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Environmental and epigenetic determinants of child adipokines in a Mexican-American population.

by

Vitaly A. Volberg

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Health Sciences

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Nina Holland, Co-Chair

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Professor John Balmes

Professor Lisa Barcellos

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Environmental and epigenetic determinants of child adipokines in a Mexican-American population.

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Abstract

Environmental and epigenetic determinants of child adipokines in a Mexican-American population.

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Professor Nina Holland, Co-Chair

Professor Brenda Eskenazi, Co-Chair

In the last 30 years there has been a sharp increase in obesity among children, and minority populations are particularly vulnerable. Although etiology of obesity is thought to be multifactorial with causes stemming from diet, environment, genetics and their interaction, no clear molecular pathways have been identified. Underlying obesity development are changes in critical energy balance hormones, adiponectin and leptin (adipokines), however their development and determinants over the childhood period remain poorly understood.

Previous studies indicate that certain features of the early life environment may have lasting effects on future child metabolic health and highlight the potential obesogenic role of Bisphenol A (BPA) – a high volume production chemical detectable in 93% of the United States population. Mechanisms of BPA action remain uncertain however a leading hypothesis argues that BPA exposure may result in epigenetic changes, such as altered deoxyribonucleic acid (DNA) methylation, affecting expression of adipogenic genes.

To address these data gaps, we proposed the following specific aims:
(1) To measure plasma adiponectin and leptin in Mexican-American children from the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort at birth and again at 2, 5, and 9 years, examining heterogeneity in adipokine growth patterns and their association with candidate perinatal factors.
(2) To determine whether maternal or concurrent urinary BPA concentrations are associated with adiponectin and/or leptin levels in children.
(3) To characterize DNA methylation structure of peroxisome proliferator-activated receptor gamma *(PPARγ*) - the master regulator gene in adipogenesis, determine whether *PPARγ* methylation is associated with child adipokines and/or body size and whether prenatal or concurrent BPA may influence *PPARγ* methylation.

Our results highlight several developmental differences in adiponectin vs. leptin over the childhood period. While leptin levels closely and positively correlated with child body size at all ages, adiponectin had inverse and weaker associations with body mass index (BMI) at 2, 5, and 9 years. Further, adjusting for BMI, adiponectin reflected an improved lipid profile while leptin was directly related to systolic and diastolic blood pressure in 9-year-old children.

Of the candidate perinatal factors examined, we identified maternal consumption of sugar-sweetened beverages (SSB) during pregnancy and increased rate of growth during the first 6 months of life as significant risk factors for altered adiponectin levels during childhood. Further, children with greater birth weight had rapidly-rising leptin levels over the birth to 9-year period and highest BMI and waist circumference at 9 years.

Our BPA analyses indicated sexually dimorphic responses similar to those previously reported in animal studies. While BPA concentrations during early pregnancy were directly associated with adiponectin levels in 9-year-old girls (β=3.71, P=0.03, N=131), BPA concentrations during late pregnancy were associated with increased plasma leptin in 9-year-old boys (β=0.06, P=0.01, N=179), controlling for sociodemographics, dietary variables and child BMI.

Finally, using the Infinium Illumina 450K Array, we examined DNA methylation in 23 sites spanning the *PPARγ* promoter and gene body region in discovery (N=117 at birth, N=108 at 9 years) and validation (N=116 at birth, N=131 at 9 years) sets of children. We report that methylation in site 1 was significantly and negatively associated with child size at birth (β=-2.5, P=0.04) and at 9 years (β=-4.8, P<0.001) in the discovery set, and these relationships were replicated in the validation set. Overall our research adds evidence in support of the hypothesis the children's metabolic health may be programmed during early life and suggests that epigenetic mechanisms may play an important role in determining child size.

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DEDICATION

I dedicate my dissertation to Dr. Nina Holland

and my parents, Alex and Olga Volberg,

for their unwavering support and encouragement

throughout the process of growing up.

ACKNOWLEDGEMENTS

I want to thank my parents, Alex and Olga Volberg, for their continual support, wisdom and advice.

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Finally, I would like thank the following organizations for financial support of this doctoral research including: UC-Berkeley Public Health Alumni Association, National Institutes of Health and the Environmental Protection Agency.

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CHAPTER 1

HYPOTHESES AND ORGANIZATION OF DISSERTATION

This thesis investigated the hypothesis that characteristics of the perinatal environment, including fetal exposure to Bisphenol A (BPA), may affect child levels of biomarkers of obesity, adiponectin and leptin (adipokines). Further, we hypothesized that mechanisms accounting for this early life – metabolic health link involve epigenetic modification of peroxisome proliferator-activated receptor gamma *(PPARγ)* – the master regulator of adipogenesis. Given current literature, the following hypotheses and specific aims were designed:

Hypothesis 1: Children belong to specific adipokine trajectories from birth, in part determined by characteristics of the perinatal environment.

> ***Aim 1:*** *To measure plasma adiponectin and leptin in children from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) at birth and again at 2, 5, and 9 years, examining adipokine growth patterns and their relationship with perinatal factors.*

Hypothesis 2: Prenatal exposure to BPA is associated with decreased adiponectin and increased leptin in 9-year-old children.

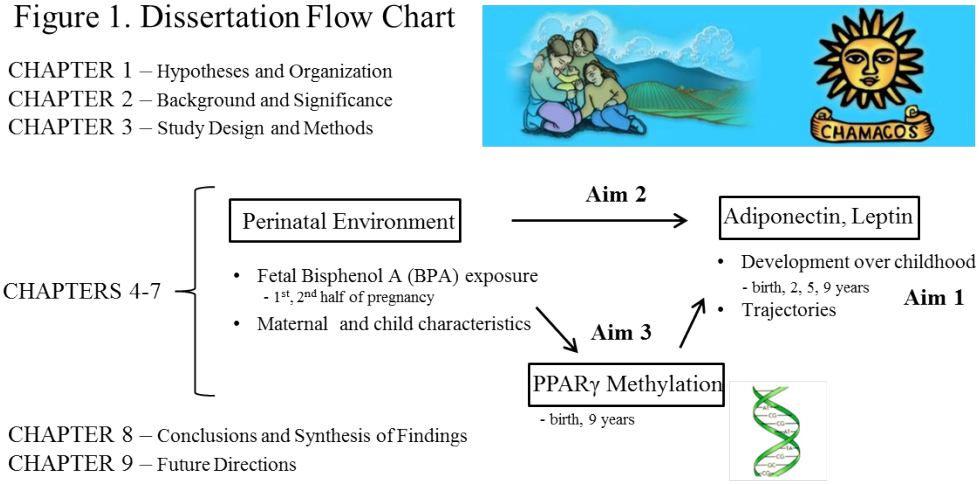
> ***Aim 2:*** *We will analyze whether maternal urinary BPA levels during pregnancy are associated with adiponectin and/or leptin levels in 9-year-old CHAMACOS children.*

Hypothesis 3: *PPARγ* methylation is associated with adipokine levels and child size and is influenced by perinatal factors, including BPA.

> ***Aim 3:*** *To characterize PPARγ methylation in children at birth and 9 years of age using the Infinium Illumina 450K array in discovery and validation sets, examining its associations with adipokines, child body size and perinatal factors.*

Figure 1 illustrates the organizational structure of this dissertation, with chapters 4, 5, 6, and 7 specifically addressing the research aims.

Figure 1. Dissertation Flow Chart



Chapter 2 discusses the current obesity epidemic in children, identifying data gaps in our understanding of adiponectin and leptin development over childhood and the factors that may affect them. Further, a plausible mechanism linking features of the perinatal environment with later life metabolic health is outlined, highlighting the need for human data.

Chapter 3 presents the CHAMACOS study design and methods used in this dissertation. In brief, the CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of pesticides and other exposures on child growth and development. Pregnant mothers were enrolled during 1999-2000 and mother-child pairs have been followed through the present (2013).

Chapter 4 describes results of characterizing adiponectin and leptin levels in 146 9-year-old children, their relationships with child weight, lipid profile (glucose, cholesterol, triglycerides, very low-density lipoprotein, low-density lipoprotein and high-density lipoprotein) and blood pressure. We also examined associations between features of the perinatal environment, including maternal diet, gestational weight gain, pre-pregnancy body mass index (BMI), birth weight and length and growth rate during the first 6 months of life, and children's 9-year adipokine levels, adjusting for their body mass index (BMI).

Chapter 5 presents results on development of adiponectin and leptin over the birth to 9-year period, examining their associations with child size within the birth, 2, 5, and 9-year time points, determining the extent to which adipokine levels are correlated with each other over time and exploring their different growth patterns (trajectories) using the recently-developed vertically-shifted mixture modeling statistical analysis. Further, we identified whether features of the perinatal environment (listed above) are associated with child membership to a particular trajectory group.

Chapter 6 reports on our multivariate linear regression analyses, assessing whether urinary BPA concentrations during first and/or second halves of pregnancy and in children at 9 years, were associated with child adiponectin and/or leptin at 9 years. In our models, we tested for effect modification by sex and controlled for maternal pre-pregnancy body mass index (BMI), pregnancy soda consumption and smoking, years in U.S. prior to pregnancy, maternal education, household poverty status, child BMI and 9-year child soda, fast food and sweet snack consumption.

Chapter 7 describes our data on methylation throughout the *PPARγ* gene. We measured methylation of 23 *PPARγ* CpG sites in blood samples from birth and 9 years, examining their relationships with adipokines and child size and determining their associations with perinatal factors, including prenatal BPA measures. We initially identified sites with significant association with adipokines and/or child size in a 'discovery' set containing 117 children at birth and 108 children at 9 years. We then sought to replicate these findings in a separate validation set containing 116 children at birth and 131 children at 9 years.

Chapter 8 summarizes our findings, highlighting data gaps outlined in current literature with respect to mechanisms of obesity etiology and our efforts to address them.

Chapter 9 provides future research directions, building on our results of examining adipokine development over childhood, their relationships with epigenetic changes and possible influence by perinatal factors.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

The Obesity Epidemic

Data from the National Health and Nutrition Examination Survey (NHANES) show a 2.5-fold increase in prevalence of childhood obesity in youth of all ethnicities since 1980 (Ogden et al. 2010). Further, in NHANES 2009-2010, Mexican-American children aged 2-19 had significantly higher obesity prevalence compared to their white counterparts (21.2% vs. 14.0%). This disparity was present even in the youngest age group, with Mexican-American infants and toddlers having the greatest prevalence of high weight-for-recumbent-length of any US ethnicity (15.7%) (Ogden et al. 2012). While the obesity epidemic is on the rise, critical questions about its etiology, potential early life origins, and environmental contributions remain difficult to address.

