component of cellular metabolic reactions as well as an important ligand in post-
translational modification and activation of histone deacetylase proteins such as SIRT1
[30, 31].

While NAM is critical for NAD synthesis, excess NAM can also inhibit SIRT1’s
activity through non-competitive substrate inhibition [32, 33]. SIRT1 catalyzes the
deacetylation reaction by cleaving NAD+ into two intermediate products, NAM and O-
acylamide (Oa). OA then removes the histone or proteins’ acetyl group forming the
compound, cADPribose [29-31] (Figure 2). During this reaction excess NAM can react
with Oa and re-form NAD+, reversing the reaction and inhibiting SIRT1’s deacetylase
action.

![Figure 2: Depiction of SIRT1 de-acetylation reaction with NAD+ and the products, NAM and Oa.](image)

Post-translational control of SIRT1 by NAM is a direct method of inhibiting
SIRT1 protein function and the metabolic consequences of this inhibition vary by tissue.
In mesenchymal stem cells - the multipotent pre-cursor cells to adipocytes, myocytes, and
osteocytes - SIRT1 suppression results in increased adipocyte differentiation [2, 34].
Furthermore, NAM has been shown to drive bone marrow-derived MSCs toward adipogenesis as opposed to osteogenesis in a SIRT1-dependent mechanism [35]. Given this, excess NAM from the diet may promote fetal fat accretion and could present a unique mechanism by which maternal diet influences fetal growth and adiposity, specifically. NAM’s direct involvement as a driver of the adipogenic mechanism may also compound the effect of a maternal high-fat diet that would provide the fat substrate for fetal adipose development, suggesting a potential nutrient-nutrient interaction in-vivo.

To date this nutrient-nutrient interaction has not been investigated in-vitro or in-vivo, therefore presenting the opportunity to study a novel mechanism by which macro- and micronutrients from the pregnancy diet impact fetal growth. The overarching goal of this thesis is to explore the relationship between maternal diet during pregnancy and neonatal adiposity, specifically testing whether nicotinamide and fat from the diet interact to increase adiposity at birth.
CHAPTER III

METHODS

Overview of Approach

The proposed study will use the infrastructure provided by the ongoing longitudinal pre-birth cohort, the Healthy Start Study. Figure 3 shows the design and expected number of participants for each of the 3 Aims proposed by this study.

![Figure 3: Proposed sample sizes for study aims and hypotheses.](image)

The Healthy Start study enrolled 1,410 pregnant women as of September 2014. We excluded mothers with gestational diabetes (n = 53) and infants born at less than 32 weeks of gestational age or with missing neonatal body composition (n = 278) leaving us with approximately 1,079 mother-child dyads to conduct analyses for Aim 1 and hypothesis 1 of Aim 2 of this study. For our primary endpoints in Aim 1 and hypothesis 1
of Aim 2, we used existing dietary data collected on all Healthy Start mothers and neonatal body composition collected on the infants at birth.

We additionally collected umbilical cord tissue to address hypotheses 2 and 3 of Aim 2 and all hypotheses in Aim 3. We collected umbilical cord tissue on 200 mother-child dyads and tested for the second part of Aim 2 (SIRT1 analysis) using the sub-cohort of 200 mother-child dyads (Sub-Cohort #1). To address Aim 3 we randomly selected 46 dyads for experiments from the total 165 dyads (Sub-Cohort #2) from which we cultured mesenchymal stem cells from the umbilical cord tissue.

**Study Population: The Healthy Start Study**

The Healthy Start Study began recruitment in August 2009 and completed recruitment in September 2014 (Phase 1) with continued follow-up of infants through 5 years of age (Phase 2).

The overall goal of Healthy Start Phase 1 was to study how maternal demographic, metabolic, and behavioral factors during pregnancy influence the development of body composition and cardiovascular and metabolic risk factors in the offspring at birth and up to 18 months of age. Healthy Start Phase 2 aims to use the pregnancy information and early life outcomes from Phase 1 and follow the health and growth trajectories of the infants out to age 5. Healthy Start Phase 1 collected data on pre-pregnancy BMI, gestational weight gain, state of the art self-reported dietary intake throughout pregnancy, and cord blood at each delivery. Offspring measurements included birth weight, length and ponderal index, skinfolds and circumferences. Neonatal body composition (percent fat mass, fat mass, and fat-free mass) was measured within 48 hours after birth using air displacement plethysmography (PEA POD). The protocol included
two pre-natal visits with the mother, an in hospital delivery visit, a visit when the offspring was 4-6 months of age and a phone interview when the offspring was 18-24 months of age. As of September 2014, 1,410 women had been enrolled into the study and their infants delivered. The retention rate at delivery remained high throughout the study period (93%).

**Maternal and Offspring Data**

*Maternal Characteristics*

Information on maternal age, race, socioeconomic status, parity, smoking status and history, physical activity, blood glucose and insulin, and blood inflammatory markers was collected during each of the two pregnancy visits.

*Maternal Body Weight*

At each of the two pregnancy visits, participants were weighed and skin fold measures obtained. Pre-pregnancy BMI was obtained from maternal medical records and by self-report where information from medical records was missing. Weight during pregnancy was obtained from clinical and research measures (on average 8 per participant), and gestational weight gain (GWG) was estimated using absolute weight gain (model-predicted weight at delivery minus model predicted weight at conception).

*Maternal Diet During Pregnancy*

The Healthy Start study employed a state of the art dietary assessment, the Automated Self-Administered 24-hour (ASA24) dietary recall [36]. The ASA24 dietary recall was developed by investigators at the National Cancer Institute to simulate an in-person 24-hour dietary recall in an online format. Data from the ASA-24 was collected and processed by the Nutrition Coordinating Center at the University of North Carolina.
(UNC) at Chapel Hill where micro- and macronutrient components of each dietary recall were calculated using nutrient values provided by the United States Department of Agriculture’s (USDA) most current Food and Nutrient Database for Dietary Studies (FNDDS). Healthy Start participants were asked to complete up to six ASA24 dietary recalls throughout their pregnancy and beginning at their first study visit. Nearly 76% of women completed at least two dietary recalls during the course of the Healthy Start study (Phase 1).

Whereas the ASA24 generates information about observed micro- and macronutrient intake, the Healthy Start study also employed a Food Propensity Questionnaire (FPQ) to capture information about food items and groups that were eaten over the course of pregnancy. Estimated daily micro- and macronutrient values were derived from combining the observed nutrient values obtained from all ASA24 recalls taken throughout the study with the food items reported in the FPQ using a measurement error modeling method developed by Tooze et al. [37] and implemented by Carroll et al. [38]. Briefly, rates of consumption of food items measured in the FPQ were calculated and used as covariates in the measurement error model with ASA24 repeated observed values as the model outcomes. This combined method improves the accuracy of the estimated daily intakes of episodically consumed nutrient (e.g. vitamin D) by accounting for food groups containing the specific macro- or micronutrient of interest.

We applied the appropriate modeling techniques to the macro- and micronutrient values obtained from the ASA24 data, including niacin, and used the estimated usual daily intakes for each participant as our measure of maternal dietary exposure during gestation.
**Maternal Diet Quality**

In addition to the macro-and micronutrient data generated by the ASA24, we also obtained MyPyramid Food Equivalents values. These values represent the numbers of servings of the major food groups consumed at each 24-hour diet recall (e.g. red meat, fish, vegetables, fruit, etc.) and were used to generate the Healthy Eating Index total and sub-scores. The Healthy Eating Index is a measure of overall diet quality and is based on the United States recommendations for Healthy People 2010 [39]. These scores were used for testing the hypothesis in Aim 1.

**Maternal High-Fat Diet**

High-fat diet was defined as obtaining greater than 35% of total energy (kcal) from fat and greater than 12% of total fat from saturated fat (American Heart Association, 2013).

**Offspring Data**

Infant gender, birth weight, birth length, and gestational age (GA) were obtained by research nurses at the time of delivery. Neonatal body composition was measured within 48 hours of birth on all infants using whole body air plethysmography (PEA POD, Life Measurement, Inc.). This 2-compartment method uses the principles of buoyancy similar to that of underwater weight measurements. PEA POD provides measurements of neonatal fat mass, fat-free mass, body mass, fat density, and fat-free mass density, among other data. Moreover, the PEA POD has shown excellent accuracy and reliability in infants when compared to a four-compartment method [40].
**SIRT1 Protein Content**

Neonatal umbilical cord tissue was collected at delivery from a convenience sample of 200 neonates (Aim 2; Sub-Cohort #1) by the University of Colorado Pediatric Clinical and Translational Research Center (PCTRC) (NIH/NCATS Colorado CTSI Grant #: UL1 TR000154). Samples were snap frozen and stored at -80°C. SIRT1 protein content was measured in tissue lysate using Simple Western, a high-throughput Western Blotting technique (Protein Simple, San Jose, California).

**Cell Culture of Umbilical Cord-Derived Mesenchymal Stem Cells (uMSCs)**

Fresh umbilical cord tissue was used for the cell culture component of this study (Aim 3). At delivery a 3-inch section of umbilical cord tissue was collected by the PCTRC. The fresh tissue samples were processed and incubated in Lonza MSCGM™ growth media (Product #: PT-3001) at 37°C for three weeks with media changes occurring every other day. Cells were harvested once they reached 90-100% confluence and stored in liquid nitrogen to await experimentation. For the purposes of Aim 3, 46 cell sets from infant born to non-obese mothers were randomly selected from the 165 cell sets collected and processed (Sub-Cohort #2). Random selection is intended to improve the variability of maternal niacin intake across the samples. uMSCs from each of the 46 samples were differentiated over a period of 21 days using adipogenic media [41].

To address Aim 3, endpoints of adipogenic potential as well as SIRT1 protein concentration and activity were measured in cells harvested on day 21 when terminal adipocyte differentiation is evident [41, 42]. Characterization of adipocytes included standard qPCR and In-Cell ELISA techniques to measure mRNA and protein content of FABP4 and PPARγ. Oil-Red-O (ORO) staining was also conducted to determine cellular
lipid content, an indicator of terminal adipocytes. SIRT1 protein concentration was measured using In-Cell ELISA and SIRT1 activity was quantified using fluorometric assay (Abcam, #ab156065).

uMSCs from each of the 46 cell sets were exposed to 4 treatment conditions: control, nicotinamide (NAM) only, lipid only, and lipid + NAM. We determined the concentration for NAM exposure to be 3 mmol through pilot experiments looking at a concentration curve of NAM exposure resulting in optimal cellular PPARγ protein content.

**Statistical Methods and Power**

*Aim 1*

Infants born to mothers with poorer diet quality will have greater adiposity compared to infants born to mothers with higher diet quality. To test this hypothesis we fit three models; the first model was a general linear multivariate model with neonatal fat-free mass (g) (FFM) and fat mass (g) (FM) as our outcomes. The second and third models were general linear multivariable regressions with percent fat mass (%FM) and birth weight as our outcomes. The interaction between maternal diet quality, as measured by total Healthy Eating Index 2010 (HEI-2010) score, and obesity was considered as our main predictor of interest in all models. We adjusted for the following covariates: maternal age, race/ethnicity, infant sex, gestational age at birth, household income, usual daily energy intake (kcal/day), smoking in pregnancy, and average energy expenditure (METS per week) over the pregnancy period. We also adjusted for pregnancy complications that could impact neonatal body composition such as chronic hypertension.
(yes/no), gestational hypertension (yes/no) and preeclampsia (yes/no) as diagnosed by a physician and reported in the medical record.

We conducted an added last test for the interaction between maternal diet quality and maternal obesity in the best fitting model. If the beta coefficient for the interaction was significant, we concluded that the synchronized action of maternal diet quality during pregnancy and maternal obesity contributed to the prediction of neonatal body composition. In this case, we reported and interpreted the beta coefficient for the interaction with asymptotic 95% confidence intervals. If the interaction was not significant, we tested the main effects of maternal diet quality and obesity. For all analyses, we evaluated the distribution of the jackknifed, studentized residuals for normality. Distributions appeared normal and no data transformations were made.

**Aim 2**

**Hypothesis 1**

Increased consumption of niacin during pregnancy is associated with increased neonatal adiposity and this association is further increased or apparent only in the presence of excess substrate as provided by a maternal HFD or maternal obesity (Figure 4). For the first part of this aim, using data from the full Healthy Start cohort we fit three models, with outcomes exactly as described above in Aim 1. However, here the interactions between maternal daily niacin (mg/day) and HFD/maternal obesity were included in all models as the main predictors of interest. We adjusted for the following covariates: total caloric intake (kcal/day), maternal age, gestational age (GA), prepregnant BMI, gestational weight gain, and birth weight. We conducted an added last test for the interaction between maternal niacin and maternal HFD/obesity in the best fitting
model. If the beta coefficient for either of the interactions was significant, we concluded that the synchronized action of niacin and HFD, as well as maternal niacin and obesity contributed to the prediction of neonatal body composition. In this case, we reported and interpreted the beta coefficient for the interactions with asymptotic 95% confidence intervals. If the interactions were not significant, we tested the main effects of maternal daily niacin intake, HFD and obesity. For all analyses, we evaluated the distribution of the jackknifed, studentized residuals for normality. Distributions appeared normal and no data transformations were made.

For Hypotheses 2 and 3 of this aim, analyses were conducted using the 200 mother-child dyads (Sub-cohort #1) described in the methods above.

**Hypothesis 2**

Increased maternal dietary niacin intake is associated with decreased neonatal SIRT1 protein concentration in the umbilical cord tissue and this relationship is

![Figure 4: Proposed relationships between maternal dietary niacin, high-fat diet (HFD)/obesity and neonatal SIRT1, and between neonatal SIRT1 and percent fat mass (%FM).](image-url)
compounded by HFD or maternal obesity. We fit a general linear univariate model with SIRT1 protein as the outcome. The interactions between maternal dietary niacin (mg/day) and HFD/obesity were our main predictors of interest and we adjusted for the same covariates listed above in Hypothesis 1. We conducted an added last test for the interactions between niacin and HFD/obesity in the best fitting model. If the beta coefficient for either interaction was significant, we concluded that it contributed to the prediction of neonatal SIRT1 protein in umbilical cord tissue. We then reported and interpreted the beta coefficient for the interactions with asymptotic 95% confidence intervals. If the interactions were not significant, similar methods were followed as described above for Hypothesis 1.

For all analyses, we evaluated the distribution of the jackknifed, studentized residuals for normality.

**Hypothesis 3**

Decreased SIRT1 protein concentration in the umbilical cord tissue is associated with increased neonatal fat mass (FM) and this relationship is enhanced in the presence of a maternal HFD or obesity. We fit a general linear univariate model with neonatal %FM as the outcome. As the primary predictor we used the interactions between SIRT1 protein and maternal HFD/maternal obesity and adjusted for all covariates listed in Hypotheses 2 and 3 for Aim 1. As described above we conducted an added last test for the interactions and reported beta values for the interactions, if significant, and for the main effects if the interactions were not statistically significant. Again, for all analyses, we will evaluate the distribution of the jackknifed, studentized residuals for normality. If the distribution is
significantly skewed, we will consider Box-Cox transformations of the outcomes to ensure normality.

**Aim 3**

In each hypothesis, adipogenic response is the main outcome of interest. Oil Red-O staining (ORO) is frequently used as an indicator of adipogenic potential [41, 42]. However, adipocyte differentiation is also characterized by the presence of several standard intracellular proteins, FABP4 and PPARγ. The role that these proteins play in adipogenic differentiation and lipid accumulation is important and related to ORO staining and should be considered in the estimation of adipogenic response. Furthermore, the experimental design requires replicates of each measure therefore inducing correlation between replicates and within cell sets.

Multivariate approaches account for intra-subject variance induced by repeated measures experiments. When outcome measures are correlated with each other, multivariate methods [43] should be employed and are capable of reducing the Type II error rate and improving the power for the analysis. For the reasons just outlined, we propose multivariate methods for Hypotheses 1 and 2 in Aim 3. From here forward we define adipogenic response as the outcomes of FABP4, and PPARγ relative protein levels.

**Hypothesis 1**

To address Hypothesis 1, that adipogenic response of MSCs will be greater in those treated with nicotinamide, lipid, or both compared to vehicle-control, we will fit a general multivariate linear model (MANOVA) for each protein outcome. We used the Hotelling-Lawley trace to test the interaction between nicotinamide and lipid treatments.
and tested the main effects of these treatments if the interaction was not significant. We reported the overall F test and p-values for the response of each of the two protein markers to adipogenic induction with and without nicotinamide or lipid.

In testing ORO and other markers of adipogenic response between the vehicle-control and nicotinamide-only conditions we used Wilcoxon rank sum t-tests for repeated measures. For all analyses the significance threshold was set at p<0.05.

**Hypothesis 2**

We hypothesized that treatment with nicotinamide would significantly decrease the SIRT1 enzyme activity of the MSCs. To address this hypothesis we used a Wilcoxon rank sum t-test for repeated measures to test the difference in enzyme activity between the nicotinamide and vehicle-control treatment conditions. Again, to declare a significant difference we set the threshold at p<0.05.

**Hypothesis 3**

To examine the association between adipogenic response (FABP4 and PPARγ) and neonatal adiposity at birth we fit a general univariate model with %FM as our outcome and FABP4 and PPARγ protein as our main predictors. We also tested the correlation between FABP4 and PPARγ to ensure that collinearity between predictor variables would not be an issue (r =0.10, p =0.56). We then reported and interpreted the beta coefficient for FABP4 and PPARγ with asymptotic 95% confidence intervals.

Power calculations were conducted using the methods of Muller et al. [44] and SAS 9.4 software (Cary, NC, SAS Corporation). Our initial proposed power is described below. Updates to the analysis are also included to describe changes in the predicted samples sizes and what we anticipated are the results of those changes. Power for Aim 1
(Diet Quality and Infant Adiposity) was not calculated as this Aim was added later in the dissertation project timeline. Aim 1 listed here corresponds to Aim 2 above; Aim 2 listed here also corresponds to Aim 2 above. Aim 3 here represents the same Aim 3 as above.

**Power for Aim 1**

Based on preliminary data from the Healthy Start study, we estimated that we would have greater than 98% power to detect a R2 change as small as 1% for the contribution of the niacin by fat interaction to the prediction of neonatal fat mass at an alpha-level of 0.05 in a linear univariate model that included the covariates described in Hypothesis 1 for Aim 1.

