

The Intervention Nurses Start Infants Growing on Healthy Trajectories
(INSIGHT) Study

PENNSSTATE



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INSIGHT: First-Born

I. PRINCIPAL HYPOTHESES TO BE TESTED

Principal Hypotheses: An intervention program designed to provide developmentally appropriate guidance to parents of infants on responsive parenting and healthy lifestyle will prevent rapid weight gain in infancy and overweight at age 3 years. Further, compared with control infants, intervention infants will have lower BMI percentiles at age 3. We also hypothesize that control infants will gain weight more rapidly over time, adjusting for trait-stable and time-varying covariates (e.g., maternal pre-pregnancy BMI, percent of feedings that are breastmilk vs. formula, sleep duration, and feeding frequency).

The Intervention **Nurses Start Infants Growing on Healthy Trajectories** (INSIGHT) Study is designed to test these hypotheses in a two-arm randomized trial where participants in a program to prevent childhood obesity will be compared with those in a child safety control program. Nurses will deliver interventions to first-time parents and their infants in both study groups at four home visits in the first year after birth followed by annual clinical research center visits until age 3. The obesity prevention program focuses on messages of responsive parenting and healthy lifestyle, extending from infancy through age 3 years. The intervention will teach first-time parents to interact with their infants in a way that is prompt, emotionally supportive, contingent, and developmentally appropriate. Behavioral states (alert and awake, fussy, drowsy, sleeping) will be used as a foundation for messages as several portions of the intervention will focus on transitioning infants out of the fussy state and into either the alert and awake or the sleeping state with appropriate methods.

More specific portions of the intervention will instruct parents: a) to recognize infant hunger and satiety cues and use feeding more selectively in response only to hunger, b) to use alternatives to feeding to soothe a fussy, but non-hungry infant and toddler, c) to provide children appropriate portions of healthy foods and allow children to determine the amount consumed, d) how to improve acceptance of developmentally appropriate foods such as vegetables by using a “repeated exposure” procedure and positive role modeling, e) how to develop good sleep hygiene and f) to actively engage their infants in play time in order to reduced sedentary behaviors.. In addition to these messages, intervention parents will be given education on growth charts, the meaning of growth chart percentiles, and healthy growth patterns during early life. The intervention program is hypothesized to show efficacy in both breast and formula fed infants as measured by **the primary outcome, body mass index (BMI) percentile at age 3 years**. This outcome provides significant insight into long-term obesity risk.

ADDITIONAL HYPOTHESES TO BE TESTED

Compared with safety control parents, obesity intervention group parents will show increases in responsive parenting, alternative soothing techniques, and parenting self-efficacy. Over time, when compared to control infants, intervention infants will have a) longer sleep bouts, b) fewer nighttime and daily feedings, c) a lower proportion of crying episodes followed by feeding in early infancy, d) improved acceptance of novel foods, and healthier diets from birth to age 3. These outcomes will be linked to healthier patterns of weight gain from birth to age 3 and lower BMI percentiles at age 3 years.

Specific secondary hypotheses include:

Weight Status.

- 1) Obesity intervention children will be less likely to demonstrate accelerated weight gain during infancy defined as a change in weight-for-age Z-score of +0.67 between birth and 4 months and/or birth and 1 year.
- 2) Obesity intervention children will exhibit less conditional weight gain between birth and 6 months, 6 months and 1 year, and birth and 1 year.
- 3) Fewer children in the obesity intervention will have BMIs at or above the 85th percentile and 95th percentile at ages 2 years, 3 years, and beyond.

- 4) Mothers in the obesity intervention group will have greater and more rapid postpartum weight loss
- 5) Mothers in the obesity intervention group will have BMIs closer to pre-pregnancy BMI 1, 2, and 3 years postpartum.

Infant Behavior - Sleep.

- 1) Obesity intervention children will have earlier bedtimes, greater total daily sleep duration, greater total nocturnal sleep, longer sleep bouts, fewer nocturnal awakenings, and fewer nocturnal feedings at all assessment points.
- 2) Obesity intervention children will be less likely to be put down for bed asleep, less likely to be picked up at night, and less likely to sleep in bed with parents at all assessment points.
- 3) Mothers in the obesity intervention will be less likely to report that their child's sleep is a problem and more confident in their ability to manage their child's sleep at all assessment points.
- 4) Mothers in the obesity intervention will be less likely to report that they have sleep problems or sleep disturbances.
- 5) Safety intervention children will be less likely to sleep in cribs with toys, stuffed animals, pillows, blankets, and sleep positioners during the first year after birth.

Infant Behavior – Fussing, Crying, and Soothing.

- 1) Mothers in the obesity intervention group will be less likely to use feeding and/or food to soothe their children at all assessment points.
- 2) Mothers in the obesity intervention group will be more likely to use other soothing techniques besides feeding as their first response to infant distress (ex. Swaddling, shushing or white noises, swinging).
- 3) Mothers in the obesity intervention group will respond differently to infant distress in the daytime compared with nighttime (less active responding at night).
- 4) Infants in the obesity intervention group will have fewer crying bouts at night, compared to the safety control group.
- 5) Mothers in the obesity intervention group will be less likely to use food to manage their child's behavior at ages 1, 2, and 3.
- 6) Mothers in the obesity intervention group will report greater confidence in their ability to soothe their children at all assessment points.
- 7) Infants that use pacifiers or fingers for non-nutritive sucking will have lower weight-for-length percentiles at age 1 year and lower BMI percentiles at 2 and 3 years. There will also be a lower prevalence of overweight and obesity among those that use pacifiers or fingers for non-nutritive sucking.

Infant Behavior – Temperament.

- 1) Mothers in the obesity intervention group will be more in tune with their infant's temperaments: mother-reported infant temperament will be more strongly correlated with observed temperament in this group.
- 2) Infants in the obesity intervention group will have higher self-regulation scores at 1 year and 3 years.

Infant Feeding and Eating.

- 1) Infants in the obesity intervention will be introduced to solid food later and show increases in acceptance of novel foods with repeated exposure. In addition, at 1 year, infants in the obesity intervention will be less likely to reject new foods than safety controls.
- 2) Infants in the obesity group will have lower scores on a maternal-report neophobia measure administered at 1, 2 and 3.
- 3) Mothers in the obesity intervention group will be less likely to endorse obesogenic feeding practices, including putting cereal in the bottle and frequently feeding juice or sweetened beverages.
- 4) Mothers in the obesity intervention group will have a more responsive feeding style (i.e. paying attention to infant cues), as shown by coded videotapes of infant feedings at 28 and 40 weeks.
- 5) Infants in the obesity intervention group will be weaned off the bottle earlier than the control group and will begin to use a sippy cup earlier than the control group.

Maternal Eating Behaviors.

- 1) Mothers in the obesity intervention will have healthier eating habits.

Parenting and Maternal Mental Health.

- 1) Mothers in the obesity intervention group will have greater parenting self-efficacy and greater satisfaction as measured by the Parenting Sense of Competence Scale compared with the control group.
- 2) After the home visits, mothers in the obesity intervention group will have increased knowledge about what they should feed their infant, how they should soothe their infant, and how to get an infant to sleep through the night.
- 3) After the home visits, mothers in the safety control group will have increased knowledge about creating safe environments for babies, including sleep safety, bath safety, and keeping baby safe on changing tables and in infant chairs.

Physical Activity.

- 1) Infants in the obesity intervention will be less likely to have a television in their room.
- 2) Children in the obesity intervention will be more likely to engage in active play.

Home safety.

- 1) Mothers in the home safety intervention will be more likely to have working smoke detectors, carbon monoxide detectors, and fire extinguishers in the home and more likely to have a fire safety plan.
- 2) Mothers in the home safety intervention will have lower bath water maximum temperatures and will be less likely to use bath rings.
- 3) Children in the home safety group will be more likely to have had their car seats inspected by a certified car seat technician, and will be more likely to remain rear facing in their car seats through age 2 years.
- 4) Mothers in the home safety intervention will be more likely to know how to find the phone number for Poison Control Centers and more likely to keep household medications in a locked container or in a location that is up and away from where children can reach them.

II. BACKGROUND AND RATIONALE

A. Introduction

In recent decades the prevalence of obesity has increased dramatically among all age groups, including infants and young children.¹⁻³ National Health and Nutrition Examination Survey (NHANES) data demonstrate the need for early intervention to prevent obesity: from 2007 to 2008, over 10% of children less than 2 years were already obese and over 20% of children between ages 2 and 5 years were overweight or obese.⁴ Because infancy and early childhood are periods of developmental plasticity when environmental effects can have long-lasting metabolic and behavioral consequences, interventions delivered during this period have the potential to alter growth trajectories and long-term risk for obesity and co-morbidities. The proposed research will test the impact of an innovative intervention promoting responsive parenting and healthy lifestyle to prevent rapid infant weight gain and obesity. Responsive parenting involves prompt, emotionally supportive, contingent, and developmentally appropriate responses to infant cues.⁵ Responsive parenting is associated with a range of positive social, emotional, cognitive and behavioral outcomes in children, but until recently, has not been applied to early obesity prevention. With regard to eating behaviors and obesity prevention, responsive parenting applies these general principles to parent responses to their child's hunger and satiety cues.^{6,7} Our proposed intervention utilizing this conceptual framework is informed by 30 years of research by PI Birch that provides insight on how caregivers shape children's eating environments, what children are learning from early experiences with food and eating, and how this learning shapes the developing behavioral controls of food intake and obesity risk. Birch's findings are consistent with the extensive literature revealing that responsive parenting is associated with positive outcomes in children:^{8,9} Responsive parenting in feeding

can promote the development of behavioral self-regulation and patterns of preference and eating behavior associated with healthy growth and weight outcomes. In contrast, feeding that is coercive, restrictive and controlling can promote heightened responsiveness to environmental cues and reduced responsiveness to hunger and satiety cues as controls of food intake, which can promote excessive intake and increase obesity risk in the current obesogenic environment. Our innovation is to apply the tenets of responsive parenting to feeding.

The obesity epidemic has been attributed in part to an increasingly obesogenic environment that promotes positive energy balance, overweight, and obesity. While numerous environmental features may promote weight gain by affecting energy intake or energy expenditure, the focus of our intervention is to prevent excessive energy intake and minimize the threats to health posed by large portions of palatable, available, inexpensive, energy-dense foods. Although too much food constitutes the current threat, our biology evolved to promote survival in the context of food scarcity and biases us to eat opportunistically and lay down fat stores essential for survival in times of scarcity.¹⁰ Traditional infant feeding practices, which evolved when food scarcity and disease were major threats to child health also promote food and fluid intake and are still in use today. Because loss of appetite is often a symptom of illness and because adequate intake is critical to child survival, traditional feeding practices are non-responsive in that they are not contingent on child hunger, rather feeding is the “default” response to infant distress.¹¹⁻¹³ Traditional feeding practices include a range of strategies intended to both soothe and promote intake, including feeding in response to any and all distress to control infant/toddler behavior, providing palatable, preferred foods, promoting intake in the absence of hunger, offering large portions and coercing children to eat beyond satiety. Evidence from PI Birch’s laboratory reveals that such traditional practices can exacerbate the effects of obesogenic environments, promoting development of eating behaviors that may persist and affect intake throughout the lifespan, including increased responsiveness to food cues,¹⁴⁻¹⁶ reduced responsiveness to hunger and satiety, eating in the absence of hunger, and excess weight gain and overweight.^{17, 18} We propose an innovative intervention to alter traditional feeding practices by teaching parents to interact with their child in ways that are responsive, appropriate, and contingent. This approach is supported by extensive evidence showing that responsive parenting has positive effects on social, emotional, cognitive, and health outcomes.^{8, 9}

A.1. Changing Clinical Care to Promote Responsive Parenting Feeding and Shared Feeding Responsibility

Currently, clinicians pay limited attention to obesity prevention and often fail to recognize obesity during infancy.¹⁹ While infant growth is monitored and most clinicians promote breastfeeding, suggest avoidance of infant cereal in bottles, and advise against early introduction of complementary foods and fruit juice, guidance aimed at early life obesity prevention often stops there. While initially appropriate, the focus for newborns and infants is typically on promoting adequate weight gain rather than preventing excessive weight gain. For example, immediately after birth mothers are typically instructed to wake their infants to feed at least every 3-4 hours for several weeks.²⁰ While this is necessary initial advice to help newborns regain weight lost shortly after birth, clinicians are far less consistent in instructing parents when to stop this practice and how to identify and rely on infant hunger and satiety cues to guide feeding. Typical anticipatory guidance in pediatric healthcare also does not discourage the use of feeding to soothe for fussiness or boredom or advise against feeding as a reward for positive behaviors later in infancy and childhood unless obvious overweight or obesity develops.

Building on PI Birch’s prior research and findings from our pilot study,²¹ the proposed research will improve the evidence base needed to alter clinical and parenting practices to reduce early life obesity risk by focusing on responsive parenting and shared feeding responsibility. The first potential change from current practice recognizes the traditional use of feeding as a routine first response to infant and toddler distress by promoting parental responsiveness to their child’s needs. When feeding is responsive to children’s needs, shared responsibility in feeding begins to develop. This shared responsibility can be succinctly summarized as “parents provide, children decide” and can be used in the context of healthy, developmentally appropriate foods. As in other areas of development, this approach provides opportunities for the development of self-regulation as children assume an increasing role in determining when and how much to consume. For future clinical application, this “stealth” approach to obesity prevention²² has the advantage of allowing healthcare providers to promote positive parenting behaviors associated with responsiveness, which can promote other positive child outcomes, as opposed to the more negative theme of prevention of obesity and its comorbidities.

A.2. Changing Clinical Care to Improve Infant and Toddler Diets

Current clinical care is based upon the timing of introduction of foods – solids at 4 to 6 months, finger foods at 6 months, no cow’s milk until 1 year, etc. Alternatively, little evidence-based guidance is given on how to feed infants to promote liking and acceptance of healthy choices in our obesogenic environment. Numerous studies from PI Birch’s lab however suggest that interventions during infancy can produce healthier eating behaviors. For example, it is accepted that infants have innate preferences for sweet and salty tastes and are “neophobic”, rejecting new foods that are not sweet or salty.²³ Given this set of predispositions, normal infants will readily accept sweet and salty foods such as sweetened drinks and French fries. In contrast, healthy foods such as pureed vegetables, meats, infant cereals, and dairy products, which are not high in sugar or salt, are likely to be initially rejected when first introduced. PI Birch’s laboratory research demonstrated that infants typically need several opportunities to sample new foods before intake increases.^{24, 25} The liking for complex flavors that are not dominated by sweet or salty tastes must be learned.²³ Interventions emphasizing repeated opportunities to try healthy, developmentally appropriate foods can have lasting positive effects on acceptance of healthy foods.

Current advice to parents to make healthy food choices later in infancy is failing to produce the desired outcomes and highlights the significant need for changes to clinical practice. Data from the Feeding Infants and Toddlers Study (FITS) revealed that unhealthy habits start early; energy intakes among infants and toddlers exceeded requirements by 20-30%.²⁶ In addition to consuming too much energy, children 4 to 24 months old ate significant amounts of developmentally inappropriate foods, high in energy density and sometimes deficient in key nutrients, and consumed too few of the foods that should form the basis of a healthy weaning diet.^{27, 28} For example, in children aged 7 and 24 months, 18% and 33% consumed no servings of vegetables, respectively, during a given 24-hour period. Twenty-three percent of 7-month old and 33% of 24-month old children did not consume any fruits. By 15 to 18 months, French fries were the most common vegetable consumed. Clearly, parents of infants and toddlers need better direction on what to feed, how much to feed, and how to promote acceptance of a variety of healthy foods during the transition to the modified adult diet. Evidence that these outcomes can be promoted in a real-world setting, such as the home could transform how parents view “picky eaters” while promoting acceptance of healthy diets.

A.3. Importance of Studying Mother/Infant Dyads Intending to Breastfeed and Formula Feed

Infant feeding mode is relevant to obesity prevention because growth patterns of formula fed and breast fed infants differ, with formula fed infants at elevated risk for rapid growth, excessive weight gain, and subsequent obesity risk.²⁹⁻³⁵ Both exclusivity and duration of breastfeeding strengthen its association with reduced obesity risk.^{32, 33, 35-39} Infant feeding mode is also strongly related to the messages of responsive parenting, division of feeding responsibility, and healthy dietary choices that are the focus of this application. Briefly, formula feeding can be less responsive to infant hunger and fullness cues and more parent-driven than breastfeeding, when the infant must play an active role in feeding. Formula fed infants, as well as those fed pumped breastmilk, are fed on a more regular schedule and the volume of feeds is more consistent, suggesting that parents or other caregivers may be more likely to drive infant intake patterns.⁴⁰ Also, common bottle feeding practices, such as “emptying the bottle” and serving larger volumes of formula at feedings are associated with greater infant intake and excess weight gain in the first six months after birth.⁴¹ Despite the behavioral differences associated with breastfeeding and formula feeding, we predict that our intervention will be effective for both feeding modes. However, because obesity risk differs by feeding mode and because many families use multiple feeding modes for their infants, we aim to assess the impact of our intervention with a sample that includes both intent to formula feed and intent to breastfeed dyads to examine how intervention effects are moderated by infant feeding mode in order to provide much needed guidance on responsive parenting for both groups.

A.4. Importance of Improving Infant Sleep Hygiene for Obesity Prevention

During the past 40 years, sleep duration in the U.S. has decreased by 1 to 2 hours per day while the prevalence of obesity has markedly increased.⁴²⁻⁴⁴ It is estimated that children, a group with a rapid rise in the prevalence of obesity, are currently sleeping 1 to 2 hours less than they require, and that approximately 15 million American children are affected by inadequate sleep.^{45, 46} The link between short sleep duration and childhood obesity was first shown in a study of French 5-year olds where investigators found a significant risk for overweight among children who slept less than 11 hours per

day.⁴⁷ Since the publication of that study, several investigations have shown that short sleep duration during early childhood (ages 3-5 years) is associated with overweight, obesity, and higher body fat during school age.^{29, 48-51} Most recently, Taveras et al. demonstrated that sleep duration of less than 12 hours during infancy is a risk factor for overweight and adiposity in preschool-aged children.⁵²

There are several mechanisms by which shorter sleep duration may lead to overweight even among the youngest of children. The first two months after birth represent a critical period in the development of sleep patterns, a period when feeding and sleeping are inextricably linked with infants waking every 2-4 hours, typically to feed.⁵³⁻⁵⁶ These first months are also central for the development of normal circadian rhythms.⁵⁷ As a result of these rhythms, infants have periods of arousal from sleep, and how parents handle the infant's night waking represents a source of variability in infants' developing nighttime sleep patterns.^{58, 59} A potential physiologic mechanism for a relationship between sleep and obesity also exists. Adult data have demonstrated that sleep restriction results in a significant reduction in the anorexigenic hormone, leptin, and an increase in the appetite stimulating peptide, ghrelin.⁶⁰ Reduced leptin and increased ghrelin were associated with a significant increase in hunger and appetite. The relationship between short sleep duration, reduced leptin, and increased ghrelin was also found in another investigation with over 1000 participants where the links were shown to exist independent of BMI.⁶¹ Though limited data on this subject exist for infants and children, lower cord blood ghrelin levels have been linked to slower weight gain from 0 to 3 months of age.⁶² These findings suggest that efforts to increase sleep duration for children could result in lower ghrelin levels, which could limit rapid weight gain during infancy.

A.5. The Next Frontier: The Relationship between Genetics, Appetite, Temperament, and Obesity Prevention

It has been well established that obesity has a strong genetic component.⁶³ However, as summarized by Manco and Dallapiccola, childhood obesity is a complex phenotype, modulated by unique gene-environment interactions which occur during sensitive periods of early life that set the stage or "program" an individual for later obesity.⁶⁴ These authors acknowledge that while the field is rapidly advancing, the genes identified in genome-wide association studies (GWAS) as being associated with obesity,⁶⁵ individually explain a very small amount of the inter-individual differences in BMI. Nonetheless, large studies including the Avon Longitudinal Study of Parents and Children, the Severe Childhood Onset Obesity Project, and the European Youth Heart Study have identified numerous gene variants associated with pronounced differences in weight status.⁶⁶⁻⁶⁸ Genes associated with two traits that are related to obesity, appetite and temperament are also showing promise both for identification of at-risk children as well as areas for intervention. For example, the *FTO* gene has been associated not only with BMI but specifically with satiety sensitivity and food responsiveness, which are key to the theoretical model described for INSIGHT.⁶⁹⁻⁷¹ Data from the Gemini study, a population-based birth cohort designed to investigate genetic and environmental influences on weight and development from birth to age 5 years, have demonstrated significant correlations between obesity-related genes and parent-reported measures of appetite (slowness in eating, satiety responsiveness, and appetite size) beginning at age 3 months.⁷² Indeed, Carnell and Wardle, in describing the behavioral susceptibility theory of obesity have proposed that intervention strategies aimed at improving satiety responsiveness and reducing food cue responsiveness in high-risk individuals could help in preventing the development of obesity.⁷³ Thus it is essential to obtain genetic information on INSIGHT family members, with a focus on the genetics of obesity, appetite, and food responsiveness which are expected to moderate effects of parenting and birth order.

Temperament, an early form of personality, is easily measured in early childhood and can be defined as individual differences in reactivity and regulation which are constitutionally-based, develop over time, and modifiable by the environment.⁷⁴ Temperament is known to be highly heritable,⁷⁵ and its interaction with the environment is emerging as an intriguing variable related to obesity risk as obese adults score higher in novelty seeking and impulsivity with extraversion.⁷⁶⁻⁸⁰ Among infants, those characterized as "difficult" due to high negativity and low adaptability, were found to be more likely to gain weight faster than less "difficult" infants.^{81,82} Further, infants who were more anger-prone, rather than fear-prone, showed the fastest weight gain.^{63, 64} This same profile is evident in childhood predictions of adult weight; negative emotionality in childhood was the most robust predictor of adult BMI, even when controlling for variables related to BMI.⁶⁵

Evidence from PI Birch's lab has recently shown that temperament moderates the impact of parenting on feeding and weight status.¹¹⁸ It is possible that the dopamine reward system underlies these links. Dopamine, a neurotransmitter, regulates reward via the meso-limbic circuitry with connections to neural areas involved in

emotional responding and memory processing. The combination of these elements results in a circuit that increases the probability that whatever behavior triggers activity in this system will be repeated. Food is one example of a natural reward that triggers dopamine release, and dopamine is released in the brain at the sight and smell of food, the anticipation of eating, and the ingestion of food.⁶⁶ Research on the dopamine receptor genes that regulate the reward system, DRD4, has been linked to the personality trait of novelty seeking.^{67, 68} Another dopamine receptor gene, DRD2, has been linked to both obesity and personality. Studies examining obesity and DRD2 found the availability of D2 receptors was decreased in obese individuals suggesting that they seek to compensate (reward deficiency syndrome) through overeating to get the reward that is missing. Alternatively, it has been proposed that fewer D2 receptors represent down regulation to compensate for dopamine increases caused by chronic over-stimulation from feeding.⁹⁰ However, so far GWASs targeting temperament have been unsuccessful.^{69, 70}

A recent study by Belsky and colleagues has informed the approach of this proposal aiming to examine candidate genes associated with obesity-risk, appetite, and temperament with weight-for-length z-score at age 1 year.⁹³ Belsky et al. utilized GWAS-identified obesity-associated single-nucleotide polymorphisms (SNPs) and the data from the Dunedin Multidisciplinary Health and Development Study in order to derive a multilocus genetic risk score (GRS) that was validated against another large cohort. Their GRS was found to be associated with rapid weight gain in childhood, early adiposity rebound, and high BMI from age 3 years to adulthood. No association was found at birth, and the critical interval between birth and age 3 was not examined. Utilizing a GRS is particularly useful for evaluating genetic effects in smaller cohorts, like the one in the INSIGHT study, for which conducting a separate GWAS is impractical due to lack of power. Notably, previous studies neither used genetic risk as a covariate for obesity together with other factors, nor performed gene-environment and gene-intervention interaction analyses. Collecting DNA on study participants will allow us to do so based upon our current knowledge of candidate genes associated with adult and childhood obesity-risk.⁹⁴ Moreover, it will allow us to examine these samples using genome-wide sequencing approaches in the future, which is imperative given the rapid advance in genome sequencing techniques and fast decline in their costs.