Currently, development of obesity is mainly framed as a caloric imbalance, where adipogenesis must happen in order to store excess energy. Because of this paradigm, there has been great focus on children's dietary and exercise variables, such as daily total energy intake, % total energy from fat, soda and high calorie snack consumption and physical vs. sedentary activity (Spear et al. 2007). However, large prospective studies on children aimed at identifying key lifestyle components associated with obesity (focusing on measures of nutrition and physical activity) have had difficulty identifying consistent associations (te Velde et al. 2012).

While the caloric imbalance paradigm is an important component of obesity etiology, several lines of evidence suggest that adipogenesis may be determined by more complex metabolic disturbances. An increasing body of literature argues in support of the Developmental Origins of Health and Disease (DOHAD) hypothesis – describing the potential for perinatal exposures to have lasting effects on later-life metabolism and obesity development (Barker 1995; Barker 1998).

One of the earliest studies supporting this idea of 'obesity programming' came from a cohort of men born during the Dutch Famine of 1944-1945, showing that boys under-nourished during fetal development developed a 'thrifty phenotype', storing energy when possible, and, as a result, had higher risk of obesity in adulthood. Building on the DOHAD framework, recent data suggest that prenatal exposure to certain environmental chemicals may lead to altered metabolic health in later life (Grun et al. 2006; Grun et al. 2009). In particular, Bisphenol A (BPA), due to its endocrine disrupting activity, has gained attention as a potential 'obesogen' – a chemical that promotes adipogenesis in animal models (Dolinoy et al. 2007; Grun et al. 2009; Vom Saal et al. 2012).

While the perinatal environment, including *in utero* characteristics such as maternal glucose levels, gestational weight gain and/or chemical exposures, may contribute to child obesity, the exact molecular mechanisms remain unclear (Oken et al. 2003; Lustig 2011). Importantly, underlying obesity development are changes in 'biomarkers of obesity', adiponectin and leptin – also termed adipokines (Kadowaki et al. 2005; Mantzoros et al. 2011). These two hormones have critical roles in regulating energy balance, insulin sensitivity, fat storage and overall metabolism (Considine et al. 1996; Margetic et al. 2002; Kadowaki et al. 2005; Mantzoros et al. 2011). However, there remain large data gaps in our understanding of how these adipokines change over childhood and their early life determinants.

Biomarkers of Obesity: Adiponectin

Use of adiponectin and leptin has given deeper insight into mechanisms of obesity development. Adiponectin is a 244 amino acid protein hormone secreted almost exclusively by adipose tissue. It targets muscle and liver to increase uptake and catabolism of fatty acids and carbohydrates, promoting insulin sensitivity (Kadowaki et al. 2005). In children, lower adiponectin levels have been associated with both metabolic syndrome and type 2 diabetes (Cruz et al. 2004; Shaibi et al. 2007). Further, while there is a well-established inverse relationship between adiponectin and BMI in school-age children, this is less clear in newborns. Several studies report a positive relationship between adiponectin and birth weight but others do not (Lindsay et al. 2003; Sivan et al. 2003; Mantzoros et al. 2004; Bozzola et al. 2010).

Changes in adiponectin levels through the prenatal and early childhood periods are not fully understood. The Butte et al (2005) cross-sectional study, encompassing a variety of ages (4-19 years), showed that plasma adiponectin levels declined from 4 to 10 years of age, then tended to plateau from 10 to 19 years of age (Butte et al. 2005). The trend of high levels of adiponectin at younger ages, followed by a decrease to a stable value is echoed in separate studies that together span the entire birth to puberty period, including perinatal (Mantzoros et al. 2009), early childhood (Iniguez et al. 2004), pre-puberty (Stefan et al. 2002) and puberty (Bottner et al. 2004). However, no one study has collected longitudinal data over this entire period.

As a result, the important question of whether early life adipokine measures can predict future adipokine levels and obesity-related outcomes has been relatively unaddressed. To date, only two prospective cohorts have explicitly examined such relationships, with both studies limited to narrow age ranges, Mantzoros et al (2009) – from birth to 3 years and Nishimura et al (2012) – from 9 to 12 years. (Mantzoros et al. 2009; Nishimura et al. 2009). Adiponectin levels were associated with one another only comparing 9 to 12-year values. While these data may suggest greater plasticity during early life and constraint to specific adipokine levels at older ages, these relationships remain unclear over the childhood period.

Biomarkers of Obesity: Leptin

Leptin is a 167 amino acid hormone synthesized primarily by adipose tissue but also by the placenta, stomach, bone marrow, skeletal muscle and liver (Margetic et al. 2002). It acts on the hypothalamus to convey satiety and regulate long-term energy balance (Margetic et al. 2002; Mantzoros et al. 2011). Plasma leptin levels correlate positively with adiposity in newborns, children and adults (Considine et al. 1996; Ong et al. 1999). However, obese individuals commonly develop 'leptin resistance' or tolerance – a hyperleptinemic state with a lack of leptin's regulatory effects (Considine et al. 1996).

Cross-sectional studies show that leptin levels in girls rose from 2–15 years in parallel with body weight. Boys had lower leptin levels than girls but they rose in parallel with weight until 10 years, when a decrease was observed until 15 years (Garcia-Mayor et al. 1997; Sharrock et al. 2008). This gender difference is thought to be due to the suppressive effect of androgens on adipokine release (Bottner et al. 2004). Importantly, no one longitudinal study has looked at changes in leptin levels over the entire birth-puberty period. The Mantzoros et al (2009) and Nishimura et al (2009) prospective studies did examine leptin's associations over time, showing, in contrast to adiponectin, that leptin levels are significantly related with one another over both the birth to 3 and 9 to 12-year ranges (Mantzoros et al. 2009; Nishimura et al. 2009). While these results highlight possible developmental differences between adiponectin and leptin, additional data over the entire childhood period are needed.

Known Determinants of Adiponectin and Leptin

Few factors, other than concurrent child size, have been identified that are associated with cord or later-life adiponectin or leptin. Further, most studies have focused on predictors of adipokine levels only at birth. Maternal gestational diabetes, type 1 diabetes and obesity during pregnancy have all been shown to influence cord blood adipokine levels, but the long term effects remain unknown (Lindsay et al. 2004; Cortelazzi et al. 2007; Pirc et al. 2007; Koebnick et al. 2008; Ortega-Senovilla et al. 2011). Importantly, while numerous risk factors for later life obesity have been identified, including maternal nutrition, weight gain during pregnancy, child birth weight and growth rate during infancy, their relationship with adipokines over childhood remains unexamined (Lindsay et al. 2002; Li et al. 2007; Mantzoros et al. 2010; Ortega-Senovilla et al. 2011; Pryor et al. 2011; Carter et al. 2012).

Perinatal Environment - Bisphenol A.

In addition to these perinatal factors, exposure to certain environmental chemicals during fetal development may also affect children's adipokine levels in later life. Of particular concern, BPA ranks among the highest volume production chemicals in the US (Shelby 2008). BPA, in its polymerized form, is used to make epoxy resins and polycarbonate plastics – present in household applications such as water bottles, food containers, canned food, dental fillings and household electronics (Vandenberg et al. 2007). Hydrolysis of the ester bond in these plastics leads to leaching of BPA into the environment. Consistent with its ubiquitous environmental presence, urinary BPA is detected in 93% of the population in the United States (U.S.) (Calafat et al. 2009). In the general U.S. population of children, consumption of soda, school lunches and meals prepared outside of the home, have all been related to increased urinary BPA concentrations (Lakind et al. 2011).

Large cross-sectional epidemiological studies have consistently shown that higher urinary BPA concentrations are positively associated with body mass index (BMI) (Trasande et al. 2012; Wang et al. 2012). Nevertheless, whether higher BPA concentrations actually precede the onset of obesity remains uncertain (Becker et al. 2009; Lakind et al. 2011; Trasande et al. 2012; Wang et al. 2012). In animal models, results are somewhat inconsistent – while some studies find prenatal BPA exposure linked to increased offspring weight (Rubin et al. 2001; Miyawaki et al. 2007; Somm et al. 2009; Hiyama et al. 2011), others find no association (Ryan et al. 2010) or an inverse relationship (Honma et al. 2002; Negishi et al. 2003; Nakamura et al. 2012). Additionally, most animal studies find that responses to prenatal BPA exposure may vary by sex, and this has been explicitly presented in both the Miyawaki et al (2007) and Somm et al (2009) reports, showing increased weight in female rodents only (Miyawaki et al. 2007; Somm et al. 2009).

There are increasing data that BPA exposure may lead to changes in adiponectin and/or leptin levels as well. Miyawaki et al (2007) exposed pregnant mice to BPA-contaminated drinking water, showing that female mice had increased postnatal levels of plasma leptin (Miyawaki et al. 2007). Further, Wei et al (2011) found that both male and female rat offspring had increased leptin levels as a result of perinatal exposure to BPA (Wei et al. 2011). Additionally, in *in vitro* cell models, BPA was found to suppress adiponectin release from adipose tissues, albeit displaying a U-shaped dose-response curve at higher BPA concentrations (Hugo et al. 2008).

To data, only one study has examined these associations in humans, reporting that mothers in the highest quartile of BPA blood concentrations at delivery had boys with increased odds of low adiponectin and high leptin at birth (Chou et al. 2011). However, results of this study are severely limited, given that BPA concentrations were measured in blood collected near time of birth and are likely biased due to contamination from hospital medical interventions (Calafat et al. 2009; Vandentorren et al. 2011; Duty et al. 2013).

Mechanisms

Taking into account rising evidence that prenatal BPA exposure, and other candidate maternal factors, may influence offspring adipokine levels, there is a pressing need to understand molecular mechanisms responsible for this mother – child link. A leading hypothesis involves *in utero* programming of epigenetic patterns, modifying gene transcription and resulting in potentially long-lasting health effects (Waterland et al. 2007; Lustig 2011). The term 'epigenetics' refers to heritable changes to deoxyribonucleic acid (DNA) that do not affect the DNA code but may affect gene expression. Perhaps the best understood and most widespread epigenetic mark is that of DNA methylation. This phenomenon involves attachment of a methyl group to the cytosine base within cytosine-guanine dinucleotides, also known as 'CpG methylation'. Higher CpG methylation within the promoter region of a gene is thought to reduce gene expression (Jaenisch et al. 2003).

A variety of prenatal exposures, including maternal starvation (Heijmans et al. 2008), exposure to lead (Pilsner et al. 2009), polyaromatic hydrocarbons (Perera et al. 2009), and tobacco smoke (Breton et al. 2009), have all been associated with changes in offspring DNA methylation. With respect to BPA, several studies indicate that it can decrease methylation (hypomethylate) in gene promoter regions of estrogen receptor α (Monje et al. 2007) and phosphodiesterase type 4 variant 4 (Ho et al. 2006), leading to altered estrogen signaling.