**Update to Aim 1 Power**

Our initial power analysis was run using the variation within niacin and total fat estimates generated from a smaller sample than that used for the final analysis. However, even given the larger sample size of the full Healthy Start cohort used in the final model our power analysis could not fully account for the effects of the covariates included in the model. Therefore, despite the power to detect a contribution of niacin and fat from the maternal diet accounting for as little as 1% of the variance in neonatal adiposity, other covariates included in the model may account for even more variance, possibly resulting in a null effect of the main predictors of interest.

**Power for Aim 2**

Information regarding SIRT1 protein concentration in human umbilical cord tissue does not currently exist in the literature. However, a study investigating SIRT1 as a factor in Alzheimer’s dementia (AD) compared serum levels of SIRT1 between young controls and elderly individuals with and without AD [45]. Here, we used the standard
deviation of SIRT1 concentration in the serum of the young controls from the Kumar study to estimate the smallest change in SIRT1 protein concentration given 80% power and a total sample size of 450 for Hypothesis 1. We used the standard deviation for FM from preliminary Healthy Start data to estimate the smallest change in FM detectable with 80% power and a total sample size of 450 for Hypothesis 2.

In the absence of existing univariate linear models that investigate the relationship between dietary niacin and SIRT1 and between SIRT1 and neonatal FM, we employed power analyses using two-sample t-tests to estimate the change in SIRT1 protein concentration and in neonatal FM that we could achieve given 80% power. Preliminary data on maternal dietary niacin intake from the Healthy Start study suggested that niacin was normally distributed and it was therefore reasonable to assume equal group sizes when dichotomizing niacin intake at the mean. With our projected sample size of 450 dyads we expected that SIRT1 protein concentrations in the umbilical cord would be normally distributed also, again making it reasonable to assume equal group sizes when dichotomizing at the mean of SIRT1 concentration.

For Hypothesis 1, we estimated that we would be able to detect a change in SIRT1 protein concentration as small as 0.23 ng/µl at the alpha-level of 0.05 with 80% power. For Hypothesis 2, we estimated that we would be able to detect a change in fat mass as small as 0.04 g at the alpha-level of 0.05 with 80% power.

**Update to Aim 2 Power**

We were unable to collect the projected number of samples (N =450) and instead achieved a sample size that was 44% of what we expected (N =200). This could significantly reduce the power to detect the differences that we projected. Further, in
measuring SIRT1 protein in the umbilical cord tissue we were unable to generate a standard curve for estimation of SIRT1 concentration (ng/µl) and instead had used the area under the curve (AUC) values provided by the Simple Western method. These values represent the relative amount of protein in the sample. The initial power calculated for all hypotheses is not relevant for AUC values; therefore it does not represent the power of the final analyses that were conducted.

Power for Aim 3

We conducted an internal pilot analysis using the methods of Gurka et al. [46]. Once 10% of our intended sample size (n=10) was cultured, we assessed what the final sample size would need to be to achieve 80% for testing the interaction between treatment of MSCs with nicotinamide and lipid and maternal dietary niacin intake on the adipogenic response of the MSCs.

Update to Aim 3 Power

The original Hypothesis 1 of Aim 3 was to test whether there was a significant association between maternal dietary intake of niacin and markers of adipogenic response (FABP4, PPARγ, ORO) to nicotinamide and lipid treatment in the MSCs cultured from the corresponding neonates. Before beginning the pilot we set an upper limit of 100 samples given the significant resources required to culture and treat the MSCs. We treated 10 cell sets with nicotinamide, lipid, or both nicotinamide and lipid and calculated the standard deviation of the mean in each treatment condition and across niacin intakes in the mothers and calculated the sample size needed to test the interaction proposed with 80% power. Given these parameters, and the large standard deviations in protein markers we estimated a needed sample size of 20,000, far above the set limit of 100. Therefore,
we moved forward without testing the interaction between treatment condition and maternal dietary niacin intake and instead tested the main effect of treatment condition on the adipogenic response of MSCs. For this we did not calculate a post-hoc power analysis.
CHAPTER IV
MATERNAL DIET QUALITY IN PREGNANCY INFLUENCES NEONATAL ADIPOSY: THE HEALTHY START STUDY

Abstract

Poor maternal diet in pregnancy may influence fetal growth and development through delivery of excess fuels such as glucose and lipids. We tested the hypothesis that poor maternal diet quality during pregnancy would increase neonatal adiposity (percent fat mass, %FM) at birth by increasing the fat mass (FM) component of neonatal body composition. Our analysis was conducted using a pre-birth observational cohort of 1,079 mother-offspring pairs. Pregnancy diet was assessed via repeated Automated Self-Administered 24-hour dietary recalls, from which Healthy Eating Index 2010 (HEI) scores were calculated for each mother. HEI was dichotomized into scores ≤ 66 and scores > 66, with low scores representing poor diet quality. Neonatal %FM was assessed within 72 hours after birth with PEA POD. Using univariate and multivariate linear models, we analyzed the relationship between maternal diet quality and neonatal %FM, FM, and fat-free mass (FFM) while adjusting for pre-pregnancy body mass index (BMI), physical activity, maternal age, smoking, daily energy intake, preeclampsia, hypertension, infant sex, and gestational age. Total HEI score ranged between 38.2 and 88.8 (mean: 62.7, SD: 10.3). An HEI score ≤ 66 was significantly associated with higher neonatal %FM (β = 0.7, 95% CI 0.2, 1.2, p=0.01) and FM (β=24.9; 95% CI 5.7, 44.0; p=0.01) but unchanged FFM. Our data provide evidence that poor diet quality during pregnancy increases neonatal adiposity independent of maternal pre-pregnancy BMI and

1 This chapter is under review with the *International Journal of Obesity*.  

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total caloric intake. This further implicates maternal diet as a potentially important exposure for fetal growth.

**Introduction**

In the United States over 60% of women of reproductive age are overweight or obese [47]. A significant focus of the research on developmental origins of health and disease has been on the impact of maternal overweight and obesity during pregnancy on infant outcomes. Large prospective cohort studies have consistently shown maternal overweight and obesity during pregnancy to be significant risk factors for higher birth weight and neonatal adiposity [48-51] and for childhood obesity and later life metabolic dysregulation [52, 53]. However, while effective interventions have been developed to promote healthy weight loss in the general adult population [53-55], it is challenging to implement interventions that can quickly and successfully help women to lose weight before pregnancy, due in large part to a significant number of pregnancies being unplanned. Therefore, a shift in research focus from reducing maternal weight before and during pregnancy to other interventions that could also impact fetal overgrowth and offspring adiposity, such as improving maternal diet and nutrition is warranted.

Nutrition as measured by levels of macro- and micronutrients consumed, as well as dietary patterns (e.g. Western, Mediterranean) during pregnancy has demonstrated significant relationships with birth outcomes implicating maternal nutritional exposures during pregnancy as important factors in fetal growth and development [15, 16, 56, 57]. Specifically, high-fat content in the diet during pregnancy has been shown to increase offspring birth weight and adiposity in several animal studies [57, 58]. Evidence from nutrient-specific and dietary pattern analyses in human pregnancies remains inconsistent.
Some studies report increased offspring adiposity given a maternal diet with a low protein-to-carbohydrate ratio [59]. Other studies report growth restriction given a maternal “Western” dietary pattern [15], or no relationship between maternal dietary patterns and offspring growth [60, 61]. This lack of consistency may be due in part to the difficulty in replicating data-driven dietary patterns across different populations [62] and the use of different infant size and growth outcomes.

Measures of diet quality that are based on national or international recommendations are a potential alternative for measuring the impact of nutrition during pregnancy on neonatal body composition. These standardized diet quality indices are generalizable across different cohorts. One such index, the Healthy Eating index, measures the inadequacy, adequacy or excess of recommended intakes of food groups (e.g. whole grains) and nutrients (e.g. sodium), therefore quantifying the quality of the total diet. In the few studies that have used a diet quality index in developed countries, higher diet quality scores were positively associated with growth parameters such as birth weight and birth length [63, 64]. However, no studies have used markers of offspring body composition such as adiposity.

The present analysis aimed to fill this information gap using the Healthy Start cohort, a pre-birth, multi-ethnic cohort of 1,410 mother-offspring pairs. Our goal was to test the hypothesis that neonates born to women with low diet quality during pregnancy have increased adiposity compared to those born to women with higher diet quality. We also tested whether diet quality modifies the effect of pre-pregnancy BMI on neonatal adiposity.
Subjects and Methods

Study Population

Mother-infant pairs included in this analysis were enrolled in the Healthy Start study, an observational, longitudinal pre-birth cohort study of ethnically diverse mothers. The Healthy Start study recruited 1,410 pregnant women ages 16 and older prior to 24 weeks gestation from the obstetrics clinics at the University of Colorado Hospital during 2010-2014. Women were excluded if they had prior diabetes, a history of prior premature birth or fetal death, asthma with active steroid management, serious psychiatric illness, or a current multiple pregnancy. The Healthy Start study protocol was approved by the Colorado Multiple Institutional Review Board and all women provided written informed consent prior to the first study visit.

As of July 2014, N=1,410 women were enrolled in the Healthy Start cohort. Healthy Start participants were eligible for the current analysis if they had at least one dietary recall (N=1,366). Women who had been diagnosed with gestational diabetes mellitus (n=53) were excluded, as these women are encouraged to adopt special diets after diagnosis. Neonates born at less than 32 weeks gestation or those without body composition measures at birth were further excluded from the eligible cohort to give a final sample size of 1,079 for the analytic cohort used in this report.

Comparison of the analytic cohort to those who were excluded revealed no significant differences in maternal race/ethnicity (p=0.35), maternal age at delivery (p=0.67), pre-pregnancy BMI (p=0.25), and household income (p=0.31). As expected, the analytical cohort had significantly higher birth weight compared to the excluded...
participants (3,255 g vs. 3,007 g, p<0.001) given the exclusion of infants born very preterm (gestational age <32 weeks).

Data Collection

Healthy Start mothers were invited to participate in two research visits during pregnancy. The first visit occurred between 8 and 24 weeks of gestation (median = 17 weeks) and the second between 24 and 32 weeks of gestation (median = 27 weeks). Maternal fasting blood samples were collected at each of the two pregnancy visits and demographic, behavioral, physical activity (energy expenditure) and dietary surveys were administered. A third visit occurred in the hospital, after delivery during which women were asked to complete surveys identical to those from the second pregnancy visit. Offspring’s birth length, weight, head circumference, and skin-fold thickness were measured within 72 hours after delivery, and neonatal body composition, fat mass (FM) and fat-free mass (FFM) were estimated from total mass and volume using air displacement plethysmography (PEA POD). Body composition was measured twice for each neonate with a third measurement taken if the first two percent body fat values were greater than two percentage points apart. Values used in this report are the average of the two closest measures.

Maternal pre-pregnant body mass index (BMI) was calculated using maternal height measured at the first research visit and pre-pregnant weight obtained from medical records (83.7%) or self-reported at the first research visit (16.2%). Pre-pregnant BMI was categorized as normal weight (BMI<25 kg/m2), overweight (25<BMI<30 kg/m2), and obese (BMI>30 kg/m2). Physical activity in pregnancy was measured using the
Pregnancy Physical Activity Questionnaire [65] from which metabolic equivalent task (MET) values were estimated as described in detail elsewhere [65, 66].

Infant sex, birth weight, and gestational age at birth were abstracted from medical records. Race/ethnicity, household income, smoking during pregnancy, and gravidity were obtained from surveys administered to participants. Race/ethnicity was categorized into non-Hispanic white, non-Hispanic black, Hispanic, and other. Household income was categorized into five levels: < $20,000, $20,000 - $40,000, $40,000 - $70,000, income > $70,000, and “don’t know”. Maternal age at delivery was calculated based on offspring delivery date and maternal date of birth.

**Dietary Assessment**

Maternal diet was assessed throughout pregnancy using the Automated Self-Administered 24-hour dietary recall (ASA24), an online platform developed and hosted by the National Cancer Institute (ASA24-Beta and ASA24-2011, Bethesda, MD: National Cancer Institute). Healthy Start participants were asked to complete up to six ASA24 dietary recalls beginning at their first pregnancy visit (approximately one per month). On average participants completed 2 recalls over the pregnancy period (range: 1 - 8) with 76% (n = 1,038) of the eligible cohort (n = 1,366) having at least 2 diet recalls. Monthly reminder calls were made by the Nutrition Coordinating Center at the University of North Carolina (UNC) at Chapel Hill to facilitate participants’ at-home dietary recalls. Trained, bilingual study staff members administered recall for Spanish-speaking participants.

Data from the ASA-24 were collected and processed by the UNC Nutrition Coordinating Center. MyPyramid Food Equivalents (MPFE) and macro- and
micronutrient components for each dietary recall were calculated using nutrient values provided by the United States Department of Agriculture’s MPFE Database (Versions 1.0 and 2.0) Food and Nutrient Database for Dietary Studies (Versions 1.0 and 4.1).

*Estimating Usual Intake of Macro- and Micronutrients*

In this study we used repeated ASA 24-hour dietary recalls to capture dietary intake across the pregnancy period. Due to the mixture of single and repeated dietary recalls and the non-linear trajectories of nutrients across the pregnancy period we implemented the National Cancer Institute’s (NCI) measurement error model (NCI method) [37, 67, 68]. The NCI method is a two-part non-linear mixed effects model from which individual estimates of usual nutrient intake can be generated using a combination of single and multiple dietary recalls.

For use in the NCI models, an a-priori list of covariates was generated from the diet and pregnancy literature; this included smoking at any time during pregnancy (yes/no), pre-pregnancy BMI (normal weight, overweight, obese), and gravidity (0 vs. any pregnancies). Observed total daily energy (kcal/day) was also included as a covariate in all NCI models. Using the NCI method we generated predicted usual intake of total energy (kcal/day), total saturated fat (g/day), total monounsaturated fat (g/day), total polyunsaturated fat (g/day), and total sodium (mg/day) for use in calculating the Healthy Eating Index scores for the present analysis. Additional predicted usual intakes were generated for total cholesterol, protein, calcium, fiber, folate, iron, and niacin.

*The Healthy Eating Index*

The Healthy Eating Index 2010 (HEI) is a diet quality scoring system developed by the United States Department of Agriculture, Center for Nutrition Policy and
Promotion designed to assess adherence to the Dietary Guidelines for Americans. This tool has been validated as a reliable measure of diet quality [39] and consists of twelve components (total fruit, whole fruit, total vegetables, greens and beans, whole grains, dairy, total protein foods, seafood and plant proteins, fatty acids, refined grains, sodium, and empty calories) [69]. The twelve components are scored per 1,000 kcal to give a maximum possible total HEI score of 100. Publically available NCI SAS macro-code from the NCI website (http://appliedresearch.cancer.gov/hei/tools.html) were used to generate the HEI total and component scores from the average MPFE values for each participant.

Statistical Analysis

Different studies have used varying ways to categorize the HEI total score. However, many analyses use quintile categorization [70, 71]. We plotted the observed mean neonatal %FM against the quintiles of the HEI total score (Q1: HEI < 53; Q2: 53 < HEI < 60; Q3: 60 < HEI < 66; Q4: 66 < HEI < 72; Q5: HEI > 72) and observed a clear, threshold effect of HEI total score on %FM that appeared between the lower three (HEI < 66) and upper two quintiles (HEI > 66). Based on the quintile plot we dichotomized the HEI total score at 66 for descriptive comparisons and all analyses reported hereafter.

Maternal and neonatal descriptive statistics were generated and differences between HEI total score categories were tested using Satterthwaite t-tests for continuous variables and Cochran Mantel-Haenszel tests for categorical variables. Average predicted estimates of usual intake of macro- and micronutrients and average servings of My Food Pyramid food groups were compared between the HEI categories using t-tests to
demonstrate the differences in nutrient and food group intakes related to the two levels of diet quality.

We fit a general linear multivariable model, and a planned, backwards stepwise approach to examine the effects of HEI total score category (< 66 versus > 66), maternal pre-pregnancy BMI category, and their interaction on neonatal %FM. Covariates for inclusion in all models were chosen based on the literature and included maternal age, race/ethnicity, infant sex, gestational age at birth, household income, usual daily energy intake (kcal/day), smoking in pregnancy, and average energy expenditure (METS per week) over the pregnancy period. We also adjusted for pregnancy complications that could impact neonatal body composition such as chronic hypertension (yes/no), gestational hypertension (yes/no) and preeclampsia (yes/no) as diagnosed by a physician and reported in the medical record. As a sensitivity analysis we included gestational weight gain as a covariate to test whether the relationship between diet quality and infant adiposity was independent of this pregnancy exposure.

Given that no significant interaction between HEI and maternal pre-pregnant BMI was noted, we then tested the main effect of the HEI categories, and the main effect of BMI categories, while controlling for covariates. This same modeling approach was applied for the general multivariate linear model that tested the effect of HEI and BMI categories on FM and FFM. An alpha-spending approach was used to control the overall Type I error. We used the Hotelling-Lawley test to assess the significance of the association between BMI and HEI with both FM and FFM at p<0.03, and, if the overall test was significant, planned to use p<0.01 for each step-down test of association between BMI and HEI, and FM and FFM, respectively.
Results

The HEI total score in the Healthy Start cohort ranged between 38 and 89 with a mean of 62.7 (SD=10.3). Maternal and neonatal characteristics are presented by HEI category in Table 1. Women with an HEI total score < 66 (lower diet quality) were significantly more likely to be obese, to have reported smoking during pregnancy, and to have a household income of less than $20,000 (p<0.001 for all, respectively). Lower diet quality was also significantly related to younger maternal age (p<0.001), shorter length of gestation (p=0.03), higher gravidity (p<0.01), and higher energy expenditure (p=0.01). Furthermore, neonates born to women with an HEI total score < 66 had significantly lower birth weight (3,231 g vs. 3,292 g, p=0.03) and FFM (2,805 g vs. 2,870 g, p<0.01) and higher %FM (9.3% vs. 8.7%, p=0.04). There was no difference in either birth head circumference (p=0.09) or birth length (p=0.47) between the two HEI groups.