A.6. Microbiome

In addition to a genetic component of obesity, the environmental factors to the pathophysiology of obesity are also important to the development of this condition. One important environmental factor lies within our own bodies – our microbiome.

Once thought to be sterile, the gut of the neonate is colonized by a distinctive community of microbes¹⁷⁶ which colonizes the fetus through the amniotic fluid and thus influenced by the microbiome of the mother¹⁷⁷ Several studies have shown that the microbiota of the early human gut changes and the make up of that community may influence the development of obesity later in life. A study by Kalliomäki et al.¹⁷⁸ showed that for two types of bacteria (Bifidobacteria and *S.aureus*) in the gut show differences during infancy of children that would remain normal weight and those that become overweight. Similarly more recent studies have shown that there are compositional differences in the communities of certain gut bacteria between obese and lean children¹⁷⁹ beginning in infancy.¹⁸⁰ These studies provide great insight on the importance of the gut microbiome and its effect on weight outcome, but focus on a select group of micro-organisms by employing culture techniques prior to community characterization.

Another important aspect of the human microbiome is the oral microbiome. This has been studied in infants with regards to influence of mode of delivery on the establishment of the oral micro community¹⁸¹, colonization differences between low-birthweight and normal-birthweight infants¹⁸² and how the infant oral microbiome compares to that of their mothers/caregivers.¹⁸³ And while there have been some studies in adults relating oral microbiome to obesity and type-2 diabetes¹⁸⁴ there is no study relating the establishment of the oral microbiome in early life with risk for obesity or weight outcome.

Fecal and oral samples from the child and maternal oral samples will be characterized for the whole composition of microbial communities using 16s rDNA metagenomics. First noted in 1965 for its unique composition of conserved and variable bases¹⁸⁵, the 16s rRNA gene is the gold standard for genetic microbial profiling from samples.

B. SPECIFIC AIMS

Specific Aim 1: To test the efficacy of an intervention designed to prevent rapid weight gain in infancy and overweight at age 3 years by providing guidance on responsive parenting, division of feeding responsibility, and healthy dietary choices. We hypothesize that compared with control infants; intervention infants will have lower BMI percentiles at age 3. Also, we hypothesize that control infants will gain weight more rapidly over time, adjusting for trait-stable and time-varying covariates (e.g., maternal pre-pregnancy BMI, percent of feedings that are breastmilk vs. formula, sleep duration, and feeding frequency).

Specific Aim 2: To test how intervention effects on primary growth and weight outcomes are mediated by parental and infant behaviors and infant diet. We hypothesize that compared with control parents, intervention group parents will show increases in responsive parenting, alternative soothing techniques, and parenting self-efficacy. Over time, intervention infants will have a) longer sleep bouts, b) fewer daily feedings, c) a lower proportion of crying episodes followed by feeding in early infancy, and d) healthier diets from birth to age 3 compared with controls. These outcomes will be linked to healthier patterns of weight gain from birth to age 3 and lower BMI percentiles at age 3 years.

C. RATIONALE FOR INTERVENTIONS

C1. Obesity Intervention Program

Most attempts to address childhood obesity have tried to impact the obesity epidemic through interventions in schools, communities, and childcare settings. Unfortunately, a Cochrane analysis of these efforts found limited success in preventing or treating obesity for children in these age groups.⁷¹ An innovative approach to the epidemic is to begin preventive efforts before age 2, a period of metabolic and behavioral plasticity as recently summarized by PIs Paul, Birch and colleagues.⁷² Our approach was piloted in a successful NIDDK-funded trial as is described below, and we are now prepared to apply refined and improved interventions in a second longitudinal study.

C.1.a. Pilot Study Using Innovative Interventions to Promote Healthy Growth: “SLIMTIME”— SLeeping and Intake Methods Taught to Infants and Mothers Early in life

We selected two promising interventions for obesity prevention based on PI Birch’s prior research, recruited first-time mothers who intended to breastfeed, and followed their infants delivered at the Penn State Hershey Medical Center (HMC) from birth to 1 year. In this NIDDK-funded pilot (R56 DK72996), research nurses delivered interventions in the home using a 2 x 2 randomized experimental design. The first intervention, “Soothe/Sleep”, began when infants were 2-3 weeks old. This intervention focused on promoting aspects of responsive parenting to alter infants’ feeding and sleeping during the first 4 months after birth using techniques that trained parents to use feeding and other soothing approaches appropriately and selectively to calm a fussy infant.⁷³ The goal was to help mothers learn to discriminate their infant’s hunger from other distress cues and to use the appropriate soothing response for the infant’s distress. Our view was that by helping parents respond appropriately and contingently, this would reduce the use of feeding as the “default” response to infant fussing and crying, reducing the risk of overfeeding and overweight. Following previous research,⁷³ the intervention was constructed to promote the development of infant self-soothing and longer sleep duration as well as to reduce feeding frequency. The second intervention, “Introduction of Solids”, began later and recognized the role of dietary choices on obesity. It focused on the transition to solids, providing information on which foods to offer or limit, information about portion sizes, and strategies for promoting liking and acceptance of healthy complementary foods. Specifically, it was based on PI Birch’s research showing the effectiveness of repeated exposure at reducing neophobia and promoting infants’ acceptance of new vegetables^{24, 25} and toddlers’ acceptance of new table foods. 110 subjects completed the year-long study.

Efficacy of interventions for weight status at age 1 year. Participants receiving both interventions had a lower weight-for-length percentile with a mean at the 33rd percentile whereas those in other study groups were higher ($p=.006$; “Soothe/Sleep” intervention only – 50th percentile, “Introduction of Solids” intervention only – 57th percentile, Control group – 50th percentile), when adjusted for covariates.. To our knowledge, ours is the first trial to show positive effects of behavioral interventions on weight status during infancy.

Safety of interventions. Because behavioral interventions aimed at obesity prevention could theoretically cause infants to gain insufficient weight, this outcome was evaluated using two standard definitions, downward crossing of two major weight-for-age growth chart percentile lines between birth and age 1 year and weight less than the 5th percentile at age 1 year.⁷⁴ Both definitions were evaluated using Centers for Disease Control and Prevention (CDC) growth charts.⁹⁹ Nine (8.2%) participants had weight-for-age <5th percentile at age 1 year, and 16 (14.6%) had downward crossing of two major percentile lines. No significant differences were detected among treatment groups for either definition of insufficient weight gain. Further, while the combined interventions had a significant impact on weight-for-length percentile, there was no impact on linear growth. Lastly, all participants were those whose mothers intended to breastfeed. The CDC growth charts contain reference values for a largely formula fed group of infants. It is well-established that breastfed infants gain weight more slowly during infancy than formula fed infants.

Effects of promoting soothing on sleeping and feeding. The findings from PI Birch's previous research showing increased sleep and reduced feeding frequency among breastfed infants were replicated in SLIMTIME. As in the earlier study,⁷³ breastfed intervention infants slept significantly longer at night during the 16 weeks after birth ($p=.04$). Compared with control, the intervention also reduced total number of feedings per day ($p=.008$) and nocturnal feedings ($p=.003$) for breastfed infants. Intervening in this fashion is novel from an obesity prevention perspective because it targets several key developmental areas in addition to feeding and does not focus parents on concerns about obesity per se. First, it helps parents learn to be responsive while calming infants without feeding in the absence of hunger, promoting parenting competence. Next, it aids in the infant's development of self-regulation by allowing infants opportunities to self-soothe.⁷⁵ Third, given the consistently demonstrated relationship between short sleep duration and overweight, obesity, higher body fat, and hyperglycemia for children of all ages including infants,^{29, 47-52, 76-78} an intervention that effectively lengthens sleep duration during infancy is potentially preventive in the short term and later in childhood given that infant sleep difficulty predicts sleep problems later in childhood.^{79, 80} The importance of research studying potential links between sleep and obesity in children was emphasized in a recent editorial in the *Archives of Internal Medicine*: "It is now critical to determine the importance of a lack of sleep during the early formative years in putting our youth on a trajectory towards obesity and the metabolic syndrome – a trajectory that could be altered if sleep loss is indeed playing a role in this epidemic."⁸¹

Effects of repeated exposure on acceptance of healthy, developmentally appropriate foods. Data from FITS, revealing that French fries are the most commonly consumed vegetable by 15 month olds, illustrate that unhealthy eating habits begin early, and parents need more detailed instruction on what to feed and how much to feed their infants and toddlers as they transition to table foods. Results from PI Birch's lab^{24, 25, 82} were used to guide interventions to improve acceptance of healthy, developmentally appropriate foods. In one often cited study, 4-6 month old infants increased vegetable consumption following repeated opportunities to try them even after initial rejection.²⁴ In a subsequent study, repeated exposure to one pureed vegetable produced a generalized acceptance of other, similar pureed vegetables.²⁵

In SLIMTIME, the "Introduction of Solids" intervention focused on the timing of and effective approaches for introducing solid foods. Parents were instructed to delay the introduction of solid foods until their infant was at least 4 months of age, and once they did introduce infant cereal, a second home nurse visit occurred. At this visit, parents in the intervention group received instructions for repeated exposure to introduce new foods to improve liking and acceptance of initially unfamiliar foods. Nurses demonstrated this technique at the home visit. The intervention was effective in delaying the introduction of solids; only 17% of mothers in the intervention group gave their infant rice cereal prior to 4 months, compared with 34% of control subjects ($p=.06$). The positive effects of the repeated exposure instructions were demonstrated by significant increases in consumption of initially novel pureed vegetables, including green beans ($p=.001$), peas ($p=.02$), and squash ($p=.04$) from the first to the last day of exposure. We also noted that the intervention affected acceptance of new table foods at 1 year, when mothers offered infants an unfamiliar food (hummus, cottage cheese, or yogurt) at the laboratory visit. Based on coding of videos, only 10% of intervention infants rejected an unfamiliar food at age 1 year compared with 25% of those in the control group ($p=.05$).

C.1.b. Home Visitation for Obesity Prevention

As in our pilot study, we propose to use nurse home visits to deliver our intervention program. This choice is based on the unique potential of home visits to intervene in the environment where many obesogenic behaviors originate.¹³ Home visiting nurses also have the time and knowledge to accommodate each family's unique environment. Other home visitation programs such as The Nurse-Family Partnership developed by

Olds and collaborators have shown multiple positive effects for new mothers and their infants.^{83, 84} Women visited in both the prenatal and postnatal periods tend to have improved knowledge about contraception, fewer subsequent pregnancies, and more time between subsequent pregnancies.^{9, 85-88} Their infants also have fewer Emergency Department (ED) visits, unintentional injuries, ingestions, and poisonings.^{87, 89, 90} Similarly, there is a reduced incidence of child abuse and neglect in those infants participating in such programs.^{86, 89, 91} Importantly, this model of home visitation, though initially expensive, has been proven to be highly cost-effective over time.⁹² Notably, obesity prevention is not a current component of the Nurse-Family Partnership. While home visitation itself is not “innovative”, a well-constructed early intervention program focusing on promoting responsive parenting and healthy eating behaviors to reduce early obesity risk is innovative.

C.2. Child Safety Program

The control group will receive a child safety program based upon numerous standard pediatric sources as well as previous studies including: a) The Injury Prevention Program (TIPP) from the American Academy of Pediatrics (AAP),⁹³ b) the Academy’s guide for health supervision, *Bright Futures*,⁹⁴ and the Home Study from the University of Cincinnati.⁹⁵ TIPP is an educational program for parents of children newborn through 12 years of age to help prevent common injuries from: motor vehicles, firearms, bicycle crashes, drowning, poisoning, choking, burns, falls, and pedestrian hazards.

Much of the information covered by the safety intervention is typically presented as part of standard pediatric office care, but will be delivered in a more hands-on fashion at the home visits. It is suitable as a control intervention because like the intervention group, it contains developmentally appropriate guidance for parents that builds upon prior lessons.

III. PROTOCOL OVERVIEW

The proposed research seeks to deliver and test a program to prevent childhood obesity in a two arm randomized trial that includes a child safety control program. The obesity prevention program focuses on developmentally appropriate messages of responsive parenting, division of feeding responsibility, and healthy dietary choices that extend from infancy through age 3 years. As such, the intervention will teach first-time parents a) to recognize infant hunger and satiety cues and use feeding more selectively in response only to hunger, b) to use alternative soothing strategies to feeding for the fussy, but non-hungry infant and toddler, c) to provide children with appropriate portions of healthy foods and allow children to determine the amount consumed, d) how to improve acceptance of developmentally appropriate foods such as vegetables through repeated exposure and positive role modeling, e) how to develop good sleep hygiene, and f) to actively engage their infants in play time in order to reduced sedentary behaviors. The control group will receive child safety instructions adapted from accepted reference sources.

Nurses will deliver interventions to first-time parents and their infants at four home visits in the first year after birth followed by clinical research center visits at ages 1, 2, and age 3. Included in the informed consent will be permission to access the infant’s medical record, to review the outpatient chart, and to converse with the infant’s primary care physician (PCP). Information and data regarding parental participation in other parenting, breastfeeding, or family programs will be collected. Study personnel from the Hershey campus will be responsible for all face-to-face visits as well as the data collected at those visits. Study personnel from the University Park campus will be responsible for all phone interviews and web-based data collection with the exception of the 2 week randomization phone call.

Parents will be asked to participate in a long-term follow-up of their child until their 18th birthday. Nurses will meet with participants at ages 5, 10, 14, 17 years old, measuring height and weight and collecting data about diet, activities, and family life.

A. STUDY GROUPS AND SUBJECTS

We expect to enroll approximately 300 mother-infant dyads from the maternity floor at HMC where 1500 deliveries occur yearly. We will randomize 276 participants to either the obesity prevention program or the child safety control group, stratifying on birth weight for gestational age (<50th percentile or ≥50th percentile – sex specific)⁹⁶ and intended feeding mode (breastfeeding or formula feeding) 10-14 days after delivery. The

difference between number enrolled and randomized is due to the expectation that some mothers will either withdraw from the study during the first 14 days or be lost to follow-up prior to randomization.

B. INCLUSION CRITERIA

Eligible mother-infant dyads for this trial will meet the following criteria:

- 1) full-term infant ($\geq 37\ 0/7$ weeks gestational age) without significant morbidity
- 2) singleton infant
- 3) nursery/NICU/maternity stay of 7 days or less
- 4) primiparous mother
- 5) English speaking mother
- 6) reside within 50 miles (1 hour) of HMC
- 7) a working telephone number

C. EXCLUSION CRITERIA

(not explicitly obvious from the inclusion criteria)

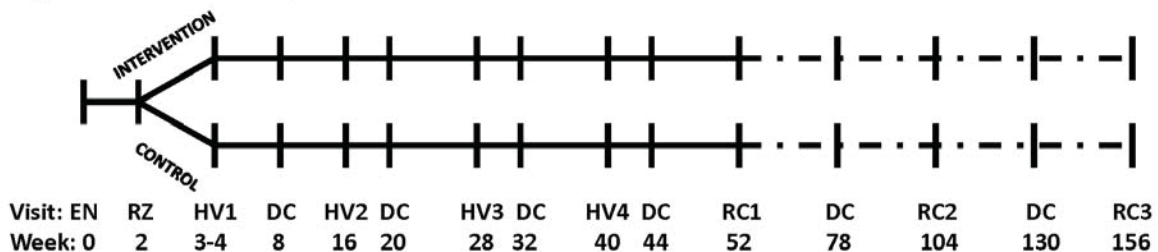
- 1) maternal age <20 years
- 2) prenatal ultrasound presence of intrauterine growth retardation (IUGR)
- 3) infant birth weight <2500 grams
- 4) presence of a congenital anomaly or neonatal condition that significantly affects a newborn’s feeding (e.g. cleft lip, cleft palate, metabolic disease)
- 5) any major maternal morbidities and/or pre-existing condition that would affect postpartum care or her ability to care for her newborn such as cancer, multiple sclerosis, lupus, etc.
- 6) plan for newborn to be adopted
- 7) plan to move from Central Pennsylvania within 3 years
- 8) inability to complete contact form with name, address, phone numbers, etc.
- 9) Practicing Pediatrician or Pediatric Resident

D. STUDY VISITS

The obesity intervention program contains messages on responsive parenting, division of feeding responsibility, healthy dietary choices, and reduction in sedentary behaviors designed for the prevention of obesity that extend from infancy through age 3 years. The intervention program will be delivered at 4 home visits and 2 CRC visits over the first 24 months after birth (Figure 1). A final CRC visit will also occur 36 months after birth.

Figure 1. INSIGHT First-Born Study Visit Schedule

Figure 1. INSIGHT Study Visit Schedule



Legend: EN – Enrollment, RZ – Randomization, HV – Home Visit, DC – Data Collection only, RC – Research Center Visit

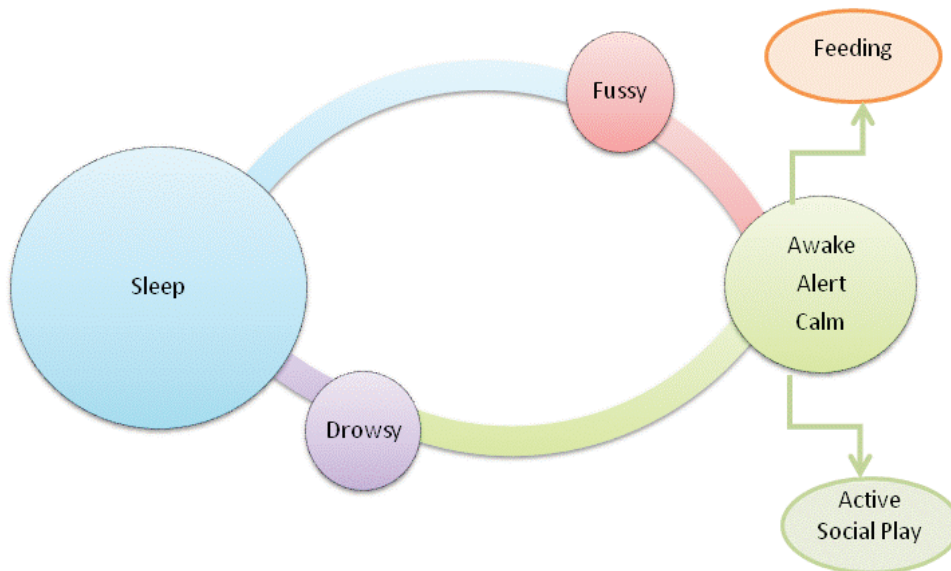
The control group will receive an equal number of visits, but the content will be a child safety intervention with messages focused on the infant’s environment and interactions with parents. They will be guided by The Injury Prevention Program (TIPP) from the AAP⁹³ as well as the Academy’s guide for health supervision, *Bright Futures*.⁹⁴ The information covered by the safety intervention is typically presented as part of standard pediatric office care, but will be delivered in a more hands-on fashion at the home visits. Parents will be encouraged to modify the environment to prevent falls, suffocation, motor vehicle-related injuries, burns, etc.

For both study groups, visit content and intervention messages will be mapped onto infant behavioral states: sleep, fussy, drowsy, and alert and calm (Figure 2). Within alert and calm are two subcategories, feeding and active social play. Key components of the obesity prevention intervention will focus on helping infants to transition between behavioral states such as fussy to asleep, fussy to alert and calm, and drowsy to asleep.

The majority of data collection will be separated from intervention visits. Data collection will occur via phone interviews and web-based software. Anthropometrics will be measured at each in-person visit, and as specified below, certain other objective measurements will occur (e.g., home observation data and a blood sample (500 µL) will be collected one time during from the child (by heel or finger stick) and both parents (by finger stick), if available).

Long term follow-up of first-borns will be completed by a nurse visit at ages 5, 10, 14, and 17 years old. Anthropometrics will be measured along with minimal data collection. No new educational materials will be delivered to families during follow-up.

Figure 2. Infant behavioral states for interventions in both study groups



1) Enrollment Visit - Maternity/Nursery Hospital Stay (0-7 days after birth)

- a. Newborn and maternal medical chart review in newborn nursery/maternity floor and/or NICU to determine eligibility based on inclusion and exclusion criteria (Screening form)
- b. Obtain maternal informed consent for mother-baby dyad (for those declining consent, attempt to complete brief Demographics – Non Participants form)
- c. Obtain paternal informed consent for his own participation (height and weight measurement, blood sampling)
- d. Complete enrollment data collection forms
- e. Chart abstraction form (coordinator completed)
- f. Demographics and Health History form,(coordinator interview with mother)
- g. Contact form (mother self-completed – if unable to complete, excluded from study for presumed illiteracy)

- h. Measure weight and height of mothers and fathers (if father not present, will attempt to measure at subsequent study visits)
- i. Collect blood samples from fathers (if father not present, will attempt to collect at subsequent study visits) and mothers (if mother chooses, her sample will be collected at the 1 year visit)
- j. Provide mothers with a copy of the book, *Your Baby's First Year*, 3rd edition, which is published by the American Academy of Pediatrics (AAP).

2) Randomization Telephone Call (10-14 days)

- a. Brief interview with mothers (Randomization form) completed by Hershey study coordinators
- b. Assess if participants have a DVD player or other means to watch videos (e.g. computer)
- c. Schedule first home visit
- d. Instruct mothers to complete on-line surveys (Babies Need Soothing – short version, Brief Infant Sleep Questionnaire – short version, Infant Feeding Style Questionnaire – short version, Babies Need Feeding – Short Version, Knowledge of Infant Developmental Inventory – adapted, Karitane Parenting Confidence Scale) and mailed surveys (Food Frequency Questionnaire - will be picked up at home visit) prior to first home visit) *Obesity Intervention Group*
- e. Mail incentive materials (The Happiest Baby on the Block DVD and White Noise CD) – ask mothers to view DVD before first home visit. Also in mailing are a welcome letter with their appointment date, the Food Frequency Questionnaire, and handouts on infant stomach size, feeding do's and don'ts, lactation support, and postpartum depression.