Using the *Agouti* mouse model, Dolinoy et al (2007) showed that prenatal BPA exposure lead to hypomethylation of the intracisternal A particle (controlling expression of the *Agouti* gene), shifting mouse coat color toward the yellow, commonly obese, phenotype (Dolinoy et al. 2007). Additionally, a recent study reported that treatment of the F0 generation of pregnant rats with a combination of plastics derivatives including BPA resulted in F3 generation methylation changes, accompanied by increased F3 obesity (Manikkam et al. 2013).

An important consideration in examining the DNA methylation – obesity relationship is that virtually no epigenetic marks have displayed strong signals or have been replicated across different studies. For example, Relton et al (2012) measured CpG methylation for 24 genes that were differentially expressed in children with higher vs. lower BMI (Relton et al. 2012). However, of the 44 CpG sites across these genes, none were associated with BMI or fat mass and only one CpG site (within the alkaline phosphatase gene) was associated with child height. Wang et al (2010) screened 27,000 CpG sites for associations with BMI, identifying CpG sites in only two genes (ubiquitin associated gene – *UBASH3A* and tripartite motif gene - *TRIM3*) that withstood replication and multiple testing adjustment (Wang et al. 2010).

An alternative approach, one with a more proof-of-concept nature, involves *a priori* selection of candidate genes. With respect to obesity development, the gene peroxisome proliferator-activated receptor gamma *(PPARγ*) is thought to play a critical role, functioning as the only gene that is both necessary and sufficient for fat cell production. *PPARγ* upregulation has been linked to increased adiponectin, improved insulin sensitivity and decreased leptin at the expense of greater body weight in adults (Maeda et al. 2001) and animals (Kubota et al. 1999;

Larsen et al. 2003; Toruner et al. 2004). Additionally, to date, two *in vitro* studies have reported that BPA exposure may lead to increased *PPARγ* expression (Kwintkiewicz et al. 2010; Wang et al. 2012). However, whether this happens through an epigenetic mechanism remains unknown.

Several studies have examined the role of *PPARγ* CpG methylation in obesity development. Using cultured adipose stem cells, Noer et al (2006) showed that CpG hypomethylation in the *PPARγ* promoter region was associated with adipocyte differentiation (Noer et al. 2006). Additionally, *PPARγ* promoter hypermethylation was found in diabetic mice compared to wild-type mice (Fujiki et al. 2009). While these studies support the idea that CpG methylation regulates *PPARγ* activity and subsequent adipogenesis, no data are available on *PPARγ* methylation or its relationship with obesity in humans.

In conclusion, while literature outlines plausible mechanisms by which the perinatal environment programs altered metabolic health in later life through epigenetic changes, large data gaps remain. The goals of this dissertation were to first understand adiponectin and leptin development over childhood and then evaluate effects of the perinatal environment, possibly through alterations in *PPARγ* methylation, on adipokine levels. We focused on prenatal BPA exposure as separate animal studies show that it may hypomethylate DNA, dysregulate *PPARγ* expression and alter weight and adipokine levels in offspring.

CHAPTER 3

DESCRIPTION OF STUDY DESIGN AND METHODS

CHAMACOS study design

The CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of pesticides and other exposures on growth and development in children living in Salinas Valley, CA (Eskenazi et al. 2004; Eskenazi et al. 2005). Mothers were enrolled during pregnancy between October 1999 and October 2000, with 537 mother-child pairs in the study at delivery and 327 pairs remaining at the 9-year visit. Eligible women were ≥18 years of age, <20 weeks gestation at enrollment, English or Spanish speaking, Medi-Cal eligible and planning to deliver at the county hospital. Women were interviewed at ~13 weeks gestation, ~26 weeks gestation, shortly after delivery, and when their children were 6 months, and 1, 2, 3½, 5, 7, and 9 years of age. During the course of this dissertation project, we have also conducted a 10.5-year visit and are currently performing the 12-year visit. Importantly, our research sought to take advantage of the 1) numerous biological samples (both urine and blood) collected for both mothers and children, 2) detailed child growth measures over time, and 3) extensive assessment of other factors of maternal and child health, including socio-demographic characteristics, lifestyle exposures and diet. Interviews were conducted in Spanish or English by bilingual, bicultural trained interviewers. All study activities were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley. Protocol ID: 2010-03-949.

CHAMACOS – questionnaire data

Sociodemographic information, including maternal age at pregnancy, years of living in US prior to pregnancy, education, and household poverty category was gathered during the first prenatal interview at 14±5 weeks gestation. Data on maternal weight at delivery, infant birth weight, length, and gestational age were obtained from delivery medical records abstracted by a registered nurse. Children were categorized as 'small for gestational age' if their birth weight was $<10^{th}$ percentile for gestational age, controlling for ethnicity, parity and infant sex from national data (Overpeck et al. 1999). Children were considered as 'at term' if they were born at or after 37 weeks of gestation. Gestational weight gain was calculated as mother's weight at delivery from medical records minus self-reported pre-pregnancy weight.

During the second prenatal interview at 26±3 weeks gestation, we used a previously validated food frequency questionnaire (FFQ) to estimate maternal calorie, protein, total fat, saturated fat, fiber and sugar-sweetened beverage consumption during pregnancy (Harley et al. 2005; Warner et al. 2006). The FFQ is based on the Spanish-language Block 98 Questionnaire which was specifically modified for this Mexican-American population, including focus groups to gather information on local foods and food use (Block et al. 1990). Participant mothers were asked about how often they ate a particular food item in the previous 3 months and what the usual portion was using a 72-item questionnaire. This information was then converted into average daily energy and nutrient intake using values from the USDA Nutrient Database for Standard Reference (USDA 1992).

Maternal sugar-sweetened beverage consumption was calculated based on answers in the FFQ and includes number of drinks per week of 100% orange, grapefruit, apple, grape, or other real 100% fruit juice; fruit drinks (Tampico, Sunny D, lemonade, Kool-Aid); or soda. Additionally, we asked about child soda, fast food, and sweet snack consumption and calculated

sugar-sweetened beverage intake when the children were 9 years of age. Household food security (food secure, low food security, and very low food security) was assessed at the 9-year visit using the USDA Spanish short form food security measure (Harrison et al. 2003).

CHAMACOS – anthropometric data

Children' weight and height were measured at the 2, 5, and 9-year visits using a calibrated electronic scale (Tanita Mother-Baby Scale Model 1582, Tanita Corp.) and wall-mounted stadiometer (Seca 222), respectively. Child height was measured in triplicate and the average of measurements was used. Child waist circumference was measured at the 9-year-visit with a tape placed above the crest of the ileum. Measurements were recorded to the nearest 0.1 cm after the child exhaled, performed in triplicate and the average of the measures was used. BMI was calculated as mass in kilograms divided by height in meters squared. Children were categorized as normal weight, overweight or obese using the sex and age-specific BMI cut-offs (85^{th} and 95^{th} percentile, respectively) provided by the 2000 Centers for Disease Control and Prevention (CDC) child growth data (National Center for Health Statistics 2005). Mothers were categorized as normal weight (18.5–24.9kg/m^2), overweight (25–29.9kg/m^2) or obese ($\geq$30kg/m^2) using the standard CDC BMI cutoffs for adults.

Monthly rate of weight gain during the first 6 months of life was calculated as weight at the 6-month visit minus birth weight divided by exact age in months at the 6-month visit and reported in 100 grams/month. This approach to examining infancy weight gain has been previously used and validated by Stettler et al (2002) (Stettler et al. 2002). Monthly rate of length gain during the first 6 months was calculated similarly as length at the 6-month visit minus birth length divided by exact age at the 6-month visit and expressed in centimeters/month.

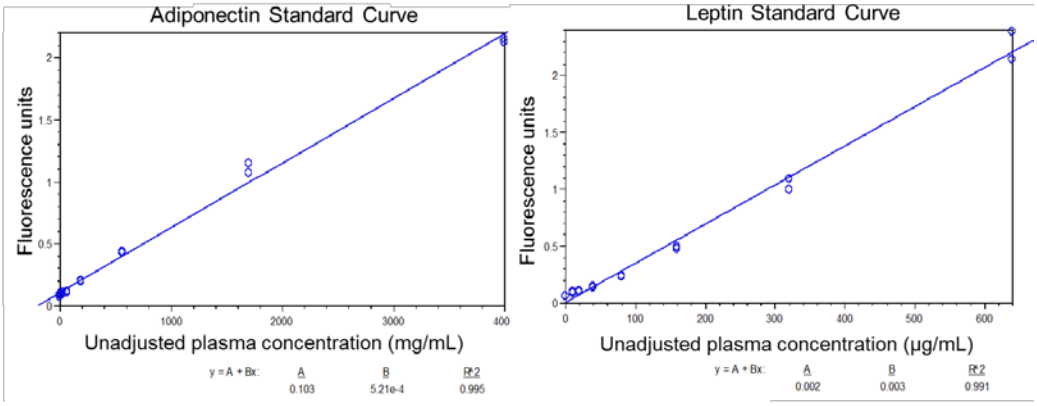
Child 9-year systolic and diastolic blood pressure (SBP and DBP, respectively) were measured at rest in triplicate using a Dinamap 9300 sphygmomanometer. A subgroup of children from the 9-year-visit (N=116) also volunteered to provide an additional fasting blood sample for measurement of blood glucose, cholesterol, triglycerides, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) at 9 years of age. These samples were collected from a convenience sample of volunteers who agreed to return for a second visit during health fairs held between August 2010 and February 2011. Fasting blood was drawn early in the morning and 1ml aliquots of serum were sent to Quest Diagnostics (Salinas, CA) for glucose and lipid profile measurement.

Plasma adiponectin and leptin measurements

Plasma adiponectin and leptin were measured in banked, non-fasting blood samples collected from 146 mother-child pairs at the time of the 9-year visit using enzyme-linked immunoassay (ELISA) RayBio Human Adiponectin and Human Leptin kits. The manufacturer recommended protocol was used with two exceptions: 1) the standard curve for adiponectin was narrowed to smaller values for better resolution while 2) the standard curve was widened for leptin. These changes were necessary to tailor the ELISAs towards the adipokine levels observed in this population. The minimum detectable concentrations for adiponectin and leptin ELISAs were 10 pg/ml and 6 pg/ml respectively. All samples were run in duplicate and the values were averaged. The intra- and inter-plate coefficients of variance (CV) were 3% and 12%, respectively, for adiponectin and 3% and 13%, respectively, for leptin.

Figure 2 shows typical standard curves used for the ELISAs:

Figure 2. Example ELISA Standard Curves for Adiponectin and Leptin



To assess inter-plate variability, we included plasma samples from the same control aliquot of blood for every ELISA plate. An additional measure of quality control included checking for linearity in the standard curve (indicated by high R^2 values – Figure 2).