The pattern of macro- and micronutrient average daily intakes (Table 2) as well as HEI component scores (Table 3) were as expected across the HEI categories. Total energy (p=0.02), percent of total energy as fat (p<0.001), as saturated fat (p<0.001) and as carbohydrate intake (p<0.01) were significantly higher in the group with an HEI total score < 66, as expected. Moreover, average HEI component scores for most of the components were significantly lower in the group with an HEI total score < 66 (p<0.001 for all, respectively).
Table 1: Characteristics of study participants by maternal Healthy Eating Index (HEI) total score (n = 1,079)

<table>
<thead>
<tr>
<th></th>
<th>HEI Total Score</th>
<th></th>
<th></th>
<th>P¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEI &lt; 66 (n=648)</td>
<td>HEI &gt; 66 (n=431)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at delivery in years, mean (SD)</td>
<td>26.2 (6.0)</td>
<td>30.2 (5.6)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Race/ethnicity, n (%):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHW²</td>
<td>298 (46.0)</td>
<td>290 (67.3)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>176 (27.2)</td>
<td>88 (20.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHB²</td>
<td>131 (20.2)</td>
<td>32 (7.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>43 (6.6)</td>
<td>21 (4.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravidity, mean (SD)</td>
<td>1.4 (1.6)</td>
<td>1.2 (1.4)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Pre-pregnancy BMI, n (%):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>329 (50.8)</td>
<td>276 (60.1)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Overweight</td>
<td>166 (25.6)</td>
<td>104 (24.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>153 (23.6)</td>
<td>51 (11.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IOM recommended GWG, n (%):</td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Inadequate</td>
<td>153 (23.6)</td>
<td>93 (21.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adequate</td>
<td>167 (25.8)</td>
<td>138 (32.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excessive</td>
<td>328 (50.6)</td>
<td>200 (46.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA at birth (weeks), mean (SD)</td>
<td>39.4 (1.3)</td>
<td>39.6 (1.3)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Cesarean section, n (%)</td>
<td>137 (21.4)</td>
<td>82 (19.2)</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Smoking in pregnancy, n (%)</td>
<td>85 (13.1)</td>
<td>11 (2.5)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Household income, n (%):</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt; $20,000</td>
<td>126 (19.4)</td>
<td>31 (7.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$20,000 - $40,000</td>
<td>100 (15.4)</td>
<td>51 (11.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$40,000 - $70,000</td>
<td>112 (17.3)</td>
<td>92 (21.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; $70,000</td>
<td>148 (22.9)</td>
<td>214 (49.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Don’t know</td>
<td>162 (25.0)</td>
<td>43 (10.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy expenditure (mets/week), mean (SD)</td>
<td>192.1 (90.7)</td>
<td>179.1 (72.1)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><strong>Offspring</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>330 (50.9)</td>
<td>199 (46.2)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Birth weight (g), mean (SD)</td>
<td>3231 (469)</td>
<td>3292 (431)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Head circumference (cm), mean (SD)</td>
<td>34.1 (2.3)</td>
<td>34.3 (1.8)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Length (in), mean (SD)</td>
<td>19.4 (1.2)</td>
<td>19.4 (1.0)</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>% FM, mean (SD)</td>
<td>9.3 (4.0)</td>
<td>8.7 (3.9)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>FM (g), mean (SD)</td>
<td>298 (156)</td>
<td>285 (149)</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>FFM (g), mean (SD)</td>
<td>2805 (360)</td>
<td>2870 (342)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

¹ P-value generated using a Satterthwaite t-test for continuous variables and Cochran Mantel-Haenszel test for categorical variables.
² NHW: non-Hispanic white; NHB: non-Hispanic black
Table 2: Average daily dietary intakes of macro and micronutrients by maternal Healthy Eating Index (HEI) total score

<table>
<thead>
<tr>
<th>HEI Total Score</th>
<th>HEI Total Score</th>
<th>HEI Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEI &lt; 66 (n=648)</td>
<td>HEI &gt; 66 (n=431)</td>
<td>P^1</td>
</tr>
<tr>
<td>Total energy (kcal)</td>
<td>2087 (412)</td>
<td>2032 (348)</td>
</tr>
<tr>
<td>% fat from total energy</td>
<td>33 (10)</td>
<td>31 (7)</td>
</tr>
<tr>
<td>% saturated fat from total energy</td>
<td>12 (4)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>% carbohydrates from total energy</td>
<td>48 (10)</td>
<td>47 (7)</td>
</tr>
<tr>
<td>% protein from total energy</td>
<td>15 (3)</td>
<td>15 (3)</td>
</tr>
<tr>
<td>Monounsaturated fat (g)</td>
<td>29 (15)</td>
<td>25 (9)</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>15 (8)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>299 (142)</td>
<td>263 (90)</td>
</tr>
<tr>
<td>Protein:Carbohydrate ratio</td>
<td>0.31 (0.04)</td>
<td>0.32 (0.05)</td>
</tr>
<tr>
<td>Added sugar (tsp)</td>
<td>67 (44)</td>
<td>53 (30)</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>3544 (1427)</td>
<td>3127 (997)</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>16 (7)</td>
<td>20 (6)</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>1085 (468)</td>
<td>1092 (343)</td>
</tr>
<tr>
<td>Folate (ug)</td>
<td>457 (181)</td>
<td>452 (131)</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>16 (7)</td>
<td>15 (5)</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>22 (8)</td>
<td>20 (5)</td>
</tr>
</tbody>
</table>

^1 p-value generated using Sattherwaite t-test.

Table 3: Mean component scores of the Healthy Eating Index (HEI) by maternal HEI total score dichotomized

<table>
<thead>
<tr>
<th>HEI Component Score 1-12, mean (SD)^1</th>
<th>HEI Total Score</th>
<th>HEI Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEI &lt; 66 (n=648)</td>
<td>HEI &gt; 66 (n=431)</td>
<td>P^2</td>
</tr>
<tr>
<td>Total vegetables^1</td>
<td>2.78 (1.33)</td>
<td>3.75 (1.59)</td>
</tr>
<tr>
<td>Greens and beans^2</td>
<td>1.35 (1.77)</td>
<td>3.38 (1.79)</td>
</tr>
<tr>
<td>Total fruit^3</td>
<td>2.94 (1.86)</td>
<td>4.37 (1.08)</td>
</tr>
<tr>
<td>Whole fruit^3</td>
<td>2.11 (2.17)</td>
<td>4.20 (1.59)</td>
</tr>
<tr>
<td>Whole grains^4</td>
<td>2.24 (2.14)</td>
<td>4.90 (2.96)</td>
</tr>
<tr>
<td>Dairy^4</td>
<td>6.45 (2.99)</td>
<td>7.49 (2.46)</td>
</tr>
<tr>
<td>Total protein foods^5</td>
<td>4.06 (1.23)</td>
<td>4.52 (0.87)</td>
</tr>
<tr>
<td>Seafood and plant protein^5</td>
<td>1.95 (1.91)</td>
<td>3.82 (1.65)</td>
</tr>
<tr>
<td>Fatty acid ratio (unsaturated:saturated)^2</td>
<td>2.83 (1.04)</td>
<td>3.35 (1.30)</td>
</tr>
<tr>
<td>Sodium^6</td>
<td>4.18 (3.40)</td>
<td>5.25 (3.09)</td>
</tr>
<tr>
<td>Refined grains^4</td>
<td>5.07 (3.24)</td>
<td>7.77 (2.34)</td>
</tr>
<tr>
<td>Empty calories^4</td>
<td>20.0 (0)</td>
<td>20.0 (0)</td>
</tr>
</tbody>
</table>

^1 P-value <0.001 for all but empty calories (NS); p-value generated using Sattherwaite t-test.

^2 Component score maximum is 10.

^3 Component score maximum is 5.

^4 Calories from added sugars and solid fats. All participants consumed ≤ 19% of their calories from empty calories therefore receiving maximum points for this component score. Alcohol was not included because participants were all under 13 g/1,000 kcal of alcohol consumption for each recall which is the threshold for inclusion of calories from alcohol.
Table 4 shows the results of the regression analyses assessing the relationship between HEI total score and neonatal body composition, adjusting for covariates. Having an HEI score < 66 was significantly associated with higher %FM (p=0.01), independent of maternal BMI. Among women with an HEI total score < 66 during pregnancy, the %FM of their neonate was on average 0.67 percentage points higher compared to neonates born to women with an HEI total score > 66 (β=0.67, 95% CI 0.17, 1.18). Furthermore, the HEI score < 66 was significantly associated with significantly higher FM (grams) (β=24.9; 95% CI 5.7, 44.0; p=0.01) but not with significantly lower FFM (β=-0.6; 95% CI -37.9, 36.7; p=0.97), indicating that the increased %FM associated with lower maternal diet quality reflects an increase in neonatal FM rather than a decrease in FFM. This compartmentalization of the effect of diet quality on infant body composition is further supported by the non-significant effect of HEI on overall birth weight (β=23.6; 95% CI -25.6, 72.8; p=0.35). Maternal pre-pregnancy BMI was a significant predictor of higher %FM, FM, FFM, and birth weight independent of maternal diet quality (p<0.01, p<0.001, p<0.05, and p<0.001). Other independent predictors of increased FM and/or %FM were older maternal age and lower household income, while gestational smoking was independently associated with lower FM. Interestingly, females had higher FM (and %FM) but lower FFM, compared with male infants while infants of non-Hispanic Black women (NHB) had both lower FM and FFM, compared with infants born to non-Hispanic white women (NHW). Additionally, our sensitivity analysis showed no significant change in the results when including gestational weight gain in the model.
## Table 4: Multivariate regression model assessing the relationship between HEI total score and neonatal body composition and birth weight.

<table>
<thead>
<tr>
<th></th>
<th>Neonatal Body Composition</th>
<th>Birth Weight</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFM (g)</td>
<td>FM (g)</td>
<td>% FM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>β (95% CI)</td>
<td>β (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>HEI Total Score:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 66</td>
<td>-0.6 (-37.9, 36.7) ref.</td>
<td>24.9 (5.7, 44.0)&lt;sup&gt;2&lt;/sup&gt; ref.</td>
<td>0.7 (0.2, 1.2)&lt;sup&gt;2&lt;/sup&gt; ref.</td>
<td>23.6 (-25.6, 72.9) ref.</td>
</tr>
<tr>
<td>&gt; 66</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td></td>
</tr>
<tr>
<td><strong>Pre-pregnancy BMI:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
</tr>
<tr>
<td>Overweight</td>
<td>31.7 (-9.6, 73.1)</td>
<td>14.7 (-6.5, 36.0)</td>
<td>0.3 (-0.2, 0.9)</td>
<td>51.1 (-3.6, 105.8)</td>
</tr>
<tr>
<td>Obese</td>
<td>52.3 (5.0, 99.7)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>47.5 (23.1, 71.8)</td>
<td>1.2 (0.6, 1.8)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>106.6 (43.3, 169.8)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>GA at birth (weeks)</strong></td>
<td>135.3 (122.2, 148.3)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>27.1 (20.4, 33.8)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.4 (0.2, 0.6)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>167.1 (149.8, 184.5)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Maternal age (years)</strong></td>
<td>2.5 (-1.1, 6.1)</td>
<td>3.7 (1.9, 5.6)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.1 (0.05, 0.1)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>7.1 (2.3, 11.9)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Smoking in pregnancy:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-131.8 (-194.4, -69.3) ref.</td>
<td>-38.8 (-71.0, -6.6)&lt;sup&gt;2&lt;/sup&gt; ref.</td>
<td>-0.7 (-1.5, 0.2) ref.</td>
<td>-194.5 (-277.4, -111.6)&lt;sup&gt;1&lt;/sup&gt; ref.</td>
</tr>
<tr>
<td>No</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td></td>
</tr>
<tr>
<td><strong>Household income:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; $20,000</td>
<td>78.2 (11.3, 145.1)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>51.7 (17.3, 86.1)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.2 (0.3, 2.1)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>129.8 (41.0, 218.6)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>$20,000 - $40,000</td>
<td>25.5 (-33.6, 84.6)</td>
<td>38.4 (8.0, 68.8)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.1 (0.3, 1.9)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>59.2 (-18.8, 137.3)</td>
</tr>
<tr>
<td>$40,000 - $70,000</td>
<td>19.8 (-30.6, 70.2) ref.</td>
<td>24.2 (-1.7, 50.1) ref.</td>
<td>0.6 (-0.05, 1.3) ref.</td>
<td>25.3 (-41.4, 92.1) ref.</td>
</tr>
<tr>
<td>&gt; $70,000</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>-19.6 (-83.9, 44.7)</td>
<td>16.0 (-17.1, 49.1)</td>
<td>0.5 (-0.4, 1.3)</td>
<td>-10.9 (-96.2, 74.4)</td>
</tr>
</tbody>
</table>

1: p<0.001
2: p<0.01
3: p<0.05
Table 4: (continued)

<table>
<thead>
<tr>
<th></th>
<th>Neonatal Body Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFM (g) (\beta) (95% CI)</td>
</tr>
<tr>
<td>Race/ethnicity:</td>
<td></td>
</tr>
<tr>
<td>NHW</td>
<td>ref.</td>
</tr>
<tr>
<td>Hispanic</td>
<td>-0.3 (-48.3, 47.7)</td>
</tr>
<tr>
<td>NHB</td>
<td>-161.6 (-217.3, -105.8)(^1)</td>
</tr>
<tr>
<td>Other</td>
<td>-41.6 (-115.7, 32.5)</td>
</tr>
<tr>
<td>Infant sex:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>ref.</td>
</tr>
<tr>
<td>Female</td>
<td>-188.2 (-222.1, -154.4)(^1)</td>
</tr>
<tr>
<td>Average energy expenditure (mets/week)</td>
<td>-0.005 (-0.3, 0.2)</td>
</tr>
<tr>
<td>Preeclampsia:</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>55.6 (-4.8, 116.0)</td>
</tr>
<tr>
<td>No</td>
<td>ref.</td>
</tr>
<tr>
<td>Chronic hypertension:</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-0.07 (-0.5, 0.4)</td>
</tr>
<tr>
<td>No</td>
<td>ref.</td>
</tr>
<tr>
<td>Gestational hypertension:</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-55.5 (-115.9, 4.9)</td>
</tr>
<tr>
<td>No</td>
<td>ref.</td>
</tr>
</tbody>
</table>

1: \(p<0.001\)
2: \(p<0.01\)
3: \(p<0.05\)
Discussion

In this prospective, multi-ethnic, pre-birth cohort we have shown that diet quality has a significant impact on neonatal adiposity, and that this effect is independent of the mother’s pre-pregnancy BMI. Furthermore, the association between lower maternal diet quality and increased neonatal adiposity is primarily due to an effect of lower diet quality on the fat compartment of neonatal body composition.

While maternal nutrition during pregnancy has been previously studied in relation to birth outcomes, to the best of our knowledge this is the first study to investigate the effect of maternal diet quality during pregnancy on neonatal body composition. Furthermore, few studies of maternal diet during pregnancy have used the HEI as a measure of overall diet quality that adheres to the Dietary Guidelines for Americans. Additionally, these studies have not targeted neonatal adiposity as the outcome, specifically; rather they have used birth weight. For example, Rodriguez-Bernal and colleagues found that increasing quintiles of the Alternative Healthy Eating Index for Pregnancy (AHEI-P) score [72] was associated with higher birth weight in a cohort of Spanish women [64].

However, in a similar study of pregnant Spanish women Gesteiro and colleagues did not see a significant difference in birth weight of infants born to mothers with an HEI total score < 70 compared to those with an HEI total score > 70 [73]. This cut-point is similar to that used in the current analysis and the inconsistency between findings may be due to differences in dietary composition of an HEI total score < 70 in Spanish women compared to the composition of an American diet with an HEI total score < 66 (e.g. servings of grains, meat, etc.). Poon and colleagues also did not see a significant
association between maternal diet quality and infant birth weight or other markers of
growth using the AHEI-P [61].

While the greatest strength of using the HEI as a tool to measure diet quality is its
comparability across populations, further explanation for the inconsistency between
studies could be the use of the HEI (score range 0-100) versus the AHEI-P (score range
0-90) and the timing of the dietary assessment during the pregnancy period. The
difference between the HEI and the AHEI-P is the incorporation of the micronutrients
folate, iron and calcium and the exclusion of alcohol in the latter. However, in our study
we show that there is no difference across the HEI categories in folate or calcium intake
and, while statistically significant, only a very small difference in iron intake was
observed, suggesting that the dietary intake of these micronutrients is relatively consistent
among the women enrolled in Healthy Start and would not contribute to the variability
among individual HEI total scores. Therefore, we do not believe the use of the different
indices to be a significant contributor to the differences in results.

The discrepancies may also be due to the timing of dietary assessment or a
combination of differences in the methods of dietary assessment (use of a single Food
Frequency Questionnaire versus repeated ASA-24 dietary recalls) and model adjustment
for potential confounders. In the Spanish cohort used by Rodriguez-Bernal (2010) and
Gesteiro (2012) diet was assessed only once and in the first trimester and Poon also
measured diet once but in the third trimester, whereas the Healthy Start study assessed
diet repeatedly (82% of sample used in this analysis had 2 or more diet recalls in
pregnancy) to estimate the HEI total score over the observed pregnancy period. The
degree to which we adjusted for additional potential confounders above that of these
other studies may also explain differences in the findings. Given the lack of studies that parallel our dietary assessment methods and analysis, our findings warrant further investigation and replication in other large, diverse birth cohorts.

The HEI categorization used in this analysis to denote a “poor quality diet” reflects what we would expect for such a diet given current dietary standards in that women with an HEI score < 66 could be considered as having a high-fat diet with high carbohydrate intake (lower protein-to-carbohydrate ratio) and high added sugar intake (Table 2). The contribution of these specific macronutrients from the maternal diet to offspring size and adiposity has been shown predominantly in animal studies of high-fat diet-fed dams [56, 58]. However, the evidence supporting the in-utero effect of these specific nutrients is less well established in humans [59]. The lack of human studies showing a deleterious effect of total fat or added sugar on neonatal size and body composition suggests that the impact on the offspring may be the result of multiple nutrients interacting. This highlights the importance of using a measure of diet quality that reflects the whole diet, likely accounting for the effects of nutrient-nutrient interactions on neonatal body composition that may not be explained by a single nutrition factor.

Maternal BMI is also an established risk factor for accelerated fetal growth and increased birth weight and size [49, 50, 74, 75]. Further, our group recently reported that the %FM at birth of neonates born to women with a pre-pregnancy BMI within the overweight or obese categories was significantly higher than the %FM of neonates born to women within the normal BMI category, a finding that sustained when further adjusted by total energy and diet quality in this analysis, further supporting the link between
maternal BMI and infant adiposity [51]. The consistent and robust data supporting the independent effects of maternal diet during pregnancy (broadly measured) and maternal BMI on fetal growth and size provide clues to potential pathways and mechanisms that need to be further explored.

We purposefully did not include gestational weight gain as a covariate in our model, hypothesizing that it may be part of the causal pathway linking maternal diet quality to neonatal adiposity. Interestingly, sensitivity analyses adjusting for maternal weight gain during pregnancy resulted in similar findings, suggesting that the effect of maternal diet quality on neonatal adiposity is independent of gestational weight gain.