3) Home Visit 1 (18-24 days)

- a. *All Participants*
 - i. Infant weight and length measured
 - ii. Review developmental milestones
 - iii. Review with mother how life has gone over the past 3-4 weeks
 - iv. Provide study binder for handouts, notes, etc., including lactation support information and postpartum depression contact/resources
 - v. Instruct mothers on use of diary cards to monitor response to fussiness
 - vi. Administer Edinburgh Postnatal Depression Survey to mothers
 - vii. Complete nurse implementation quality survey after the end of visit
 - viii. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park
- b. *Obesity Intervention Group Visit Content*
 - i. Provide DVD with instructional videos and group- specific visit handouts (Do's and Don'ts, Baby Belly Size)
 - ii. Provide infant swaddle blanket
 - iii. Provide overview of infant temperament and uniqueness of each infant, discuss how guidance over course of the study will be developmentally appropriate Sleep domain
 - 1. Instruct on establishing bedtime routine
 - 2. Instruct on components of bedtime routine
 - 3. Discuss dream feeds – parent waking baby to feed before parent goes to bed
 - 4. Discuss creating day/night environmental differences in the home
 - iv. Fussy domain
 - 1. Discuss reasons for infant crying View *The Happiest Baby on the Block* video clip to show alternate methods to calm a fussy baby. Work with parents to practice technique.
 - 2. Emphasize that soothing strategies may be different day vs. night as the goal at night is often to return the baby to sleep rather than to an alert and calm state
 - a. Allow baby to learn to self-soothe at night, quick responses to fussiness are important during the day
 - b. Look for hunger signs before feeding especially at night

- v. Growth
- vi. Child's weight and length will be entered into the WHO anthropometric software. Alert, and calm domain
 - 1. Feeding sub-domain
 - a. Review norms for breastfeeding and formula feeding depending on the current infant feeding mode
 - b. Discuss delaying solids (including infant cereal) until 4-6 months fruit juice until 6 months and the reasons for it
 - c. Discuss bottle and nipple size recommendations
 - d. Review infant hunger and satiety cues
 - 2. Active and social play sub-domain
 - a. Recommend playing on the ground with baby each day
 - b. Instruct on awake tummy time
 - c. Advise outdoor play time when possible

4) **Diary Data Collection (3-4 weeks, 8 weeks)** Diary cards will be distributed to mothers at the 3-4 week home visit for the purpose of collecting data on each infant's fussy events and total number of daily feeds. The data collection will occur at 2 different time points (following the first home visit and at 8 week phone call). Parents will have complete diary cards for 2 consecutive days beginning either on a Friday or a Sunday, in order to capture one weekday and one weekend day. Mothers will be reminded to complete the 8 week diary cards both when they receive their emailed survey and at their 8 week phone call.

5) **On-line Survey Completion (8 weeks, 16 weeks, 32 weeks, 40 weeks, 44 weeks, 1 year, 1.5 years, 2 years, 2.5 years, 3 years)**. Mothers will be contacted via e-mail and asked to complete study-related surveys at various time points over the 3 years (Table 1). Arrangements will be made for surveys to be mailed if internet access is not available for mothers.

6) **Mailed Survey Completion (16 weeks, 28 weeks, 40 weeks, 1 year, 2 years, 3 years)**. A few selected surveys are not conducive to web-based completion. These surveys will be mailed to mothers, and will be picked up at in-person visits.

7) **Study phone calls (8 weeks, 20 weeks, 32 weeks, 44 weeks, 1.5 years, 2.5 years)**. Finally, two surveys necessitate an interview, and thus participants will be contacted by study personnel to answer these questions via phone.

8) **Home Visit 2 (16 weeks)**

a. *All Participants*

- i. Infant weight and length measured
- ii. Review and discuss whether mothers achieved the goals that were set during the 3-4 week visit and how the past 12 weeks have gone
- iii. Review developmental milestones
- iv. Collect completed paper surveys from mothers
- v. Complete home environment assessment and data collection
- vi. Complete nurse implementation quality survey at end of visit
- vii. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park

b. *Obesity Intervention Group Visit Content*

- i. Provide DVD with group-specific instructional videos and visit handouts
- ii. Provide teether, teaspoon, and tablespoon
- iii. Provide vegetables and instruction for repeated exposure tastings and Baby Food Log.
- iv. Sleep domain
 - 1. Review customized sleep profile based on the work by Mindell and Sadeh⁹⁷
 - 2. Review norms for napping and nighttime sleep

3. Review sleep hygiene and bedtime routine
 - a. Discourage rocking, singing, bouncing, feeding baby to sleep
 - b. Encourage soothing activities come between feeding and putting a baby to bed drowsy, but awake
4. Review methods to be responsive at night without picking baby up for every fussy episode at night
5. Encourage cessation of dream feeds
- v. Fussy domain
 1. Discuss reasons for infant crying and the natural history of infant crying
 2. Review *The Happiest Baby* – what worked to calm baby before may not still be working
 3. Review soothing strategies for 4-6 month olds
- vi. Growth
 1. Plot child's weight and length on growth chart
 2. Review with parents what percentiles on the growth chart indicate
 3. Review normal weight gain during the first several months after birth
 4. Child's weight and length will be entered into the WHO anthropometric software.
 5. Review what influences how fast babies grow and that percentiles on growth chart are not the same as those used for academic achievement – higher isn't necessarily better, lower may be ok
- vii. Alert, and calm domain
 1. Feeding sub-domain
 - a. Review norms for breastmilk and/or formula consumption
 - b. Review appropriate bottle and nipple size, reasons to change
 - c. Review obesogenic bottle feeding behaviors (e.g. propping a bottle) and discourage them
 - d. Nurse will view mother bottle feeding and provide feedback on her responsiveness to hunger/satiety cues.
 - e. If solid foods have been introduced, nurse will observe a feeding and provide feedback on developmental readiness and how mother responds to hunger/satiety cues
 - f. Discourage infant cereal in the bottle
 - g. Discuss signs that a baby is ready for solid foods
 - h. Discuss how to introduce cereal
 - i. Discuss progression to other solid foods, such as vegetables, and to introduce small volumes of different foods in successive days, but with repeated exposure to the same foods over the course of many days (note – infant vegetables and fruits have very low allergic risk making this acceptable practice though different than traditional guidance).
 - j. Discuss avoidance of fruit juice until at least 6 months and avoidance of other sweet beverages and unhealthy foods now and later
 - k. Discuss the importance of modeling healthy food consumption for children
 2. Active and social play sub-domain
 - a. Recommend playing on the ground with baby each day
 - b. Instruct on awake tummy time
 - c. Advise outdoor play time when possible
 - d. Advise that parents minimize the use of restrictive equipment for babies and allow them to explore their environments more
- c. *Safety Intervention Group Visit Content*
 - i. Provide DVD with instructional videos and visit handouts
 - ii. Provide bathtub spout cover
 - iii. Baby Food Log
 - iv. Sleep domain

1. Review associations with Sudden Infant Death Syndrome and how to create a safe sleep environment for infants
2. Discuss features of safe playpens and pack 'n plays
- v. Fussy domain
 1. Discuss Shaken Baby Syndrome and normal infant crying
 2. Discuss things to look for in a child care provider and/or child care center
 3. Discuss safe medication dosing
- vi. Alert, and calm domain
 1. Feeding sub-domain (food safety)
 - a. Discuss safe preparation of infant solid foods
 - i. Baby food jars – dish out portion with clean spoon
 - ii. Avoid sharing spoons
 - iii. Perishable foods and time outside of refrigerator
 - iv. Foods to avoid with young children for safety reasons (e.g. honey, home canned food)
 - b. High chair safety
 - i. Discuss features of safe high chairs
 - ii. Never leave baby unattended
 2. Active and social play sub-domain
 - a. Preventing falls
 - i. Nurses view changing table and look for proximity to diapers, and other items needed for changing baby?
 - ii. Nurse reviews features of changing table (straps, concavity, guardrail)
 - b. Preventing burns
 - i. Advise against table cloths
 - ii. Advise to cook on back burners, keep hot liquids/foods away from edge of table
 - c. Bath safety
 - i. Avoid using bath rings
 - ii. Never leave a bathing baby unattended
 - iii. Spout covers can reduce traumatic injuries to babies
 - d. Cutting nails
 - i. Review how to safely cut baby's nails
- vii. Growth
 1. Plot child's weight and length on growth chart
 2. Child's weight and length will be entered into the WHO anthropometric software.

9) Home Visit 3 (28 weeks)

a. *All Participants*

- i. Infant weight and length measured
- ii. Review and discuss questions that they have from the 16 week and how the past 12 weeks have gone
- iii. Review developmental milestones
- iv. Collect completed paper surveys from mothers
- v. Complete home environment assessment and data collection
- vi. Complete nurse implementation quality survey at end of visit
- vii. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park

b. *Obesity Intervention Group Visit Content*

- i. Provide DVD with group-specific instructional videos and visit handouts
- ii. Provide child cup
- iii. Sleep domain
 1. Review norms for napping and nighttime sleep
 2. Review sleep hygiene and bedtime routine

- a. Discourage rocking, singing, bouncing, feeding baby to sleep
 - b. Encourage soothing activities come between feeding and putting a baby to bed drowsy, but awake
 - 3. Review methods to be responsive at night without picking baby up for every fussy episode at night
 - 4. Discuss naptime routine
 - a. Review methods to be responsive to fussiness during naptime
- iv. Fussy domain
 - 1. Discuss temperament
 - 2. Discuss emotions of 6-9 month olds including frustration/anger and fear
 - 3. Discuss teething as a source of fussiness
 - 4. Discuss strategies for responding to a fussing 6-9 month old.
 - 5. Modeling a good mood example
- v. Growth
 - 1. Plot child's weight and length on growth chart
 - 2. Review with parents what percentiles on the growth chart indicate
 - 3. Review normal weight gain during the first several months after birth
 - 4. Child's weight and length will be entered into the WHO anthropometric software.
 - 5. Review what influences how fast babies grow and that percentiles on growth chart are not the same as those used for academic achievement – higher isn't necessarily better, lower may be ok
- vi. Alert, and calm domain
 - 1. Discuss milestones for 6-9 month olds
 - 2. Gross motor milestones including rolling over and crawling
 - a. Ways to support crawling skills
 - b. Things to avoid
 - 3. Fine Motor Skills
 - a. Ways to support development of fine motor skills
 - 4. Social and Emotional Development at 6-9 months
 - a. Ways to support emotional and social development
 - 5. Discuss limit setting and routines
 - a. Limit TV time
 - b. Discuss the beginnings of discipline
 - 6. Parent Modeling
 - a. Teach parents about being a positive role model for physical activity
- vii. Feeding
 - 1. Discuss bottle and nipple size recommendations for 6-9 month olds
 - 2. Review average intake of breast milk and/or formula for this age
 - 3. Discuss introduction of solid foods.
 - a. If solid foods have not been introduced, review and booster information on starting solids provided at the 16 week visit
 - 4. Discuss developing skills associated with feeding like using the spoon
 - 5. Review signs of fullness
 - 6. Discuss division of responsibility: Parents provide, babies decide
 - 7. Discuss age appropriate portion sizes
 - 8. Share with parents information about meals
 - a. When are babies ready for meals
 - b. Examples of healthy age-appropriate meals
 - 9. Discuss limit setting and meal time routines
 - a. Do not add ingredients like salt and sugar
 - b. Avoid a lot of juice and grazing
 - 10. Discuss Table and Finger foods
 - a. Provide parents with suggestions of good and not good choices
 - b. Discuss self-feeding

11. Give parent information on starting use of a cup
12. Parent Modeling
 - a. Healthy eating and tips
 - b. Family meal time
- c. *Safety Intervention Group Visit Content*
 - i. Provide DVD with instructional videos and visit handouts
 - ii. Provide Medication Lock Box
 - iii. Sleep domain
 1. Review associations with Sudden Infant Death Syndrome and how to create a safe sleep environment for infants
 2. Discuss teething as a source of night waking
 - a. Provide parents with home care advice for teething
 - iv. Fussy domain
 1. Discuss Distracted Driving
 - a. Provide tips to reduce child distractions while driving.
 - v. Alert, and calm domain
 1. Feeding sub-domain
 - a. Discuss Choking
 - i. Discuss common choking hazards and prevention tips
 - b. Parents will be taught the Baby Heimlich
 - vi. Active and social play sub-domain
 - a. Discuss fall prevention
 - i. Provide parents with advice on baby safety gates and answer questions on placement or installation of gates in the home
 - b. Discuss Poison Prevention
 - i. Talk to parents about safe storage of cleaners and hazardous materials
 - vii. Growth
 1. Plot child's weight and length on growth chart
 2. Child's weight and length will be entered into the WHO anthropometric software.

10) Home Visit 4 (40 weeks)

- a. *All Participants*
 - i. Infant weight and length measured
 - ii. Review and discuss questions that they have from the 28 week and how the past 12 weeks have gone
 - iii. Review developmental milestones
 - iv. Collect completed paper surveys from mothers
 - v. Complete home environment assessment and data collection
 - vi. Complete nurse implementation quality survey at end of visit
 - vii. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park
- b. *Obesity Intervention Group Visit Content*
 - i. Provide DVD with group-specific instructional videos and visit handouts
 - ii. Sleep domain
 1. Review norms for napping and nighttime sleep
 2. Review sleep hygiene and bedtime routine
 - a. Discuss increased nightwakings as new motor milestones develop and separation anxiety begins
 3. Review methods to be responsive at night without picking baby up for every fussy episode at night

- iii. Fussy domain
 - 1. Discuss separation and stranger anxiety
 - a. Included ways to comfort baby during episodes of anxiety
 - 2. Introduce Emotion Coaching
 - a. Teach parents techniques for using emotion coaching.
 - iv. Growth
 - 1. Plot child's weight and length on growth chart
 - 2. Review with parents what percentiles on the growth chart indicate
 - 3. Review normal weight gain during the first several months after birth
 - 4. Child's weight and length will be entered into the WHO anthropometric software.
 - 5. Review what influences how fast babies grow and that percentiles on growth chart are not the same as those used for academic achievement – higher isn't necessarily better, lower may be ok
 - v. Alert, and calm domain
 - 1. Discuss language development for 10-12 month olds
 - a. Ways to support language development
 - 2. Gross motor milestones including walking and grasping
 - a. Ways to support motor skills
 - 3. Discuss limit setting and routines
 - a. Limit TV time
 - 4. Parent Modeling
 - a. Teach parents about being a positive role model for physical activity
 - vi. Feeding
 - 1. Discuss introducing healthy table foods.
 - 2. Review steps for starting use of a cup
 - 3. Discuss weaning from the bottle
 - a. Provide steps for weaning and
 - 4. Review steps for starting use of a spoon
 - 5. Review responsive parenting to promote healthy eating
 - a. Portion size for 10-12 month olds
 - b. Limiting unhealthy food choice offered to baby
 - 6. Discuss mealtime routines
 - a. Timing, portions, distractions
 - 7. Discuss feeding snacks
 - 8. Discuss "all done" signing
 - 9. Discuss feeding options when eating away from home
 - 10. Discuss feeding practices
 - a. Negative impact of using food to control child's behavior
 - b. Pressuring child to eat.
- c. *Safety Intervention Group Visit Content*
- i. Provide DVD with instructional videos and visit handouts
 - ii. Provide PackIt cooler lunch box
 - iii. Sleep domain
 - 1. Discuss Carbon Dioxide poisoning and prevention
 - 2. Discuss window safety and fall prevention
 - iv. Fussy domain
 - 1. Discuss Car Seats
 - a. Reinforce the American Academy of Pediatrics recommendations on use of rear-facing car seats until age 2. .
 - 2. Fevers: discuss with parents what to do if their child has a fever
 - a. Discuss proper medications for this age and how to give them.
 - v. Alert, and calm domain
 - 1. Feeding
 - a. Discuss Food poisoning due to improper temperatures.

- i. Provide tips for packing lunches
- vi. Active and social play sub-domain
 - a. Discuss Furniture safety
 - i. Provide parents with advice on use of furniture straps
 - b. Discuss Electrical outlet safety
 - c. Discuss Toy Safety
- vii. Growth
 - 1. Plot child's weight and length on growth chart
 - 2. Child's weight and length will be entered into the WHO anthropometric software.

11) Research Center Visit 1 (1 Year)

a. All Participants

- i. Child weight and length measured
- ii. Collect blood sample from child (finger or heel stick)
- iii. Collect blood sample from mother and father (if not collected at a previous visit)
- iv. Review and discuss questions that they have from the 40 week and how the past 12 weeks have gone
- v. Review developmental milestones
- vi. Complete and data collection
- vii. Toy Removal Task
 - 1. Length of task: 6 minutes
 - 2. Child is seated in high chair and presented with an attractive toy with a detachable part. Mother is instructed to take toy away from child and place it on a chair in child's view but out of reach and engage in a conversation with research coordinator. Mother then returns toy without detachable part and continues conversation with research coordinator. To end the task, mother returns both parts of toy to child and continues to engage with the research coordinator.
- viii. Self-feed Observation
 - 1. Length of task: 2 minutes
 - 2. Bib will be worn/given as incentive gift
 - 3. Research coordinator will observe child attempt to self-feed with a spoon (mom chooses desirable, familiar food) and to drink from a cup without a lid (water).
- ix. Novel Feeding Task
 - 1. Length of task: 10 minutes
 - 2. Mother will select three novel foods for child to try. Research coordinator will instruct the mother to feed 2 tastes of each food to the child. Child reactions will be recorded.
- x. Complete nurse implementation quality survey at end of visit
- xi. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park

b. Obesity Intervention Group Visit Content

- i. Provide DVD with group-specific instructional videos and visit handouts
- ii. Provide visit handouts
- iii. Provide baby book & Happiest Toddler on the Block DVD
- iv. Introduction to self-regulation material
 - a. Definition
 - b. Why is self-regulation important
 - c. Timeline of toddler self-regulation skills
- v. Fussy Domain
 - 1. Helping child learn to regulate emotions/feelings
 - 2. Tantrums
 - a. Why they occur

- b. Tips for prevention
 - c. Show a portion of the Happiest Toddler on the Block DVD by Dr. Harvey Karp
 - d. Tips to stopping a tantrum
 - e. Time-out
- vi. Feeding Domain
 - 1. Beverages: cow's milk, water, juice recommendations
 - 2. Review weaning off the bottle, if needed
 - 3. Self-regulation when eating – eat when hungry and stop when full
- vii. Sleep Domain
 - 1. Transitioning from 2 to 1 nap per day
 - 2. Bedtime stalling
 - 3. Self-regulation and sleep – fall asleep on own, self-soothe
- viii. Active Social Play Domain
 - 1. Language development
 - 2. Make play part of every day
 - 3. TV reminder – no screen time is still recommended until age 2
- ix. The back of the Conceptual Model will be used to review the content delivered in all domains with respect to self-regulation.

c. *Safety Intervention Group Visit Content*

- i. Provide visit handouts
- ii. Provide baby book and frame
- iii. Sleep domain
 - 1. Discuss reduced SIDS risk
- iv. Fussy domain
 - 1. Discuss Discipline
 - a. Reinforce the American Academy of Pediatrics recommendations on not using spanking for discipline.
- v. Alert, and calm domain
 - 1. Feeding
 - a. Discuss dangers of leaving a child unattended in a high chair
 - b. Discuss food allergies
 - i. Provide examples of how to read food labels
- vi. Active and social play sub-domain
 - a. Discuss Gun safety
 - b. Discuss Fluoride use and age-appropriate amount to use
 - c. Give parents seasonal safety information
 - i. Spring:
 - 1. Use of Insect Repellant
 - 2. Use of Sunscreen
 - ii. Summer:
 - 1. Water Safety
 - iii. Fall:
 - 1. Candy and trick-or-treating safety
 - iv. Winter:
 - 1. Holiday decorations and lighting safety
- vii. Growth
 - 1. Plot child's weight and length on growth chart
 - 2. Child's weight and length will be entered into the WHO anthropometric software.

12) Mailing (1.5 years)

a. *All Participants*

- i. *Will receive a letter thanking them for their continued participation.*
 - ii. *“Who Does What” survey.*
- b. *Obesity Intervention Group Handouts*
 - i. Fussy Domain
 - 1. Disrupted routines
 - 2. Temper tantrums
 - ii. Feeding Domain
 - 1. Picky eating
 - 2. Healthy Snack ideas
 - iii. Sleep Domain
 - 1. Tips for good sleep
 - 2. Bedding and things to remember
 - iv. Active and social play sub-domain
 - 1. Make play part of everyday-tips for playing with toddlers
 - 2. Language development games
 - v. Provide DVD with instructional videos
- c. *Safety Intervention Group Handouts.*
 - i. Active and social play sub-domain
 - 1. Playground safety
 - 2. Insect repellent
 - 3. Holiday safety
 - 4. Water safety
 - 5. Back to school
 - 6. Car seats-toddlers

13) Research Center Visit 2 (2 Years)

- a. *All Participants*
 - i. Child weight, length, and height measured
 - ii. Collect blood sample from child (finger or heel stick) –if not collected at 1 year and parent consents
 - iii. Collect blood sample from mother and father (if not collected at a previous visit)
 - iv. Collect buccal swabs from child, if parent consents
 - v. Collect buccal swab from mother, if parent consents
 - vi. Collect child’s stool sample. Parents are asked to bring sample in (using the provided tube and biohazard bag) frozen within 48 hours of study visit.
 - vii. Review and discuss questions that they have from the 1 year visit and how the past 12 months have gone
 - viii. Review developmental milestones
 - ix. Complete and data collection
 - x. Complete nurse implementation quality survey at end of visit
 - xi. Mother given implementation survey to be completed after visit and returned to Penn State Research Team at University Park
- b. *Obesity Intervention Group Visit Content*
 - i. Provide visit handouts
 - ii. Provide My Plate gift
 - iii. Sleep domain
 - 1. Discuss transitioning from a crib to a bed
 - 2. Discuss nightmares and nighttime fears
 - iv. Fussy domain
 - 1. Discuss toddler’s emotions

- 2. Discuss tantrums and avoiding tantrums and attention seeking behavior.
- v. Feeding domain
 - 1. Discuss healthy beverages
 - 2. Discuss importance of making healthy food choices both at home (snacktime) and away from home
 - 3. Using MyPlate
- vi. Active Social Play domain
 - 1. Discuss new screen time recommendations and good screen time habits
 - 2. Discuss play, modeling, and motor/language development.
- c. *Safety Intervention Group Visit Content*
 - i. Provide visit handouts
 - ii. Provide Handwashing gift and first aid kit
 - iii. Sleep domain
 - 1. Discuss toddler bed and baby proofing toddler's room
 - iv. Fussy domain
 - 1. Discuss First Aid treatments and provide information from the AAP
 - v. Alert, and calm domain
 - 1. Feeding
 - a. Discuss poisonings: Medicine vs. Candy
 - b. Discuss stove safety
 - 2. Active and social play sub-domain
 - a. Discuss Pedestrian Safety
 - b. Discuss importance of getting car seat checked
 - c. Discuss importance of proper hand washing and teaching toddlers
 - vi. Participants in the safety intervention will also be given time to explore the Safety Center in the new Children's Hospital.
- d. Growth
 - i. Plot child's weight and length on growth chart
 - ii. Child's weight and length will be entered into the WHO anthropometric software.