Maternal and child urinary BPA measurement

Urinary spot samples were collected in sterile, polypropylene urine cups during the first and second half of pregnancy and from children at 9 years of age. Samples were stored at -80^{0}C and shipped to the CDC (Atlanta, GA) for analysis using online solid-phase extraction coupled with isotope-dilution high-performance liquid chromatography tandem mass spectrometry with peak focusing (Ye et al. 2005). Total urinary concentration of BPA (free and conjugated) was measured with a limit of detection (LOD) at 0.4 µg/L. For concentrations below the LOD, we used the instrument-reported values. When no signal was detected, we imputed values at random based on a log-normal probability distribution whose parameters were determined by maximum likelihood estimation (Lubin et al. 2004). Blanks, low (~2.8 µg/L) and high (~10 µg/L) quality control materials were included in all runs. To account for urinary dilution, we corrected maternal BPA concentrations using specific gravity, assessed using a hand-held refractometer (National Instrument Company, Baltimore, MD). Child 9-year BPA concentrations were corrected using urinary creatinine determined by the Vitros CREA assay (Ortho Clinical Diagnostics, NJ).

Child DNA Methylation Measurement

To measure DNA methylation, we used the Infinium Illumina 450K array, which is based on multiplexed genotyping of bisulfite converted genomic DNA. This technology is currently considered as a leading method to measure genome-wide methylation, providing both broad and dense coverage, in total interrogating 485,577 CpG sites over 99% of RefSeq genes. The workflow involves bisulfite conversion of DNA, performed using Zymo Bisulfite Conversion Kits (Zymo Research, Orange, CA). Subsequently, each sample is whole-genome amplified, enzymatically fragmented, purified and applied to the BeadChips according to the Illumina methylation protocol. BeadChips were processed with robotics and analyzed using the Illumina Hi-Scan system at the UC-Berkeley Genomics Core (Barcellos-Director). The relative methylation beta (β, %methylation) for each CpG site is calculated as the ratio of methylated-probe signal to total (methylated + unmethylated) fluorescent signal intensity.

CHAPTER 4

Associations between perinatal factors and adiponectin and leptin in 9-year-old Mexican American Children.

ABSTRACT

Objectives: To 1) determine whether perinatal factors (including maternal anthropometry and nutrition and early life growth measures) are associated with adiponectin and leptin levels in 9-year-old children, and 2) assess relationships between adiponectin, leptin and concurrent lipid profile in these children.

Methods: We measured plasma adiponectin and leptin for 146 mother - 9-year-old child pairs from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS). Data on perinatal factors, including sociodemographics, maternal anthropometry and nutrition, and early life child growth were collected during pregnancy, birth and 6-month visits.

Results: Greater rate of weight and length gain during the first 6 months of life were associated with lower adiponectin in 9-year-olds (β=-2.0, P=0.04; β=-8.2, P=0.02, respectively) adjusting for child BMI. We found no associations between child adipokine levels and either maternal calorie, protein, total fat, saturated fat, fiber, sugar-sweetened beverage consumption during pregnancy or children's concurrent sugar-sweetened beverage and fast food intake. Lipid profile in 9-year-old children closely reflected adiponectin but not leptin levels after adjustment for child BMI. Additionally, we report that child adipokine levels were closely related to their mothers' levels at the 9-year-visit.

Conclusion: Overall, our results support the hypothesis that early life factors may contribute to altered adipokine levels in children.

Introduction

Data from the National Health and Nutrition Examination Survey (NHANES) show a 2.5-fold increase in the prevalence of childhood obesity from 1976-1980 to 2007-2008 for youth of all ethnicities (Ogden et al.). Further, the obesity epidemic disproportionately affects minority populations. In NHANES 2009-2010, Mexican-American children aged 2-19 had significantly higher obesity prevalence compared to their white counterparts (21.2% vs. 14.0%). This disparity was apparent among the youngest age group, with Mexican-American infants and toddlers having the greatest prevalence of high weight-for-recumbent-length of any US ethnicity (15.7%) (Ogden et al. 2012). While the obesity epidemic is on the rise, critical questions about its etiology, potential early life programming, and maternal contribution remain difficult to address.

Studies show that obesity development is accompanied by changes in important metabolism-related hormones, adiponectin and leptin – also known as adipokines (Koerner et al. 2005; Rosen et al. 2006). Adiponectin, a protein hormone secreted almost exclusively by adipose tissue, acts to increase the uptake and catabolism of fatty acids and carbohydrates, promoting insulin sensitivity. In children, hypoadiponectinemia has been associated with the metabolic syndrome and type 2 diabetes (Cruz et al. 2004; Ogawa et al. 2005). Leptin, a hormone synthesized primarily by adipose tissue but also by the placenta, stomach, bone marrow, skeletal muscle and liver, (Green et al. 1995; Margetic et al. 2002) acts on the hypothalamus to convey satiety, thereby regulating the body's energy intake and expenditure (Koerner et al. 2005). Both obese children and adults have been documented to have 'leptin resistance' – a state of hyperleptinemia without leptin's beneficial regulatory control (Considine et al. 1996; Fleisch et al. 2007). Whether adiponectin or leptin disturbance precedes obesity development or is merely a reflection of adipose tissue amount remains unknown. However, there is increasing evidence that adiponectin and leptin may be prenatally determined by the *in utero* environment (Lindsay et al. 2004; Cortelazzi et al. 2007; Pirc et al. 2007; Mantzoros et al. 2010; Ortega-Senovilla et al. 2011). Examining whether candidate factors during pregnancy are associated with later life adipokine levels may provide deeper insight into molecular mechanisms of obesity.

Existing studies have focused on relationships between maternal parameters during pregnancy and adipokine levels at birth. Mothers with gestational and type 1 diabetes, respectively, tended to have infants with lower adiponectin and higher leptin levels in umbilical cord blood (Lindsay et al. 2004; Cortelazzi et al. 2007; Pirc et al. 2007; Koebnick et al. 2008; Ortega-Senovilla et al. 2011). Additionally, one study examined effects of maternal nutrition with respect to cord adipokines, showing that maternal protein intake was inversely related with both leptin (β=-0.22, P=0.03) and adiponectin (β=-0.25, P=0.03) at birth (Mantzoros et al. 2010). Further, comparing adipokine levels between maternal serum collected during pregnancy and cord blood, Weyermann et al (2006) reported a small but highly significant correlation for leptin (r=0.16, P<0.0001) and a weaker association for adiponectin (r=0.07, P=0.07) (Weyermann et al. 2006). There remains a data gap on relationships between maternal and/or early life factors and adiponectin or leptin levels in children as they age (Mantzoros et al. 2009). Finally, while certain perinatal characteristics, including maternal gestational weight gain and infancy growth rate, have been consistently associated with greater later life body mass index (BMI), few data are available on their relationship with child adipokines at older ages (Ong et al. 2006; Oken et al. 2007).

To further characterize the maternal and early life contribution to child metabolic health, we examined whether: 1) maternal anthropometric measures or 2) child growth measures during infancy are associated with children's adiponectin and leptin levels at 9 years of age. We also

aimed to confirm relationships between lipid profiles and adiponectin and leptin levels in 9-year-old children. We examined these associations in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS), a Mexican-American cohort with a high prevalence of child overweight and obesity.

Methods

Subjects and study design

The CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of pesticides and other exposures on child growth and development (Eskenazi et al. 2004; Eskenazi et al. 2005). Mothers were enrolled during pregnancy, with 537 mother-infant pairs in the study at delivery and 327 remaining at the 9-year visit. We selected a random sub-sample of 146 mother-child pairs for analysis of adiponectin and leptin. Mothers in the study were primarily young (mean age of 26.3±5.1 years), married, low-income, Mexican-born, Spanish-speaking women from farmworker households. No differences were seen comparing maternal and child socioeconomic (SES) and lifestyle factors, anthropometric measures or lipid profile between the sub-sample in these analyses and the overall CHAMACOS cohort.

Women were interviewed at ~13 weeks gestation, ~26 weeks gestation, shortly after delivery, and when their children were 6 months, and 1, 2, 3½, 5, 7 and 9 years of age. Developmental assessments of children, including anthropometrics, were conducted at birth and at the time of each maternal interview. All interviews and assessments were conducted in Spanish or English by bilingual, bicultural interviewers. Details are provided below.

Questionnaire data

Sociodemographic information, including maternal age at pregnancy (18 through 29 years and ≥ 30 years), years of living in US prior to pregnancy (<1 year, 1-10 years, >10 years) and education (≤6th grade, 7-12 grade, and ≥high school) was gathered at the initial prenatal visit (~13 weeks gestation). Additionally, women were asked to report their pre-pregnancy weight at the 13-week visit. At the second pregnancy visit (~26 weeks gestation), we used a previously validated food frequency questionnaire (FFQ) to estimate maternal calorie, protein, total fat, saturated fat, fiber and sugar-sweetened beverage consumption during pregnancy (Harley et al. 2005; Warner et al. 2006). The FFQ is based on the Spanish-language Block 98 Questionnaire which was specifically modified for this Mexican-American population, including focus groups to gather information on local foods and food use (Block et al. 1990). Participant mothers were asked about how often they ate a particular food item in the previous 3 months and what the usual portion was using a 72-item questionnaire. This information was then converted into average daily energy and nutrient intake using values from the USDA Nutrient Database for Standard Reference (USDA 1992). The FFQ used here is described in detail in Harley et al (2005) (Harley et al. 2005).

Maternal sugar-sweetened beverage consumption was calculated based on answers in the FFQ and includes number of drinks per week of 100% orange, grapefruit, apple, grape, or other real 100% fruit juice; fruit drinks (Tampico, Sunny D, lemonade, Kool-Aid); or soda. This variable was categorized into tertiles of 0-8, 9-16 and 17+ drinks per week. Additionally, we assessed child fast food and sugar-sweetened beverage intake when the children were 9 years of age. Household food security (food secure, low food security, and very low food security) was

assessed at the 9-year visit using the USDA Spanish short form food security measure (Bickel et al. 2000; Harrison et al. 2003).

Data on pregnancy weight gain, child birth weight, length, and gestational age were obtained from delivery medical records abstracted by a registered nurse. Children were categorized as small-for-gestational age if their birth weight was <10th percentile for gestational age, adjusting for ethnicity, parity and infant sex from national data (Overpeck et al. 1999). Children were considered to be 'at term' if they were born at or after 37 weeks of gestation.

Information about smoking during pregnancy was obtained at each pregnancy interview. Since 140 of the 146 mothers reported no use of tobacco at the prenatal visits, associations of maternal smoking during pregnancy on with adipokines were not examined.