In our large pre-birth cohort we have demonstrated that lower maternal diet quality, characterized by higher energy intake and higher percent dietary fat and saturated fat has a statistically significant impact on neonatal adiposity and that this effect is independent of pre-pregnancy BMI. While the clinical relevance of this finding is unclear at this time, it is important to note that neonates of women with lower diet quality had, on average, 24.9 g more fat mass, compared with those whose mothers had higher diet quality. This compares with an average 47.5 g more fat mass in neonates of obese vs. normal weight mothers in this same analysis. Longitudinal follow up of this cohort, now ongoing, will provide much needed data on the clinical significance of increased adiposity at birth with respect to childhood obesity and other relevant outcomes.

Our analysis is not without limitations. Our dichotomization of the HEI total score at 66 does not precisely align with other reported cut-off thresholds for the HEI (e.g. HEI < 70). Therefore, we are not able to compare our results directly to studies that employ other categorizations. However, given our results comparing the HEI categories to dietary...
characteristics we are confident that our categorization is appropriately capturing the thresholds of diet quality that are relevant within our cohort and are generalizable to the pregnant population in the United States.

In conclusion, our study suggests that poor diet quality during pregnancy may lead to increased neonatal adiposity regardless of maternal BMI. This highlights the potential importance of dietary interventions during pregnancy, which is likely a more accessible time for clinicians and public health practitioners to communicate the importance of healthy eating to pregnant women. Future studies of diet in pregnancy and neonatal outcomes should employ the Healthy Eating Index so to allow comparison across pregnant populations and for replication of the findings from this analysis.
CHAPTER V
EXPLORING THE ROLE OF MATERNAL NIAacin INTAKE AND NEONATAL SIRT1 PROTEIN EXPRESSION IN ADIPOsITY AT BIRTH: THE HEALTHY START STUDY

Abstract

Fetal over-nutrition by maternal obesity or unhealthy diet during pregnancy can result in excess fetal fat accretion. Excess micronutrients, such as nicotinamide, a form of niacin, may also contribute to higher infant adiposity through adipogenic mechanisms involving the Sirtuin proteins. We hypothesized that high maternal dietary niacin intake during pregnancy, especially in combination with either maternal obesity or high-fat diet (HFD) would significantly increase adiposity at birth and decrease Sirtuin 1 (SIRT1) protein in umbilical cord tissue. We included 1,040 participants from a pre-birth cohort of mother-infant pairs. Pregnancy diet was assessed using Automated Self-Administered 24-hour dietary recalls from which daily niacin and fat intake were derived. Neonatal body composition (% fat mass [%FM], fat mass [FM], fat-free mass [FFM]) was measured by PEAPOD. SIRT1 protein was measured in umbilical cord tissue of 200 neonates. We used multivariate regression models to assess the joint effects of maternal dietary niacin with pre-pregnancy body mass index (BMI) and maternal HFD on %FM, FM, and FFM. We also tested the associations between niacin and SIRT1 protein, and SIRT1 protein and %FM using univariate regression. All interactions and the main effect of dietary niacin were non-significant. Both maternal HFD and BMI were significantly associated with %FM and FM (p<0.01). Maternal niacin was not associated with SIRT1 protein, nor was SIRT1 protein associated with %FM. Maternal obesity and HFD both contribute to
higher infant adiposity. However, we found no evidence that maternal niacin intake is associated with infant adiposity.

**Introduction**

Adequate nutrition during pregnancy has been shown to positively impact fetal growth and development. Specifically, increasing maternal intake during pregnancy of micronutrients such as folic acid, iron, and several B vitamins have demonstrated consistent effects on preventing low birth weight, pre-term birth and decreasing postnatal mortality [76, 77]. However, studies showing these effects are mostly observed in developing nations where nutrition is poor. In the United States where maternal obesity in pregnancy is prevalent and is itself an over-nourishing state for the fetus, it is likely that the resulting excess availability of nutrients contribute to detrimental outcomes such as macrosomia, high birth weight and excess neonatal adiposity [51].

In addition, maternal high-fat diet (HFD) in particular has been shown to be causally associated with higher offspring adiposity and size at birth in animal models [19, 56, 58]. However, the evidence supporting this relationship in humans is less consistent, suggesting that excess dietary fat during pregnancy may not be the sole culprit in increasing fetal growth and adiposity. Furthermore, while fat from the maternal diet during pregnancy or maternal fat stores may provide the substrate for fetal fat accretion, micronutrients may act on specific adipogenic mechanisms to compound the effect of maternal obesity or maternal dietary fat on development of infant adiposity.

The peroxisome proliferator activator gamma (PPARγ) protein is a central controller of progenitor cell differentiation into adipocytes [78-80], a process called adipogenesis. Higher levels of PPARγ protein and gene expression have been linked to
greater adiposity in humans [81, 82]. Importantly, PPARγ is itself controlled by several mechanisms that are sensitive to the energy and nutrition status of the organism. Sirtuin 1 (SIRT1), a Class III histone and protein deacetylase enzyme, is involved in one such control mechanism as it is an inhibitor of PPARγ gene expression and protein activity [2, 35, 83, 84]. Activation of SIRT1 in mouse mesenchymal stem cells (MSCs) significantly reduced terminal differentiation into adipocytes and this corresponded with significantly lower PPARγ mRNA levels [35]. Within this mechanism where PPARγ is down regulated by SIRT1, resulting in less fat accretion, it would follow that restriction of SIRT1 could potentially induce greater adipogenesis.

Nicotinamide (NAM) is a form of niacin or vitamin B3 consumed predominantly through animal-based foods, enriched grain products, and vitamin supplements. NAM is an essential micronutrient and contributes to production of nicotinamide adenine dinucleotide (NAD+), a major energy substrate used in whole-body metabolism and deacetylase enzyme reactions. Dietary niacin, in general has been shown to increase PPARγ gene expression in adipose tissue of rabbits fed a high-cholesterol diet [85]. NAM is also a potent, non-competitive inhibitor of SIRT1 [4, 86]. Treatment of human mesenchymal stem cells (MSCs) with nicotinamide inhibited SIRT1 protein activity and increased PPARγ protein levels and promoted cell commitment toward an adipogenic fate (Shapiro et al., 2015, unpublished data) suggesting that nicotinamide/niacin could drive adipogenesis through a SIRT1 and PPARγ dependent mechanism.

Given the evidence supporting a potential link between NAM/niacin, SIRT1, PPARγ, and adipogenesis we hypothesized that high dietary niacin intake (as a proxy for dietary NAM) in conjunction with maternal obesity or a HFD diet during pregnancy
would increase infant adiposity at birth. Furthermore, to gain a window into the hypothesized mechanistic pathway, we investigated the specific associations between maternal dietary niacin and neonatal SIRT1 protein levels in umbilical cord tissue, and how umbilical tissue SIRT1 protein levels related to infant adiposity at birth, in a subset of participants.

**Subjects and Methods**

*Study Population*

In this analysis we used mother-infant pairs who participated in the Healthy Start study, a longitudinal, pre-birth cohort of ethnically diverse mothers which aims to investigate maternal metabolic and behavioral exposures in pregnancy and their impact on offspring obesity and related outcomes. Details of the Healthy Start study recruitment methods and protocol have been published elsewhere [51, 66]. Briefly, the Healthy Start study recruited 1,410 pregnant women ages 16 and older prior to 24 weeks gestation from the obstetrics clinics at the University of Colorado Hospital during 2010-2014. Women were excluded if they had prior diabetes, a history of prior premature birth or fetal death, asthma with active steroid management, serious psychiatric illness, or a current multiple pregnancy. Participants provided written informed consent prior to the first study visit. The Healthy Start study protocol was approved by the Colorado Multiple Institutional Review Board.

*Data Collection*

Healthy Start mothers were invited to participate in two research visits during pregnancy. The first visit occurred at a median of 17 weeks gestation (range 8 – 24 weeks) and the second visit was at a median of 27 weeks gestation (range 24 – 32 weeks).
At each of the two pregnancy visits maternal fasting blood samples were collected and demographic, behavioral, physical activity (energy expenditure) and dietary surveys were administered.

A third visit occurred at delivery in the hospital during which women were asked to complete surveys identical to those from the second pregnancy visit. Neonatal anthropometrics (e.g. birth length, weight, head circumference, and skin-fold thickness) were measured within 72 hours after delivery, and neonatal body composition, fat mass (FM) and fat-free mass (FFM) were estimated from total mass and volume using air displacement plethysmography (PEA POD). Body composition was measured twice for each neonate with a third measurement taken if the first two of the %FM values were greater than two percentage points apart. Values used in this report are the average of the two closest measures.

Umbilical cord tissue was also collected at time of birth in a sub-cohort of infants (N = 200) from the parent Healthy Start study. Upon delivery of the placenta, trained clinical research nurses excised a 1-inch section of the umbilical cord from the area furthest from the cord’s attachment to the placenta. The tissue was cut in half along the length of the excised section and washed with chilled distilled water to remove any remaining blood. The clean tissue was then snap frozen in liquid nitrogen and stored at -80°C until tissue processing.

Maternal pre-pregnant body mass index (BMI) was calculated using maternal height measured at the first research visit and pre-pregnant weight obtained from medical records (83.7%) or self-reported at the first research visit (16.2%). Pre-pregnant BMI was categorized as normal weight (BMI<25 kg/m²), overweight (25≤BMI<30 kg/m²), and
obese (BMI $> 30 \text{ kg/m}^2$) for all analyses. Physical activity in pregnancy was measured using the Pregnancy Physical Activity Questionnaire [65] from which metabolic equivalent task (MET) values were estimated as described in detail elsewhere [65, 66].

Infant sex, birth weight, and gestational age at birth were abstracted from medical records. Race/ethnicity, household income, smoking during pregnancy, and gravidity were obtained from surveys administered to participants. Race/ethnicity was categorized into non-Hispanic white, non-Hispanic black, Hispanic, and other. Household income was categorized into five levels: $< \$20,000$, $\$20,000 - \$40,000$, $\$40,000 - \$70,000$, income $> \$70,000$, and “don’t know”. Maternal age at delivery was calculated based on offspring delivery date and maternal date of birth.

*Dietary Assessment*

The Healthy Start study collected detailed dietary information on women throughout their pregnancies using the Automated Self-administered 24-hour dietary recall, an online platform developed and hosted by the National Cancer Institute (ASA24-2014. Bethesda, MD: National Cancer Institute) as well as a Food Propensity Questionnaire (FPQ). Detailed dietary assessment methods used in Healthy Start have been previously described (Chapter IV).

Estimation of usual intake of non-episodically consumed macro- and micronutrients was conducted using the “NCI method”, a measurement error model that applies a two-part non-linear mixed effects modeling approach [37, 67, 68]. This approach resulted in a single individual estimate of average daily nutrient intake (e.g. niacin, total fat, saturated fat, etc.) for each participant. For detailed modeling methods please refer previous publications (Chapter IV).
Snap-frozen umbilical cord tissue was segmented into pieces weighing approximately 30 mg and thawed in a 200 ul solution of tissue lysis buffer (Sigma, #C3228) with 5 ul/ml protease inhibitor (Sigma, #P8340). Thawed samples were homogenized and spun at 16,000xg for 10 minutes. The supernatant was collected and stored at -80°C until protein analysis.

SIRT1 protein content was analyzed using the Simple Western (WES) method (Protein Simple, San Jose, CA). SIRT1 antibody (Santa Cruz Biotechnology, #sc-15404) was optimized in-house for use on WES at a 1:50 dilution with a protein concentration of 0.4 mg/ml. For assurance of quality control a pooled sample was run on each WES plate (%CV =11%). The area under the curve (AUC) value was used for SIRT1 quantification; all AUC values were normalized to the pooled sample. Normalized SIRT1 values were square root-transformed to achieve a normal distribution and used for all analyses.

**Statistical Analysis**

Maternal and infant characteristics were generated by BMI status of the mother. Means (SD) and frequencies of these characteristics were compared across BMI categories (normal weight, overweight, and obese) by ANOVA for continuous variable or Cochran Mantel-Haenszel tests of general association for categorical variables.

Average daily dietary niacin intake was applied in all models as a continuous variable while we defined a HFD using the American Heart Association criteria where a diet with >30% of caloric energy derived from fat (total fat) and >12% of total fats consumed as saturated fat qualifies as an HFD. Due to the potential for collinearity between nutrient variables such as niacin and total energy (kcal/day) we estimated the
residual value for total energy regressed on niacin. This value was then used in all models to adjust for total kcal/day.

To test the hypothesis that a maternal high-fat diet modifies the effect of maternal dietary niacin consumption on neonatal body composition we fit a general linear multivariable model, and a planned, backwards stepwise approach to examine the effects of maternal high-fat diet (yes/no), estimated average daily dietary niacin intake (mg/day), and their interaction on neonatal %FM. Covariates for inclusion in this model were chosen based on their potential as confounders and included maternal age, race/ethnicity, infant sex, gestational age at birth, and usual daily energy intake (kcal/day). We did not observe a significant interaction between maternal HFD and dietary niacin (p >0.1), therefore we tested the main effect of HFD, and the main effect of dietary niacin, while controlling for covariates. We applied this same modeling approach for the general multivariate linear model that tested the effect of maternal HFD and dietary niacin on FM and FFM.

For each of the above models we also tested the two-way interaction between pre-pregnancy BMI and dietary niacin. We used an alpha-spending approach to control the overall Type I error and the Hotelling-Lawley test to assess the significance of the association between pre-pregnancy BMI and dietary niacin with both FM and FFM at p<0.03. If the overall test was significant, we used p<0.01 for each step-down test of association between BMI and dietary niacin intake, and FM and FFM, respectively. Pre-pregnancy BMI did not significantly modify the relationship between dietary niacin and body composition (p >0.1). Therefore, BMI was included in the final models as a covariate.
We analyzed the relationships among maternal HFD, dietary niacin, umbilical cord tissue SIRT1 protein levels and neonatal body composition using general linear multivariable models. Specifically, the first of these models tested the association between dietary niacin and umbilical cord tissue SIRT1 protein levels while controlling for maternal age, pre-pregnant BMI, total energy (kcal/day), and gestational age at birth. In our second model we tested the association between SIRT1 protein levels and neonatal %FM and whether a maternal HFD modified this relationship. Here we controlled for the same group of covariates as in our first model. When a statistically significant interaction was not found (p >0.1), we then tested the independent associations of maternal HFD and SIRT1 protein levels with %FM.

**Results**

At the conclusion of recruitment in September 2015, a total of 1,410 pregnant women had been enrolled in the Healthy Start cohort. Healthy Start participants were eligible for the current analysis if they had at least one dietary recall (N=1,366). Neonates born at less than 37 weeks gestation or those without body composition measures at birth were also excluded. Women who had been diagnosed with gestational diabetes mellitus (n=53) were further excluded from the eligible cohort to give a final sample size of 1,040 for the full analytic cohort used in the first of our analyses investigating the relationship between maternal dietary niacin and neonatal body composition. In our analysis of maternal diet, umbilical cord tissue SIRT1 protein levels, and neonatal body composition we included 173 of the total 200 mother-infant pairs from whom we were able to collect umbilical cord tissue at birth as these individuals also met the above inclusion criteria.
Table 5 presents mother and infant characteristics by pre-pregnant BMI status in the full analytic cohort of 1,040 pairs. Mothers who were classified as having an obese pre-pregnancy BMI were more likely to be non-Hispanic black, have a higher gravidity, gain excessive weight over the gestation period, have an annual household income of less than $27,000, and smoke during pregnancy (p<0.001 for all, respectively). These women were also more likely to deliver by cesarean section (p=0.01). Infants of mothers with obesity prior to pregnancy were significantly heavier overall (p=0.04) with higher absolute FM and %FM (p<0.001 for both, respectively).

In the full analytic cohort, both maternal HFD and maternal obesity, but not maternal dietary niacin intake were significantly associated with %FM and FM (Table 6). On average mothers who ate a HFD during pregnancy gave birth to neonates who had, on average 0.8 percentage points greater %FM (β=0.8, 95% CI: 0.1, 1.4, p<0.05) and 32.4 g higher absolute FM (β=32.4, 95% CI: 6.7, 58.0, p<0.01). However, FFM was not associated with a HFD. Independent of maternal HFD, infants of obese mothers had, on average 1.5 percentage points greater %FM (β=1.5, 95% CI: 0.9, 2.2, p<0.001), 60.5 g higher absolute FM (β=60.5, 95% CI: 35.9, 85.0, p<0.001), 53.4 g higher absolute FFM (β=53.4, 95% CI: 5.7, 101.1, p<0.001) and 116.7 g higher birth weights (β=116.7, 95% CI: 53.6, 179.8, p<0.001) compared to infants of normal weight mothers.
Table 5: Demographic and descriptive characteristics by pre-pregnant BMI status of the full analytic cohort (N = 1,040)