14) Mailing/Phone call (2.5 years)

- a. *All Participants*
 - i. *Will receive a letter thanking them for their continued participation.*
 - ii. *Will receive mailed packets and a phone call*
- b. *Obesity Intervention Group*
 - i. *Handouts*
 - 1. Fussy Domain
 - a. Acting out and discipline
 - b. Rewarding good behavior
 - c. Bribing vs. Rewarding
 - 2. Feeding Domain
 - a. Kids in the Kitchen
 - b. Grocery shopping
 - 3. Active and social play sub-domain
 - a. Playing every day and how to make a sensory bin
 - b. Creativity and free play
 - ii. *Discussion*
 - 1. Fussy Domain
 - a. Discipline
 - b. Rewarding good behavior-reward chart
 - 2. Feeding Domain
 - a. Picky eating

- b. "I tried it" chart
 - 3. Sleep Domain
 - a. Sleep discussion based on responses to sleep survey
 - 4. Active and social play sub-domain
 - a. Playing every day
- c. *Safety Intervention Group:*
 - i. *Handouts.*
 - 1. Stranger Danger
 - 2. Putting items in noses and ears.
 - ii. *Discussion*
 - 1. *Outdoor Safety: lawn mower, snow blowers, trampolines*
 - 2. Safety reminders: bath safety, bed safety, climbing

15) Research Center Visit 3 (3 Years):

- a. *All Participants*
 - i. Child weight, length, and height measured
 - ii. Mother weight measurement
 - iii. Mother/Father height measurement (if not collected at a previous visit)
 - iv. Child Blood Pressure measurement
 - v. Collect blood sample from mother and father (if not collected at a previous visit)
 - vi. Mom completes surveys-see construct for detailed list
 - vii. Free play task
 - 1. Length of task~6 minutes
 - 2. After a few minutes of play, when child seems to be comfortable in the room, the research assistant will give mom an auditory signal (knock on door) that cues mother to ask child to pick up toys and put them back into the box.
 - viii. Delay of gratification task
 - 1. Length of task ~10 minutes
 - 2. Child offered small and a large pile of their choice of either M&Ms or mini-marshmallows and told that they can have the small pile now or if they can wait for the research assistance to return then they can have the big pile. If they cannot wait they will knock on the door to the study room and can eat the small pile. The longest wait time is 5 minutes. If the child is able to wait the full 5 minutes then they may eat the large pile.
 - ix. Taste test of "novel" foods
 - 1. Length of task ~5-10 minutes
 - 2. Child is shown 3 faces (one for yummy, one for yucky, and one in between). Child is given examples and asked to use the faces to identify yummy, yucky, middle foods. They are then asked to try 3 novel foods and indicate which face they think fits the new food.
 - x. Child consumes preload drink and snack
 - 1. Either a measured yogurt or no-dairy smoothie drink and some dry cereal. Given to ensure that none of the children are hungry.
 - xi. Anger/frustration task
 - 1. Length of task~5 minutes
 - 2. Mother will encourage the child to play with the toy. After approximately one minute of play the research assistant will nod to the mom as signal to terminate play. The mom then takes the toy cars and track away from the child and puts it behind her back. The mom will retain the toy for 30 seconds. After 30 seconds the mom will then give the cars and track back to the child and encourage child to play with the toy for 30 seconds so that baseline can be reached.

- xii. Taste test of Eating in the Absence of Hunger (EAH) foods
 - 1. Length of task~10 minutes
 - 2. Using the same Yummy, Yucky, and middle faces children will be asked to try about 5 snack foods (depending on allergies) and indicate how they taste.
 - xiii. Eating in the Absence of Hunger (EAH) task
 - 1. Length of task~10 minutes
 - 2. The research assistant shows the child a tray of snack foods that they can eat (the snack foods from the Taste test of Eating in the Absence of Hunger (EAH) foods) The child is also shown some toys he/she may play with (he/she played with these same toys during the free play task). The toy bin is opened and a few options are pointed out. Then the bin is placed on one side of the room. The research assistant tells the child that he/she can play with any of the toys, *or* eat any of the foods on the table while she finishes some work in the other room. The research assistant leaves and sets a timer for 7 minutes.
 - 3. The food is pre and post weighed to see how much food the child consumes in the absence of hunger.
- b. At this visit there are no differences between groups. No intervention is given.
 - c. Growth
 - i. Plot child's weight and length on growth chart
 - ii. Child's weight and length will be entered into the WHO anthropometric software.

16) Study Evaluation phone call:

- a. After 3 year visit is completed participants will receive a phone call asking questions evaluating the study. Phone call will take about 15-20 minutes.

17) 5 year Birthday Card

- a. *All Participants*
 - i. A birthday card wishing the participating child a happy 5th birthday with a \$10 Target card enclosed will be mailed to each family within approximately 1 month of the child's 5th birthday. The card will thank the families for participating and reminds them to contact the study team should any of their information change.

18) 5 year visit

- a. *All Participants*
 - i. Infant weight and height measured
 - ii. Ask mothers about any health or contact changes/updates

19) Home/clinic visits (6, 10, 14, 17 years old): long term follow-up

- a. *All Participants*
 - i. Infant weight and length measured
 - ii. Plot child's weight and length on growth chart
 - iii. Ask mothers about how they and their child are eating and sleeping.

20) Phone calls or emails (3.5, 4, 4.5, 5, 5.5, 6):

- a. *All Participants*
 - i. Research staff will call and/or email participants to remind them about the sibling study and to ask them to let study staff know if there are any changes. –no data or information is given at these phone calls.

21) Audio Recordings to prevent drift:

To prevent drift of messages delivered at the multiple study visits, nurses will ask approximately the 20th mother (17th-23rd) she is assigned for each of the visits (3-4, 16, 28,40 weeks and 1,2,3 years), if she would allow the visit to be audio recorded, using the IRB approved verbal consent form. Mothers may refuse to be recorded so the nurses should try to get a recording completed for a range from her 17th to 23rd mother receiving each visit. Each nurse would be recorded approximately 3-4 times per visit type throughout the study. Audio recordings will be sent to the Center for Childhood Obesity for assessment and feedback.

E. OUTCOME MEASURES AND THE RATIONALE FOR CHOOSING THEM

Per current CDC and AAP recommendations, all growth related outcomes will use the World Health Organization (WHO) growth references for child outcomes <2 years and the CDC growth reference for child outcomes 2 years and older.⁹⁸

Primary Outcome: Numerous outcome measures will be assessed over the 3-year study period with the primary outcome of BMI percentile at 3 years. This outcome provides significant insight into long-term obesity risk.

Secondary Outcomes: We will evaluate outcomes related to weight status in numerous ways and these will serve as the secondary outcomes for the study. They include:

- Weight-for-length percentile at several intervals in the first 12 months after birth
- Conditional weight gain between birth and 6 months, 6 months and 1 year, birth and 1 year
- BMI percentile at age 2 years
- Proportion of infants with BMI \geq 85th and 95th percentiles at ages 2 and 3 years
- Proportion of infants with accelerated weight gain (0.67 difference in BMI Z-scores) between numerous study intervals (e.g. birth to 4 months, birth to 1 year, birth to 3 years, 1 year to 3 years, etc.)

Other Outcome Measures to be Studied:

- Sleep duration during first year after birth
- Number of daily feedings
- Proportion of crying episodes followed by feeding
- Use of alternative soothing strategies
- Timing of introduction of solid foods
- Breastfeeding duration (for those who choose to breastfeed)
- Dietary content
- Parent feeding style
- Infant temperament
- Infant acceptance of healthy solid and table foods

Outcomes Related to Control Group Intervention:

- Proportion of correct responses on child safety related questions that are incorporated into other study surveys
- Objective home safety data collected as part of home observation data collection

A conceptual model depicting the hypothesized relationships between study group, behavioral mediators, and weight outcomes is shown in Figure 3.

Constructs	Form #	MEASURES	WEEKS												YEARS					
			0	2	3-4	8	16	20	28	32	40	44	1	1.5	2	2.5	3	5		
		Food allergy checklist																	P	
Neophobia		Neophobia (Pliner)													O					
Baby Appetite		Baby Eating Behavior Questionnaire												O						
Child Eating Behavior		Child Eating Behavior Questionnaire (Wardle)															O			
Sign Language Use		Sign Language Survey (created in-house)													O					
Motor Development																				
Motor Milestones		Motor Milestones (see Neelon and Oken)											O			P				
(3) PARENTING																				
Soothing																				
Fussing/soothing events, # feeds		Smart phones (<i>only thru Participant 289</i>)			S#	S														
		Response diaries (replaces Smart Phones)			D#	D														
Soothing/use of food to soothe		Babies Need Soothing (adapted from Stifter)	O+		O	O				O		O		O		O		O		
Infant/child feeding																				
Infant feeding practices and styles		Infant Feeding Style Questionnaire (Thompson)					O+		M											
Parental feeding styles		Caregivers Feeding Styles Questionnaire (Hughes, et al.)																	O	
Child feeding practices		Child Feeding Questionnaire (Birch)-CFQ																		O
Infant feeding mode		Babies Need Feeding (from IFPS + our items)	O+			C		C		C				P	O			C		
Responsive feeding; food as comfort		Routines (JFS and LLB)												O		O			O	
Parenting self-efficacy																				
Parenting self-efficacy		Parenting Sense of Competence (Gibaud-Wallston) (PSOC)												O						O
Domain-specific self-efficacy		Karitane Parenting Competence Scale (KPCS)	O			O					O									
(4) MATERNAL PSYCHOSOCIAL VARIABLES AND BEHAVIOR																				
Maternal psychosocial variables																				
Post-partum depression		Edinburgh Postnatal Depression Survey (Cox)			P									P						
Anxiety		State Trait Anxiety Index – T-Scale													O					
Maternal sleep																				
Maternal sleep		PROMIS Scale (items 109 and 11)						O											O	
Maternal eating behaviors																				
Restrained and disinhibited eating		Three-Factor Eating Questionnaire (Stunkard)						O												
Dietary intake		Maternal Food Frequency Questionnaire												O					M	
Maternal eating habits		Maternal Eating Behaviors (10-item survey)													O					
(5) FAMILY CONTEXT																				
+																				
Family variables																				

Constructs	Form #	MEASURES	WEEKS											YEARS												
			0	2	3-4	8	16	20	28	32	40	44	1	1.5	2	2.5	3	5								
Division of labor		Adapted Who does What (Cowan)																								
Family functioning		Family Assessment Device general functioning scale (Epstein)				O					M															
Home environment																										
Home observations (*at 3wks is only safety group)		Generated items (safety, obesity)			N*		N:		N:		N:															
Home environment		Generated items (safety, obesity) about general home environment					O																			
Safety pre-test		Generated safety items		O																						
TV viewing and family meals		Items from various sources							C					O		O		O								
Physical Activity																										
Playtime and physical activity		Items from Pate (#: parent items only) C=Active Social Play				C			C							O#		O								
Yard and Recreational Space		Items from various sources											O													
(6) BACKGROUND, DEMOGRAPHICS, AND COVARIATES																										
Screening		Screening form Chart abstraction Contact form (also used as literacy screen)	P P P																							
Randomization		Randomization form		C																						
Demographics and Health History (w/ food insecurity items)		Demographics and Health History Demographics/Health History Update	P													O		O		O		O				
Maternal employment and childcare		Maternal employment and childcare survey (our items, IFPS items) Maternal employment follow-up				C		C								O		O				O				
Implementation quality		Created by our laboratory: filled out by nurses and moms after each visit			N# M:		N# M:		N# M:		N# M:			N# M:		N# M:		N# M:								
Knowledge about infant development and behavior		Adapted KIDI, modified version (a few items will be administered as part of the mom implementation quality measure)		O	P:		P:		P:		P:													P:		
		New Vital Sign-literacy evaluation																						P		
Reason left study early		Early Exit Survey																								
															X (once, if and when we can get it)											

Key:

X = administer this measure at this time point, modality not yet determined or n/a

? = Some question as to whether to administer this measure or when

Modality codes:

M = mail (and pick up at next visit; exception – implementation quality: leave there after visit, and they mail back)

O = online

D=Diary cards

C = phone call

P = paper in person

V = video in person

N = nurse fills out

+ Short version

When:

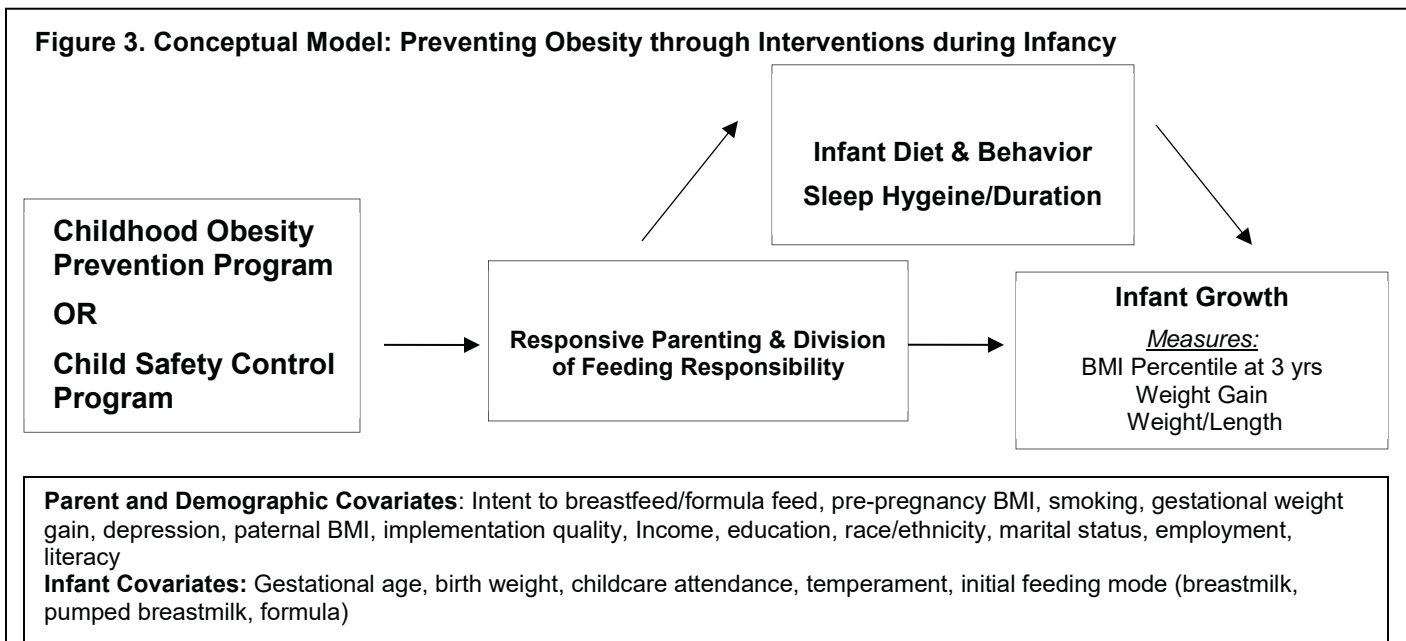
Bold vertical lines = home visits

* = Administer 2 weeks before visit; then enter their responses into the Sleep Profile tool. Email/bring feedback to next home visit.

Assessment is right AFTER home visit.

: Assessment is DURING home visit.

Rule: Assessments that are listed under a bolded (home visit) time point take place before the visit UNLESS marked with a number sign or a colon. E.g., wherever there is an M right before the black line indicating a home visit it means that the forms are mailed a few weeks before the visits and are picked up at the visit.



Growth Measures.

Infant weight and length. At birth and at each home/RC visit, infant weight and recumbent length/height will be measured by nurses trained in obtaining anthropometrics, and will be used to calculate weight-for-length, BMI percentile, and BMI Z-scores (2 and 3 years) based on percentiles for age and sex established by the WHO (for outcomes <2 years) and CDC (for outcomes 2 years and up).^{98,99} Infant and toddler weights will be obtained using Seca Model 354 scale. Recumbent lengths and standing heights will be obtained in triplicate with Shor boards.

Parent weight and height. Mothers height, pre-pregnancy weight and weight prior to delivery will be obtained from chart abstraction, if available. Should any of these data be missing from charts, it will be collected via survey. At subsequent home and CRC visits, mothers will again be weighed using calibrated scales (Adult Seca 876).. Their height will be measured once using a stadiometer or Shor board. Fathers will be weighed and measured once during the first year after the infant's birth.

Sleep and Soothe Outcomes. Infant sleep. The Brief Infant Sleep Questionnaire (BISQ)¹²⁵ will be used to assess infant sleep

Infant soothing. See smartphone section above. Soothing will also be measured using the Babies Need Soothing questionnaire, which was modified from Stifter and colleagues' Babies Basic Needs survey, designed to assess the use of food (and other soothing strategies) to soothe infant distress.¹⁰⁰

Infant and child temperament. The validated, revised Infant Behavior Questionnaire (IBQ-R)¹⁰¹ and the Child Behavior Questionnaire (CBQ-VSF)¹⁰² will assess temperament as greater infant fussiness, difficulty soothing, and lower self-regulation have been related to rapid weight gain¹⁰³ and body composition.¹⁰⁴

Energy Balance Related Outcomes.

Babies Need Feeding. The survey addresses intent to breastfeed, current feeding mode (breastmilk and/or formula), and timing and reasons for introduction of solid foods. Items were generated by our laboratory or adapted from the Infant Feeding Practices II survey.

Modified Harvard Food Frequency Survey. The Harvard Food Frequency Questionnaire (HFFQ) was developed to assess the diets of low-income women and subsequently modified as a dietary assessment tool for children and youth. It is a total of 103 items, including 84 foods and 19 questions about food habits, supplements, and services. The HFFQ is useful for health service programs as well as research. Program uses include individual client diet assessment (eligibility for programs, nutrition counseling, and change in diet over time) as well as population data (nutrition surveillance and program planning). Research uses include relation of diet to wellness or disease, program evaluation, and descriptive studies. It will be used to assess the diets

of mothers, and a modified version will be used to assess infant diets. Maternal eating habits will also be assessed using 9 items that were created in our laboratory and assess frequency of obesogenic eating behaviors like fast food and sweetened beverage intake.

Repeated exposure evaluation. Parents in the obesity intervention group will be provided with 6 jars each of 3 different pureed foods at the 16 week home visit: green beans, squash, and peas) and will receive instructions on feeding these foods. Once infants have consistent success eating infant cereal, parents in the intervention group, will be instructed to offer the infant green beans at a meal on day 1, squash at a meal on day 2, and then peas at a meal on day 3, and to repeat the order until each vegetable has been offered 6 times. Parents will be instructed to refrain from offering other new foods during this time, and to only offer one of the vegetables at each meal. The meal should be one at which the parents would be serving other solid foods such as cereal. Parents will receive a Baby's Food Log so that they can record when new foods were offered and what the child's reaction was each time the food was initially tasted. In the safety control group, participants will fill out a Baby Food Log for the first five solid foods that they introduce. For the subjects in the safety control group, the instructions on the daily food log are those that address how to fill in the log, whereas the intervention group will receive much more detailed instructions reminding them how to introduce and serve each new vegetable

Unfamiliar food tasting evaluation. To test the long-term effects of the repeated exposure protocol, all mothers will offer their infant a healthy, unfamiliar food that is developmentally appropriate at the yearly CRC visits (plain yogurt, hummus, cream of wheat). In addition, acceptance of the foods provided at the 4 month visit will be assessed. This food tasting will be video recorded, and blinded coders will assess food acceptance using the behavioral observation measure developed in our pilot study. Videos of parents feeding children at the CRC will be used to code parental feeding behaviors and infant eating, including introducing a new food. The categories of behavior include positive and negative facial expressions, vocalization, body movements, and food acceptance behaviors.^{105, 106} Foods will be pre/post-weighed to assess intake. Parents will also complete a paper and pencil measure of food neophobia at eat of these time points (Pliner six question instrument).

Physical activity. To assess parent and child physical activity, Pate and colleagues' measure of physical activity in preschool children will be used at age 1, 2, and 3 (parent items only at age 1).¹⁰⁷ To assess sedentary behavior, we will also administer items that we generated to assess television viewing (this instrument also assesses the frequency of family meals).

Responsive Parenting and Related Measures.

Maternal feeding practices and attitudes. The Infant Feeding Styles Questionnaire (IFSQ),¹⁰⁸ and the Child Feeding Questionnaire (CFQ)^{109, 110} are validated self-report instruments that assess maternal feeding beliefs and behaviors. Maternal sleep. Two items were selected from the PROMIS scale¹¹¹ and will be used to assess quality and duration of maternal sleep, as we believe that increasing infant sleep will impact maternal sleep, and in our intervention messages, we are promoting the idea that being well-rested can help you to be a better parent.

Parenting Competence. Parenting Sense of Competence Scale (PSOC) will assess maternal satisfaction and parenting self-efficacy.¹¹² The validated PSOC¹¹³ will allow us to test whether mother's feelings of self-efficacy moderate intervention effects and whether parenting self-efficacy relates to the behavioral variables of interest. In SLIMTIME, at the 1 year visit, positive relationships existed between maternal self-efficacy and effective use of soothing strategies as an alternative to feeding.

Karitane Parenting Confidence Survey. This survey will be used to assess domain-specific parenting efficacy to test whether our intervention affected mothers' parenting efficacy in domains like feeding and soothing¹¹⁴.

Maternal depression. The Edinburgh Postnatal Depression Scale (EDPS) is valid for postnatal women¹¹⁵ and those with toddlers.¹⁴⁰ It is included for similar reasons as the PSOC.

Family functioning. The general family functioning subscale of the Family Assessment Device will be administered, as the overall health and pathology of the family could be a covariate or moderator of intervention effects.¹¹⁶

Demographics and Health.

Family demographics and maternal/infant health. As in SLIMTIME, we will abstract medical charts and administer a survey during the maternity/nursery stay to collect demographic and maternal/infant health data including those on key covariates such as maternal type 2 or gestational diabetes mellitus, smoking during pregnancy, pre-pregnancy BMI, gestational weight gain, paternal BMI, self-reported race and ethnicity, and gestational age at delivery. The demographic forms will also contain two items that have been validated to screen for food insecurity¹¹⁷ Ongoing assessments will continue to follow these and include maternal employment and child enrollment at childcare facilities.

Maternal literacy. The New Vital Sign¹¹⁸ will be used to assess maternal literacy at 1 year.

Eating Inventory. The validated Eating Inventory¹¹⁹ assesses eating cognitions and human eating behaviors across cognitive restraint (conscious attempt to regulate intake), dietary disinhibition (dysregulation of eating for hunger) and hunger (perceptions), and is related to child feeding attitudes and practices.¹⁷

Paternal Involvement.