Anthropometric measurements

An electronic scale (Tanita Mother-Baby Scale Model 1582, Tanita Corp.) was used to measure recumbent infant weight at the 6-month visit and child and mother weight at the 9-year visit. Infant length and child height were measured in triplicate using a measuring mat and stadiometer, respectively, and the average of measurements was used. BMI was calculated as mass in kilograms divided by height in meters squared. Children were categorized as normal weight, overweight or obese using the sex and age-specific BMI cut-offs (85^{th} and 95^{th} percentile, respectively) provided by the 2000 Centers for Disease Control and Prevention (CDC) child growth data.

Monthly rate of weight gain during the first 6 months of life was calculated as weight at the 6-month visit minus birth weight divided by exact age in months at the 6-month visit and reported in 100 grams/month. This approach to examining infancy weight gain has been previously used and validated by Stettler et al (2002) (Stettler et al. 2002). Monthly rate of length gain during the first 6 months was calculated similarly as length at the 6-month visit minus birth length divided by exact age at the 6-month visit and expressed in centimeters/month. Child 9-year systolic and diastolic blood pressure (SBP and DBP, respectively) were measured at rest in triplicate using a Dinamap 9300 sphygmomanometer.

Plasma adiponectin and leptin measurements

Plasma adiponectin and leptin were measured in banked, non-fasting blood samples collected from 146 mother-child pairs at the time of the 9-year visit using enzyme-linked immunoassay (ELISA) RayBio Human Adiponectin and Human Leptin kits. The manufacturer recommended protocol was used with two exceptions: 1) the standard curve for adiponectin was narrowed to smaller values for better resolution while 2) the standard curve was widened for leptin. These changes were necessary to tailor the ELISAs towards the adipokine levels observed in this population. The minimum detectable concentrations for adiponectin and leptin ELISAs were 10 pg/ml and 6 pg/ml respectively. All samples were run in duplicate and the values were averaged. The intra- and inter-plate coefficients of variance (CV) were 4% and 12%, respectively, for adiponectin and 3% and 15%, respectively, for leptin.

Fasting blood lipid profile measurements

A subgroup of 56 of the 146 children also volunteered to provide an additional fasting blood sample for measurement of blood glucose, cholesterol, triglycerides, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) at 9 years of age. These samples were collected from a convenience sample of volunteers who agreed

to return for a second visit during health fairs held between August 2010 and February 2011. Fasting blood was drawn early in the morning and 1ml aliquots of serum were sent to Quest Diagnostics (Salinas, CA) for glucose and lipid profile measurement.

Statistical analyses

Adiponectin levels were normally distributed, but leptin levels were right-skewed. Thus, analyses using leptin levels as the dependent variable report geometric means or use base-ten log-transformed values. We used analysis of variance (ANOVA) to examine differences in child adipokine levels by child's sex, weight status and maternal descriptive characteristics (Table 1). We then used multivariate linear regression to characterize associations between perinatal factors and child adipokine levels (Table 2) and between child adipokines and lipid profiles (Table 3), controlling for 9-year child BMI. P-values < 0.05 were considered statistically significant. All statistical analyses were conducted using STATA 12 (College Station, TX) for Windows.

Results

Maternal and child characteristics

Of the 146 9-year-old children in this study, there were a similar number of girls (N=74) and boys (N=72) (Table 1). Children were primarily delivered at term (≥37 weeks, N=138) and appropriate for gestational age (N=119). Few children were small for gestational age (<10th percentile, N=9) There was a very high prevalence of obesity (41%) in children and overall 54% were overweight or obese (Table 1). Additionally, we observed a bimodal distribution for child BMI categories, with most children either normal weight (46%) or obese (41%). The average age of the mothers was 26.3 years. Nearly half (47%) had ≤6th grade education and, at the time of their pregnancy, 77% had resided in the US 10 years or less. Low or very low food security was documented in 37% of families.

Table 1: Study cohort characteristics and 9-year-old child adiponectin and leptin levels (N = 146).					
		9 Year Child			
		Adiponectin (µg/ml)		Leptin (ng/ml)	
	N (%)	Mean (95% CI)	P	Mean[1] (95% CI)	P
Child Sex					
Boy	72 (49)	42.9 (38.2, 47.6)		6.4 (4.9, 8.2)	
Girl	74 (51)	45.4 (41.3, 49.4)	0.43	9.1 (6.8, 12.1)	0.07
Child Gestational Age at Birth					
34 - 37 Weeks	8 (5)	37.3 (26.1, 48.5)		9.0 (4.2, 19.1)	
≥ 37 Weeks	138 (95)	44.6 (41.4, 47.8)	0.29	7.5 (6.2, 9.2)	0.69
Child Birth Size					
Small for gestational age (<10th %ile)	9 (6)	35.1 (18.0, 52.3)		6.3 (1.9, 21.1)	
Appropriate for gestational age	119 (82)	45.8 (42.5, 49.1)		7.6 (6.2, 9.4)	
Large for gestational age (> 90th %ile)	18 (18)	37.8 (28.7, 46.9)	0.08	8.4 (4.5, 15.6)	0.84
Child BMI at 9 Years[2]					
Normal (≤ 85th %ile)	67 (46)	49.2 (45.6, 52.7)		3.3 (2.7, 3.9)	
Overweight (> 85th, < 95 %ile)	19 (13)	52.1 (42.0, 62.3)		7.8 (5.3, 11.4)	
Obese (≥ 95 %ile)	60 (41)	36.1 (31.0, 41.1)	<0.001	19.4 (15.3, 24.6)	<0.001
Maternal Age at Pregnancy					
18-23 Years Old	46 (32)	44.8 (38.8, 50.7)		8.7 (6.2, 12.2)	
24-29 Years Old	63 (43)	46.5 (42.0, 51.0)		6.9 (5.2, 9.2)	
30-41 Years Old	37 (25)	39.4 (33.4, 45.5)	0.19	7.6 (4.9, 11.8)	0.59
Maternal Yrs in US at Pregnancy					
<1	31 (21)	44.9 (38.0, 51.7)		7.5 (4.8, 11.5)	
1-10	81 (56)	46.1 (42.0, 50.2)		7.2 (5.6, 9.3)	
>10	34 (23)	38.9 (32.2, 45.7)	0.17	8.8 (5.5, 13.9)	0.72
Maternal Education at Pregnancy					
<= 6th Grade	68 (47)	45.3 (40.6, 49.9)		7.5 (5.6, 9.9)	
7-12 Grade	50 (34)	43.3 (38.1, 48.5)		7.4 (5.2, 10.6)	
>= High School	28 (19)	43.0 (35.5, 50.4)	0.8	8.4 (5.6, 12.5)	0.89
Maternal Sugar Sweetened					
Beverage Use in Pregnancy					
0-8 Drinks/Week	44 (31)	44.8 (38.5, 51.0)		8.3 (5.8, 12.0)	
9-16 Drinks/Week	51 (35)	44.5 (39.9, 49.2)		6.8 (4.7, 9.7)	
17+ Drinks/Week	49 (34)	43.6 (37.9, 49.4)	0.96	8.0 (5.9, 10.8)	0.66
Household Food Security[3]					
Food Secure	92 (63)	43.1 (39.5, 46.7)		6.6 (5.2, 8.5)	
Low Food Security	41 (28)	46.4 (39.3, 53.6)		9.0 (6.3, 12.9)	
Very Low Food Security	13 (9)	44.5 (34.8, 54.1)	0.64	11.7 (6.7, 20.4)	0.15

1: Geometric mean. 2: Child's weight status was determined using age and sex adjusted body mass index cut offs for 85th and 95th percentiles from CDC child growth charts. 3: At 9 year visit.

Child adipokines by study cohort characteristics

Overall, mean child adiponectin concentrations were 44.2 µg/ml (95% CI 41.1, 47.2) ranging between 8.2 and 92.5 µg/ml. Mean child leptin concentrations were 7.6 ng/ml (95% CI 6.3, 9.2), ranging between 0.3 and 93.3 ng/ml. Boys tended to have slightly lower adiponectin (42.9 vs. 45.4 µg/ml, p=0.43) and leptin levels (6.4 vs. 9.1 ng/ml, p=0.07) compared to girls. Neither adipokine was different across gestational age categories. There was a suggestion that appropriate for gestational age children had the highest levels of adiponectin (P=0.08). As expected, we observed significantly lower adiponectin (P<0.001) and higher leptin (P<0.001) over increasing BMI categories in 9-year-old children. Children's adiponectin and leptin levels were not related to maternal age, years in US, education, sugar-sweetened beverage consumption during pregnancy or household food security measured at the 9-year visit. Additionally, other maternal dietary variables during pregnancy, including calorie, protein, total fat, saturated fat, and fiber consumption or 9-year child fast food or sugar-sweetened beverage intake were also not associated with child adiponectin or leptin (data not shown).

Associations of pregnancy and early life parameters with 9-year-old child adipokines

Relationships between maternal and early life anthropometric parameters and child adipokines at 9 years are summarized in Table 2. While birth weight and length were not associated with adipokine levels, increased weight and length gain in the first 6 months of life were negatively related to adiponectin (β=-2.8, P=0.007; β=-9.7, P=0.007, respectively) but not leptin levels in 9-year-olds. After adjustment for 9-year child BMI, these associations with adiponectin were slightly attenuated but remained significant (β=-2.0, P=0.04; β=-8.2, P=0.02).

Higher maternal pre-pregnancy BMI was associated with both lower adiponectin (β=-0.8, P=0.006) and higher leptin (β=0.03, P<0.001) in 9-year-old children. However, adjusting these relationships for child BMI eliminated these associations (β=-0.3, P=0.28; β=0.001, P=0.84, for adiponectin and leptin, respectively). We found no associations between maternal weight gain during pregnancy and either adiponectin or leptin in the 9-year-olds.

Additionally, mother's adiponectin and leptin levels (measured at the 9-year visit) were strongly related to concurrent measures of their child's adiponectin (β=0.4, P<0.001) and leptin (β=0.42, P<0.001) levels, respectively, and these associations remained significant after adjustment for 9-year child BMI (β=0.3, P=0.001, β=0.23, P=0.002, respectively) (Table 2).