<table>
<thead>
<tr>
<th></th>
<th>Pre-pregnant BMI Status</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (n=582)</td>
<td>Overweight (n=258)</td>
<td>Obese (n=200)</td>
</tr>
<tr>
<td>Age at delivery in years, mean (SD)</td>
<td>27.9 (6.1)</td>
<td>27.8 (6.2)</td>
<td>27.9 (6.0)</td>
</tr>
<tr>
<td>Race/ethnicity, n (%) :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHW¹</td>
<td>373 (64.1)</td>
<td>120 (46.5)</td>
<td>77 (38.5)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>98 (16.8)</td>
<td>90 (34.9)</td>
<td>68 (34.0)</td>
</tr>
<tr>
<td>NHB¹</td>
<td>72 (12.4)</td>
<td>37 (14.3)</td>
<td>44 (22.0)</td>
</tr>
<tr>
<td>Other</td>
<td>39 (6.7)</td>
<td>11 (4.3)</td>
<td>11 (5.5)</td>
</tr>
<tr>
<td>Gravidity, mean (SD)</td>
<td>1.0 (1.3)</td>
<td>1.5 (1.7)</td>
<td>2.1 (1.8)</td>
</tr>
<tr>
<td>IOM recommended GWG, n (%) :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inadequate</td>
<td>146 (25.1)</td>
<td>36 (13.9)</td>
<td>48 (24.0)</td>
</tr>
<tr>
<td>Adequate</td>
<td>198 (34.0)</td>
<td>57 (22.1)</td>
<td>42 (21.0)</td>
</tr>
<tr>
<td>Excessive</td>
<td>238 (40.9)</td>
<td>165 (63.9)</td>
<td>110 (55.0)</td>
</tr>
<tr>
<td>GA at birth (weeks), mean (SD)</td>
<td>39.6 (1.1)</td>
<td>39.6 (1.1)</td>
<td>39.4 (1.1)</td>
</tr>
<tr>
<td>Cesarean section, n (%)</td>
<td>98 (16.9)</td>
<td>61 (23.9)</td>
<td>51 (26.3)</td>
</tr>
<tr>
<td>Smoking in pregnancy, n (%)</td>
<td>49 (8.4)</td>
<td>17 (6.6)</td>
<td>24 (12.0)</td>
</tr>
<tr>
<td>Household income, n (%) :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; $20,000</td>
<td>58 (10.0)</td>
<td>36 (13.9)</td>
<td>55 (27.5)</td>
</tr>
<tr>
<td>$20,000 - $40,000</td>
<td>68 (11.7)</td>
<td>41 (15.9)</td>
<td>35 (17.5)</td>
</tr>
<tr>
<td>$40,000 - $70,000</td>
<td>106 (18.2)</td>
<td>59 (22.9)</td>
<td>32 (16.0)</td>
</tr>
<tr>
<td>&gt; $70,000</td>
<td>241 (41.4)</td>
<td>75 (29.1)</td>
<td>35 (17.5)</td>
</tr>
<tr>
<td>Don’t know</td>
<td>109 (18.7)</td>
<td>47 (18.2)</td>
<td>43 (21.5)</td>
</tr>
<tr>
<td>Energy expenditure (mets/week), mean (SD)</td>
<td>184.5 (85.0)</td>
<td>188.3 (82.2)</td>
<td>188.9 (80.7)</td>
</tr>
<tr>
<td>HFD (yes), n (%)</td>
<td>199 (34.2)</td>
<td>85 (32.9)</td>
<td>75 (37.5)</td>
</tr>
<tr>
<td>Niacin (mg/day), mean (SD)</td>
<td>21.7 (7.6)</td>
<td>20.8 (7.1)</td>
<td>20.7 (7.9)</td>
</tr>
<tr>
<td>Offspring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>299 (51.4)</td>
<td>120 (46.5)</td>
<td>92 (46.0)</td>
</tr>
<tr>
<td>Birth weight (g), mean (SD)</td>
<td>3254 (417)</td>
<td>3311 (454)</td>
<td>3331 (444)</td>
</tr>
<tr>
<td>Length (cm), mean (SD)</td>
<td>49.3 (2.8)</td>
<td>49.5 (2.5)</td>
<td>49.5 (2.3)</td>
</tr>
<tr>
<td>% FM, mean (SD)</td>
<td>8.8 (3.9)</td>
<td>9.1 (3.9)</td>
<td>10.1 (4.1)</td>
</tr>
<tr>
<td>FM¹ (g), mean (SD)</td>
<td>282 (147)</td>
<td>299 (153)</td>
<td>334 (164)</td>
</tr>
<tr>
<td>FFM¹ (g), mean (SD)</td>
<td>2838 (324)</td>
<td>2876 (367)</td>
<td>2864 (330)</td>
</tr>
</tbody>
</table>

¹ NHW – non-Hispanic white; NHB – non-Hispanic Black; FM – fat mass; FFM – fat-free mass.
² p-values generated using Cochran-Mantel Haenszel test for categorical characteristics and ANOVA for continuous characteristics.

As expected, older maternal age at delivery, higher gestational age at birth, and female sex of the infant were all significantly associated with higher %FM and FM.
(p<0.01 for all, respectively). These covariates were also significantly associated with FFM and birth weight. Finally, infants of non-Hispanic black race/ethnicity had significantly lower FM, FFM, %FM and birth weight, compared with NHW peers (p<0.001 for all, respectively).

In our analysis of the sub-cohort, we found no association between maternal dietary niacin intake and umbilical cord SIRT1 protein levels, nor did we find that any covariates in this model related to SIRT1 protein in the umbilical cord tissue (data not shown). Neither SIRT1 (β=0.5, 95% CI: -1.3, 2.2) nor HFD (β=0.7, 95% CI: -0.6, 2.0) were associated with %FM in the sub-cohort, but as expected, gestational age at birth and female sex of the infant were significantly and positively associated with %FM (p<0.05 for both, respectively) (data not shown).

**Discussion**

In this large, diverse pregnancy cohort we did not find a significant association between maternal dietary niacin intake and infant body composition, nor were we able to provide supporting evidence that maternal obesity or HFD altered the relationship between maternal niacin intake and infant body composition. However, both maternal pre-pregnancy BMI and maternal HFD were significant and independent predictors of %FM and FM. Our finding that a maternal HFD during pregnancy was significantly associated with neonatal adiposity but not with either FFM or birth weight, suggests that the effect of maternal dietary fat intake during pregnancy may be preferential to increasing the fat compartment of the fetus during growth.
Table 6: Associations between maternal dietary niacin, high-fat diet, obesity and neonatal body composition, adjusted for covariates (N = 1,040).

<table>
<thead>
<tr>
<th></th>
<th>%FM $\beta$ (95% CI)</th>
<th>FM $\beta$ (95% CI)</th>
<th>FFM $\beta$ (95% CI)</th>
<th>Birth Weight $\beta$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary niacin (mg/day)</td>
<td>0.01 (-0.03, 0.05)</td>
<td>0.4 (-1.2, 1.9)</td>
<td>-1.2 (-4.2, 1.8)</td>
<td>-1.6 (-5.5, 2.3)</td>
</tr>
<tr>
<td>HFD:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.8 (0.1, 1.4)</td>
<td>32.4 (6.7, 58.0)</td>
<td>28.0 (-21.9, 77.9)</td>
<td>52.3 (-13.6, 118.3)</td>
</tr>
<tr>
<td>No</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
</tr>
<tr>
<td>Pre-pregnant BMI:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
</tr>
<tr>
<td>Overweight</td>
<td>0.4 (-0.1, 1.0)</td>
<td>19.6 (-2.3, 41.5)</td>
<td>34.3 (-8.4, 76.9)</td>
<td>58.1 (1.7, 114.6)</td>
</tr>
<tr>
<td>Obese</td>
<td>1.5 (0.9, 2.2)</td>
<td>60.5 (35.9, 85.0)</td>
<td>53.4 (5.7, 101.1)</td>
<td>116.7 (53.6, 179.8)</td>
</tr>
<tr>
<td>Race/ethnicity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHW</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0.1 (-0.6, 0.7)</td>
<td>4.6 (-19.1, 28.3)</td>
<td>6.4 (-39.6, 52.5)</td>
<td>-0.03 (-61.0, 60.9)</td>
</tr>
<tr>
<td>NHB</td>
<td>-0.2 (-0.9, 0.5)</td>
<td>-23.7 (-51.9, 4.5)</td>
<td>-167.6 (-222.4, 112.7)</td>
<td>-223.8 (-296.3, -151.2)</td>
</tr>
<tr>
<td>Other</td>
<td>-0.7 (-1.7, 0.3)</td>
<td>-24.1 (-63.1, 14.9)</td>
<td>-35.0 (-110.9, 40.8)</td>
<td>-83.1 (-183.4, 17.2)</td>
</tr>
<tr>
<td>Maternal age</td>
<td>0.1 (0.01, 0.1)</td>
<td>2.4 (0.8, 4.1)</td>
<td>3.0 (-0.1, 6.1)</td>
<td>6.1 (2.0, 10.3)</td>
</tr>
<tr>
<td>Total energy (kcal/day)</td>
<td>0.002 (-0.003, 0.01)</td>
<td>0.1 (-0.1, 0.3)</td>
<td>0.1 (-0.3, 0.4)</td>
<td>0.03 (-0.5, 0.5)</td>
</tr>
<tr>
<td>GA at birth (weeks)</td>
<td>0.5 (0.3, 0.7)</td>
<td>28.8 (20.6, 37.0)</td>
<td>128.7 (112.9, 144.7)</td>
<td>158.1 (137.1, 179.1)</td>
</tr>
<tr>
<td>Infant sex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.5 (1.0, 2.0)</td>
<td>35.1 (17.1, 53.0)</td>
<td>-191.0 (-225.9, -156.2)</td>
<td>-154.1 (-200.2, -108.1)</td>
</tr>
<tr>
<td>Male</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
<td>ref.</td>
</tr>
</tbody>
</table>

1 p-value < 0.001.  
2 p-value < 0.01.  
3 p-value < 0.05
Excess substrate availability (glucose, fatty acids, triglycerides) due to progressive insulin resistance is a defining characteristic of maternal metabolism during pregnancy, especially among obese pregnancies. A high-fat diet has also been shown to further promote insulin resistance [87-90]. The adoption of a diet high in fat may further increase the fuel availability for fetal growth and fat mass accretion.

We suspect that the lack of association between dietary niacin and neonatal body composition may be due, at least in part, to our dietary instrument’s inability to distinguish between the NAM form of niacin and the nicotinic acid (NA) form, both of which are derived from the diet. NA is not physiologically equivalent to NAM and does not inhibit SIRT1. Therefore, the sensitivity of using total niacin estimates derived from self-reported diet as a proxy for dietary NAM exposure may be poor. This could result in a washing-out of the effect of NAM on neonatal adiposity, potentially explaining our finding of lack of an association between maternal dietary niacin and neonatal adiposity and body composition.

Further, in our analysis of umbilical cord tissue SIRT1 protein levels in the Healthy Start sub-cohort we did not find that maternal dietary niacin was associated with umbilical cord SIRT1 levels or that umbilical cord SIRT1 levels were related to adiposity at birth. In addition to the potential lack of sensitivity of total dietary niacin as our proxy measure for NAM exposure, we suspect that SIRT1 protein levels may not accurately measure the \textit{in-vivo} impact of NAM on SIRT1 protein activity and therefore not fully capture the effect on neonatal adiposity as the activity of the SIRT1 protein is responsible for controlling PPARγ and ultimately, adipogenesis. Given the heterogeneous cell populations contained in umbilical cord tissue and the predominance of connective tissue,
it is also possible that the SIRT1 protein levels in the umbilical cord are not representative of the SIRT1 protein content in the progenitor cells (mesenchymal stem cells [MSCs]), the multipotent cell population with the capacity to undergo adipogenesis.

To test our hypothesis in-vitro, studies within our group are ongoing to investigate whether NAM has a specific effect on the adipogenic potential of MSCs derived from the umbilical cord tissue collected from the sub-cohort of Healthy Start participants. These studies are also investigating the protein activity of SIRT1 in the MSC population and how the level of activity impacts PPARγ protein expression and other adipogenic markers such as intracellular lipid accumulation in adipose tissue.

Our analysis is the first to investigate the relationships between maternal dietary niacin intake with neonatal body composition and umbilical cord SIRT1 protein. We have successfully collected longitudinal dietary information and to our knowledge are the first to implement the ASA24 hour dietary assessment tool and NCI measurement error model approach within a large pre-birth cohort in the United States. We were also able to collect umbilical cord tissue samples from a relatively large group within the Healthy Start cohort that allowed us to analyze proteins in a tissue that is likely more representative of fetal tissue than cord blood, which is a mixture of both mother and neonate.

Despite these strengths our analysis has some limitations. While maternal dietary information was collected with state-of-the-art self-reporting tools, the data remains self-reported and can be subject to recall error [91, 92]. It is well recognized that individuals underreport food and nutrient consumption when self-reporting their diets therefore leading to random error and an overall attenuation of the diet-disease relationship being
studied. Misreporting of diet can also lead to bias; here, pregnant women who recognize that they eat less healthy diets (containing more total energy, fat and niacin) may have underreported their diet because they were counseled by a physician on how an unhealthy diet could impact their infant’s size. This differential error due to misreporting of the diet and its potential relationship to the outcome of infant adiposity could compound the attenuated effect of dietary niacin as a poor proxy for NAM exposure as previously described.

In conclusion, our results suggest that maternal obesity and maternal HFD during pregnancy independently increase neonatal adiposity at birth, while we were unable to find an effect in our assessment of the relationship between dietary niacin and the umbilical cord tissue protein expression of SIRT1. Further biomarker studies that directly measure maternal NAM and SIRT1 protein activity are needed to confirm that the associations between nicotinamide and SIRT1, and SIRT1 and adiposity do not in fact exist.
CHAPTER VI

NICOTINAMIDE PROMOTES ADIPOGENESIS IN UMBILICAL CORD-DERIVED MESENCHYMAL STEM CELLS AND CORRESPONDS TO NEONATAL ADIPOSYTITY

Abstract

The cellular mechanisms whereby excess maternal nutrition during pregnancy increases adiposity of the offspring are incompletely understood. Nicotinamide (NAM) has been linked to adipogenesis through inhibition of Sirtuin 1 (SIRT1) protein deacetylase activity, which controls expression of the key adipogenic gene, PPARγ. We tested the novel hypothesis that NAM increases the adipogenic response of human umbilical cord tissue-derived mesenchymal stem cells (MSCs) through a SIRT1 and PPARγ pathway and that the adipogenic response to NAM is associated with increased adiposity among neonates. MSCs were derived from the umbilical cord of 46 neonates born to non-obese mothers enrolled in the Healthy Start study. Neonatal adiposity was measured using air displacement plethysmography (Pea Pod) shortly after birth.

Adipogenic differentiation was induced for 21 days in MSCs +/- 3 mM NAM in the presence or absence of lipid (200 µM oleate/palmitate mix). Compared to the vehicle-control condition, NAM significantly increased PPARγ protein (+24%, p <0.01) and intracellular lipid content (+51%, p <0.01) at day 21 but lipid alone had no effect (p =0.99). With NAM exposure, in day 21 differentiated MSCs, SIRT1 enzymatic activity was significantly decreased (-70%, p <0.05). Further, the percent increase in PPARγ protein in response to NAM treatment was significantly associated with %FM at birth ($\beta$ =0.04, 95% CI 0.01-0.06, p <0.001). These data suggest that chronic NAM exposure
potentiates adipogenesis in human MSCs in culture, possibly through a SIRT1 mediated pathway. Further, the association between NAM-mediated increases in PPARγ and neonatal adiposity suggests a specific role for excess dietary NAM in the development of infant adiposity, a hypothesis that requires further testing.

**Introduction**

Nutrition in pregnancy has been shown to affect various aspects of fetal growth and development [93]. A maternal high-fat diet (HFD) during pregnancy increases newborn adiposity in both rodents and in non-human primates [94-96]. In addition to increased macronutrient content, modulation of adipogenesis may also occur through pathways involving micronutrients that can interact directly with proteins involved in promoting adipose tissue accretion. Evidence from cell culture models suggests that niacin may be one such “obesogenic” micronutrient, where bone marrow-derived mesenchymal stem cells (MSCs) treated with nicotinamide (NAM), the amide form of niacin, demonstrated increased adipogenesis in vitro [35]. However, the mechanism by which niacin induces a greater adipogenic response is unclear.

One prominent nutrient sensor that governs adipogenesis is silent mating type information regulation 2 homolog 1 (SIRT1), a NAD(+) -dependent deacetylase. SIRT1 has been shown in several studies to govern adipogenesis, in a 3T3L pre-adipocyte cell model [84], and in animal models whereby adipose-specific SIRT1 knockout mice have increased PPARγ protein activity, increased adiposity, and metabolic dysfunction in-vivo [95]. SIRT1 has also been shown to inhibit adipogenic differentiation when stimulated by a pharmacologic activator, resveratrol [2]. Importantly, SIRT1 is potently inhibited by NAM suggesting that nutritional micronutrient levels govern its activity. Likewise, HFD
inhibits adipose tissue SIRT1 activity through proteolysis [97], and its activity is suppressed in the fetal livers of non-human primate offspring whose mothers consumed a HFD during gestation [20]. Taken together, these data suggest a possible pathway whereby dietary fat and nicotinamide promote adipogenesis through a SIRT1-mediated pathway. Moreover, NAM-mediated changes in SIRT1 activity may be one mechanism by which maternal diet in pregnancy impacts adipogenesis in-utero and fetal adiposity.

Micronutrients have received very little attention as modulators of fetal developmental programming. In this study we investigate the effect of nicotinamide, in combination with excess fatty acids, on SIRT1 activity and the adipogenic potential of human umbilical cord-derived mesenchymal stem cells, which represent a fetal stem cell population. We hypothesized that in-vitro NAM exposure would decrease SIRT1 activity and induce greater adipogenesis and that co-incubation with lipids would amplify these effects. We also tested the hypothesis that NAM-induced increases in adipogenic response would be associated with infant adiposity at birth, possibly reflecting the potential for NAM exposure in-utero to increase adipogenic response in infants over a range of fat mass.

**Subjects and Methods**

We cultured human umbilical cord-derived mesenchymal stem cells (MSCs) from umbilical cord tissue collected from neonates whose mothers were enrolled in the Healthy Start study, a longitudinal pre-birth cohort study of ethnically diverse women in Colorado. The Healthy Start study recruited pregnant women ages 16 and older with a gestational age less than 24 weeks from the obstetrics clinics at the University of Colorado Hospital during 2010-2014. Women were excluded if they had prior diabetes, a
prior premature birth or fetal death, asthma with active steroid management, serious psychiatric illness, or a current multiple pregnancy. The Healthy Start study was approved by the Colorado Multiple Institutional Review Board and all participants provided written informed consent prior to delivery for collection of the umbilical cord tissue for cell culture purposes.

Healthy Start data collection methods have been described in detail elsewhere [51]. Briefly, with regard to measures used in this study, infant body composition was measured within 72 hours after birth using air displacement plethysmography (Pea Pod). Fat mass and fat-free mass were estimated from total body mass and percent fat mass (%FM) was generated by calculating the proportion of fat mass over total body mass. Body composition was measured twice for each infant with a third measurement taken if the two preceding %FM measures differed by more than two percentage points. Values used in this study are the average of the two closest measures of body composition.

**Sample Collection, MSC Isolation and Culture**

We collected, cultured, and cryogenically stored 165 samples of unique MSC sets from infants that make-up the Healthy Start Baby Biology of intra-Uterine Metabolic Programming (BabyBUMP) Project, which is the mechanistic arm of the parent Healthy Start study. For the current study, MSCs from up to 46 individual infants born to non-obese (BMI <30), healthy mothers were randomly selected for experimentation.

At delivery, trained research personnel cut a 4-inch section of the umbilical cord below the placement of the clamp and rinsed the segment thoroughly with deionized water. The tissue section was then immersed in PBS with 0.01% penicillin and
streptomycin (PEN-STREP) and stored at 4°C until processing. All tissue samples were processed within 24 hours of delivery.