While paternal involvement in the study is not required for participation, fathers will be strongly encouraged to attend study visits so they too can receive the study interventions. We will collect data on their attendance and degree of participation at these visits as part of the process evaluation described below and in Appendix 3. Mothers will complete the Who Does What Survey¹²⁰ to assess paternal involvement in childcare through individual measurements for different child developmental stages as well as men's and women's perceptions of their relative responsibility for household tasks, and rearing of children. Our feeding surveys will also assess the proportion of feedings given by each parent, as well as other caregivers.

Safety Home Environment Surveys; Parents will be given a 2-week pre-test on their knowledge of infant safety that includes questions on food, burn/fire, bath, sleep, and car seat safety. Additional questions will be strategically worked into other surveys to provide attention to the safety intervention group. Lastly, a home environmental survey will be performed at each home visit for the safety group and starting at the 16 week visit for the parenting group to collect objective data on the infant sleep environment (e.g. crib safety), eating environment (e.g. high chair safety), bath safety (e.g. water temperature, spout cover), poison prevention (e.g. storage of medications). Also, factors that may be related to the obesity intervention such as backyard space, local playgrounds or parks, etc. will be examined.

Knowledge of infant development. A modified version of the Knowledge of Infant Development Inventory (KIDI)¹²¹ will be administered at 2 weeks to assess mothers' initial knowledge of infant development. Then, a selection of developmentally appropriate knowledge items from the KIDI will be incorporated into the implementation quality instruments described below to assess whether the information given during the home visits increased participants' knowledge.

Process Evaluation and Implementation Quality Assessment.

SLIMTIME gave us the opportunity to evaluate participants' responses to training and evaluation materials and this has led to revised and simplified training and assessment tools (see Appendices 1 and 2), suitable for those reading at a 4th grade level. In addition to our previous qualitative measures where participants reported on fidelity, satisfaction, burden, attitudes, motivation to continue in intervention, and barriers that interfere with intervention, we have added new measures to systematically assess the quality of nurses' implementation of the intervention and participants' comprehension of the intervention's main messages after the study visits. These data will provide information on overall implementation quality in our sample and will also allow implementation quality to be investigated as moderator of intervention effects, to test whether the intervention was effective regardless of implementation quality, or if it was only effective in the case of optimal implementation by nurses and/or complete comprehension by parents. We will use parallel measures to assess implementation quality of and participant fidelity with the Child Safety Program.

IV. PROTOCOL

A. RECRUITMENT

During the maternity and newborn hospital stay at HMC, study personnel will identify eligible babies born to first-time mothers through a review of hospital charts, recording that information on a screening log to track

eligible mothers vs. the mothers delivering at Hershey Medical Center. Once identified as meeting each of the inclusion criteria, informed consent will be obtained from the infant's mother with an additional optional section for paternal consent.

Because our pilot study suggested that some attrition occurs between the maternity/nursery stay and the first study home visit 2-3 weeks after delivery, we seek permission to enroll up to 350 dyads. Once 276 dyads have successfully completed the first study home visit, enrollment will cease.

B. PARTICIPANT ADHERENCE

We will systematically assess the quality of nurses' implementation of the intervention and participants' comprehension of the intervention's main messages both after the study visits. These data will provide information on overall implementation quality in our sample and will also allow implementation quality to be investigated as moderator of intervention effects, to test whether the intervention was effective regardless of implementation quality, or if it was only effective in the case of optimal implementation by nurses and/or complete comprehension by parents.

C. PARTICIPANT RETENTION

The transition to parenthood represents a major life change for first time parents. In our pilot study, the attrition rate for the one-year study was 31%. Because dropouts were mostly likely to occur either in the two weeks between enrollment and the first home visit or as a result of the burden of the study requirements later in the year after delivery, in the current project, we have addressed both issues. We plan to replace early dropouts and reduce the study burden by decreasing the survey burden. We also have eliminated diary card completion as this was a burden for participants. Nonetheless, this project seeks to have a 3 year duration of participation for each participant and we have accounted for this in our sample size calculation with a 30% dropout rate *after* randomization.

D. RISKS/BENEFITS

Risks to the subjects. The following are categories of potential risk:

Human subjects' involvement and characteristics. This study will involve a total of 276 mother-infant dyads who are patients on the maternity floor and newborn nursery at the Penn State Milton S. Hershey Medical Center (HMC). Inclusion criteria are outlined in the study methods and generally require being a healthy term newborn of a healthy, primiparous mother. Because the objective of the study is prevent obesity through very early intervention, inclusion of the vulnerable population of newborns is necessary for this research.

Sources of materials. The initial source of information regarding study eligibility will come from a brief review of the infant medical record. Because Dr. Paul has clinical responsibilities as an attending physician in the HMC newborn nursery, he and his research staff are permitted by the Institutional Review Board (IRB) to review these records and are compliant with HIPAA regulations. Only Dr. Paul and his clinical research staff will be permitted to review the medical records, and non-clinical investigators will not have access to patient charts. Numerous paper and/or electronic surveys and diaries related to infant diet, sleep, and activity will be completed by participating mothers as will maternal mental health, parenting, and relationship assessments, which will be accessible by all study investigators. Surveys will be marked with a subject ID number as opposed to identifiable information to protect participant confidentiality.

Potential risks. All participants will continue to receive their standard care from their medical providers. Though our pilot study found no increased risk of adverse events or specifically, failure to thrive, among those infants randomized to receive study interventions, it is possible that infants randomized to interventions aiming to prevent excessive weight gain could have insufficient weight gain and/or failure to thrive. As such a detailed growth monitoring plan is in place for all study participants regardless of treatment group that will combine growth assessment for study purposes combined with the frequent growth monitoring that routinely occurs as part of frequent well child visits during the first 3 years after birth. Specific details regarding this growth monitoring and communication between the investigators and primary care physicians is included in the Data Safety and Monitoring Plan.

Potential benefits of the proposed research to the subjects and others. Participants may experience improved health outcomes such as acceptance of healthy, developmentally appropriate foods, healthier weight status, and longer sleep duration during the first three years after birth. Those in the safety control arm may benefit from the home-based safety interventions. Childhood obesity may be prevented in some subjects in the study. Because mothers will have formal surveys completed for postpartum depression, there may be improved identification of this condition and subsequent increased intervention for new mothers. Pilot study revealed positive effects on parenting self-efficacy.

Importance of the knowledge to be gained. The findings of this study will provide insight into the benefits of an early intervention program to prevent childhood obesity. If successful, these procedures could easily be replicated in other non-research settings.

E. ANTICIPATED RESULTS

We hypothesize that compared with control infants; intervention infants will have lower BMI percentiles at age 3. Also, we hypothesize that control infants will gain weight more rapidly over time, adjusting for trait-stable and time-varying covariates (e.g., maternal pre-pregnancy BMI, percent of feedings that are breastmilk vs. formula, sleep duration, and feeding frequency). We also hypothesize that compared with control parents, intervention group parents will show increases in responsive parenting, alternative soothing techniques, and parenting self-efficacy. Over time, intervention infants will have a) longer sleep bouts, b) fewer daily feedings, c) a lower proportion of crying episodes followed by feeding in early infancy, and d) healthier diets from birth to age 3 compared with controls. These outcomes will be linked to healthier patterns of weight gain from birth to age 3 and lower BMI percentiles at age 3 years.

V. ADVERSE EVENTS

A. DEFINITIONS

An adverse event shall be defined as any detrimental change in the patient's condition, whether it is related to study interventions, study outcomes, or to another unrelated illness.

B. ADVERSE EVENTS UNRELATED TO STUDY INTERVENTIONS

Adverse events due to illnesses unrelated to study interventions may be grounds for withdrawal if the illness is considered significant by the study investigators or if the participant is no longer able to effectively participate in the study. A significant illness would be one that would compromise the child's ability to function normally and thrive such as the diagnosis of a malignancy, illness characterized by growth problems, disease requiring ongoing and intensive treatment, and/or one requiring repeated hospitalizations or physician visits. Subjects experiencing routine, minor, self-limited acute illnesses that typically occur during infancy and do not affect long-term growth will not be recorded, and the child will continue to participate in the study. Examples of minor illnesses include acute otitis media, bronchiolitis, upper respiratory infections, urinary tract infections, and gastroenteritis. Medications for acute, self-limited illnesses such as those stated above will not be recorded, but chronic medication use (≥ 1 month) will be recorded.

Other adverse events that could affect growth include a milk-protein allergy, other food allergies, or physician-diagnosed gastroesophageal reflux disease (GERD) requiring medication. Surgical conditions such as intestinal malrotation and pyloric stenosis also would impact infant feeding and weight gain. Therefore, these will be recorded and if the participant's physician determines that these conditions can affect long-term growth, the participant will be withdrawn from the study.

Documentation of an adverse event unrelated to study interventions that are not considered minor illnesses of childhood and those that can significantly affect growth will be recorded on an Adverse Event Report Form and will include the following information:

1. Description of the illness
2. Dates of illness
3. Treatment of illness and dates (medications, doses, and dose frequency)
4. Whether emergency treatment or hospitalization was required

C. ADVERSE EVENTS POTENTIALLY RELATED TO STUDY INTERVENTIONS

It is theoretically possible that behavioral interventions designed to prevent early life obesity could result in underfeeding by parents and insufficient growth. The NIH-funded pilot study that serves as the foundation for the current project did not find an association between study interventions and these adverse events. However, reviewers raised concerns regarding our procedures in the pilot study for monitoring growth to identify cases of potentially insufficient growth. The data presented in the grant application were from two screening criteria for potentially insufficient growth used to evaluate this outcome: weight-for-age <5th percentile at 1 year and downward crossing of two major centile lines. Both criteria were presented to the reviewers using the Centers for Disease Control and Prevention (CDC) growth charts. In the study sample of 110 infants that completed the pilot study and were available for analysis, 9 (8.2%) of infants were below the 5th percentile at age 1 year on the weight-for-age CDC growth chart and 14.6% showed downward percentile crossing although neither screening criteria had differences in incidence by study group. Further, while as hypothesized the combined interventions had a significant impact on weight-for-length percentile, there was no impact on linear growth.

Despite the reassuring data from the pilot study, the concern raised in the grant review has led to further analyses that will inform growth monitoring for the current project where we will use the same screening criteria for potentially insufficient growth:

1. Weight <5th percentile: It is well-established that formula fed and breastfed infants grow and gain weight differently. The CDC growth charts were based upon data from a predominantly formula-fed cohort of infants. These charts have been contrasted with the newer 2006 World Health Organization (WHO) growth charts that were developed using a multinational cohort of exclusively breastfed infants. Because the pilot study enrolled only mother/infant dyads intending to breastfeed, it was logical for us to chart and recalculate the incidence of growth patterns meeting our thresholds for review using the WHO growth charts. Using the CDC growth charts, 9 infants (8.2%) from the pilot study cohort, had a weight-for-age <5th percentile at age 1 year, but this number falls to only 5 (4.5%) participating infants when using the WHO charts. Notably, this is exactly what would be expected using a population-based sample where approximately 5% of all children would have weights below the 5th percentile. Further, the children below the 5th percentile on the WHO growth charts had mothers with a lower pre-pregnancy body mass index than the overall cohort (22.6 kg/m² vs. 25.1 kg/m²) indicating that they may have a genetic pre-disposition to being of lower weight.
2. Downward crossing of 2 major centile lines: In order to understand the growth trajectories of infants that met this screening criterion, we evaluated where these infants began on the growth chart at birth. We found that each child meeting this criterion had a birth weight above the 50th percentile on both the CDC and WHO growth charts. Therefore, because of the well-established phenomenon of regression to the mean, it is not surprising that some of the infants born at higher percentiles on the growth chart would move closer to the mean. Next, whereas 14.6% met this criterion using the CDC charts, only 10.0% fulfilled it using the WHO charts. Lastly, only a single participating infant met both screening criteria for potential growth concerns, and this child's PCP elected to watch this child clinically without intervention due to an overall healthy disposition. Follow-up of this child has continued through age 3 years and has demonstrated good health without any growth concerns.

While the results of these additional analyses are reassuring, in the proposed project, weight status and growth will be monitored and evaluated at frequent intervals in order to monitor the study intervention for potential adverse events. Importantly, as of September 2010, the WHO growth charts became the standard reference for children in the US through age 2, and will be used for all safety monitoring assessments through the 2 year visit after which the CDC chart will be used as is current standard of care. In the current study there are several ways a potential adverse event related to growth will be identified:

1. a diagnosis by a treating primary care provider (PCP) of insufficient growth or failure to thrive
2. weight-for-age below the 5th percentile using growth charts from the WHO

3. downward crossing of two major percentile lines between any two study visits on the WHO weight-for-age growth chart statistically evaluated as a -1.34 Z-score change in order to provide a consistent measure across subjects

For the PCP diagnosis portion of identifying potential adverse events, the study's informed consent document will include information indicating that the study team will communicate with the participant's PCP. Because all participants are infants, it is expected that they will all have a PCP or office where they receive their medical care as is typical for infants. The study team will ensure lines of communication exist with PCPs in addition to the growth monitoring performed as part of the study. To establish the lines of communication between the study team and the PCPs, upon participant enrollment PCPs will be notified that their patients are participating in a study that includes an intervention arm designed to promote healthy growth trajectories during the first 3 years after birth. A brief description of the study will be shared with them as well as a contact number to call the study team should the PCP become concerned about insufficient growth by their patient. Recognizing that growth monitoring is typically performed at all regularly scheduled primary care appointments, we will ask PCPs for their collaboration in monitoring infant growth in that we would like to be alerted to any concerns that their patient is demonstrating insufficient weight gain. In turn, the study team will report to the PCP if we determine that their patient meets one of the two screening criteria above when assessed by research nurses at study visits at 3-4weeks, 4 months, 7 months, and 1, 2, and 3 years.

During each study visit, nurses will calculate weight and length percentiles, and each child's growth chart will be plotted to allow for identification of potentially concerning growth patterns in real time. The infant's growth will be plotted on the WHO charts. The PIs will review the growth charts at least twice monthly and then contact the primary care physicians when appropriate after each individual is closely evaluated as described below.

For any individual child that meets initial screening criteria for growth concerns, numerous factors will be considered in determining whether the child's growth is problematic and/or related to study interventions. Examples of such factors include genetic potential based upon parental size, the participant's linear growth, feeding mode (breastmilk vs. formula), and interval illnesses. For predominantly breastfed infants, the WHO growth charts will be used to assess growth as these charts were designed specifically to monitor breastfed newborns and infants though the study's outcomes will be reported based upon percentiles on the CDC charts as is currently standard of care in the US. If either the primary care provider or the study investigators believe that it is possible that these growth patterns are a negative result from study participation, the child will be withdrawn from the study.

D. CRITERIA FOR DISCONTINUING SUBJECTS FROM THE STUDY

Any participant with a physician-diagnosed serious adverse event related to the study interventions will be discontinued from the study. Should insufficient weight gain be detected and the cause of this weight gain is potentially related to the study interventions as determined by the PIs after consultation with the participant's primary care provider, the participant will be discontinued from the study. Other non-minor concurrent illnesses or major changes in the family social environment also would also lead to discontinuation as determined by the investigators alone or in consultation with the PCP.

E. CRITERIA FOR STOPPING THE TRIAL

As for stopping the trial, it is exceedingly unlikely that any new, outside information will become available during this trial that would necessitate stopping the trial. There are two situations, however, that could potentially necessitate stopping the trial: 1) poor recruitment and/or retention and 2) disparate serious adverse events related to growth.

Poor recruitment and/or retention. The sample size calculation for this study determined that 276 participating mother/infant dyads were required to participate with the anticipation that there would be a 30% drop-out rate over the course of the study. The rate of expected recruitment is approximately 15-20 dyads per month. Importantly, we noted that a significant portion of the attrition in our pilot study occurred in the first 2-3 weeks after childbirth. Therefore, all subjects withdrawing from the study prior to the first study home visit (which occurs 3-4weeks after delivery) will not be counted as among the 276 participating mother/infant dyads.

Should attrition exceed 20% at any point during the study, the required sample size will be reviewed along with retention strategies in collaboration with the study statistician. All attempts will be made to salvage the study and achieve the sample size required for analysis. The NIH program officer will be involved in these discussions so that in the event of extreme difficulties, a decision regarding the stoppage of the trial can be made.

Another recruitment issue relates to minority group participation. We expect to enroll at least 30% of newborns that are classified by their parents as non-White, Hispanic, or bi-racial. Because our pilot study enrolled only 24% minorities, this will need to be reviewed closely as the trial progresses and addressed accordingly should poor minority enrollment occur.

Disparate serious adverse events related to growth. As described in detail above, we will closely monitor rates of insufficient growth for individual infants and by study treatment group. In addition to individual level monitoring, the study statistician will analyze rates of insufficient growth by treatment group on a quarterly basis. If there are significantly more children with inadequate growth in the intervention group at any point, these data will be presented to the HMC IRB, the study's DSMB, and NIH/NIDDK so that a decision can be jointly made as to whether the trial needs to be suspended.

We acknowledge that there are other situations that could occur that might warrant stopping the trial. We have a section on the study's safety report entitled 'Other situations that have occurred since the last safety report that warrant discussion' to allow for communication of concerns to the study PIs, statistician, and the safety officer.

F. DROPOUT STATUS

Any family who withdraws consent to participate will be assigned dropout status.

VI. SAFETY MONITORING

A. PROTECTION OF HUMAN SUBJECTS

Recruitment and Informed Consent.

Informed consent will be obtained using forms and protocols approved by at the HMC IRB. The investigation will be initiated solely on the maternity floor at this center. Study procedures and the risks of normal newborn life will be explained and written informed consent will be obtained from mothers of the newborns in accordance with the requirements of the IRB. Those obtaining informed consent will include the investigators, nurse study coordinators, and project manager.

Protection against risk.

As the current study is not blinded, the investigators will monitor for an increase in morbidity in each of the treatment groups. Drs. Birch and Paul will assume all responsibility for addressing adverse events. Because the study will include children, all IRB requirements for the protection of children will be fulfilled.

Data and safety monitoring plan.

The data and safety monitoring plan (DSMP) for this trial focuses on close monitoring of growth by the principal investigators (PIs), in conjunction with a safety officer and data and safety monitoring board (DSMB), along with prompt reporting of excessive adverse events and any serious adverse events to the NIH/NIDDK and to the Institutional Review Board (IRB) at the Penn State Milton S. Hershey Medical Center (HMC). Because behavioral interventions aimed at preventing obesity could theoretically result in insufficient weight gain by study participants, individual participant growth will be closely monitored by the investigators in conjunction with the participants' primary care providers as described below in detail. The adverse event form meets the goals of this plan. Safety reports will be sent to the study statistician, the PIs, and the safety officer quarterly. At least once per year, the DSMB will convene to review study progress and issues related to the safety of the study interventions. Meetings may be more frequently than once per year if the need for such meetings becomes apparent to the safety officer, PIs, IRB, or NIH/NIDDK. The project manager will be

responsible for assembling the data and producing these reports in conjunction with the study statistical team, as well as assuring that all parties obtain copies of these reports.

ClinicalTrials.gov Requirements.

This study will be registered at clinicaltrials.gov prior to the enrollment of the first participant.

B. INCLUSION OF WOMEN AND MINORITIES

Half of the newborn patients enrolled are expected to be female, and maternal co-participation is a study requirement. Approximately 30-40% of the participants enrolled in the prospective trial are expected to be minorities as we will attempt to over-sample for minority families from our center. Several minority groups are relatively under-represented in this recruitment plan because of the geographic location of the study and the patient demographics of the local population. Attempts to recruit minority patients will be planned for subsequent investigations that will be specifically powered to evaluate the effect of ethnicity and race of the study outcomes.

B1. Infant Enrollment

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	17	17	34
Not Hispanic or Latino	121	121	242
Ethnic Category: Total of All Subjects *	138	138	276
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	16	16	32
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	22	22	44
White	100	100	200
Racial Categories: Total of All Subjects *	138	138	276

B2. Maternal Enrollment*

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	13	13	26
Not Hispanic or Latino	125	125	250
Ethnic Category: Total of All Subjects *	138	138	276
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	13	13	26
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	17	17	34
White	108	108	216

Racial Categories: Total of All Subjects *	138	138	276
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*While maternal participation is a requirement for the study, paternal involvement will be voluntary. Further, the absence of an identified father to participate will not be a study exclusion. Thus, no paternal enrollment table is included.

C. INCLUSION OF CHILDREN

Improving outcomes for newborns and infants is the main objective of this trial and therefore this population will be primarily studied. Only those born at term (≥ 37 weeks gestation) will be included, and those discharged from the neonatal intensive care unit will not be included because of an increased likelihood of neonatal morbidity in that population.

PI Paul and the pediatric clinical research nurses involved in the study have extensive clinical experience providing medical care for newborns and children of all ages.

VII. COST, LIABILITY, AND PAYMENT

All home visits and tests will be performed without cost to the participating subjects. Since this trial overlaps a period of frequent physician visits especially for the infant, liability for patient care costs incurred by patients during the course of the trial will be borne by the family or their insurer. Details of the NIH policies concerning this issue can be found in NIH Documents #5305 and 6352-2, Research Patient Care Costs Supported Agreements.

Given the significant time and effort required for participating families in this proposal, a participant stipend is warranted. Participants will receive \$375-\$415 (depending on diary card completion incentives) for participation plus numerous visit specific gifts (detailed within each study visit) which have a retail value of approximately \$100.

Participants enrolling into the long term follow-up will receive \$100 for each of the follow-up visits that they attend.

VIII. STATISTICAL DESIGN AND ANALYSIS

A. DATA RECORDING AND DATA MANAGEMENT

The staff in the Center for Childhood Obesity Research (University Park, PSU) will coordinate the creation of all paper data collection forms that will be distributed to research subjects and subsequently returned to the Center.

Whenever possible, the data entry system will be designed to prevent illogical values from being entered into the data files. In addition, error checks will be programmed to identify potential data errors, such as missing values, out-of-range values or illogical, inconsistent values within or across data collection forms, duplicate forms and missing forms.

Study research staff will record participant data on the data collection forms, review the forms for completeness and legibility, make a copy of the forms for their files and forward the original data collection forms to the Center for data entry and processing. The study research staff may be required to send copies of laboratory reports in order to verify the accurate transcription of the laboratory values from the reports to the data collection forms. The research staff will be required to send their completed forms on a weekly basis to the Center in order that the data can be entered and processed in a timely fashion. The Center staff will date stamp all forms received from the study research staff. The forms will be inspected visually for problems that cannot be detected electronically. Center staff will perform independent, double-data entry on each data collection form. The data will be stored on a College of Health and Human Development server, maintained by the College Information Systems and Services (ISS) staff. All paper forms will be stored in locked filing cabinets in the Center for Childhood Obesity Research.