Table 2: Associations of maternal and early life anthropometric parameters with child adiponectin and leptin levels at age 9.						
			9 Year Child			
			Adiponectin (µg/ml)		Leptin (logged)	
Characteristic	**N**	**Mean[1] ± SD (95% CI)**	**Beta[2] (95% CI)**	**P**	**Beta (95% CI)**	**P**
Birth Weight (kg)						
Crude[3]	146	3.5 ± 0.5 (3.4, 3.6)	-4.7 (-12.0, 2.6)	0.2	0.12 (-0.08, 0.31)	0.26
Adjusted[3,4]	146		-1.6 (-8.6, 5.3)	0.64	-0.06 (-0.19, 0.08)	0.4
Birth Length (cm)						
Crude[3]	146	50.5 ± 2.6 (50.0, 50.9)	-0.2 (-1.5, 1.2)	0.83	0.02 (-0.01, 0.06)	0.23
Adjusted[3,4]	146		-0.1 (-1.4, 1.2)	0.88	0.02 (-0.01, 0.05)	0.12
Weight Gain in First 6 Months of Life (100g/month)						
Crude	133	7.4 ± 1.6 (7.1, 7.7)	-2.8 (-4.8, -0.8)	0.007	0.04 (-0.01, 0.1)	0.15
Adjusted[4]	133		-2.0 (-1.79, -0.5)	0.04	0.01 (-0.05, 0.03)	0.61
Length Gain in First 6 Months of Life (cm/month)						
Crude	133	2.6 ± 0.5 (2.5, 2.7)	-9.7 (-16.7, -2.8)	0.007	-0.001 (-0.19, 0.19)	0.99
Adjusted[4]	133		-8.2 (-15.0, -1.5)	0.02	-0.1 (-0.23, 0.03)	0.14
Child 9Y Adiponectin (µg/ml)						
Crude	146	44.2 ± 18.8 (41.1, 47.2)	-----		-0.007 (-0.01, -0.002)	0.003
Adjusted[4]	146		-----		0.001 (-0.002, 0.004)	0.56
Child 9Y Leptin						
Crude	146	7.6 ± 3.2 (6.3, 9.2)	-8.9 (-14.7, -3.0)	0.003	-----	
Adjusted[4]	146		2.5 (-5.9, 10.8)	0.56	-----	
Pre-Pregnancy BMI						
Crude	146	27.4 ± 5.4 (26.5, 28.2)	-0.8 (-1.4, -0.2)	0.006	0.03 (0.01, 0.04)	<0.001
Adjusted[4]	146		-0.3 (-0.9, .3)	0.28	0.001 (-0.01, 0.01)	0.84
Weight Gain During Pregnancy (kg)						
Crude	146	13.2 ± 5.1 (12.4, 14.0)	-0.1 (-0.8, 0.5)	0.65	0.01 (-0.01, 0.02)	0.43
Adjusted[4]	146		0.03 (-0.5, 0.6)	0.91	-0.003 (-0.01, 0.01)	0.61
Maternal Adiponectin At 9 Year Visit (µg/ml)						
Crude	146	29.2 ± 13.7 (26.9, 31.4)	0.4 (0.2, 0.6)	<0.001	-0.0002 (-0.01, 0.01)	0.95
Adjusted[4]	146		0.3 (0.1, 0.6)	0.001	0.003 (-0.002, 0.01)	0.23
Maternal Leptin At 9 Year Visit						
Crude	146	22.3 ± 2.4 (19.3, 25.7)	-9.3 (-17.4, -1.2)	0.02	0.42 (0.21, 0.63)	<0.001
Adjusted[4]	146		-5.9 (-13.7, 1.8)	0.13	0.23 (0.08, 0.38)	0.002

1: Geometric mean for leptin. 2: Linear regression estimate. 3: Model adjusted for gestational age. 4: Model adjusted for 9-year child BMI.

Relationships between adiponectin, leptin and lipid profile in children

Table 3 shows the mean, standard deviation and range for lipid profile and blood pressure measurements for the 9-year-olds. The mean levels were within recommended guidelines for children: glucose (89±6 mg/dL), cholesterol (151±30 mg/dL), triglycerides (83±42 mg/dL), VLDL (17±8 mg/dL), LDL (82±26 mg/dL), HDL (52±12 mg/dL), SBP (97.4±11.1 mmHg) and DBP (53.4±5.9 mmHG).

As expected, 9-year child BMI was positively associated with triglycerides (β=4.8, P<0.001), VLDL (β=1.0, P<0.001), SBP (β=1.6, P<0.001), DBP (β=0.5, P<0.001), and negatively related to HDL (β=-1.5, P<0.001) (not shown). We did not find any differences in lipid profile between sexes, except that boys had slightly higher fasting blood glucose compared to girls (90.9 vs. 87.6 mg/dL, P=0.05).

Increased adiponectin levels were associated with lower triglycerides, VLDL, systolic and diastolic blood pressure and higher HDL in crude analyses. After adjusting for 9-year child BMI, associations of adiponectin with triglycerides (β=-0.5, P=0.03), VLDL (β=-0.1, P=0.03) and HDL (β=0.1, P=0.02) persisted (Table 3). Increased leptin was associated with higher triglycerides, VLDL cholesterol, systolic and diastolic blood pressure and lower HDL cholesterol in crude analyses. After controlling for child BMI, the associations with SBP (β=7.6, P<0.001) and DBP (β=3.6, P=0.02) remained.

Table 3: Associations between child adiponectin, leptin and metabolic parameters, including lipid profile (N = 56) and blood pressure (N = 122).

	Child Metabolic Parameters							
	Glucose (mg/dL)	**Cholesterol (mg/dL)**	**TG (mg/dL)**	**VLDL (mg/dL)**	**LDL (mg/dL)**	**HDL (mg/dL)**	**SBP (mmHg)**	**DBP (mmHg)**
Mean ± SD, Range	89±6, 73-101	151±30, 94-228	83±42, 30-195	17±8, 6-39	82±26, 35-156	52±12, 27-98	97.4±11.1, 74.5 - 133	53.4±5.9, 37.5-70
Child Adiponectin								
Crude Beta (P)	-0.0004 (0.99)	-0.04 (0.81)	-0.8 (<0.001)	-0.2 (<0.001)	-0.1 (0.44)	0.2 (<0.001)	-0.2 (0.004)	-0.1 (0.04)
Adjusting for 9Yr BMI (P)	0.01 (0.81)	-0.1 (0.63)	-0.5 (0.03)	-0.1 (0.03)	-0.1 (0.44)	0.1 (0.02)	-0.01 (0.86)	-0.02 (0.58)
Child Leptin (logged)								
Crude Beta (P)	1.5 (0.38)	-6.3 (0.47)	31.9 (0.007)	6.4 (0.007)	1.1 (0.88)	-13.8 (<0.001)	15.1 (<0.001)	5.3 (<0.001)
Adjusting for 9Yr BMI (P)	1.8 (0.53)	-7.3 (0.59)	-13.8 (0.38)	-2.9 (0.4)	-0.5 (0.97)	-4.9 (0.23)	7.6 (<0.001)	3.6 (0.02)

Abbreviations: BMI, Body mass index; TG, Triglycerides; VLDL, Very low-density lipoprotein; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

Discussion

In this Mexican-American cohort with a high prevalence of obesity, we aimed to fill the data gap on relationships between maternal and early life parameters on 9-year-old child adiponectin and leptin. Our analyses show that children with an increased rate of weight or length gain in the first 6 months of life tend to have lower levels of adiponectin at 9 years and these associations remained after adjusting for 9-year child BMI. Data on relationships between infancy growth rate and later life adipokines are limited and our results are in agreement with one of the only studies currently available. Larnkjaer et al (2010). showed that increased weight gain during the first 3 or 9 months of life was negatively associated with adiponectin, but not leptin, in 17 year olds, adjusting for body fat (N=60) (Larnkjaer et al. 2010). Taken together, these findings add support to the hypothesis that early life growth rate may be an important contributor to altered adiponectin levels at older ages.

However, it remains a challenge to determine whether such associations are due to direct effects on adipokine levels or merely reflective of underlying obesity and increased fat mass. Further, the biological mechanisms linking infancy growth rate and later life adipokine levels are poorly understood. Early life programming of energy balance and regulation may underlie both increased size gain and alterations in adipokine levels which persist into childhood and additional research is needed to elucidate these relationships (Lustig 2011). Finally, it is important to note that in our study, calculation of weight gain is in accordance with methods used in previous publications and is not adjusted for accompanying length gain (Stettler et al. 2002; Ong et al. 2006). Whether it is excess weight gain relative to length gain during infancy that may affect future adipokine levels remains an important question to answer.

While several reports show weak or no correlation between maternal and fetal adipokines, we found a strong relationship between maternal (at the 9-year visit) and 9-year-old child adiponectin and leptin levels in the CHAMACOS cohort (Lepercq et al. 2001; Sivan et al. 2003; Weyermann et al. 2006). In addition, large studies (HERITAGE, Framingham Heart and the National Heart, Lung and Blood Institute Family cohorts) have consistently reported heritability of obesity or obesity-related traits to be in the 40-80% range (Rice et al. 1997; Borecki et al. 1998; McQueen et al. 2003). Currently, data on adiponectin and leptin heritability in Mexican-American children are available from only one cross-sectional cohort, showing a moderate heritability of leptin (36%) and high heritability of adiponectin (97%) (Cai et al. 2008). Given that heritability of BMI may vary with age, it is important to examine the relative genetic, developmental programming and environmental/cultural contributions to adipokine levels over childhood (Haworth et al. 2008).

We did not find significant associations between birth weight or length and adipokine levels in 9-year-old Mexican-American children. While several studies have focused on the relationship between birth weight and adipokine levels in cord blood, few data are available tracking the relationship of birth size on adiponectin and leptin through childhood. Bozzola et al (2010) found that both small and appropriate for gestational age infants had statistically similar adiponectin levels over the birth to 1-year-old period (Bozzola et al. 2010). However, two other studies have shown a positive relationship between cord adiponectin and birth weight (Sivan et al. 2003; Tsai et al. 2004). These conflicting results suggest that the relationship between child weight and adipokine levels may change over childhood, from no or weak associations at birth to strong correlations at later years, as observed in this report.

Previously, several large prospective cohorts have identified a relationship between excess maternal gestational weight gain and increased risk of obesity in their offspring (Oken et al. 2007; Schack-Nielsen et al. 2010). While we did not find similar associations with adipokines in 9-year-old children, this may be due to the specific characteristics of the CHAMACOS cohort or related to the relatively modest sample size. Additionally, pre-pregnancy weight was self-reported and likely an underestimate of true weight (Engstrom et al. 2003). This would skew gestational weight gain to larger values, potentially biasing effect estimates toward the null.

We found no significant associations between maternal sugar-sweetened beverage, calorie, protein, total fat, saturated fat or fiber intake and adipokine levels in 9-year-old children. Available evidence shows that malnutrition during pregnancy increases risk of obesity in the offspring but data on child adipokines are limited (Tarry-Adkins et al. 2011). To date, only one study is available, reporting that maternal protein consumption was associated with marginally lower adiponectin and leptin levels at birth (Mantzoros et al. 2010). An earlier report on CHAMACOS 5-year-old children found no associations between either soda or fast food intake and child weight status (Rosas et al. 2011). Echoing this, we found that neither children's sugar-sweetened beverage use nor fast food consumption at 9 years were related to their adipokine levels. Overall, we suggest that the complex biological and environmental interactions in older children may mask the relatively smaller effects of diet on adipokines.