Umbilical cord tissue was cut into 12, 50-100 mg pieces with 6 pieces arranged equidistant, Wharton’s Jelly side down, on each of two 10 cm dishes with bovine serum albumin (BSA) (Sigma, #A7970) to assist with adhesion of the tissue to the plate. Tissue was immersed in low-glucose (4.5 g/L) Dubelcco’s Modified Eagle’s Serum (DMEM) supplemented with MSC growth factors (MSCGM; SingleQuot, Lonza, Walkersville, MD, USA). Media changes were completed twice per week until cells had visibly grown out around the perimeter of the tissue explants. Tissue explants were removed from the 10cm dishes and cells were passed into two 10cm dishes using 0.05% trypsin (Corning, #25-051-CI) for removal. Cells were then cultured to 80-100% confluence in MSCGM at which point they were stored in liquid nitrogen with 0.05% DMSO for cryogenic preservation. We have shown this method to yield cells >98% positive for MSC markers (Boyle K.E. et al., under review).

**Adipogenic Induction of MSCs**

For adipogenesis experiments, cells were thawed and sub-cultured to confluence into 10 cm dishes. At 100% confluence, cells were plated at an approximate cell density of 12.8x10^4 per well onto standard 96-well culture plates. Day 0 of experimentation occurred when experimental plates were 90–100% confluent. Differentiation was induced on Day 0 by changing growth media to Adipogenic Induction Media (AIM) consisting of low-glucose DMEM supplemented with 5% fetal bovine serum (FBS), 0.01%-STREP, 1.0 uM dexamethasone (DEX), 0.2 mM indomethasone (INDO), and 170 nM insulin [41]. After 3 days of AIM, media was changed to Adipogenic Maintenance Media.
(AMM) consisting of low-glucose DMEM, 5% FBS, 0.01% PEN-STREP, and 170 nM insulin. Cells were exposed to AIM twice more with a rest period of AMM in between. See S1 Table for induction schedule and media recipes.

For adipogenesis in the presence of NAM and/or lipid, adipogenic induction was performed as described above, with or without 3 mM NAM and/or lipid (1 mM carnitine + 200 μM oleate and palmitate in a 2:1, oleate:palmitate ratio, bound to BSA at a molar concentration of 2.5:1). Prior to experimentation, a concentration dose-response curve of NAM (2.5, 3.0, 4.5 mM) was performed to determine the optimal concentration for PPARγ induction (S1 Figure). Ethanol was used as vehicle-control. On day 21 plates were rinsed twice in PBS and then fixed to culture plates with 4% formalin diluted in PBS for 5 min at room temperature. Plates were stored in PBS at 4°C until assays were performed.

*In-Cell ELISA (ICE)*

**Antibody Selection and Optimization**

We quantified adipogenic differentiation response by measuring protein content of PPARγ (Cell Signaling, #C26H12) and FABP4 (Cell Signaling, #3544). We also measured intracellular SIRT1 protein content (Santa Cruz, #sc-15404). Beta(β)-actin was measured for total protein normalization (Cell Signaling, #13E5.). Primary antibodies for these proteins were selected based on their successful application by the manufacturer to either immunohistochemistry or in-cell ELISA platforms. Goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Goat ≠ Rabbit; Abcam, #ab97051) was also selected based on this criterion.
Antibody optimization for the ICE was performed on purchased human bone marrow-derived mesenchymal stem cells (hBM-MSC; Lonza, PT-2501). hBM-MSCs were grown in 96-well plates for 21 days according to the adipogenic differentiation protocol above (without treatment conditions). Following the ICE assay procedures below accepting for a 30 minute colorimetric kinetic read, each antibody was tested at multiple dilutions that were selected so to flank the manufacturers’ recommended dilution. The slope profiles for each antibody dilution were analyzed. Dilutions presenting with multiple stable slope values over 5 minute intervals within the 30-minute read time were chosen as optimal dilution factors for the respective antibody (S2 Table).

**ICE Assay Procedure**

Cells were permeabilized at room temperature rotating for 1 hour using 100 μL/well permeabilization/blocking buffer (0.1% Fraction V BSA, 5% goat serum, 0.3% Triton X-100, and 0.2% sodium azide in PBS). Wells were rinsed at room temperature for 2x5 minutes with rotation using 100 μL/well rinse buffer (0.01% Fraction V BSA and 1% goat serum in PBS). Primary antibodies were diluted in antibody buffer (0.1% Fraction V BSA and 5% goat serum in PBS) and 50 μL was added to all wells, except the no primary control (NPC) wells. Plates were incubated overnight at 4°C with rotation.

After primary antibodies were removed, wells were rinsed at room temperature for 7x5 minutes with rotation using 100 μL/well rinse buffer. Fifty μL/well of the optimal dilution of Goat ≠ Rabbit secondary antibody (Supplemental Table 2) was added to all wells and plates were incubated at room temperature for 1 hour with rotation. Wells were then rinsed at room temperature for 3x5 minutes with rotation using with 100 μL/well rinse buffer, then for 2x5 minutes with rotation using with 100 μL/well PBS. Wells were
emptied and blotted dry on absorbent paper, 75 μL/well of HRP developing solution (Thermo Scientific, #N301) were added to all wells. Color change was measured at 1-minute intervals for 10 min at 650 nM. Stable slopes for 5 consecutive minutes were used to quantify the content of proteins of interest. All data were normalized to β-actin protein content.

ICE Validation in MSC vs. 3T3-L1 Cells

To help validate the protein measurements for differentiation response obtained from the MSC experiments we compared MSCs to the 3T3-L1 mouse fibroblast cell line (ATCC, #CL-173) during differentiation under control conditions. MSCs were grown and differentiated in standard 96-well plates according to the protocol above and 3T3-L1 cells were differentiated using published methods [98]. Plates were fixed with 4% formalin as described above on days 0, 7, and 14. PPARγ, FABP4, and β-actin protein content was measured by ICE assay (S2 Fig). In the MSCs we also measured PPARγ protein content at day 21 in the vehicle-control condition by Simple Western size-based protein assay (WES, ProteinSimple, Santa Clara, CA) following manufacturer’s protocol. Results from WES were analyzed using ProteinSimple Compass software. Antibodies were optimized in-house for this system and antibody specifics are listed in S2 Table.

Lipid Accumulation and Oil-Red-O Staining

Intracellular lipid accumulation was measured on cells fixed in 4% formalin and stained with 0.2% Oil Red-O dissolved in 85% propylene glycol for one hour. Cells were rinsed with fresh propylene glycol (2x) followed by two rinses with deionized water. Pictures were then taken of representative plate wells using a phase contrast microscope with a 10x objective lens. The ORO stain was then solubilized from the cell by
submergence in isopropanol for 5 minutes. Lipids were measured on a spectrophotometer (520 nm) with endpoint analysis.

**SIRT1 Activity Assay**

Cells were grown to confluence in 6 cm petri dishes, differentiated for 9 days (peak adipogenesis in the second AIM induction) and 21 days (endpoint of adipogenic induction) using the adipogenic protocol with and without NAM treatment and harvested with 100 ul of CellLytic lysis buffer (Sigma, #C3228). Cell lysate was sonicated and centrifuged; the supernatant was flash frozen and stored at -80°C until assay.

SIRT1 protein was extracted from cell supernatant using immunoprecipitation. Briefly, 1 ul of a 1:50 dilution of SIRT1 antibody (Abcam, #ab7343) was added to each sample of cell supernatant and incubated overnight at 4°C with agitation. The antibody-supernatant mixture was added to a 50% slurry of protein A agrose bead (Cell Signaling, #9863S) and incubated for 3 hours at 4°C with agitation. SIRT1 protein activity was then measured by fluorometric assay at 350 nm (Abcam, #ab156065) according to the manufacturer’s protocol for quantification of SIRT1 activity.

**Acetylation of SIRT1 Target Proteins**

PPARγ and β-catenin are directly de-acetylated by SIRT1 [83, 99]. Therefore, as an additional measure of SIRT1 de-acetylase activity we quantified acetylated protein content of these specific proteins in the same representative sample of 9 cell sets described above at day 9 of adipogenic differentiation. Total acetylated protein was immunoprecipitated from cell supernatant using an antibody to acetylated lysine as described above and the content of PPARγ (Cell Signaling, #C26H12) and β-catenin
(Cell Signaling, #9582P) in the immunoprecipitant was measured on WES, and expressed relative to total PPARγ and β-catenin. Antibody specifics are listed in S2 Table.

Quantitative PCR Analysis

Cells were rinsed with PBS (x2) and then harvested in Buffer RLT (Qiagen, Valencia, CA) with 1% 2-mercaptoethanol (Bio-Rad, #161-0710). Total RNA was isolated using RNeasy Plus mini kit (Qiagen). Total RNA (0.5 μg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using primer sets for genes of interest and reference genes (designed using NCBI’s Primer3/BLAST) and iTaq Universal SYBR Green Supermix (Bio-Rad) following manufacturer's protocols. Reactions were run in duplicate on an iQ5 Real Time PCR detection system (Bio-Rad) along with a no-template control per gene. Validation experiments were performed to demonstrate that efficiencies of target and reference genes were approximately equal. Data were normalized to three reference genes using the comparative Ct method. Gene names and primer sequences are listed in S3 Table.

Statistical Analysis

All analyses were conducted with Type I error rate set to 0.05, using SAS 9.4 (SAS Institute, Cary, North Carolina).

We conducted MANOVA analyses by fitting a separate general linear multivariate model for each protein marker (day 21 PPARγ, SIRT1, and FABP4). We describe the analysis for PPARγ; the analysis for the two additional protein markers is exactly parallel. The general linear multivariate model allows accounting for correlation between the repeated measurements within one individual cell set. The four treatment conditions correspond to the elements of a complete two-by-two factorial design. The
two factors, each with two levels: NAM+/− and lipid +/−. The NAM-/lipid- condition is the vehicle-control condition.

We used the Hotelling-Lawley trace to consider, in turn, the NAM by lipid interaction, and then, if the interaction was non-significant, the main effects of NAM and lipid separately. We did not account for multiple comparisons, because we conducted a planned sequence of a priori hypothesis tests for only three outcomes. We used regression diagnostics to examine the assumptions of multivariate normality of the residuals.

In our sub-sample (n = 9) of the full dataset (N = 46), we used the Wilcoxon signed rank t-test for matched pairs to compare vehicle-control to NAM for the outcomes of ORO, SIRT1 activity, acetylated SIRT1 target proteins, and gene expression.

The percent change in PPARγ protein content in response to NAM was expressed relative to the vehicle-control cells and defined as 100% times the ratio of PPARγ protein content for the NAM+/Lipid- condition relative to the PPARγ protein content for the vehicle-control condition (the NAM-/Lipid- condition). The percent increase for FABP4 was calculated similarly.

To examine the association between adipogenic response in the MSCs and the outcome of %FM at birth in the neonate, we fit a general linear univariate model. The two predictors were percent increase in PPARγ and FABP4. We produced 95% confidence intervals for the beta coefficients of the models, as measures of the association between adipogenic response to NAM treatment and adiposity in the neonate.
Results

*NAM Increased PPARγ on Day 9 in MSCs*

We investigated the effects of NAM at day 9 of differentiation, during the second hormonal induction with AIM. As expected, we observed a significant increase in PPARγ mRNA expression between the vehicle-control and NAM conditions (Figure 5A). SIRT1 activity was unchanged between the vehicle-control treated cells and the NAM-treated cells on day 9 of adipogenic differentiation (Figure 5B) as were protein levels of acetylated PPARγ and β-catenin (Figure 5C-D). However, interestingly, NAMPT, the rate limiting enzyme in the NAD+ salvage pathway that normally increases SIRT1 activity [100] was significantly lower in the NAM-treated cells compared to the vehicle-control treated cells (-34%, p <0.01) (Figure 5E).

**Figure 5:** Effects of NAM (3mM) incubation during adipocyte cell differentiation in human MSC. Cells were harvested at day 9 of differentiation and mRNA expression of PPARγ (A), SIRT1 enzyme activity (B) and acetylation of protein targets, PPARγ (C) and β-catenin (D), and mRNA expression of NAMPT (E) in the vehicle-control and NAM treatment conditions as described in Methods. N =9 per group. *p<0.05 vs. control.
**NAM Incubation Decreased SIRT1 Activity in Differentiated MSCs**

We compared the effects of NAM and lipid treatment over 21 days of differentiation, with the hypothesis that increasing lipid concentration would suppress SIRT1 protein. NAM increased SIRT1 protein by 20% (p<0.01) (Figure 6A), with no effects of lipid treatment alone. Nevertheless, SIRT1 enzyme activity was suppressed significantly at day 21 of differentiation in NAM-treated cells by an average of 71% (p=0.01) (Figure 6B).

![Figure 6: Effects of NAM (3mM) and lipid (200uM) during adipose differentiation on SIRT1 protein and enzyme activity. SIRT1 protein content in MSCs measured by ICE assay at day 21 in all four treatment conditions in the full experimental cohort of 46 cell sets (A). A representative sample of 9 sets of cells from the 46 experimental sets was used for SIRT1 enzyme activity at day 21 in vehicle-control and NAM conditions only (B). *p<0.05 vs. control.](image)

**NAM and Not Lipid Increases Adipogenic Markers in Terminally Differentiated MSCs**

To test the effects of lipid and NAM on differentiation, we measured PPARγ and its down-stream target FABP4 on day 21 of differentiation. Cells treated with NAM or NAM-plus-lipid had significantly greater PPARγ protein content compared to vehicle-control treated cells (PPARγ +24%, p <0.05 for both, respectively), whereas there was no significant difference in PPARγ protein content in lipid-only treated cells compared to the vehicle-control condition (Figure 7A). Similarly, FABP4 protein content was
significantly higher in cells with the NAM and NAM-plus-lipid treatment compared to the vehicle-control condition (+57%, p <0.05 for both, respectively) (Figure 7B). Further, lipid accumulation, as measured by Oil-Red-O was significantly greater in cells treated with NAM, compared to vehicle-control (+51%, p <0.05) as shown in Figure 7 C-E.

**Figure 7:** NAM increases PPARγ, FABP4, and lipid content in MSC-derived adipocytes. MSC were incubated with standard differentiation media for 21 days +/-NAM (3mM) as described in Methods. Protein content at day 21 was measured by ICE assay in all treatment conditions (A and B) (N = 46). A representative sample of 10 sets of cells from the 46 experimental sets was used for day 21 Oil-red O staining (ORO); lipid accumulation measured by ORO in vehicle-control and NAM conditions only (C and D). A representative sample is shown in (E).
Adipogenic Response to NAM in MSCs is Associated with Infant Adiposity at Birth

We compared the NAM-induced adipogenic response in MSCs with the %FM of the neonate from which the cells were derived. The percent increase in PPARγ protein between the NAM and vehicle-control condition was significantly and independently associated with neonatal %FM at birth ($\beta =0.04$, 95% CI 0.01-0.06, $p <0.001$) while the percent increase in FABP4 protein was not associated with %FM ($p =0.53$).

Discussion

The present study tested the hypothesis that SIRT1 may be involved in micronutrient-induced enhancement of adipogenic differentiation of human MSCs. Our results demonstrated that NAM inhibits SIRT1’s activity during adipogenic differentiation and increases adipogenic protein markers in differentiated human MSCs. Others have found that SIRT1 inhibitors, such as resveratrol, may control adipogenesis in different species and cell lines [35, 86]. However, this is the first study to demonstrate NAM’s effect in human MSCs and suggests that this micronutrient could be involved in controlling MSC cell fate toward adipogenesis.

We have also shown in this study that lipid exposure did not increase adipogenic protein markers in our MSCs; rather NAM exposure appeared to account entirely for the increased adipogenesis in the NAM-only and co-incubated NAM and lipid conditions. Intracellular lipid accumulation in the NAM condition was also significantly higher than the vehicle-control condition. Together these results suggest that NAM may be a strong driver of adipogenesis even in the presence of lipids. Our failure to induce greater adipogenesis with lipid treatment may be due in part to our attempt at mimicking a
physiologically relevant concentration of excess lipid exposure [101] whereas other studies of fatty acid-induced adipogenesis have used much higher lipid doses [102, 103].

Interestingly, despite an increase in PPARγ, we found a potentially delayed effect of chronic exogenous NAM exposure on reducing SIRT1 activity during adipogenesis in that SIRT1 activity was not significantly reduced until terminal adipogenic differentiation (day 21). Our early analysis on day 9 showed that NAM reduced the expression of the key NAD+ salvage pathway enzyme, NAMPT. The intracellular \textit{de-novo} synthesis of NAD+ from NAM is dependent on the NAD+ Salvage Pathway that involves the rate-limiting enzyme, NAMPT. The availability and activity of NAMPT is essential for maintaining the NAD+- to -NAM ratio and is therefore important for regulating SIRT1 activity [104-106]. In a murine model of MSC cell fate, Li and colleagues demonstrated that the NAMPT inhibitor, FK866, increased intracellular NAM levels and decreased SIRT1 activity which subsequently increased PPARγ expression, lipid accumulation, and adipocyte formation [107].

In our experimental model, the early reduction in NAMPT expression would potentially diminish the intracellular pool of NAD+ and increase the endogenous levels of NAM, thereby favoring decreased SIRT1 activity. This decreased NAD-to-NAM ratio could compound the direct inhibitory effects of the exogenous NAM, possibly reaching a threshold later in the adipogenic induction, which resulted in the significant decrease in SIRT1 activity toward the end of adipogenic differentiation. However, in several studies NAMPT was reportedly increased during adipogenic induction of 3T3-L1 cells [105, 108], therefore suggesting that under normal adipogenic conditions NAMPT is up-regulated potentially to accommodate the increased demand for NAD+. The increase in
PPARγ in the early phases of differentiation without a change in SIRT1 activity suggests that the effects of NAM on PPARγ expression in early adipogenesis are not straightforward, and could be governed by other early factors that affect PPARγ expression, e.g. C/EBPs, CREB, or OCT1. It remains unknown how NAM directly impacts NAMPT expression and activity and therefore this finding and proposed mechanism requires further investigation.

Importantly, we were able to demonstrate a significant association between in-vitro markers of adipogenesis in the MSCs treated with NAM and in-vivo neonatal adiposity at birth in the infants from which the cells were derived; infants with greater adiposity at birth had correspondingly larger percent changes for cellular PPARγ in response to NAM treatment. This finding, notably in cells derived from infants born to non-obese mothers, suggests a potential adipogenic susceptibility of MSCs to excess NAM exposure in-utero, possibly resulting in the adiposity levels that we observed in the corresponding infants. Further, given that the MSC population is retained throughout an individual’s lifetime, this MSC model of adipogenesis and NAM exposure also highlights the potential for postnatal sensitivity to dietary NAM-induced adipogenesis. As this is the first study to demonstrate this relationship, future studies are needed to support our findings.