The staff at the Center for Childhood Obesity Research will also coordinate the creation of online data collection tools and the management of the corresponding data. Qualtrics software will be used to upload selected surveys, and links to the surveys will be sent to participants via email, prompting them to complete the surveys. All online surveys at a given time point will be merged together, so that they appear as one survey to

the participant. Participants will be prompted to enter an identification number that allows their responses to remain anonymous. The participant may close the program and resume answering the rest of the questions at a later time. The participant will be given one week to complete a set of online surveys after receiving the link via email. After that, online data will be downloaded onto the College of Health and Human Development Server.

The security of the project data will be maintained through the ISS network hardware and user authentication (usernames and passwords). The project directory will be restricted to only staff in the Center working on the project. Back-ups of the project data files will occur every night, with user data backed-up incrementally Monday through Thursday and complete back-ups every Friday. Archival back-ups, stored indefinitely, are cut on the last weekend of every month. All back-up data are stored in a secure off-site location. The number and variety of back-ups ensure ample data redundancy and protection. In addition, Center staff will never receive any identifying personal information on the research participants. Each participant will be assigned a unique subject identification number. Only the study research staff will have the log linking this number with the participant's personal information.

B. RANDOMIZATION AND STRATIFICATION BY “AT RISK” STATUS

We will randomize participants to either the obesity prevention program or the child safety control group, stratifying on birth weight for gestational age (<50th percentile vs. ≥50th percentile)⁹⁶ and intended feeding mode (breastmilk or formula feeding) 10-14 days after delivery. Mothers will be contacted by phone to confirm the date and time for the first study home visit, and asked about intended feeding mode (see Appendix 2 for questions). The second stratification variable, birth weight for gestational age, was selected given our experience in the SLIMTIME study as described in the innovation section. Our randomization scheme will use permuted blocks, as stratified randomization without blocking would not guarantee balance beyond that of simple randomization.

Another statistician unaffiliated with the study will prepare the final randomization list. The randomization will be administered using a secure Microsoft Excel application. Mother-infant dyads will be entered consecutively and stratified as described above. When a mother-infant dyad is eligible for the trial, informed consent has been obtained, and they complete the brief questionnaire 10-14 days after delivery, a unique identification number will be assigned to the mother-infant dyad. After entering the secure Microsoft Excel application and entering the birth weight for gestational age quartile and intended feeding mode information, a randomization assignment will be returned for the mother-infant dyad.

C. SAMPLE SIZE, POWER CALCULATIONS, AND STATISTICAL ANALYSIS

Sample Size.

The primary outcome for this study will be BMI percentile at 3 years. As we are interested in the effect of the intervention program on formula fed and breastfed infants, prior to the initial home visit at 3-4 weeks, participants will be randomized to one of 2 treatment arms (control/intervention) based on mother's intended feeding mode (breast milk/formula) and birth weight for gestational age quartile, to provide a balanced distribution based on infant size and feeding mode. A 0.67 difference in BMI Z-scores represents the distance between major centile lines displayed on infant growth charts. This difference is clinically meaningful, and upward percentile crossing of this magnitude has been the operational definition frequently used for rapid weight gain.¹²² For this reason we have powered the study to detect a 0.67 difference in BMI Z-scores between the safety control and the obesity intervention groups within each of the feeding modes (intent-to-breast feed or formula feed). To detect this difference with 90% power and a 5% Type 1 error rate, 276 participants are required (69 in each of 2 arms for each of 2 feeding modes). This includes an anticipated 30% loss to dropout.

Statistical Analyses.

Statistical support related to behavioral outcomes will be led by Eric Loken, PhD from Penn State's Methodology Consulting Center at University Park, who has expertise in the analysis of longitudinal data, including analyzing complex models to test mediation hypotheses. Dr. Loken has also applied advanced statistical models to the study of infant temperament as well as studies of child weight gain. Statistical support related to the genetic analyses will be led by Francesca Chiaromonte, PhD from Penn State's Department of Statistics. Dr. Chiaromonte has extensive expertise in the statistical analysis of genomic data. Michele Marini,

MS, the data analyst for the Center for Childhood Obesity Research, will provide statistical support for conducting the analyses. All primary statistical analyses will invoke the intent-to-treat paradigm, analyzing data based on randomized assignment.

Aim 1 Analyses. For BMI percentile at age 3 years, a repeated measures analysis, incorporating BMI percentile at age 2 years, will be used. A linear mixed-effects model, a generalization of regression, will be used to examine the relationship between BMI and intervention group within each of the feeding modes.¹²³ The linear mixed-effects model will include intervention group, feeding mode, their interaction, and initial birth weight adjusted for gestational age. Contrasts will be constructed to assess within feeding mode intervention effects. BMI is generally assumed to be the standard growth measure for assessing obesity-risk in children age 2 years and older, but there is not one universally accepted measure for children younger than age 2 years. We will consider growth models that examine weight for length z-scores, percentiles and weight for age z-scores for children from birth to age 1 year. A repeated measures mixed-effects model will be used to evaluate intervention effects on growth trajectories of these measures from birth to age 1 year. The model will include intervention group, feeding mode, time of growth measure, and all interaction effects, as well as initial birth weight adjusted for gestational age. Time-varying covariates to be examined include percent of feedings that are breastmilk, infant sleep and feed durations, as well as infant temperament scores (e.g. difficultness). Contrasts will be constructed to address within feeding mode intervention comparisons.

Additionally, because previous studies have demonstrated that rapid weight gain during the first months after birth is a predictor of childhood and adult obesity, rapid weight gain will also be assessed.^{48, 124-130} Weight gain Z-scores will be calculated for each measurement during the first year of life, and rapid weight gain scores will be calculated as described by Ong and Loos,¹²² as an increase in weight-for-age Z-score $\geq +0.67$. This increase corresponds to crossing adjacent major centile lines on the standard CDC growth charts. The primary analysis for rapid weight gain scores will be a repeated measures analysis, assessing the intervention effects on rapid weight gain scores up to one year, and the moderating effect of initial intent to breast or formula feed. The analysis will use generalized estimating equations (GEE) with a logit link, an extension of logistic regression that allows for repeated measurements on binary data, and will include maternal pre-pregnancy BMI and birth weight adjusted for gestational age as covariates. This analysis will be extended to identify factors contributing to absence of rapid weight gain. We will also examine the relationships between these early rapid weight gain scores and BMI at age 3 years.

Aim 2 Analyses. Continuous secondary parental and infant behavior outcomes assessed at multiple time points, including sleep duration, number of feeds, fussing and crying frequency, infant soothing methods, feeding practices and attitudes and parenting sense of competence, will be analyzed using appropriate mixed-models, path modeling, and time series methodologies. These methods will allow us to create summary variables to identify patterns of change. These patterns will be predicted by the effects of the model, namely intervention, initial feeding mode intent, and their interaction. These secondary outcomes will be also be included as moderators in analyses of primary growth outcomes. The moderator hypothesis represents the interaction between these variables and the main effects of the model while including each of these variables as additional main effects. We will also test whether these secondary variables function as mediators; that is, are these variables predicted by the effects of the model and, in turn, predict the primary outcomes. Using path modeling to test these models, we will be able to determine the direction of influence among the variables in the model.

Repeated measures analysis will be used to assess the effect of the intervention on acceptance of vegetables. The introduction and acceptance of healthy but unfamiliar foods will be analyzed by a chi-square test to determine difference in acceptance rate by intervention group. Repeated measures analysis using generalized estimating equations with a log-link and Poisson distribution will be used to assess the relationship between number of nocturnal and daily feeds during infancy and intervention.¹³¹ For secondary outcomes that are a result of survey instruments, composite variables will be created based on factor analysis, and repeated measures analysis of variance will be used to assess the effects of the intervention on the changes over time of these outcomes.

All models considered allow inclusion of relevant covariates. Covariates to be considered in refining the analyses can be grouped into categories that include maternal and paternal biologic variables, socioeconomic and demographic variables, infant behavioral variables, and parental behavioral variables. Examples are included in the conceptual model shown in Figure 2.

Genetic analyses. To interrogate the effect of genetics on childhood obesity, SNP typing will occur, which will be followed by the construction of the Genetic Risk Score (GRS) as done by Belsky et al. described in the innovation section of this proposal.¹⁵⁸ In that study, the GRS for childhood obesity was based on 32 SNPs associated with adult BMI from two large GWAS studies.^{159,160} In addition to typing these 32 SNPs, we will also type 56 SNPs identified to be associated with obesity in more recent GWASs⁹⁴ for a combined 88 SNPs. We anticipate that during the initial years of the proposed funding period, additional SNPs associated with obesity will be discovered by ongoing GWASs in other labs. Therefore, conservatively, we envision typing 120 SNPs. Notably, many of the 88 SNPs already discovered to be associated with obesity are located within or in the vicinity of genes that act on regulation of food intake, i.e. determine appetite.⁶⁶ This will require processing of 276 trios of parents and children. SNPs will be typed with the commercially available array (iPLEX/Taqman, Sequenom). A subset of SNPs will be validated with allele-specific PCR.¹³² We will thus construct the GRS using the same method as in Belsky et al., but based on 120 SNPs: we will group the SNPs into linkage disequilibrium (LD) blocks using a linkage threshold of $R^2 \geq 0.95$ and data from the International HapMap Consortium's CEU sample.¹⁶² LD blocks including SNPs associated with obesity-related phenotypes will be retained, and one tag SNP from each such LD block will be included in the GRS. For these SNPs, the obesity-associated alleles will be weighted by the effect size reported in the corresponding GWAS.⁹⁴ The GRS will thus be the sum across these SNPs of the weighted counts of obesity-associated alleles as done by Peterson et al. and Li et al.^{133, 134}

To investigate whether genetics modulates the dependence of weight-related outcomes on the INSIGHT intervention program, we will consider the 276 first-born children of the families enrolled in the study and develop multiple regression models for WLZ (at birth and age 1 year) and BMI Z-score (at age 2 and 3 years), that comprise as potential predictors a dichotomous variable for intervention, the GRS, a family history score computed from parental BMIs as in Belsky et al.,⁹³ and a number of covariates (some of which are “environmental” and some of which may be “mediating” the effect of genetics on WLZ and BMI z-score. Possible covariates could include birth weight adjusted for gestational age, infant sex, timing of introduction of solids, family income, or maternal education. This analysis will require appropriate data preprocessing (outlier detection and removal, variable transformation and normalization) followed by model selection – during which we will assess the explanatory power of first and second order terms, including interactions, in the predictors. In particular, this will allow us to evaluate how GRS modulates the effects of the intervention (gene-intervention interactions), as well as the effects of environmental covariates (gene-environment interactions). Notably, the use of a multiple regression setting allows us to evaluate the role of each predictor and predictor interaction in the context of all others considered. Nevertheless, if some of the covariates utilized are likely to mediate the effects of genetics on WLZ and BMI z-score we may implement the mediation analysis suggested in Belsky et al.¹³⁵ to carefully separate direct and indirect effects of GRS on the response.

One of the “environmental” covariates for genetics is the composition of the microbiome. We will be determining oral and gut microbiome compositions of the children involved in this study using 16s rDNA sequencing (targeting the variable 3 and variable 4 region) on the Illumina MiSeq platform. Sequence reads will be compared to the GreenGenes Database for taxonomic designation. Alternatively, Qiime (Quantitative Insights Into Microbial Ecology) will be used to process raw sequencing reads to determine taxonomic representation. Using this metagenomic technique and workflow we will be able to determine the composition of the microbial community down to the genus level. Using regression analysis we can estimate if there is a relationship among the GRS (as well as other covariates mentioned previously) and the microbiome.

In addition to the development of the regression models described above, we will use availability of the parental GRSs to investigate the interdependence structure of the genetic risk score for obesity of parents and children. For this purpose, we will use principal components analysis after appropriate preprocessing of the scores; the triads from the current INSIGHT study will give us 276 points in 3D. In the analysis we expect to uncover independence of the parental scores from one another, but a strong dependence of the offspring scores on parental scores, and the second will allow us to also investigate the degree of dependence between offspring scores. Notably, “independent” GRSs from the parental cohort will also be useful in validating the effectiveness of the offspring GRSs as a proxy for genetic risk. Scores of individual SNPs for fathers and their children will also be utilized to validate paternity.

Similar analyses relating parent-reported as well as observer-rated temperament and appetite to infant weight status will be performed as we have done previously,¹³⁶ this time including the effects of birth order as it impacts those relationships.

E. MISSING DATA

Every attempt will be made to determine the full set of outcome values for each infant. Regardless of this effort, we expect there will be some missing data due to dropouts and missed visits. Most analytic methods are robust to small amounts of missing data. And, where possible, we will consider analytic methods that assume the data are missing at random (MAR), often referred to as ignorable missingness. If the number of cases with missing data should exceed 5%, we will explore approaches that allow for non-ignorable missingness. These methods included selection models and pattern-mixture models.¹³⁷

F. MULTIPLE TESTING

As our planned analyses are based upon defined hypotheses, we have not included corrections for multiple testing. While the issue of p-value adjustment for multiple testing has long been a topic of statistical debate, it generally is accepted that p-values should not be the sole criterion for assessing significance of relations. Conclusions will be based on the preponderance of scientific evidence related to each hypothesis, considering point estimates and confidence intervals, as well as statistical significance. Nonetheless, findings with marginal statistical significance (p-values in the range 0.01 to 0.05) will be interpreted cautiously, taking into consideration whether multiple testing could have contributed to a type I error.

INSIGHT Study Second-Born

I. PRINCIPAL HYPOTHESES TO BE TESTED

- A. Parental responsiveness and responsive feeding will be improved among parents with their second child, and this will be associated with lower weight-for-length z-score at age 1 year and lower BMI throughout childhood.
- B. After collection of biological specimens from siblings and parents, known candidate genes associated with obesity-risk, appetite, and temperament will be associated with weight-for-length z-score at age 1 year and BMI beginning at age 2 years adjusting for trait-stable and time-varying covariates (e.g., sex, birth weight). Similar associations are expected for parent-reported and laboratory-observed appetite and temperament.
- C. We will test a hypothesis that weight status at the age of 1 year can be predicted from the microbiome diversity at this age or even from the microbiome diversity at an earlier age.
- D. We will perform statistical analyses to test the hypotheses (1) that the oral microbiome is a predictor of childhood obesity, (2) that there is an association between the developing oral and gut microbiomes, and (3) that they interact in affecting childhood obesity.
- E. We will test the hypothesis that epigenetic regulation of the gastrointestinal tract by miRNA is influenced by (1) the microbiome, (2) maternal breast milk factors (including coding and noncoding nucleic acids such as miRNA and metabolic components such as sodium, zinc, and potassium) and (3) childhood diet and weight.

II. BACKGROUND AND RATIONALE

A. Differences in Obesity-Related Parenting During Infancy Between First and Second Siblings Remains Largely Unexplored

Beginning with Sir Francis Galton's *English Men of Science* published in 1874, researchers have examined relationships between birth order and outcomes related to health, achievement, behavior, and intelligence.¹³⁸⁻¹⁴⁰ Yet despite observations over the past 140 years regarding birth order, few have prospectively evaluated differences in parenting between successive siblings as a source of the disparities between first- and second-born children. What is established is that while mothers spend more time interacting with their first-born during infancy,^{141, 142} they are more likely to use restrictive or coercive parenting strategies as first described by Lasko in 1954.¹⁴³ Hilton later found that first-time mothers are significantly more interfering, extreme in response, and inconsistent with parenting response than mothers with their later born children.¹⁴⁴ *These findings suggest that parents are less likely to use responsive parenting practices with their first-borns than with later-borns.*

A recent review of studies examining the effect of birth order on parenting by Kaley et al.,¹⁴⁵ reports that evidence is scant and that to date, no studies have prospectively examined differences in parenting of first- and second-born siblings within the same family. However, the review identified several potentially modifiable postnatal factors affecting infant obesity risk: sleep duration, feeding style, and parental regulation of distress.¹⁴⁶ Evidence for differences in these factors for first-borns and non-first-borns is discussed below:

Sleep. In the only study to date examining birth order and sleep duration, Kaley et al. studied a small sample of 74 infants aged 4-10 weeks;¹⁴⁵ first-born infants had shorter nap and total sleep durations. Given this paucity of data, at our request, INSIGHT's pediatric sleep expert consultant, Jodi Mindell, PhD (see letter of support) performed an analysis comparing first-born children's sleep with non-first-borns from her cohort of over 29,000 infants and toddlers aged 0-3 years.¹⁴⁷ The data show that while total daily sleep time was similar between first and non-first-borns (12.45 vs. 12.65 hours), as were mean number of night wakings per night (1.55 vs. 1.50), parents of first-borns characterized their child as having a sleep problem 49% of the time compared with 31% of those describing non-first-borns ($p < .001$). *This suggests major differences between*

first-time parents and more experienced parents in what constitutes an infant sleep problem, and possible differences in parenting behavior.

Feeding Style. A series of studies from the early 1970s compared feeding behaviors of first-born and later-born infants, showing marked differences in maternal feeding behavior between primiparous and multiparous mothers beginning immediately after childbirth.¹⁴⁸⁻¹⁵⁰ Thoman et al. found that the total time the mother spent using the bottle in both feeding and non-feeding activities was significantly longer for mothers of first-borns than later-borns, even though their infants consumed less formula. These authors then compared mother-infant feedings with nurse-infant feedings of the same infants and found that when the nurses did the feedings there were no differences due to birth order in terms of feeding frequency, duration of feed, and volume of formula consumed but when the mothers did the feeding, birth-order differences were apparent. The authors concluded that, "...infants provide clues in the feeding situation to which the nurses respond. Inexperienced, primiparous mothers apparently respond less to cues of the infants ..."¹⁴⁹ *This suggests maternal differences in feeding style between primiparous and multiparous mothers with less responsiveness to cues with first-borns and more responsiveness with later-born children.* The already referenced study by Kaley et al. reported similar findings with older infants. Primiparous mothers were found to feed their infants significantly more frequently and for longer duration than multiparous mothers,¹⁴⁵ again suggesting that mother-infant feeding interactions are affected by birth order.

Among school-aged children, Webber et al. demonstrated that mothers attribute differences in appetite and diet quality between siblings to genetic differences between their own children, but describe the cause of poor diets by other children as a lack of parental control.¹⁵¹ In fact, while the mothers perceived themselves as feeding their children similarly, observations of their interactions revealed differing feeding practices in response to each child's appetite and preferences. This finding was further developed by Farrow et al., who found that parents use restrictive or pressuring feeding practices differentially between siblings based upon each child's eating behavior, using restrictive practices for those who are fussy and desire to drink more, and pressure for those who eat slowly, enjoy food less, are less responsive to food, and are more responsive to internal satiety cues.¹⁵²

Parental Regulation of Distress. A portion of our INSIGHT intervention instructs parents not to use food to soothe fussiness or crying in the absence of hunger cues, and this is an important focus for obesity prevention in our view. Regarding birth order, two studies have shown no relationship between birth order and daily infant crying.^{145, 153} *However, parity has been associated with significant differences in how crying is attended to.*¹⁵⁴

B. SPECIFIC AIMS

Specific Aim 1: To characterize how differences in obesity-related parenting are associated with infant weight status at age 1 year of first- and second-born siblings. The detailed, prospective data collection conducted with first-born INSIGHT participants offers the novel opportunity to prospectively assess differences in sleep, feeding, feeding style, and parental regulation of infant distress for their second-born siblings when analyzed both within and between families. We hypothesize that parental responsiveness and responsive feeding will be improved among parents with their second child, and this will be associated with lower weight-for-length z-score at age 1 year. Potential interactions with INSIGHT intervention group will be explored. Subsequent BMI comparisons will similarly be made at later ages throughout childhood.

Specific Aim 2: To explore how genetic susceptibility to obesity and observed differences in appetite and temperament moderate associations between responsive parenting and weight status at age 1 year among first- and second-born siblings. After collection of biological specimens from siblings and parents, known candidate genes associated with obesity-risk, appetite, and temperament will be associated with weight-for-length z-score at age 1 year adjusting for trait-stable and time-varying covariates (e.g., sex, birth weight). Similar associations are expected for parent-reported and laboratory-observed appetite and temperament. Potential interactions with INSIGHT intervention group will be explored. Subsequent BMI comparisons will similarly be made at later ages throughout childhood.

III. PROTOCOL OVERVIEW

Second-born children will be invited to participate to prospectively evaluate parenting similarities and differences that occur for two consecutive siblings, comparing parenting practices and child outcomes for sibling pairs at the same time points in development. Nurses will meet parents and their second-born infants at 3 home visits in the first year after birth followed by clinical research center visits at age 1. Included in the informed consent will be permission to access the infant's medical record, to review the outpatient chart, and to converse with the infant's primary care physician (PCP). Information and data regarding parental participation in other parenting, breastfeeding, or family programs will be collected. Study personnel from the Hershey campus will be responsible for all face-to-face visits as well as the data collected at those visits. Study personnel from the University Park campus will be responsible for all phone interviews and web-based data collection.

Second-born siblings will also be asked to participate in a long-term follow-up of their child until their 18th birthday. Nurses will meet with participant at ages 2, 3, 5, 10, 14, 17 years old, measuring height and weight and collecting data about diet, activities, and family life.

A. STUDY GROUPS AND SUBJECTS

Beginning with a cohort of 276 INSIGHT families, even with a possible drop-out rate of 15% from INSIGHT among first-born children (bringing the cohort to 234 families), if 50-60% have a second child (based upon Pennsylvania fertility data) during the proposed enrollment period (2013-2019) as expected, we should be able to follow second-born siblings (estimated to be N=125-150) for at least 1 year given the investment families have already made in their participation in this research as evidenced by our low attrition rate to date.

B. INCLUSION CRITERIA*

Eligible mother-infant dyads for this trial will meet the following criteria:

- 1) full-term infant (≥ 36 0/7 weeks gestational age) without significant morbidity
- 2) singleton infant
- 3) nursery/NICU/maternity stay of 7 days or less

C. EXCLUSION CRITERIA

(not explicitly obvious from the inclusion criteria)

- 1) prenatal ultrasound presence of intrauterine growth retardation (IUGR)
- 2) infant birth weight <2250 grams
- 3) presence of a congenital anomaly or neonatal condition that significantly affects a newborn's feeding (e.g. cleft lip, cleft palate, metabolic disease)
- 4) any major maternal morbidities and/or pre-existing condition that would affect postpartum care or her ability to care for her newborn such as cancer, multiple sclerosis, lupus, etc.
- 5) plan for newborn to be adopted
- 6) plan to move from Central Pennsylvania within 1 year

D. STUDY VISITS

Similar to INSIGHT first-borns, nurse visits for enrolled second-born siblings will occur at 3 home visits and 1 CRC visit within the first year. Anthropometrics will be measured at each in-person visit, and as specified below, certain other objective measurements will occur (e.g., home observation data and a blood sample (500 μ L) will be collected one time during from the second-born child (by heel or finger stick). Maternal weight will also be assessed at the 28 week visit, while paternal weight will be assessed at any single study visit between birth and the second-born child's 1 year visit. Pending funding, INSIGHT siblings would also be followed until their 18th birthday with a nurse visit to collect anthropometrics 2, 3, 5, 10, 14, 17 years old.