In our cohort, leptin levels in children were tightly linked to participant body weight while adiponectin was more reflective of metabolic parameters. Results from our analyses on associations between adipokine levels, lipid profile and blood pressure are similar to findings from several other studies (Kavazarakis et al. 2001; Butte et al. 2005). Data from the Viva la Familia cohort showed that adiponectin was inversely associated with the homeostasis model of insulin resistance (HOMA-IR) and triglycerides/HDL ratio. With respect to leptin, reports indicate that its relationships with lipid profile are largely mediated by BMI (Kavazarakis et al. 2001; Gannage-Yared et al. 2006).

In summary, in this cohort of Mexican-American children with a high prevalence of obesity, greater infancy weight and length gain were associated with lower levels of adiponectin at 9 years, adjusting for child BMI. In turn, decreased adiponectin was related to an adverse lipid profile. Additionally, we report that child adiponectin and leptin were closely related to their mothers' levels at the 9-year visit. A strength of this study is the unique nature of the CHAMACOS birth cohort, having gathered extensive biological, anthropometric and questionnaire-based data on Mexican-American children from the prenatal period into puberty. Limitations include: 1) it remains uncertain whether the early life anthropometric measures examined are independent risk factors for later life adipokine changes and 2) this study was conducted on a cohort of largely first generation, immigrant, relatively low SES Mexican-American families from an agricultural community and results may not be fully generalizable to other populations.

CHAPTER 5

Adiponectin and Leptin Trajectories in Mexican-American Children from Birth to 9 Years of Age.

ABSTRACT

Objectives: To address molecular mechanisms underlying obesity, we examined adiponectin and leptin development over childhood.

Methods: Plasma adiponectin and leptin were measured in 80 Mexican-American children at time of birth and again at 2, 5, and 9 years from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS). We used a mixture modeling approach to identify growth patterns in adipokine trajectories from birth to 9 years.

Results: Leptin was positively related to child body size at all ages, however adiponectin had inverse and weaker associations with BMI at 2, 5, and 9 years. Correlations over the 0 – 2, 2 – 5, and 5 – 9-year periods increased for both leptin (r=0.06, 0.31 and 0.62) and adiponectin (r=0.25, 0.41 and 0.46). Our mixture modeling approach identified three trajectory clusters for both leptin (1L, 2L, and 3L) and adiponectin (1A, 2A, and 3A). While leptin groups were most separated over the 2 – 9-year period, adiponectin trajectories displayed greatest heterogeneity from birth to 2 years. Children with greater birth weight and those whose mothers consumed more sugar-sweetened beverages during pregnancy were at risk of belonging to the rapidly-rising 2L group (OR=1.21 95% CI 1.03, 1.43, compared to stable group 3L) and steep-dropping 1A group (OR=1.08, 95% CI 1.01, 1.17, compared to stable group 3A), respectively.

Conclusion: Our results highlight developmental differences in leptin and adiponectin over the childhood period. Leptin closely reflects child body size however factors affecting adiponectin and long-term consequences of its changes over infancy need to be further explored.

Introduction

Recent data show that children may follow different growth patterns and that those who display excessively rapid body mass index (BMI) gains may be at higher risk for adult obesity and adverse metabolic health (Li et al. 2007; Lammi et al. 2009; Pryor et al. 2011; Carter et al. 2012; Wen et al. 2012). However, longitudinal data demonstrating changes in critical energy balance hormones, such as adiponectin and leptin (commonly termed adipokines), are limited. Examining adipokine trajectories over childhood may help shed further light on obesity etiology and heterogeneity of growth patterns.

Adiponectin, a protein hormone secreted by adipose tissue, targets muscle and liver to increase uptake and catabolism of fatty acids and carbohydrates, promoting insulin sensitivity (Kadowaki et al. 2005). In children, lower adiponectin levels are associated with both metabolic syndrome and type 2 diabetes (Cruz et al. 2004; Shaibi et al. 2007). Further, while there is a well-established inverse relationship between adiponectin and BMI in school-age children, this is less clear in newborns. Several studies report a positive relationship between adiponectin and birth weight but others do not (Lindsay et al. 2003; Sivan et al. 2003; Mantzoros et al. 2004; Bozzola et al. 2010).

Leptin, a hormone synthesized primarily by adipose tissue but also by the stomach, skeletal muscle and liver, acts on the hypothalamus to convey satiety and regulate long-term energy balance (Margetic et al. 2002; Mantzoros et al. 2011). Plasma leptin levels correlate positively with adiposity in newborns, children and adults (Considine et al. 1996; Ong et al. 1999). However, obese individuals commonly develop 'leptin resistance' or tolerance – a hyperleptinemic state with a lack of leptin's regulatory effects (Considine et al. 1996).

An important and yet relatively unaddressed question is whether early life adipokine measures predict future adipokine levels and obesity-related outcomes. Only two prospective cohorts have explicitly examined such associations, both over a relatively narrow age range (Mantzoros et al. 2009; Nishimura et al. 2009). Data from Project Viva (N=588) showed that cord blood adiponectin was positively related to central obesity ($p=0.04$) but not with adiponectin at 3 years (Mantzoros et al. 2009). Cord blood leptin was negatively related to BMI ($p<0.001$) and positively related to leptin ($p=0.001$) at 3 years. Additionally, a report on older children (N=519) showed that adiponectin and leptin in 9 to 10-year-olds were directly and significantly correlated with their respective adipokine levels, three years later ($p<0.001$ for both) (Nishimura et al. 2009). While there is some evidence that early and later life adipokine levels are associated, these relationships remain unclear over the childhood period.

Emerging data show that the perinatal environment may influence adipokine trajectories. We have previously shown that greater rate of weight and length gain in the first 6 months of life is negatively associated with 9-year adiponectin levels, adjusting for concurrent child BMI (Volberg et al. 2012). Further, maternal nutrition, gestational diabetes, weight gain during pregnancy and child birth weight have been previously identified as risk factors for obesity and adverse metabolic health in later life, but their relationship with child adipokines remains poorly understood (Lindsay et al. 2002; Li et al. 2007; Mantzoros et al. 2010; Ortega-Senovilla et al. 2011; Pryor et al. 2011; Carter et al. 2012).

To address current data gaps on adipokine development over childhood, we measured adiponectin and leptin levels in a prospective cohort of children at birth, 2, 5, and 9 years. We determined their associations between time points and with child BMI at each age. Further, using a mixture modeling approach, we identified groups of children with distinct adipokine trajectories and examined whether demographic, fetal growth and dietary factors are associated

with these groups. Analyses were performed using data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS), a Mexican-American cohort with a high prevalence of child obesity (Warner et al. 2006; Harley et al. 2013).

Methods

Subjects and study design

The CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of pesticides and other exposures on growth and development in children living in Salinas Valley, CA (Eskenazi et al. 2004; Eskenazi et al. 2005). Mothers were enrolled during pregnancy between October 1999 and October 2000, with 537 mother-child pairs in the study at delivery and 327 pairs remaining at the 9-year visit. Eligible women were ≥18 years of age, <20 weeks gestation at enrollment, English or Spanish speaking, Medi-Cal eligible and planning to deliver at the county hospital. Women were interviewed at ~13 weeks gestation, ~26 weeks gestation, shortly after delivery, and when their children were 6 months, and 1, 2, 3½, 5, 7, and 9 years of age. Developmental assessments of children, including anthropometrics, were conducted at birth and at the time of each maternal interview. Adiponectin and leptin were measured on a convenience sample of 80 children having blood samples at birth, 2, 5, and 9 years and complete anthropometric and demographic data. No differences were observed comparing maternal and child demographic and anthropometric measures between this sub-sample and the overall CHAMACOS cohort.

Ethics Statement

We obtained signed informed consent from all women at the time of enrollment in the study. All study procedures were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley. IRB Protocol 2010-03-949.

Questionnaire data

Sociodemographic information, including maternal age at pregnancy, years of living in US prior to pregnancy, education, and household poverty category was gathered during the first prenatal interview at 14±5 weeks gestation. Interviews were conducted in Spanish or English by bilingual, bicultural trained interviewers. Maternal pre-pregnancy BMI was calculated using the mother's self-reported pre-pregnancy weight and measured height. During the second prenatal interview at 26±3 weeks gestation we used a previously validated food frequency questionnaire (FFQ) to assess maternal sugar-sweetened beverage consumption (Block et al. 1990; Harley et al. 2005). In brief, participant mothers were asked to report number of drinks in the last 3 months of 100% orange, grapefruit, apple, grape, or other real 100% fruit juice; fruit drinks (Tampico, Sunny D, lemonade, Kool-Aid); or non-diet soda (recoded as drinks per week). This FFQ is based on the Spanish-language Block 98 Questionnaire and was modified for use in the CHAMACOS Mexican-American population (Block et al. 1990). Additional details on the FFQ used here are available in Harley et al (Harley et al. 2005). Data on maternal weight at delivery, infant birth weight, length, and gestational age were obtained from delivery medical records abstracted by a registered nurse. Children were categorized as 'small for gestational age' if their birth weight was <10th percentile for gestational age, controlling for ethnicity, parity and infant sex from national data (Overpeck et al. 1999). Children were considered as 'at term' if they were born at or after 37 weeks of gestation. Gestational weight gain was calculated as mother's weight

at delivery from medical records minus self-reported pre-pregnancy weight. Associations between maternal smoking and adipokines were not examined as 76 of the 80 mothers reported no use of tobacco at the prenatal visits.

Anthropometric measurements

Children' weight and height were measured at the 2, 5, and 9-year visits using a calibrated electronic scale (Tanita Mother-Baby Scale Model 1582, Tanita Corp.) and stadiometer, respectively. Child height was measured in triplicate and the average of measurements was used. Child waist circumference was measured at the 9-year-visit with a tape placed above the crest of the ileum. Measurements were recorded to the nearest 0.1 cm after the child exhaled, performed in triplicate and the average of the measures was used. BMI was calculated as mass in kilograms divided by height in meters squared. Children were categorized as normal weight, overweight or obese using the sex and age-specific BMI cut-offs (85^{th} and 95^{th} percentile, respectively) provided by the 2000 Centers for Disease Control and Prevention (CDC) child growth data. Mothers were categorized as normal weight (18.5–24.9kg/m^2), overweight (25–29.9kg/m^2) or obese (≥30kg/m^2) using the standard CDC BMI cutoffs for adults.