In addition to its novelty, our study has several significant strengths. Most importantly is the relevance of the MSC model for studying in-utero and postnatal adipogenic mechanisms in human offspring given that they are sourced directly from fetal tissue and are precursor cells to adipose tissue as well as other metabolically active tissues. Furthermore, the parent study, Healthy Start provides extensive phenotyping of
participants both during pregnancy and postnatally. This has facilitated our ability to take
\textit{in-vitro} measures of adipogenesis and compare them to \textit{in-vivo} adiposity of the
participating infants, therefore helping to generalize our findings to living, intact human
systems. The detailed information available from Healthy Start will also allow future
mechanistic investigations using the BabyBUMP Project sample into the impact of other
pregnancy and postnatal exposures on fat, muscle, bone, and some neuronal tissue
development.

Despite our study’s strengths there are several notable limitations. Primarily, our
study was not designed to test whether NAM \textit{induces} adipogenesis in the absence of
hormonal influence from dexamethasone and insulin. Rather, we have shown that NAM
exposure during the adipogenic process \textit{enhances} differentiation. Furthermore, The
balance of NAD+ to NAM is an important regulator for SIRT1 action, and while we were
able to show a reduction in the key NAD+ salvage enzyme, NAMPT we did not measure
intracellular levels of NAD+, with which we could calculate the NAD-to-NAM ratio and
potentially estimate the activity of NAMPT. These limitations warrant further
investigation.

In conclusion, our study has shown the potentially “obesogenic” effect of excess
NAM \textit{in-vitro}. NAM is ubiquitous in the diet and may be even more prevalent in diets
with high consumption of processed grains and animal products. Among pregnant women
specifically, additional NAM, and not nicotinic acid is consumed through prenatal
vitamins [6]. This in addition to our results suggests that further investigation is needed to
determine whether maternal NAM consumption during pregnancy in a free-living human
population increases infant adiposity at birth.
Supplementary Material

**S1 Figure:** PPARγ protein content in human bone marrow-derived MSCs measured at day 21 by ICE assay at tested concentrations of nicotinamide.

**S2 Figure:** Comparison of 3T3-L1 pre-adipocytes with human MSC over 14 days of differentiation. Adipogenic induction in control media in both the 3T3-L1 cell set (A and B) and the representative set of MSCs (C and D) were carried out according to Methods and PPARγ and FABP4 measured as outlined in methods. A representative sample is shown here.
**S1 Table**: Adipogenic differentiation media recipes and induction schedule

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**S2 Table**: Antibodies and their dilutions for the different protein detection methods used in this study

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<td>Goat ≠ Rabbit</td>
<td>1:50</td>
</tr>
<tr>
<td></td>
<td>β-catenin</td>
<td>1:50</td>
<td></td>
<td>1:50</td>
</tr>
</tbody>
</table>
### S3 Table: Gene names and primer sequences for PCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ v2</td>
<td>AGCAAAACCCTATTCCATGCT</td>
<td>TGTGTCACCATGGTCATTCTTG</td>
</tr>
<tr>
<td>NAMP T</td>
<td>GTGGAGGTTTCTACAGAACT</td>
<td>TGGGTCTTTGAAGACGTTAATC</td>
</tr>
<tr>
<td>RLP13 A (ref.)</td>
<td>CCTGGAGGAGAAGGAAAGA GA</td>
<td>TTGAGGACCTCTGTTATTTGTC AA</td>
</tr>
<tr>
<td>ACTB (ref.)</td>
<td>CACTCTCAGCCTCCTTC</td>
<td>GTACAGGTCTTTGCGGATGT</td>
</tr>
</tbody>
</table>
CHAPTER VII

SUMMARY, LIMITATIONS, AND FUTURE DIRECTIONS

Fetal programming, a concept introduced over twenty years ago by the late Dr. David Barker, centers on the phenomenon of in-utero and early childhood exposures permanently altering an individual’s susceptibility to chronic diseases across the life course. Barker’s work as well as others’ has highlighted the potential detrimental role of the maternal diet during pregnancy in fetal growth and long-term health of the offspring (reviewed in [109]). Increasingly, a “poor diet” in the United States is characterized by excess carbohydrates and saturated fat [110]. Studies that have investigated this recent trend, specifically with maternal diets in relation to neonatal outcomes have identified increased neonatal size (e.g. birth weight, length) as a consequence of a poor, nutrient-rich pregnancy diet [59, 60, 63]. The research presented in this dissertation contributes additional evidence to support these observations.

In this multifaceted investigation of the relationship between maternal diet and offspring body composition we found that overall diet quality and maternal high-fat diet specifically, were significantly associated with infant adiposity at birth in the Healthy Start study, a large, diverse cohort of mother-infant pairs. Our finding regarding diet quality suggests that the foods and nutrients that make up a mother’s diet during pregnancy may act together in-vivo to promote fetal fat accretion, possibly through a general, overall excess of nutrients. This is corroborated in part by our finding that a high-fat diet contributes to greater adiposity; therefore further elucidating one potential and specific component of the diet that is in excess. However, upon testing the hypothesis that excess maternal dietary niacin (as a proxy measure for dietary nicotinamide) would
also increase adiposity, especially in the presence of excess substrate, we did not find the hypothesized association. As discussed previously in Chapter V we attribute this null association in part to the imprecision of the dietary measure of total niacin that is an amalgamation of two compounds, nicotinamide and nicotinic acid, of which nicotinamide is our hypothesized “obesigenic” micronutrient. We also did not find a significant relationship between maternal dietary niacin intake and neonatal umbilical cord tissue SIRT1 protein levels, nor did we find that SIRT1 protein levels were associated with neonatal adiposity at birth. While non-significant, given the limitations of this component of the study, our results warrant further investigation into the relationship between maternal nicotinamide consumption and umbilical cord tissue SIRT1 and adiposity in vivo.

In our investigation into the possible mechanism through which nicotinamide from the diet could induce greater adiposity we observed in-vitro that umbilical cord-derived mesenchymal stem cells exposed to nicotinamide demonstrated greater propensity towards adipogenesis that we attribute, in part, to a SIRT1 and PPARγ mediated mechanism. SIRT1 activity was significantly reduced at terminal differentiation and this corresponded with a significant increase in the main adipose cell markers, PPARγ and FABP4. Further, we found that this mechanism may be preceded by disruption of cellular energy homeostasis involving a significant reduction in cell salvaging of NAD+, a major substrate in oxidative metabolism and SIRT1 action, by the enzyme, NAMPT. NAMPT mRNA expression was significantly reduced at day 9 of differentiation suggesting that nicotinamide recycling into NAD+ by the NAD+ salvage pathway may be reduced early in the adipogenic process. Further, SIRT1 enzyme activity
was unchanged at this same point during adipogenic induction. Therefore, the effect of nicotinamide treatment on SIRT1 function may be delayed by two processes; first, endogenous nicotinamide begins to accumulate in the cell as a result of reduced NAD+ salvaging and therefore reducing NAD+ substrate availability for the NAD-dependent enzyme activity of SIRT1. This may then be further compounded by exogenous addition of nicotinamide and its direct, inhibitory effect on SIRT1.

The adipogenic response of the fetal cells to the nicotinamide treatment was also significantly correlated with neonatal adiposity at birth, suggesting a possible link between the effect of excess nicotinamide on the adipogenic potential of these progenitor adipose cells and in-utero development of adipose tissue. In-vivo, this may manifest in the fetal mesenchymal stem cell population as adipogenic induction-sensitivity followed by enhanced fetal fat accretion as a result of excess maternal dietary nicotinamide.

However, our null finding in the larger Healthy Start cohort demonstrates that further investigation is needed to draw firm conclusions about the effect of nicotinamide from the maternal diet on the in-utero development of body composition. Further, we were not able to relate maternal dietary intake of niacin to the adipogenic potential of the MSCs. This brings to light the limitations of this work, namely the challenges of accurate and precise measurement of dietary components and the implementation of nutrient-disease modeling, as well as the limitations to generalizability of in-vitro research models to free-living human populations.

To synthesize the knowledge gained by this work, we have revised the initial conceptual model (Figure 1) and present it below (Figure 8). In Figure 8, poor maternal diet quality encompasses both a high-fat diet (HFD) and high dietary niacin, evidence of
which is presented in Table 2 where a mother with a poor quality diet (HEI score < 66) consumed significantly more dietary fat and niacin. In Chapters IV and V we showed that poor maternal diet quality and HFD were significantly and independently associated with neonatal adiposity (depicted in Figure 8 by long, solid arrows). However, we were unable to provide evidence that maternal dietary niacin intake was directly related to adiposity at birth and therefore there is no direct connection depicted in Figure 8. Rather, our experimental model reported in Chapter VI demonstrated that \textit{in-vitro} manipulation of fetal stem cells with nicotinamide (NAM) increased adipogenic potential and therefore we propose that this represents a possible indirect effect of maternal niacin on adiposity through NAM which could not be measured directly in the Healthy Start cohort.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Revised conceptual model depicting the interdependence of maternal diet quality, high-fat diet (HFD), and niacin and the observed direct and proposed indirect effects of these in-utero exposures on infant adiposity.}
\end{figure}
In this work we have combined the fields of nutrition epidemiology and experimental cell biology, each of which has its own strengths and general limitations. Nutrition epidemiology has contributed significantly to the design of methods for assessment and analysis of the whole diet and its components for population-level research. However, this field has long struggled with the issue of random error in nutrient estimates caused by underreporting of dietary consumption [91]. In nutrient-disease modeling, the approach used in Aim 2, this generally leads to an attenuation of the overall effect; therefore a relationship may exist but it is not discernable amongst the noise of the nutrient estimates. However, the combination of several diet survey methods can help to reduce the errors introduced by self-report [37, 67, 68].

In our study we used this combined approach, employing repeated 24-hour dietary recalls over the entire pregnancy period coupled with food propensity questionnaires (food frequency questionnaire with only frequency of consumption and not amount), and data were analyzed in an advanced statistical modeling approach, specifically the measurement error model, designed to improve the estimation of specific nutrients while minimizing the effect of random error and potential reporting bias induced by self-report [67]. Therefore, while we found no association between dietary niacin and infant adiposity, we do not attribute this completely to random error; rather, we believe that the potential lack of sensitivity of using total niacin as a surrogate for dietary nicotinamide is the primary reason for not observing a significant association, as discussed in Chapter V. We also did not account for nicotinamide obtained from supplement use. However, in the Healthy Start cohort greater than 95% of the participants were taking prenatal vitamins
suggesting that all women were receiving a similar supplementary dose and that our diet analysis was measuring niacin/nicotinamide consumption above the baseline level.

Our use of a stem cell model to test the hypothesized pathway between nicotinamide and adipogenesis presents further strengths and also limitations for this work. Using this experimental approach we were able to directly test the cellular response to excess nicotinamide exposure and the potential in-vitro nutrient-nutrient interaction between nicotinamide and lipid. Through this approach we learned that nicotinamide decreases SIRT1 protein activity but increases protein content, highlighting a potential cellular compensatory response. Further, our stem cell model allowed us to target a relevant human adipose tissue precursor, therefore improving the generalizability of our results to our target population over what would be possible using an in-vitro model with mouse fibroblast-derived pre-adipocytes (e.g. widely used 3T3-L1 cell line for adipogenic experiments) or an in-vivo animal model.

There are also several limitations to our experimental model. Foremost, we tested the effect of excess nicotinamide and/or excess lipid treatment and not the effect of a whole diet that has excess nicotinamide and lipids. While originally designed as a proof of concept experiment, choosing to expose cells to specific nutrients limits our ability to generalize our results to a free-living maternal-placental-fetal unit who would otherwise consume a diet with multiple components. Therefore, we cannot speculate on the true effect of excess nicotinamide in the presence of all other dietary components. Our use of in-vitro methods withMSCs also limits our generalizability because these cells were not manipulated within the whole body system and therefore the impact of complimentary and compensatory responses from other tissues could not be captured by our experiments.
While our external generalizability is limited, our use of cells derived from umbilical cord tissue provides a relevant model for fetal response to potential in-utero exposures due to this tissue’s fetal origin.

The specific limitations of this study highlight future directions for investigations into the relationship between maternal micronutrients, specifically dietary nicotinamide exposure during pregnancy, and fetal fat accretion. Given the comprehensive biological sampling of the mothers in the Healthy Start study, future efforts should also focus on measuring nicotinamide in the maternal urine, of which we have two repeated samplings during the pregnancy period in mid- and late gestation. This would provide a more objective measure of maternal nicotinamide status in pregnancy and therefore potentially the exposure dose received by the fetus. Further, this biomarker assessment could be linked to the diet data for validation of the niacin and nicotinamide estimates derived from the ASA24. A biomarker for dietary fat intake such as a red blood cell membrane fatty acid analysis would give us a more durable assessment of fat intake over 3 months.

Alternative measures could also be applied to address our limitations in Aim 2 (Chapter V) surrounding the use of SIRT1 protein content in umbilical cord tissue as an in-vivo measure of the proposed pathway linking maternal dietary niacin, neonatal SIRT1 and adiposity. Here, a future direction should be to measure SIRT1 enzyme activity in the frozen umbilical cord samples given that SIRT1 activity and not necessarily protein content is the driving mechanism behind enhanced adipogenesis. However whether umbilical cord tissue is an accurate index of SIRT1 activity in progenitor cells of the fetus is unknown.
Importantly, the implications of our findings could potentially go beyond adipogenic differentiation in-utero; the consequences of reduced SIRT1 activity may impact the fetus on a systemic level given the ubiquitous expression of SIRT1 throughout the different tissue systems. Namely, several studies have demonstrated SIRT1’s role in promoting neural axonal outgrowth and cognitive function. Li and colleagues have shown that activation of SIRT1 by resveratrol promotes axonogenesis through an Akt and glycogen synthase kinase 3 (GSK3) mediated pathway in primary rat embryonic hippocampal neurons and that inhibition of SIRT1 led to significantly decreased axon length and number in-vitro [111]. Moreover, in-vivo, SIRT1 knockout (KO) mice demonstrate significant deficits in immediate and long-term memory and spatial learning, as well as neuronal dendritic branching [112]. While KO models are a severe phenotype, our study has demonstrated a 70% reduction in SIRT1 activity given excess exogenous nicotinamide exposure; therefore significant disruption can occur without gene KO or even knockdown of SIRT1. It can be hypothesized then that nicotinamide-induced inhibition of SIRT1 may also impact neurodevelopment. This has not yet been studied in humans and presents an important area for future research.

Furthermore, the impact of nicotinamide may not be isolated to SIRT1 as there are six other proteins in the Sirtuin family, all of which are NAD-dependent de-acetylase enzymes. SIRT3, specifically is also sensitive to nicotinamide inhibition [113] and is a key enzyme involved in maintaining mitochondrial energy homeostasis (reviewed in [25]). In the mitochondria SIRT3 is particularly important in reactive oxygen species detoxification (reviewed in [114]). Through direct de-acetylation and activation of antioxidant enzymes such as manganese superoxide dismutase or indirect regulation of
antioxidant systems such as the glutathione system (reviewed in [114]), SIRT3 contributes to redox homeostasis without which oxidative stress, cellular damage, and loss of function can occur. SIRT3 KO mice manifest a myriad of conditions attributed to increased oxidative stress including impairment of fatty acid oxidation in the liver and development of hepatic steatosis, insulin resistance in metabolically active tissues, and impaired insulin secretion from β cells of the pancreas [115]. This body of evidence suggests that nicotinamide-induced impairment of Sirtuin enzymes could lead to systemic disruption of cellular metabolism therefore potentially increasing the risk for chronic diseases such as metabolic syndrome, type 2 diabetes mellitus, and cardiovascular complications. This disruption during fetal and early infant growth could have significant consequences for accelerated development of these metabolically linked diseases.

The possibility that excess nicotinamide from the diet could contribute to systemic disruption of human development suggests that we re-evaluate our current public policy regarding enrichment of grains with nicotinamide [5]. Grain enrichment with nicotinamide was implemented when meat, dairy, and fresh vegetable and fruit consumption were low and nicotinic acid/nicotinamide deficiency and Pellagra, a potentially fatal disease was prevalent [120]. While no national cohort study has measured nicotinamide sufficiency in this country, our current high consumption of animal products [121], the predominant natural source of nicotinamide [5], suggests that the United States may be at or above recommendations for daily niacin/nicotinamide (16 mg/day in pregnant women). Again, further research on the unintended consequences, if any, of grain enrichment with nicotinamide is needed and the research presented in this work supports the call for studies of nicotinamide and human development.
CHAPTER VIII  
CLINICAL AND TRANSLATIONAL RESEARCH EXPERIENCE

Introduction

In partial fulfillment of the requirements for the Certificate in Clinical and Translational Research, conferred by the NIH-funded Colorado Clinical and Translational Sciences Institute (CCTSI), I shadowed Dr. Linda Barbour, my clinical research mentor, in the University of Colorado Hospital Obstetric Diabetes Clinic. Given my research interests in nutrition, metabolism, and diabetes in pregnancy and their consequences for the offspring, the overarching goal of this clinical experience was to learn about the clinical presentation of metabolic disorders in pregnancy and to observe the clinical management of these conditions. This chapter is a compilation of my observations and experiences while shadowing Dr. Barbour in the clinic and includes some literature review of specific conditions or clinical terms that I was not initially familiar with until investigating them further.

Clinical Experience #1 - High-Risk OB/GYN Diabetes Clinic

As it will be in future clinic experiences with Dr. Barbour, my morning began at 8am with the residents and fellows meeting to review case profiles of the patients scheduled to come in that day. This meeting consisted of discussing which conditions the mom was currently experiencing in pregnancy and what tests and exams were needed during her visit.

During the discussions Dr. Barbour pointed out to me a very interesting sibling pair; the older, a young adult has severe Type 2 diabetes (T2DM) with nephropathy and the younger, an adolescent also had T2DM in pregnancy. With both sisters having Type 2
diabetes at very young ages it was clear to me that there must be some element of a genetic predisposition within that family or shared environment.

One case scheduled for the morning clinic was a mystery to the team. The patient was transferring her care from another clinic and no medical records were available for the team to extract any information about her pregnancy. This was also her 14th pregnancy and would be her 10th live birth. Incredible! The team spent quite some time discussing what her issues could be given the limited information they had and also what information they needed to extract from her during her scheduled visit.

Throughout the meeting I was delighted by the number of opportunities that Dr. Barbour took to teach a new concept or offer a new factoid for the residents and fellows. It was truly a learning environment and it was clear that these physicians were being trained by someone who is dedicated to their being competent and insightful within the practice of high-risk obstetrics.