1) Enrollment Visit – Pregnancy/Maternity/Nursery Hospital Stay

- a. Mother's will be asked to consent for the sibling portion of the INSIGHT study either prior to delivery, during their maternity hospital stay, or as soon as study staff are made aware of delivery (within 2 week of delivery)
- b. Newborn and maternal medical chart review in to determine eligibility based on inclusion and exclusion criteria (Screening form)
- c. Obtain maternal informed consent for mother-baby dyad
- d. Obtain paternal informed consent for his own participation (height and weight measurement, blood sampling)
 - i. If father has already participated in INSIGHT with the first-born, only his weight will be measured.
 - ii. If the father for the second-born is different than his consent will be sought to collect height, weight, and the blood sample.
- e. Complete enrollment data collection forms
 - i. Chart abstraction form (coordinator completed)
 - ii. Demographics and Health History form, (coordinator interview with mother)
 - iii. Contact form (updated)
- f. Measure weight and height of mothers and fathers (if father not present, will attempt to measure at subsequent study visits)
- g. Collect blood samples from fathers-if not obtained for first-born or if a different father (if father not present, will attempt to collect at subsequent study visits).
- h. Collect buccal swabs from mother and baby, if mother consents to this portion of the study.
- i. Collect skin swab from mother, if mother consents to this portion of the study.
- j. Collect Breast Milk sample if mother agrees to this portion of the study.
- k. Collect meconium sample from baby, if mother consents to this portion of the study.
- l. Collect cord blood (collected per standard of care at time of delivery), if mother consents to this portion of the study.
- m. Collect placenta samples (collected via pathology), if the mother consents to this portion of the study

2) Home/Clinic Visit 1 (18-24 days):

- a. Infant weight and length measured
- b. Plot child's weight and length on growth chart
- c. Administer Edinburgh Postnatal Depression Survey to mothers
- d. Complete home environment assessment and data collection
- e. Collect buccal swabs from baby, if mother consents to this portion of the study.
- f. Collect vaginal and rectal swabs from mother (these are self-administered with the swab kit and instructions sent prior to the visit), if mother consents to this portion of the study.
- g. Collect baby's stool sample, if mother consents to this portion of the study.
- h. Collect Breast Milk samples if mother agrees to this portion of the study.

3) Home Visit 2 (16 weeks)

- a. Infant weight and length measured
- b. Plot child's weight and length on growth chart
- c. Complete home environment assessment and data collection

4) Home Visit 3 (28 weeks)

- a. Infant weight and length measured
- b. Maternal weight measured
- c. Plot child's weight and length on growth chart
- d. Complete data collection
- e. Collect buccal swabs from baby, if mother consents to this portion of the study.
- f. Collect baby's stool sample, if mother consents to this portion of the study

5) Research Center Visit 1 (1 Year)

- a. Child weight and length measured
- b. Collect blood sample from child (finger or heel stick) if not collected at a previous visit
- c. Collect blood sample from second child's father (if not collected at a previous visit)
- d. Complete data collection
- e. Collect buccal swabs from baby, if mother consents to this portion of the study.
- f. Collect baby's stool sample, if mother consents to this portion of the study.
- g. Collect blood sample from child (finger or heel stick)
- h. Toy Removal Task-Length of task: 6 minutes
 - i. Child is seated in high chair and presented with an attractive toy with a detachable part. Mother is instructed to take toy away from child and place it on a chair in child's view but out of reach and engage in a conversation with research coordinator. Mother then returns toy without detachable part and continues conversation with research coordinator. To end the task, mother returns both parts of toy to child and continues to engage with the research coordinator.
- i. Self-feed Observation-Length of task: 2 minutes
 - i. Research coordinator will observe child attempt to self-feed with a spoon (mom chooses desirable, familiar food) and to drink from a cup without a lid (water).
- j. Novel Feeding Task-Length of task: 10 minutes
 - i. Mother will select three novel foods for child to try. Research coordinator will instruct the mother to feed 2 tastes of each food to the child. Child reactions will be recorded.

6) Home/clinic visits (2, 3, 5, 10,14, 17 years old):

- a. *All Participants*
 - i. Infant weight and length measured
 - ii. Plot child's weight and length on growth chart
 - iii. Collect information on child and mother's feeding and behaviors
 - iv. Collect buccal swabs from child, if parent consents
 - v. Collect child's stool sample. Parents are asked to bring/collect a sample (using the provided tube and biohazard bag) frozen within 48 hours of study visit.
 - vi. Data collection

E. OUTCOME MEASURES AND THE RATIONALE FOR CHOOSING THEM

Per current CDC and AAP recommendations, all growth related outcomes will use the World Health Organization (WHO) growth references for child outcomes <2 years and the CDC growth reference for child outcomes 2 years and older.⁹⁸

Primary Outcome: Numerous outcome measures will be assessed over the study period with the primary outcome of weight-for-length at 1 year comparisons between first and second-born siblings.

Secondary Outcomes: We will evaluate outcomes related to weight status in numerous ways and these will serve as the secondary outcomes for the study. They include:

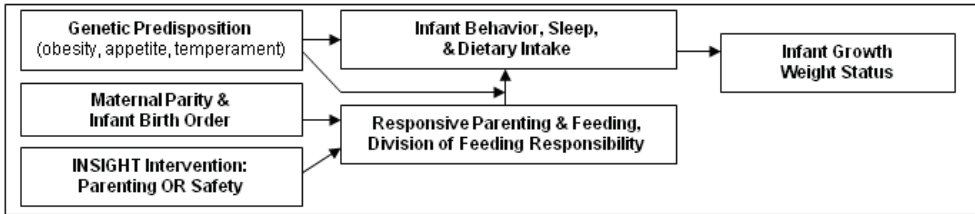
- Weight-for-length percentile at several intervals in the first 12 months after birth
- BMI percentile at age 2 years
- Proportion of infants with BMI \geq 85th and 95th percentiles at ages 2 and 3 years
- Proportion of infants with accelerated weight gain (0.67 difference in BMI Z-scores) between numerous study intervals (e.g. birth to 4 months, birth to 1 year, birth to 3 years, 1 year to 3 years, etc.)

Other Outcome Measures to be Studied:

- Sleep duration during first year after birth
- Number of daily feedings
- Proportion of crying episodes followed by feeding
- Use of alternative soothing strategies

- Timing of introduction of solid foods
- Breastfeeding duration (for those who choose to breastfeed)
- Dietary content
- Parent feeding style
- Infant temperament
- Infant acceptance of healthy solid and table foods
- Microbiome diversity within and between individuals (gut and oral)
- Epigenetic variations (miRNA) within and between individuals (gut and maternal breast milk)

Outcomes Related to Control Group Intervention:



- Proportion of correct responses on child safety related questions that are incorporated into other study surveys
- Objective home safety data collected as part of home observation data collection

A conceptual model depicting the hypothesized relationships between study group, behavioral mediators, and weight outcomes is shown in Figure 4.

Figure 4. Revised conceptual model to include birth order and genetic predisposition

F. STUDY ASSESSMENTS AND MEASUREMENT TOOLS

The assessment protocol is reduced from the first-borns but utilized many of the same instruments and data collection modalities. Data collection modalities include: 1) hospital chart abstraction, 2) web-based, paper surveys, and/or phone interviews, 3) home visits, and 4) research center (RC) visits. The study measures modalities, and times points for data collection are depicted in Table 1.

Second-BORN Table 2.

Timing of Data Collection (NV – performed at Nurse Visit, X – collected on-line or via phone survey)

CONSTRUCTS	MEASURES	WEEKS					YEARS	
		0	3-4	16	28	52	2	3
(1) OBJECTIVE MEASUREMENTS								
Child body mass index	weight and length / height	X	NV	NV	NV	NV	NV	NV
Mother body mass index	Observed weight and height	X			NV	NV	NV	NV
Biologic specimen collection	Stool samples and cheek swabs	X	NV		NV	NV	NV	NV
	Maternal cheek and skin swabs	X						
	Maternal vaginal and rectal swabs		NV					
	Cord blood	X						
	Placenta	X						
	Blood sample					X		
	Breast milk sample	X	NV					
Father body mass index	Observed weight and height	←NV (obtained x 1)→						
(2) INFANT/CHILD BEHAVIOR AND DEVELOPMENT								
Temperament								
Parent reported temperament	Infant Behavior Questionnaire-*=Revised, otherwise full			X*		M		
Observed temperament: negativity, reactivity, regulation	Toy Removal Task Procedure Arm Restraint Procedure					NV		
Child temperament	Early Childhood Behavior Questionnaire						X	
	Child Behavior Questionnaire (VSF, Putnam)							X
Sleep								
Sleep hygiene/behaviors	Adapted Brief Infant Sleep Questionnaire		X	X		X		X
	Bedtimes routines Questionnaire						X	
Feeding and eating behaviors								
Dietary intake	Modified Food Frequency Questionnaire*=abbrev.		X*	X*	M	M		
Appetite	Baby Eating Behavior Questionnaire				X			
	Child Eating Behavior Questionnaire					X		
Baby food preference	Novel Food Tasting Procedure					NV		
Neophobia	Neophobia					X		
Responsive Feeding, food a comfort	Routines (JFS and LLB)						X	X
Child feeding practices	Child Feeding Questionnaire (Birch)-CFQ							X
(3) PARENTING								
Soothing								
Soothing/use of food to soothe	Babies Need Soothing		X	X	X	X		X
Fussing/soothing/feeding events	Diary Cards		X	X				

Infant/child feeding							
Feeding practices and styles	Infant Feeding Style Questionnaire					M	
Infant feeding mode	Babies Need Feeding	X	X	X	X	X	
Child feeding practices	Toddler feeding practice				X		X
Parenting self-efficacy and knowledge							
(4) MATERNAL PSYCHOSOCIAL VARIABLES AND BEHAVIOR							
Maternal psychosocial variables							
Postpartum depression	Edinburgh Postnatal Depression Survey		NV				
Maternal sleep							
Maternal sleep	PROMIS Scale items			X		X	X
Maternal eating behaviors							
Maternal eating habits	Maternal Eating Behaviors			X		X	
(5) FAMILY CONTEXT							
Family variables							
Division of labor	Who does What (adapted)				X		
Home environment							
Home assessment	Home Observation		X	X	X		
TV viewing & family meals	Family Nutrition and Physical Activity Survey (adapted from project EAT)				X	X	
Playtime & physical activity	Active Social Play Questionnaire			X	X	X	X
	Physical Activity 1 year						X
(6) BACKGROUND, DEMOGRAPHICS, AND COVARIATES							
Screening	Screening and Eligibility	X					
	Chart Abstraction	X					
Demographics/Health History (with food insecurity items)	Demographics and Health History	X					X
	Demographics/Health History Update					X	X
Mother employment/childcare	Maternal employment/childcare follow-up			X		X	X

Refer to section 8 above for additional details on the measurement tools.

IV. PROTOCOL

A. RECRUITMENT

Sibling recruitment will be based on participating mothers reporting pregnancy and review of hospital charts. Informed consent will be obtained from the infant's mother and inclusion/exclusion criteria will be reviewed to make sure sibling is able to participate.

B. RISKS/BENEFITS: see section IV B above. The risk/benefits are the same as the first-born participants.

C. ANTICIPATED RESULTS

Weight outcomes. We expect second-born infants to be leaner at age 1 year than their older siblings, particularly among the families randomized to the parenting intervention with their first-born.

Parenting responsiveness. We expect parents to demonstrate more signs of feeding responsiveness as a result of an enhanced ability to detect their infant's hunger/satiety cues.

D. ADVERSE EVENTS: See Section V above.

All Adverse Event reporting and stopping rules are the same as for the first-borns enrolled into the study, recognizing that there are less in-person visits.

E. SAFETY MONITORING: See Section VI above.

Safety monitoring will follow the same procedures as for the first-borns enrolled; including review of growth charts and a DSMB.

F. COST, LIABILITY, AND PAYMENT

As with the first-borns, all home visits and tests will be performed without cost to the participating subjects. Since this trial overlaps a period of frequent physician visits especially for the infant, liability for patient care costs incurred by patients during the course of the trial will be borne by the family or their insurer. Details of the NIH policies concerning this issue can be found in NIH Documents #5305 and 6352-2, Research Patient Care Costs Supported Agreements.

Given the significant time and effort required for participating families in this proposal, an additional participant stipend is warranted for the second-born child. Participants will receive \$250 for participation in the first year plus numerous visit specific gifts which have a retail value of approximately \$50. Participants would also receive \$100 for each long term follow-up visit.

Parents will also receive an additional stipend for the sample collection portion of the study, totaling up to \$200 over the first year. The schedule will be:

- at the 3-4 week visit: \$100 for maternal (\$50) and child (\$50) samples collected in hospital and at the 3-4 week visit,
- at the 28 week visit: \$25 for the baby samples collected;
- at 1 year visit: \$25 for the baby samples collected;
- at 1 year visit: a \$50 bonus for completing all the samples during the first year.

V. STATISTICAL DESIGN AND ANALYSIS

A. DATA RECORDING AND DATA MANAGEMENT: See section VIII A above.

B. SAMPLE SIZE, POWER CALCULATIONS

Sample Size. The primary outcome for this study will be comparing weight-for-length z-scores (WLZ) at age 1 year of first- and second-born siblings. The World Health Organization (WHO) international growth charts¹⁵⁵ will be used for reference values as is standard practice in the US. The within-family design permits repeated measures analysis and is well-powered to detect moderate effect sizes. Based on participant data

from our SLIMTIME study,²¹ we found that a 0.3 difference in WLZ-scores was interesting and meaningful, and the pooled sample standard deviation equaled 1.0. Using those estimates and assuming a moderate sibling correlation ($r=0.5$), the power to reject the null hypothesis of zero WLZ differences group wide between first- and second-born siblings is between 80-90% with a Type 1 error rate of 5% (two-tailed). The range in power depends upon the number of second-born siblings enrolled (for 80% power – 90 second-borns are required; for 90% power – 119 second-borns are required). As stated in the Recruitment Site and Participant Enrollment section, we anticipate 100-140 second-born siblings to be born during the recruitment period based upon Pennsylvania fertility data. Other major comparisons and secondary outcomes related to Aim 1 include changes in maternal behaviors regarding infant feeding, soothing, and sleep. For analyses incorporating multiple repeated time-points (i.e. baby growth rate and maternal feeding practices) the analyses are substantially more strongly powered, and will accommodate covariate and moderator analysis.

C. STATISTICAL ANALYSES

Statistical support related to behavioral outcomes will be led by Eric Loken, PhD from Penn State's Methodology Consulting Center at University Park, who has expertise in the analysis of longitudinal data, including analyzing complex models to test mediation hypotheses. Dr. Loken has also applied advanced statistical models to the study of infant temperament as well as studies of child weight gain. Statistical support related to the genetic analyses will be led by Francesca Chiaromonte, PhD from Penn State's Department of Statistics. Dr. Chiaromonte has extensive expertise in the statistical analysis of genomic data. Michele Marini, MS, the data analyst for the Center for Childhood Obesity Research, will provide statistical support for the conduct of the analyses as will a graduate student from the Department of Statistics. All primary statistical analyses involving INSIGHT study group will invoke the intent-to-treat paradigm, analyzing data based on randomized assignment.

Aim A Analyses. For the primary outcome of WLZ at age 1 year, a linear mixed-effects model, a generalization of regression, will be used to examine the effects of study group and sibling birth order on WLZ scores.¹²³ The linear mixed-effects model will include study group, sex, and sibling birth order and relevant interactions between those factors, and will also include initial birth weight for each sibling. Other continuous secondary outcomes assessed at multiple time points, including maternal BMI, infant sleep duration, feeding frequency, and parenting sense of competence, will be analyzed as repeated measures using linear mixed-effects models. A repeated measures analysis based on a linear mixed effects model also will be used to examine the relationship between acceptance of vegetables and treatment group. Generalized estimating equations using a log-link and Poisson distribution, a generalization of Poisson regression which allows for repeated measurements, will be used to assess the relationship between number of nocturnal and daily feeds during early infancy and treatment group.¹³¹ All models considered allow the introduction of relevant covariates. Covariates to be considered in refining the analyses can be grouped into categories that include maternal and paternal biologic variables, socioeconomic and demographic variables, infant behavioral variables, and parental behavioral variables. Examples of these include but are not limited to maternal pre-pregnancy BMI, birth weight, maternal tobacco use during pregnancy, paternal BMI, sex, and family income. Other major comparisons between first- and second-born siblings include changes in maternal behaviors regarding feeding, soothing, and sleep.

Aim B Analyses. To interrogate the effect of genetics on childhood obesity and on differences between first- and second-born children, SNP typing will occur, which will be followed by the construction of the Genetic Risk Score (GRS) as done by Belsky et al. described in the innovation section of this proposal.¹³⁵ In that study, the GRS for childhood obesity was based on 32 SNPs associated with adult BMI from two large GWAS studies.^{156, 157} In addition to typing these 32 SNPs, we will also type 56 SNPs identified to be associated with obesity in more recent GWASs¹⁵⁸ for a combined 88 SNPs. We anticipate that during the initial years of the proposed funding period, additional SNPs associated with obesity will be discovered by ongoing GWASs in other labs. Therefore, conservatively, we envision typing 120 SNPs. Notably, many of the 88 SNPs already discovered to be associated with obesity are located within or in the vicinity of genes that act on regulation of food intake, i.e. determine appetite.¹⁵⁹ This will require processing of (276 trios of parents and first-borns + 100 second-born children) x 120 SNPs = 928 individuals x 120 SNPs. SNPs will be typed with the commercially available array (iPLEX/Taqman, Sequenom). A subset of SNPs will be validated with allele-specific PCR.¹³² We will thus construct the GRS using the same method as in Belsky et al., but based on 120 SNPs: we will group the SNPs into linkage disequilibrium (LD) blocks using a linkage threshold of $R^2 \geq 0.95$ and data from

the International HapMap Consortium's CEU sample.¹⁶⁰ LD blocks including SNPs associated with obesity-related phenotypes will be retained, and one tag SNP from each such LD block will be included in the GRS. For these SNPs, the obesity-associated alleles will be weighted by the effect size reported in the corresponding GWAS.¹⁵⁸ The GRS will thus be the sum across these SNPs of the weighted counts of obesity-associated alleles as done by Peterson et al. and Li et al.^{133, 134}

To investigate whether genetics modulates the dependence of weight-related outcomes on the INSIGHT intervention program, we will consider the 276 first-born children of the families enrolled in the study and develop multiple regression models for WLZ (at birth and age 1 year) and BMI Z-score (at age 2 and 3 years), that comprise as potential predictors a dichotomous variable for intervention, the GRS, a family history score computed from parental BMIs as in Belsky et al.,¹³⁵ and a number of covariates (some of which are "environmental" and some of which may be "mediating" the effect of genetics on WLZ and BMI z-score. Possible covariates could include birth weight adjusted for gestational age, infant sex, timing of introduction of solids, family income, or maternal education. This analysis will require appropriate data preprocessing (outlier detection and removal, variable transformation and normalization) followed by model selection – during which we will assess the explanatory power of first and second order terms, including interactions, in the predictors. In particular, this will allow us to evaluate how GRS modulates the effects of the intervention (gene-intervention interactions), as well as the effects of environmental covariates (gene-environment interactions). Notably, the use of a multiple regression setting allows us to evaluate the role of each predictor and predictor interaction in the context of all others considered. Nevertheless, if some of the covariates utilized are likely to mediate the effects of genetics on WLZ and BMI z-score we may implement the mediation analysis suggested in Belsky et al.¹³⁵ to carefully separate direct and indirect effects of GRS on the response.

In addition to the development of the regression models described above, we will use availability of the parental GRSs to investigate the interdependence structure of the genetic risk score for obesity of parents and children. For this purpose, we will use principal components analysis after appropriate preprocessing of the scores; the triads from the current INSIGHT study will give us 276 points in 3D. In the analysis we expect to uncover independence of the parental scores from one another, but a strong dependence of the offspring scores on parental scores, and the second will allow us to also investigate the degree of dependence between offspring scores. Notably, "independent" GRSs from the parental cohort will also be useful in validating the effectiveness of the offspring GRSs as a proxy for genetic risk. Scores of individual SNPs for fathers and their children will also be utilized to validate paternity.

Similar analyses relating parent-reported as well as observer-rated temperament and appetite to infant weight status will be performed as we have done previously,¹³⁶ this time including the effects of birth order as it impacts those relationships.

Aims C and D analyses. One of the "environmental" covariates for genetics is the composition of the microbiome. We will be determining oral and gut microbiome compositions of the children involved in this study using 16s rDNA sequencing (targeting the variable 3 and variable 4 region) on the Illumina MiSeq platform. Sequence reads will be compared to the GreenGenes Database for taxonomic designation. Alternatively, Mothur and/or Qiime (Quantitative Insights Into Microbial Ecology) will be used to process raw sequencing reads to determine taxonomic representation. Using this metagenomic technique and workflow we will be able to determine the composition of the microbial community down to the genus level. Using regression analysis we can estimate if there is a relationship among the GRS (as well as other covariates mentioned previously) and the microbiome. We will record composition from the gut and oral microbiome for 50 individuals at 4 time points. Since it has been noted that individuals who are obese have a decrease in the diversity in their gut microbiome¹⁶¹, we will start by calculating alpha diversity (diversity within a sample, Shannon diversity index or Chao1) and beta diversity (diversity between samples – e.g. computing weighted and un-weighted UniFrac distances between samples with QIIME)^{162,163,164} for the oral and gut microbiomes. These indexes will be compared (1) within each individual across time points, to assess progression of the gut and oral microbiomes (we hypothesize diversity will increase over the course of the first year after birth as the oral cavity and gut become colonized and move towards an adult-like microbiome); and (2) between individuals and across times, in a mixed-effects model to determine how age and other covariates (from SIBSIGHT: diet, feeding habits, demographics, child care, soothing habits of parents, antibiotic and other medication use) affect diversity of the gut and oral microbiomes. We will also test for correlation between gut and (separately) oral microbiome diversity and weight status at age 1 year (weight-for-length percentiles for age and sex established by the World Health Organization are the most widely accepted reference for those <2 years); a significant correlation

would indicate a link between the risk of obesity and the gut or oral microbiome. We expect to find a link between gut microbiome diversity and obesity, as it was shown in previous studies (6 and 12 months¹⁶⁵; weeks 3 and 52¹⁶⁶; years 6-16¹⁶⁷). Moreover, we will employ multivariate tools to characterize and investigate, at various points in time, the composition of oral and gut microbiomes and the relationships between them. These tools will include principal components, canonical correlations, co-occurrence networks using various similarity/dissimilarity measures, as well as multivariate, partial least square and generalized boosted linear regression techniques.^{168,169-171} A significant association between these two communities, if found, will be of great clinical interest and prompt further studies to confirm causal links. Regression techniques, and possibly supervised multivariate classification techniques such as discriminant analysis, will also be used to investigate the effects of composition of oral and gut microbiomes (along with other critical covariates) on obesity risk.

Aim E Analyses. To examine the interaction between individual genetics and the environment we will characterize epigenetic control of the gastrointestinal system by miRNA. Fecal samples from second born children and maternal breast milk samples from 0 and 1 month time-points will be analyzed for miRNA expression using high throughput small RNA sequencing techniques on the Illumina MiSeq platform. Small RNA reads (~22 nucleotides) from each sample will be aligned to Build 21 of the human genome in Illumina BaseSpace Software using the Bowtie algorithm. Data will be normalized as RPMs and individual miRNAs present in at least half the total samples will be used for analysis. Differences in fecal miRNA levels across age groups will be determined using a Mann-Whitney U test with Benjamini Hochberg FDR. Hierarchical clustering of differentially expressed microbiota taxa from Aim 1 will be performed using a Euclidian distance metric and a PCA factor analysis will be used to examine relationships between miRNA and taxa across groups. Spearman's rank correlation will be employed to examine associations between miRNAs of interest and environmental/demographic factors (anthropometrics, demographics, MBM factors (miRNAs, metabolic components such as sodium, potassium, and zinc), and measures of child behavior and parental/family function). Gene targets of differentially regulated miRNAs will be identified with IPA's MAP function and cross-referenced against genetic patterns observed in Aim B.

E. MISSING DATA: See Section VIII E above

F.MULTIPLE TESTING: See Section VIII F above

Ancillary Microbiome Collection

I. PRINCIPAL HYPOTHESES TO BE TESTED

- A.) We will be studying the development of the microbiome over the first year after birth in light of various environmental influences – e.g. mode of delivery, diet (and diet changes), antibiotic exposure, GERD medication exposure. We predict that factors that are contemporary to the microbiome collection time point will be most relevant in determining perturbations in the microbiome and that the effect of the incredible changes in exposures throughout this first year will be greater than the ability of the microbiome to form a stable and static community.
- B.) We will perform statistical analyses to test the hypotheses (1) that there is an association between the developing oral and gut microbiomes, and (2) that they interact in affecting childhood obesity.

II. BACKGROUND AND RATIONALE

A. Background

While it is generally accepted that the gut microbiome is an important contributor to weight status, less is known about the role that the oral microbiome has to play. Furthermore, less is known about the development of these two microbiomes from the initial microbiome community present at the time of birth through the establishment of adult-like communities later in life and how this succession may influence the growth of the child along the way.

The establishment of the oral microbiome (as recently reviewed by Gomez and Nelson, 2017) begins *in utero* and may be influenced by maternal oral health¹⁸⁶. The oral microbiome is further influenced by mode of delivery and early diet (breast milk vs. formula) and the diversity of the microbiome increases through time as the infant matures and is exposed to an increasingly diverse environment. However, there is a lack of understanding about the role of early life exposures in concert (diet + antibiotic exposure + acid reducing medications + etc.) as an influence on the microbiome. Furthermore, host genetics as an influence on the establishment of the oral microbiome is underexplored.

The gut microbiome development, on the other hand, is more widely studied (see Gritz et al. 2015 for a review).¹⁸⁷ Environmental influences such as mode of delivery, diet, and antibiotic exposure are studied as sources of variation between individuals.¹⁸⁸ Even host genetics have been explored as influential on the gut microbiome¹⁸⁹ While the links between the gut microbiome and obesity have been investigated¹⁹⁰ it is not understood how the gut microbiome influences the growth trajectory in early childhood. Additionally, how the interplay of environmental factors and host genetics shape the gut microbiome development in relation to the child's weight growth has not been explored.

B. Specific Aims

- 1) **Specific Aim 1: To characterize the development of the oral microbiome from birth through the first year after birth and test whether the diversity of the oral microbiome is linked to obesity as measured by growth curves.** In our analyses on previous study participants we found that there is an association between the diversity and the Firmicutes-to-Bacteroidetes ratio in the oral microbiome and the pattern of growth. We, however, do not know the pattern of the oral microbiome succession.
- 2) **Specific Aim 2: To characterize the development of the gut microbiome from birth through the first year after birth and test whether the diversity of the gut microbiome is linked to obesity as measured by growth curves.** Our analyses on previous study participants found that there was no association between gut microbiome diversity and the Firmicutes-to-Bacteroidetes ratio and the rate of growth. However, we are interested in how the gut microbiome influences the growth rate of a child in a prospective manner, rather than a retrospective manner.
- 3) **Specific Aim 3: To characterize factors that influence the succession of the oral and gut microbiomes.** We will be assessing how environmental factors (diet, antibiotic exposure, etc.) influence the microbiomes at several time points and how these impact the microbiome at 1 year of age.

III. PROTOCOL OVERVIEW

Newborn children will be invited to participate to prospectively evaluate the microbiome development over the first year after birth. Nurses will meet parents either in their home or at an HMC affiliated clinic (around a scheduled routine care visit or at the family's convenience) 3 times within the first year after birth. Included in the informed consent will be permission to access the infant's medical record and to review the outpatient chart. Study personnel from the Hershey campus will be responsible for all face-to-face visits as well as the data collected at those visits. Study personnel from the University Park campus will be responsible for all phone interviews and web-based data collection.

A. STUDY GROUPS AND SUBJECTS

Given the dropout rate from INSIGHT and the second born study (~15%) if we enroll an additional 40 individuals into the ancillary microbiome study, we expect to be able to follow 34 children from birth through 1 year.

B. INCLUSION CRITERIA

Eligible mother-infant dyads for this trial will meet the following criteria:

- 1) full-term infant (≥ 36 0/7 weeks gestational age) without significant morbidity
- 2) nursery/NICU/maternity stay of 7 days or less
- 3) participant children can be (but do not have to be) twins
- 4) English speaking mother
- 5) care provided at an HMC affiliated clinic
- 6) a working telephone number

C. EXCLUSION CRITERIA

(not explicitly obvious from the inclusion criteria)

- 1) prenatal ultrasound presence of intrauterine growth retardation (IUGR)
- 2) infant birth weight <2250 grams
- 3) presence of a congenital anomaly or neonatal condition that significantly affects a newborn's feeding (e.g. cleft lip, cleft palate, metabolic disease)
- 4) any major maternal morbidities and/or pre-existing condition that would affect postpartum care or her ability to care for her newborn such as cancer, multiple sclerosis, lupus, etc.
- 5) plan for newborn to be adopted
- 6) plan to move from Central Pennsylvania within 1 year
- 7) have 2 older siblings in INSIGHT/SIBSIGHT

D. STUDY VISITS

Similar to INSIGHT/SIBSIGHT participants, nurse visits for enrolled ancillary study participants will occur at 3 in-person visits within the first year. Anthropometrics will be measured at each in-person visit, and as specified below. Samples and survey questions will be collected as listed below.

1. Enrollment Visit – Pregnancy/Maternity/Nursery Hospital Stay

- a. Mother's will be asked to consent for the microbiome study during their maternity hospital stay
- b. Newborn and maternal medical chart review in to determine eligibility based on inclusion and exclusion criteria (Screening form)
- c. Obtain maternal informed consent for mother-baby dyad
- d. Complete enrollment data collection forms
 - i. Chart abstraction form (coordinator completed)
 - ii. Demographics and Health History form, (coordinator interview with mother)
 - iii. Contact form (updated)

- e. Collect weight and height of mother from the medical record
- f. Collect buccal swabs from mother and baby.
- g. Collect skin swab from mother.
- h. Collect breast milk sample from mom (approximately 1 mL)
- i. Collect meconium sample from baby.
- j. Collect cord blood (collected per standard of care at time of delivery).
- k. Collect placenta samples (collected via pathology)

2. Home/Clinic Visit 1 (10-24 days):

- a. Infant weight and length measured
- b. Complete data collection
- c. Collect buccal swabs from baby.
- d. Collect vaginal and rectal swabs from mother (these are self-administered with the swab kit and instructions sent prior to the visit).
- e. Collect breast milk sample from mom (approximately 1-5mL)
- f. Collect baby's stool sample.

3. Home/Clinic Visit 2 (28 weeks)

- a. Baby's weight and length measured
- b. Complete data collection
- c. Collect breast milk sample from mom. (approximately 1-5mL)
- d. Collect buccal swabs from baby.
- e. Collect baby's stool sample.

4. Home/Clinic Visit 1 (1 Year)

- a. Baby's weight and length measured
- b. Complete data collection
- c. Collect buccal swabs from baby.
- d. Collect baby's stool sample.

E. OUTCOME MEASURES AND THE RATIONALE FOR CHOOSING THEM

- 1) Microbiome outcome
 - a. Microbiome diversity within individuals (how gut and oral microbiomes are similar or different) and between individuals (e.g. are there differences between children with different growth outcomes?)
- 2) Genetic variation
 - a. Associations between genetic variation and microbiome diversity (Does an individual's genetic information influence the pattern of microbiome development?)
 - b. Genetic variation as it relates to growth outcomes (e.g. Genetic Risk Score for overweight and obesity)

F. STUDY ASSESSMENTS AND MEASUREMENT TOOLS

The assessment protocol is greatly reduced from the first- and second- born children, but utilizes some of the same instruments and data collection modalities. Data collection modalities include: 1) hospital chart abstraction, 2) emailed/paper surveys, and 3) in-person visits. The study measures modalities, and times points for data collection are depicted in Table 1.

Ancillary Study Table 1.

CONSTRUCTS	MEASURES	WEEKS			
		0	3-4	28	52
Objective Measures					
Child body mass index	Weight and length / height	X	NV	NV	NV
Mother body mass index	Observed weight and height	X			
Biologic specimen collection	Stool samples and cheek swabs	X	NV	NV	NV
	Maternal cheek and skin swabs	X			
	Maternal vaginal and rectal swabs		NV		
	Cord blood	X			
	Placenta	X			
Dietary intake	Modified Food Frequency Questionnaire		X*	M	M
Screening					
	Screening and Eligibility	X			
	Chart Abstraction	X			
Demographics/Health History	Demographics and Health History	X			
	Demographics/Health History Update			X	X
Mother employment/childcare	Maternal employment/childcare follow-up				X

Refer to section 8 above for additional details on the measurement tools

IV. PROTOCOL

A. RECRUITMENT

During the maternity and newborn hospital stay at HMC, study personnel will identify eligible babies born through a review of hospital charts. Once identified as meeting each of the inclusion criteria, informed consent will be obtained from the infant's mother.

Because our pilot study suggested that some attrition (~30%) occurs between the maternity/nursery stay and the first study home visit 2-3 weeks after delivery, we seek permission to enroll up to 43 dyads. Once 30 dyads have successfully completed the first study home visit, enrollment will cease.

B. RISKS/BENEFITS

Risks to the subjects. The following are categories of potential risk:

Human subjects' involvement and characteristics. Inclusion criteria are outlined in the study methods and generally require being a healthy term newborn of a healthy mother. Because the objective of the study is to study the development of the very early microbiome and how it relates to (and potentially influences) the growth of an infant, inclusion of the vulnerable population of newborns is necessary for this research. Sample collection is non-invasive and has risk to the participant other than loss of confidentiality.

Sources of materials. The initial source of information regarding study eligibility will come from a brief review of the infant medical record. Because Dr. Paul has clinical responsibilities as an attending physician in the HMC newborn nursery, he and his research staff are permitted by the Institutional Review Board (IRB) to review these records and are compliant with HIPAA regulations. Only Dr. Paul and his clinical research staff will be permitted to review the medical records, and non-clinical investigators will not have access to patient charts. Numerous paper and/or electronic surveys and diaries related to infant diet will be completed by participating mothers, which will be accessible by all study investigators. Surveys will be marked with a subject ID number as opposed to identifiable information to protect participant confidentiality.

Potential benefits of the proposed research to the subjects and others. None

Importance of the knowledge to be gained. The findings of this study will provide insight into the development of the infant microbiome in a healthy population and how the development is related to the growth of the infant. If significant patterns are observed, these could inform future studies of microbiome modulation to influence a healthy growth trajectory.

C. ANTICIPATED RESULTS

Microbiome Composition: We predict that children who have non-rapid weight growth will have microbiomes that are more diverse and have a higher proportion of Bacteroidetes in respect to abundance of Firmicutes. We hypothesize that these associations will be observed earlier in the oral microbiome than in the gut microbiome.

Microbiome Succession: We predict that the oral and gut microbiomes will begin with similar compositions and with through time will become increasingly different.

D. ADVERSE EVENTS

– see section V of INSIGHT: First - Born

All Adverse Event reporting are the same as for the first- and second- borns enrolled into the study, recognizing that there are less in-person visits and there is no intervention component associated with the families enrolled in the ancillary study.

E. SAFETY MONITORING

A. PROTECTION OF HUMAN SUBJECTS

Recruitment and Informed Consent.

Informed consent will be obtained using forms and protocols approved by at the HMC IRB. The investigation will be initiated solely on the maternity floor at this center. Study procedures and the risks of normal newborn

life will be explained and written informed consent will be obtained from mothers of the newborns in accordance with the requirements of the IRB. Those obtaining informed consent will include the investigators, nurse study coordinators, and project manager.

Protection against risk.

Because the study will include children, all IRB requirements for the protection of children will be fulfilled.

B. INCLUSION OF WOMEN AND MINORITIES

Half of the newborn patients enrolled are expected to be female, and maternal co-participation is a study requirement. Approximately 30-40% of the participants enrolled in the prospective trial are expected to be minorities as we will attempt to over-sample for minority families from our center. Several minority groups are relatively under-represented in this recruitment plan because of the geographic location of the study and the patient demographics of the local population. Attempts to recruit minority patients will be planned for subsequent investigations that will be specifically powered to evaluate the effect of ethnicity and race of the study outcomes.

B1. Infant Enrollment

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	2	2	4
Not Hispanic or Latino	18	18	36
Ethnic Category: Total of All Subjects *	20	20	40
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	2	2	4
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	3	3	6
White	15	15	30
Racial Categories: Total of All Subjects *	20	20	40

B2. Maternal Enrollment*

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	2	2	4
Not Hispanic or Latino	18	18	36
Ethnic Category: Total of All Subjects *	20	20	40
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	2	2	4
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	2	2	4

White	16	16	32
Racial Categories: Total of All Subjects	20	20	40

C. INCLUSION OF CHILDREN

Observing microbiome changes and outcomes for newborns and infants is the main objective of this trial and therefore this population will be primarily studied. Only those born at term (≥ 37 weeks gestation) will be included, and those discharged from the neonatal intensive care unit will not be included because of an increased likelihood of neonatal morbidity in that population.

PI Paul and the pediatric clinical research nurses involved in the study have extensive clinical experience providing medical care for newborns and children of all ages.

D. COST, LIABILITY, AND PAYMENT

All home visits and tests will be performed without cost to the participating subjects. Since this trial overlaps a period of frequent physician visits especially for the infant, liability for patient care costs incurred by patients during the course of the trial will be borne by the family or their insurer. Details of the NIH policies concerning this issue can be found in NIH Documents #5305 and 6352-2, Research Patient Care Costs Supported Agreements.

Given the significant time and effort required for participating families in this proposal, a participant stipend is warranted. Participants will receive \$10 for each sample provided at each time point: maternal skin, maternal buccal, child stool, child buccal; \$15 for each sample provided: maternal vaginal, maternal rectal.—Participants will also receive \$5 for completing their surveys at 3-4, 28, and 52 weeks.

V. STATISTICAL DESIGN AND ANALYSIS

A. DATA RECORDING AND DATA MANAGEMENT

The staff in the Center for Childhood Obesity Research (University Park, PSU) will coordinate the creation of all paper data collection forms that will be distributed to research subjects and subsequently returned to the Center.

Whenever possible, the data entry system will be designed to prevent illogical values from being entered into the data files. In addition, error checks will be programmed to identify potential data errors, such as missing values, out-of-range values or illogical, inconsistent values within or across data collection forms, duplicate forms and missing forms.

Study research staff will record participant data on the data collection forms, review the forms for completeness and legibility, and forward the data collection forms to the Center for data entry and processing. The research staff will be required to send their completed forms within a week of the study visit to the Center in order that the data can be entered and processed in a timely fashion. The Center staff will date stamp all forms received from the study research staff. The forms will be inspected visually for problems that cannot be detected electronically. Center staff will perform independent, double-data entry on each data collection form. The data will be stored on a College of Health and Human Development server, maintained by the College Information Systems and Services (ISS) staff. All paper forms will be stored in locked filing cabinets in the Center for Childhood Obesity Research.

The staff at the Center for Childhood Obesity Research will also coordinate the creation of online data collection tools and the management of the corresponding data. REDCAP software will be used to create surveys, and links to the surveys will be sent to participants via email, prompting them to complete the surveys. All online surveys at a given time point will be merged together, so that they appear as one survey to the participant. Participants will be prompted to enter an identification number that allows their responses to remain anonymous. The participant may close the program and resume answering the rest of the questions at a later time. The participant will be given one week to complete a set of online surveys after receiving the link via email.

The security of the project data will be maintained through the ISS network hardware and user authentication (usernames and passwords). The project directory will be restricted to only staff in the Center working on the project. Back-ups of the project data files will occur every night, with user data backed-up

incrementally Monday through Thursday and complete back-ups every Friday. Archival back-ups, stored indefinitely, are cut on the last weekend of every month. All back-up data are stored in a secure off-site location. The number and variety of back-ups ensure ample data redundancy and protection. In addition, Center staff will never receive any identifying personal information on the research participants. Each participant will be assigned a unique subject identification number. Only the study research staff will have the log linking this number with the participant's personal information.

B. SAMPLE SIZE, POWER CALCULATIONS, AND STATISTICAL ANALYSIS

Aims 1 & 2 analyses. One of the “environmental” covariates for genetics is the composition of the microbiome. We will be determining oral and gut microbiome compositions of the children involved in this study using 16s rDNA sequencing (targeting the variable 3 and variable 4 region) on the Illumina MiSeq platform. Sequence reads will be compared to the GreenGenes Database for taxonomic designation. A custom Galaxy workflow will be used to process raw sequencing reads to determine taxonomic representation. Using this metagenomic technique and workflow we will be able to determine the composition of the microbial community down to the genus level. Using functional data analysis we can estimate if there is a relationship among the microbiome and weight. Additionally using regression techniques we can investigate if there is a relationship between genetic risk score (as well as other covariates- diet, antibiotic exposure, etc.) and the microbiome. We will record composition from the gut and oral microbiome for 40 children at 4 time points during the first year after birth. Since it has been noted that individuals who are obese have a decrease in the diversity in their gut microbiome¹⁶¹, we will start by calculating alpha diversity (diversity within a sample, Inverse Simpson diversity index or Chao1) and beta diversity (diversity between samples – e.g. computing weighted and un-weighted UniFrac distances between samples, and Bray-Curtis diversity)^{162,163,164} for the oral and gut microbiomes. These indexes will be compared (1) within each individual across time points, to assess progression of the gut and oral microbiomes (we hypothesize diversity will increase over the course of the first year after birth as the oral cavity and gut become colonized and move towards an adult-like microbiome); and (2) between individuals and across times, in a mixed-effects model to determine how age and other covariates (diet, demographics, child care, antibiotic and other medication use) affect diversity of the gut and oral microbiomes. We will also test for correlation between gut and (separately) oral microbiome diversity and growth rate (using Conditional Weight Gain z-scores- CWG); a significant correlation would indicate a link between the risk of obesity and the gut or oral microbiome. We expect to find a link between gut microbiome diversity and obesity, as it was shown in previous studies (6 and 12 months¹⁶⁵; weeks 3 and 52¹⁶⁶; years 6-16¹⁶⁷). Furthermore, our previous research on INSIGHT children show that the *oral* microbiome diversity and composition is associated with CWG and predictive of growth curves (unpublished data). Moreover, we will employ multivariate tools to characterize and investigate, at various points in time, the composition of oral and gut microbiomes and the relationships between them. These tools will include principal components, canonical correlations, co-occurrence networks using various similarity/dissimilarity measures, as well as multivariate, partial least square and generalized boosted linear regression techniques^{168,169-171}. A significant association between these two communities, if found, will be of great clinical interest and prompt further studies to confirm causal links. Regression techniques, and possibly supervised multivariate classification techniques such as discriminant analysis, will also be used to investigate the effects of composition of oral and gut microbiomes (along with other critical covariates) on obesity risk.

Aim 3 Analyses. To interrogate the effect of genetics on childhood obesity SNP typing will occur, which will be followed by the construction of the Genetic Risk Score (GRS) as done by Belsky et al. described in the previous section of this proposal.¹³⁵ In that study, the GRS for childhood obesity was based on 32 SNPs associated with adult BMI from two large GWAS studies.^{156, 157} In addition to typing these 32 SNPs, we will also type additional SNPs identified to be associated with obesity in more recent GWASs¹⁵⁸. We anticipate that during the initial years of the proposed funding period, additional SNPs associated with obesity will be discovered by ongoing GWASs in other labs. Therefore, conservatively, we envision typing at least 120 SNPs. Notably, many of the SNPs already discovered to be associated with obesity are located within or in the vicinity of genes that act on regulation of food intake, i.e. determine appetite.¹⁵⁹ SNPs will be typed with a commercially available array (Precision Medicine Array by Affymetrix). A subset of SNPs will be validated with allele-specific PCR.¹³² We will thus construct the GRS using the same method as in Belsky et al., but based on 120 SNPs: we will group the SNPs into linkage disequilibrium (LD) blocks using a linkage threshold of $R^2 \geq$

0.95 and data from the International HapMap Consortium's CEU sample.¹⁶⁰ LD blocks including SNPs associated with obesity-related phenotypes will be retained, and one tag SNP from each such LD block will be included in the GRS. For these SNPs, the obesity-associated alleles will be weighted by the effect size reported in the corresponding GWAS.¹⁵⁸ The GRS will thus be the sum across these SNPs of the weighted counts of obesity-associated alleles as done by Peterson et al. and Li et al.^{133, 134}

In addition to the development of the regression models described above, we will use availability of the parental GRSs to investigate the interdependence structure of the genetic risk score for obesity of parents and children. For this purpose, we will use principal components analysis after appropriate preprocessing of the scores; the triads from this study will give us 40 points in 3D, which will be used in conjunction with the ~426 triads from INSIGHT (276) and SIBSIGHT (100-150) for a total of ~466 points (keeping in mind not all points are independent). The addition of the 40 points from this portion of the study will allow us to increase the statistical power of our analysis. In the analysis we expect to uncover independence of the parental scores from one another, but a strong dependence of the offspring scores on parental scores. Notably, "independent" GRSs from the parental cohort will also be useful in validating the effectiveness of the offspring GRSs as a proxy for genetic risk. Scores of individual SNPs for fathers and their children will also be utilized to validate paternity.

Similar analyses relating parent-reported as well as observer-rated temperament and appetite to infant weight status will be performed as we have done previously,¹³⁶ this time including the effects of birth order as it impacts those relationships.

C. MISSING DATA

- see section VIII E in INSIGHT: First-Born section

D. MULTIPLE TESTING

- see section VIII F in INSIGHT: First-Born section

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