We concluded the meeting and began seeing patients. I shadowed both the resident and the fellow during their appointments giving me the opportunity to observe their interactions with patients and learn what information they collect in routine visits as well as first visit intakes. Once a visit was completed, we all gathered in the resident office and Dr. Barbour reviewed the information the resident and fellow collected on the patient. Again, during this review Dr. Barbour asked challenging questions of the trainees and shared additional information about differential diagnoses and other symptoms, and lab tests/results that would help to make a primary diagnosis or decide on an appropriate treatment.
Overall, my initial experience in the high-risk OB Diabetes Clinic was enriching and has lead me to ask questions about the clinical manifestations of certain conditions in pregnancy and what the standards of care are to address those issues. One such issue that I have taken a particular interest in is preeclampsia (PE) in diabetic pregnancy and the specific diabetic pathophysiology that leads to the increased risk of PE. At the end of clinic Dr. Barbour shared with me articles pertaining to PE as well as other articles describing clinical management of diabetes (Type 1 and 2) in pregnancy. This stack of articles has kept me busy this past week.

Clinical Experience #2 - High-Risk OB/GYN Diabetes Clinic

Today I was able to observe a very complicated pregnancy. The patient is the older sister of a patient whom I met last week. Both women are pregnant with Type 2 diabetes. However, the older sister is in very poor shape. It was interesting to learn that when the patient was first seen, several providers had discussed termination of the pregnancy due to the patient’s ill health. I don’t think that the clinicians even expected her to make it to viability (24 weeks) but she is here today at 26 weeks gestation.

This particular patient is what the clinicians call a Class F T2DM because as a result of her severe, long-standing, inadequately treated diabetes she has developed diabetic nephropathy. Dr. Barbour has discussed at length how she expects this to manifest as the patient’s pregnancy advances. The clinicians are patiently waiting for this patient to develop preeclampsia, which is inevitable. Due to the severity of the nephropathy, the patient will also likely need dialysis before she reaches term. Today, in fact, Dr. Barbour began the patient on Lasix (Furosemide) because it was quite obvious that the patient had developed severe, debilitating edema in her legs. It was also
suspected that she had generalized edema (anasarca), as it was apparent in several body regions. While edema is normally seen in pregnancy – blood vessels get leaky and fluid extravasates in the tissues – this case of edema was extensive. If you pushed on the patient’s legs you would leave a large crater in her skin, and it would not go away and place her at high risk for venous insufficiency and blood clots.

I was curious to know what effect blood pressure medication, such as Lasix, or dialysis would have on the developing fetus. Dr. Barbour explained that it could slightly increase the risk of intrauterine growth restriction because the mother’s blood flow to the placenta might be mildly reduced. However, she also mentioned that in such a case where Lasix or dialysis is needed, it is likely that the fetus is already growth restricted and extravasation of fluid into the pleural space could compromise her ability to oxygenate. Dr. Barbour also said that etiology of preeclampsia is abnormal angiogenesis in which the placenta develops abnormally into a highly resistant vasculature at the beginning of pregnancy. So, I imagine that women coming into pregnancy with underlying macrovascular disease manifested as nephropathy are at the greatest risk of intrauterine growth restriction.

In the midst of learning all of this new information, I reflected on the pathogenesis of preeclampsia and how the predominant factor leading to this severe condition is inadequate or inappropriate angiogenesis on either the maternal or fetal side. Seeing that this patient would inevitably develop preeclampsia, her fetus must be at even greater risk of growth restriction. Therefore, this patient may deliver a premature neonate with severe growth restriction, which does not bode well for either individuals’ quality of life.
As part of the clinical management of diabetes in pregnancy, certified diabetes educators (CDEs) are employed to counsel patients on dietary restrictions and food alternatives as well as help the physicians read, interpret, and effectively apply the data gathered by a patient’s glucose monitor, insulin pump, or continuous glucose sensor to help with clinical decision making and medical care planning. Today, under the supervision of the clinic’s CDE I learned this very practical clinical skill of reading a glucose monitor. First, however, I was given a crash course on how intensive insulin regimens worked; this also necessitated a vocabulary lesson.

Briefly, depending on a patient’s estimated total daily insulin requirements, a carbohydrate ratio is calculated (the amount of carbohydrate that 1 unit of insulin will cover) which is programmed into the patient’s insulin pump to calculate an appropriate insulin bolus in response to the carbohydrates consumed during any one meal. The basal insulin, which covers ongoing hepatic gluconeogenesis in the fasted state, is about 50% of the total insulin dose. Both the basal insulin rates, which vary according to the insulin sensitivity and physical activity level throughout the day, and the carbohydrates ratio are programmed into the insulin pump. Importantly, different meals with variable macronutrient contents at different times of day may demand different carbohydrate to insulin ratios.

From the data collected by the glucose monitor, a specialist will look for patterns among the high glucose and low glucose values. A high glucose value suggests that the insulin bolus was not adequate to cover the amount of blood glucose generated by the grams of carbohydrates consumed. This could be due to several factors: 1) the
carbohydrate ratio used was not aggressive enough (e.g. 1 unit insulin to 20 grams carbohydrate instead of 1 unit per 10 grams); 2) inadequate basal insulin coverage given that pregnancy always results in progressive insulin resistance after the first trimester; 3) the patient did not count her meal carbohydrates correctly, underestimated the total carbohydrate, or had a meal that contained high fat (which worsens insulin resistance); or 4) the patient has an unrecognized infection or stressor causing worsening insulin resistance. A glucose value below 70 is of significant concern to a clinician because the patient may develop hypoglycemic unawareness at this level and no longer experience symptoms of hypoglycemia. In general patients are most at risk of a hypoglycemic event while they are sleeping and if they have Type I diabetes mellitus (T1DM). In the case of a “glycemic low”, it is most likely that the patient input the incorrect number of carbohydrates (too many) and the insulin bolus delivered by the pump was too large. However, increased physical activity can also play a significant role since glucose disposal into skeletal muscle with exercise does not require insulin. Both scenarios highlight the importance of the food and activity diaries that women are asked to keep consistently throughout pregnancy. Hypoglycemia in the mother often leads to significant hypoglycemia in the fetus which can be life threatening if it is extended.

Food diaries can be quite tricky and sometimes unreliable as they are dependent on the patients’ recall of the food that they ate. In the diabetes clinic, moms are encouraged to write down what they ate as they are eating with the intention that the recall will be more accurate. When the women come in for their prenatal visits they bring with them their food diaries so that the diabetes specialists and physicians can see how food affects the patient’s glucose levels and can compare that to the glucose monitor
download. This side-by-side comparison allows the diabetes specialist to pinpoint the source of a hyperglycemic or hypoglycemic event given information about the basal insulin levels and the bolus of insulin administered for the carbohydrates consumed with one meal or snack. Not only do carbohydrates affect glucose excursions but the amount and type of dietary fat can cause a delay in glucose absorption and elevated free fatty acids after a high saturated fat meal resulting in worsening insulin resistance. The food diaries also allow the specialists to counsel patients on better food choices for lowering and better matching of the insulin to the food intake. For example, eating a piece of string cheese, which has no carbohydrate does not require insulin in contrast to a cookie or piece of bread which are high in carbohydrates and would require insulin to control the blood glucose.

Given the challenges that women with diabetes face during pregnancy, I assigned myself the task of keeping a food diary to see if I could track my meals and snacks for a whole week (7 days). Right off, this was not a successful experiment as I was unable to keep up with my eating habits. I snack far too much. The carbohydrate counting was the worst. It took so much time and effort for me to look up the amount of carbohydrates in one serving of anything. Further, it was difficult for me to track how many servings of foods I ate. My experiment certainly put things into perspective.

Clinical Experience #4 – High-Risk OB/GYN Diabetes Clinic

The reality of diabetes management using insulin is much more complicated than I had (naively) expected. In pregnancy, it’s not as simple as using a bolus of insulin to adjust the blood glucose, especially when there are pregnancy complications such as preeclampsia. Dr. Barbour explained that having placental insufficiency or outright
pregestational diabetes reduces a mom’s insulin requirements due to decreasing levels of placental insulin resistance hormones and therefore, without adjustment, can lead to further complications with hypoglycemic episodes. Hypoglycemia during pregnancy in particular is dangerous for the fetus since the fetus has minimal gluconeogenic capacity. Furthermore, inadequate carbohydrate intake or prolonged fasting does not meet the glucose requirements of the fetus later in pregnancy (150 g/day) and the mother resulting in a switch from carbohydrate to fat metabolism or (lipolysis) where fats are broken down to provide energy as free fatty acids and ketones.

Hypoglycemia resulting from insufficient energy intake or an inappropriate insulin bolus can also, quite paradoxically induce hyperglycemia due to provoking a stress hormone response with increased levels of glucagon, cortisol and epinephrine to counter the hypoglycemia. DKA is due to inadequate insulin to shut down lipolysis which leads to ketone body production from fatty acids. DKA is thankfully infrequent but as high as 5-10% in women with T1DM and can occur without significant hyperglycemia (euglycemic DKA) [116]. While ketones are an important lipid substrate for fetal brain development, in the case of maternal DKA, the acidosis and electrolyte derangements can result in a fetal demise. DKA can cause decreased utero-placental perfusion of nutrients as well as fetal acidosis, and hypoxia both of which may contribute to fetal death which occurs in up to 20-30% of pregnancies complicated by DKA in the third trimester.

Hyperglycemia, the case of excess glucose (without DKA), is common in T2DM and GDM pregnancies managed by diet. In these cases, due to the fetal hyperinsulinemic response from unabated utero-placental flow of glucose, fetal overgrowth can occur. The most well studied mechanism by which fetal overgrowth occurs is mediated through fetal
pancreatic insulin production with insulin acting as a growth hormone and contributing to overall excess growth. This, says Dr. Barbour, is generally why the incidence of large for gestational age (LGA) and macrosomia is much higher among infants born to diabetic mothers. The increased pancreatic insulin production by the fetus may then result in neonatal hypoglycemia after the baby is delivered since it takes some time for the baby’s beta cells to decrease insulin production.

Clinical Experience #5 – High-Risk OB/GYN Diabetes Clinic

Today was a short day, however, as usual I learned a great deal about pregnancy and physiology, in general. In our morning meeting with the residents and fellows we briefly discussed the gluconeogenic capacity of the adult kidneys and this led me to research this phenomenon and how it could affect diabetes in pregnancy.

In the fasted state, the kidneys actually contribute 20-25% of the circulating glucose [117]. In diabetes, particularly T2DM, renal gluconeogenesis increases. This process, as in the liver is regulated by insulin; the increasing insulin resistance in pregnancy and in T2DM and GDM contributes to the hyperglycemic state. Kidney reabsorption of glucose is also increased in diabetes and is suspected to be due to increased expression of SGLT2, a high capacity co-transporter (sodium and glucose) responsible for most of renal glucose reabsorption, and GLUT2 transporter that moves glucose into the blood stream. This constitutes a double hit and further contributes to exacerbating hyperglycemia. However, given the recent understanding of the kidneys’ role in diabetes and glucose homeostasis, new therapies are being developed that involve inhibition of SGLT2 transporters so to decrease renal reabsorption and increase urinary excretion. However, pregnant women commonly have glycosuria due to the increased
GFR and thus these agents would not likely be helpful and possibly increase the risk for urinary tract infections that are already high in pregnancy.

A new, pregnancy-specific hormone was also brought to my attention today. Interestingly, the hormone called Relaxin causes the tendons to loosen so that the hips become wider in late pregnancy in preparation for birth. On a side note, while not original, the name is quite fitting to the function. As more and more of my friends go through pregnancy I feel I can actually observe this process occurring in each of them and this hormone makes complete sense. Their gate noticeably changes toward the end of the second trimester and, while it may be difficult to support my claims, I see their hips actually widen. As an observer, pregnancy sure induces some alarming changes to a woman’s body!

Clinical Experience #6 – High-Risk OB/GYN Diabetes Clinic

In diabetic pregnancies (T1DM, T2DM, and GDM) the risk of abnormal fetal growth, either overgrowth or restricted growth, is higher compared to pregnancies not complicated by diabetes. As part of standard care for pregnant moms with diabetes, clinicians routinely measure the progress of growth in-utero through ultrasonography and fetal biometry. Today I learned all about the different biometric variables measured and used in the estimation of large for gestational age (LGA) infants and intrauterine growth restricted (IUGR) infants. I received a substantial lesson in acronyms.

In estimating whether the fetus was growing too quickly, Dr. Barbour told me that clinicians use the abdominal circumference (AC) to biparietal diameter (BPD) ratio (AC:BPD) which essentially is estimating the ratio of subcutaneous fat in the abdomen to the skull diameter. If the AC:BPD ratio is disproportionate by >3 weeks, this suggests a
LGA fetus which is gaining abdominal fat too rapidly. When a LGA fetus is identified the clinicians then discuss the need for tighter glycemic control or cesarean delivery if severe. Poorly controlled of fetuses of mothers with diabetes are also at greater risk of hypertrophic cardiomyopathy which is characterized by an enlargement of the fetal heart muscles due to increased fetal demand for blood flow as a result of hypertrophy of the cardiac muscle. The LGA fetus can also “outgrow” the placenta, a scenario where the placenta can no longer transport or provide adequate nutrients for the accelerated growth of the fetus, therefore increasing the risk of fetal demise.

On the other end of the growth spectrum, in IUGR fetuses, there are two phenotypes, assymetric IUGR and symmetric IUGR. Each of these is determined differently using ultrasound biometry measures. Asymmetric IUGR is most often characterized by a disproportionate head circumference (HC) to AC ratio (HC:AC > 1.0). The asymmetry in growth of the head is hypothesized to be the result of “brain sparing” where the fetus preferentially shunts nutrients to the brain to preserve its development and function and away from other tissues. While in symmetric IUGR fetuses, the HC:AC ratio is 1.0 (hence, symmetrical), they are identified as having an AC < 5th percentile of the expected AC size for gestational age (derived from published growth charts). In this way symmetric IUGR fetuses may be constitutionally small compared to the general population rather than from placental insufficiency. The timing of growth restriction is also thought to be different between asymmetric IUGR and symmetric IUGR fetuses with asymmetric IUGR initiated in later pregnancy and symmetric IUGR initiated in early pregnancy. In general, however, both forms of IUGR result in a higher risk for stunted postnatal growth, and if also born premature, neurological and developmental delays.
It is generally accepted that, at least in the developed world, IUGR occurs primarily due to placental insufficiency. Unfortunately, it doesn’t seem as if there is much that can be done to improve growth of a IUGR fetus as interventions involving the placenta are likely a decade or more away. However, reducing the comorbid exposures such as fetal hypoxic episodes during labor and delivery may help to ameliorate the damage.

*Clinical Experience #7 – High-Risk OB/GYN Diabetes Clinic*

One of the many perks of shadowing Dr. Barbour is being privy to her extensive knowledge of metabolism and all the diseases that arise when the body’s metabolic systems go awry. She is also enthusiastic when answering any random questions that I may have regarding these diseases and unusual processes. I can’t quite remember how the subject came up – likely spurring from our discussion regarding a patient with T1DM – but today Dr. Barbour and I discussed the interesting phenomenon of latent autoimmune diabetes in adults also known as LADA.

LADA is considered a sub-type of T1DM as it is diagnosed on the basis of having developed autoimmunity to islet cell antibodies but it is also characterized by having a slow progression towards insulin dependence. While T1DM is mostly diagnosed before or during adolescence, LADA appears in mid-adulthood (>30 years old). In fact, it is not uncommon for adults to be misdiagnosed with T2DM, when in fact they meet diagnostic criteria for LADA; one rather older UK study was able to identify 10% of T2DM patients with LADA (U.K. Prospective Diabetes Study Group, 1995). This propensity towards misclassification is certainly what peeked my interest regarding this odd mixture of the two known diabetes forms. As a sprouting epidemiologist I have often considered one of
my roles in the scientific community as one who strives to develop methods to measure things accurately and precisely to minimize misclassifications. Actually, on a side note, in my master’s degree “measuring things accurately and precisely” was central to my training and was our program’s mantra. Needless to say, appropriate classification of diabetes, particularly in pregnancy, is of great interest to me.

Bringing this phenomenon of misclassification of LADA into the world of pregnancy, my question was simple; what is the incidence of LADA onset during pregnancy and how many pregnant women with LADA get misdiagnosed as T2DM or insulin-dependent GDM? To my surprise, answering my question was not difficult given the extensive literature on autoimmune diabetes in pregnancy. In the most recent review, the authors estimated the prevalence of autoantibodies from the existing literature to be between 1% and 10% among women who were diagnosed with GDM [118]. In line with the observation that developing diabetes in pregnancy increases the risk of overt T2DM postpartum, women who screen positive with islet autoantibodies in pregnancy are at increased risk of developing T1DM after pregnancy. Further, the risk increases with the number of islet autoantibodies detected during pregnancy [118].

Next question; do women with LADA in pregnancy present differently (clinically) from “traditional” GDM women in any way? And, are there differences in birth outcomes between women with GDM and women with LADA in pregnancy? Now, this is where the research isn’t as robust. Interestingly, and contrary to the phenotype of GDM moms, women who develop GDM with islet autoantibodies in pregnancy have a pre-pregnancy weight that is classified as normal and tend to gain less weight during pregnancy [119]. Given this, I recently asked Dr. Barbour about whether women with
GDM are ever tested for islet autoantibodies at UCH. She promptly responded that if they were normal weight and insulin sensitive then she has them tested and she has diagnosed at least a dozen women with LADA. However, she did make the point that it was too expensive to screen all GDM women for islet autoantibodies. As for poor birth outcomes, even less research has been conducted, but from what is available the data are quite mixed with several studies showing no difference between islet autoantibody GDM and “traditional” GDM in pre-term delivery, congenital malformations, or birth weight and one showing significant increases in preterm birth, still births, and macrosomic infants [119].

Still, there is much to be studied with regard to LADA in pregnancy highlighting yet another important part of my shadowing experience, which is the discovery of new research questions that have direct implications for clinical care of high risk pregnancies.

**Summary**

In the months that I spent shadowing Dr. Barbour I was exposed to a world that had been in the periphery of my training. I was given the opportunity to interact with patients, something that outside of a research study I was inexperienced with. The clinical environment is fluid and not regimented like the research study visits that I have conducted. It is unpredictable and decisions are made based on data within a matter of minutes. To say the least I have gained a new level of respect for clinicians. Dr. Linda Barbour’s extensive clinical experience as an endocrinologist and her role as the attending physician in the high-risk diabetes pregnancy clinic and also as a researcher provided me with an exceptional learning environment. Translation from research to practice was something that she did *every day* and I am grateful to have witnessed it.
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