Plasma adiponectin and leptin measurements

Adiponectin and leptin were measured in banked blood plasma samples stored at -80ºC using enzyme-linked immunoassay (ELISA) RayBio Human Adiponectin and Human Leptin kits (Norcross, GA). The minimum detectable concentrations for adiponectin and leptin ELISAs were 10pg/ml and 6pg/ml respectively. All samples were run in duplicate and the values were averaged. The intra- and inter-plate coefficients of variance (CV) were 3% and 12%, respectively, for adiponectin and 3% and 13%, respectively, for leptin.

Statistical analyses

Adiponectin levels were approximately normally distributed, but leptin levels were right-skewed at all four time points. Thus, all leptin-based analyses use base-ten log-transformed values. We used Pearson's correlation coefficient and linear regression to determine both associations between adipokines measured at different time points and associations between adipokine levels and child anthropometry.

To explore whether there are distinct adipokine trajectory groups, we used vertically-shifted mixture modeling (VSMM). This method is an extension to Gaussian mixture modeling, which is the basis of group-based trajectory modeling (GBTM) and growth mixture models (GMM) and focuses on clustering trajectories based on their shape over time (Nagin 1999; Muthen 2001; Nagin et al. 2010). A detailed description of this approach is provided in Heggeseth et al (Heggeseth et al. 2012). In brief, this method removes individual level means and assumes that within each group, 1) mean adipokine trajectories follow a piecewise polynomial function of time based on a 3^{rd} order B-spline with one knot at the median time, and 2) an exponential working variance-covariance matrix for the repeated adipokine measurements within participants. Overall, this approach seeks to identify mean patterns of adipokine trajectories and assigns individuals to these groups based on highest posterior probabilities using the expectation-maximization (EM) algorithm.

Best fit was assessed comparing the Bayesian information criterion (BIC) for 1 to 5 group models. Group membership was simultaneously modeled using a generalized logit function to estimate odds ratios (ORs) of group membership as a function of early life variables. Pearson's

correlation and linear regression analyses were conducted using STATA 12 (College Station, TX) for Windows. All mixture modeling analyses were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria). P-values < 0.05 were considered statistically significant.

Results

Maternal and child characteristics

Of the 80 children in this study, there were a similar number of boys (N=39) and girls (N=41) (Table 1). Children were delivered primarily at term (≥37 weeks, 95%) and appropriate for gestational age (88%). Very few children were small for gestational age (<10th percentile, 6%) or large for gestational age (>90th percentile, 6%). At 9 years, the majority of children were overweight or obese (54%). At time of pregnancy, mothers tended to be young (25.6 years), have resided in the US 10 years or less (75%) and have low educational attainment (41% with ≤6th grade). Additionally, more than half of the families (56%) were living at or below the federal threshold for poverty. Based on pre-pregnancy BMI, 55% of mothers were overweight or obese.

Table 1: Demographic characteritics of mothers and children from the CHAMACOS Study, Salinas Valley, CA (N=80).

Characteristic	N (%)
Child sex	
Boy	39 (49)
Girl	41 (51)
Child gestational age at birth	
34 - 36 Weeks	4 (5)
≥37 Weeks	76 (95)
Child birth size	
Small for gestational age (<10th percentile)	5 (6)
Appropriate for gestational age	70 (88)
Large for gestational age (>90th percentile)	5 (6)
Child BMI[1] at 9 years	
Normal (≤85th percentile)	37 (46)
Overweight (>85th, <95th percentile)	17 (21)
Obese (≥95th percentile)	26 (33)
Maternal age at pregnancy	
18-24	39 (49)
25-29	22 (27)
30-34	15 (19)
35-45	4 (50
Maternal years in US at pregnancy	
<1	20 (25)
1-10	40 (50)
>10	20 (25)
Maternal education at pregnancy	
≤6th Grade	33 (41)
7-12 Grade	34 (43)
≥High School	13 (16)
Household poverty category at pregnancy	
≤Poverty threshold	45 (56)
>Poverty level but <200% poverty level	32 (40)
≥200% Poverty level	3 (4)
Maternal pre-pregnancy BMI	
Normal (18.5–24.9kg/m^2)	36 (45)
Overweight (25–29.9 kg/m^2)	28 (35)
Obese (≥ 30 kg/m^2)	16 (20)

[1]Child's weight status was determined using age and sex adjusted body mass index cut offs for 85th and 95th percentiles from CDC child growth charts.

Child adipokines by age and gender

Children had highest levels of adiponectin at birth (mean±SD, 112.8±35.1 μg/ml), with a subsequent decrease to lower values at 2 years of age (51.3±20.4 μg/ml) (Table 2). Adiponectin levels continued to decrease, however not as sharply, until 5 years (41.9±18.5 μg/ml), and remained similar at 9-years (42.5±19.6 μg/ml). Adiponectin did not differ between boys and girls at any of the time points assessed. Child log(leptin) decreased from initially high levels at birth (1.08±0.37) to lower levels at 2 years of age (0.42±0.16). Following, there was a small but significant increase to 5-year levels (0.49±0.23), and a sharper increase to higher leptin in 9-year-olds (0.89±0.47). Girls had higher levels of leptin compared to boys at birth and at 5 years ($p<0.001$, $p=0.01$, respectively), but only the difference at birth persisted after adjustment for child's birth weight.

Table 2: Adiponectin (μg/ml), log(leptin) and child size by age and gender (N=80) .

		Birth		2 years		5 Years		9 years	
	N	**mean (SD)**	**P[1]**	**mean (SD)**	**P**	**mean (SD)**	**P**	**mean (SD)**	**P**
		A_0 (μg/mL)		A_2 (μg/mL)		A_5 (μg/mL)		A_9 (μg/mL)	
Boy	39	108.4 (33.1)		52.8 (19.4)		38.6 (16.0)		41.6 (21.0)	
Girl	41	117.0 (36.8)	0.28	49.9 (21.6)	0.53	45.1 (20.2)	0.12	43.4 (18.4)	0.69
All	80	112.8 (35.1)		51.3 (20.4)		41.9 (18.5)		42.5 (19.6)	
		L_0		L_2		L_5		L_9	
Boy	39	0.92 (0.29)		0.39 (0.14)		0.43 (0.18)		0.80 (0.41)	
Girl	41	1.23 (0.37)	<0.001	0.45 (0.18)	0.12	0.55 (0.25)	0.01	0.97 (0.50)	0.11
All	80	1.08 (0.37)		0.42 (0.16)		0.49 (0.23)		0.89 (0.47)	
		Birth weight (kg)		BMI_2 (kg/m^2)		BMI_5 (kg/m2)		BMI_9 (kg/m2)	
Boy	39	3.44 (0.41)		16.9 (1.7)		16.8 (1.82)		20.2 (3.8)	
Girl	41	3.46 (0.47)	0.84	17.5 (1.64)	0.09	17.6 (2.55)	0.1	20.4 (4.29)	0.81
All	80	3.45 (0.44)		17.2 (1.7)		17.2 (2.3)		20.3 (4.0)	

A - adiponectin, L- $\log_{10}$(leptin), BMI - body mass index.

A_0,L_0 - at birth, A_2,L_2, BMI_2 - at 2 years, A_5,L_5,BMI_5 - at 5 years, A_9,L_9,BMI_9 - at 9 years.

[1]P-value refers to t-test for differences by sex within a given time point.

Relationships between child adipokines and size (birth weight and BMI) by age

While child adiponectin levels at birth and birth weight were unrelated (β=0.01, p=0.47; r=0.08), there was a progressively significant negative association between adiponectin and BMI at 2 (β=-2.91, p=0.03; r=-0.24), 5 (β=-2.33, p=0.01; r=-0.28), and 9 years (β=-2.06, p<0.001; r=-0.42) (Table 3). Leptin levels at birth were associated with birth weight (β=0.001, p<0.001; r=0.46) and the leptin – BMI relationship strengthened over the 2 (β=0.05, p<0.001; r=0.52), 5 (β=0.07, p<0.001; r=0.73), and 9-year (β=0.09, p<0.001; r=0.78) time points.

Table 3: Relationship between adiponectin, leptin and size (birth weight and BMI) in children from birth to 9 years of age (N=80).

	Beta[1] (p)	**r^2**
Birth Weight - A_0	0.01 (0.47)	0.08
BMI_2 - A_2	-2.91 (0.03)	-0.24
BMI_5 - A_5	-2.33 (0.01)	-0.28
BMI_9 - A_9	-2.06 (<0.001)	-0.42
Birth Weight - L_0	0.001 (<0.001)	0.46
BMI_2 - L_2	0.05 (<0.001)	0.52
BMI_5 - L_5	0.07 (<0.001)	0.73
BMI_9 - L_9	0.09 (<0.001)	0.78

A - adiponectin, L - $\log_{10}$(leptin). BMI - body mass index (kg/m^2).

A_0,L_0 - at birth, A_2,L_2 - at 2 years, A_5,L_5 - at 5 years, A_9,L_9 - at 9 years.

[1]Linear regression coefficient.

[2]Pearson's correlation coefficient.

Associations between adipokine levels at different ages

Crude bivariate analyses showed that while adiponectin levels at birth were significantly but weakly associated with adiponectin at older ages, stronger correlations were observed over the 2 – 5 and 5 – 9-year periods (Table 4). Additionally, strength of correlations grew comparing birth – 2-years (β=0.14, p=0.03; r=0.25), 2 – 5-years (β=0.38, p<0.001; r=0.41), and 5 – 9-years (β=0.49, p<0.001; r=0.46). Leptin at birth did not significantly predict later life leptin levels at either 2, 5, or 9 years. Leptin at 2 years was weakly but significantly associated with leptin at both 5 (β=0.45, p=0.004; r=0.31) and 9 (β=0.63, p=0.05; r=0.22) years. The strongest correlation was found between 5 and 9-year-old leptin levels (β=1.26, p<0.001; r=0.62).

Table 4: Associations within adipokines in children over the birth - 9-year period (N=80).

	A_2		A_5		A_9	
	Beta[1] (p)	r[2]	Beta (p)	r	Beta (p)	r
A_0	0.14 (0.03)	0.25	0.17 (0.003)	0.33	0.12 (0.05)	0.22
A_2			0.38 (<0.001)	0.41	0.46 (<0.001)	0.48
A_5					0.49 (<0.001)	0.46
	L_2		L_5		L_9	
	Beta (p)	r	Beta (p)	r	Beta (p)	r
L_0	0.03 (0.58)	0.06	0.12 (0.08)	0.20	0.24 (0.09)	0.19
L_2			0.45 (0.004)	0.31	0.63 (0.05)	0.22
L_5					1.26 (<0.001)	0.62

A - adiponectin, L - $\log_{10}$(leptin).

A_0,L_0 - at birth, A_2,L_2 - at 2 years, A_5,L_5 - at 5 years, A_9,L_9 - at 9 years.

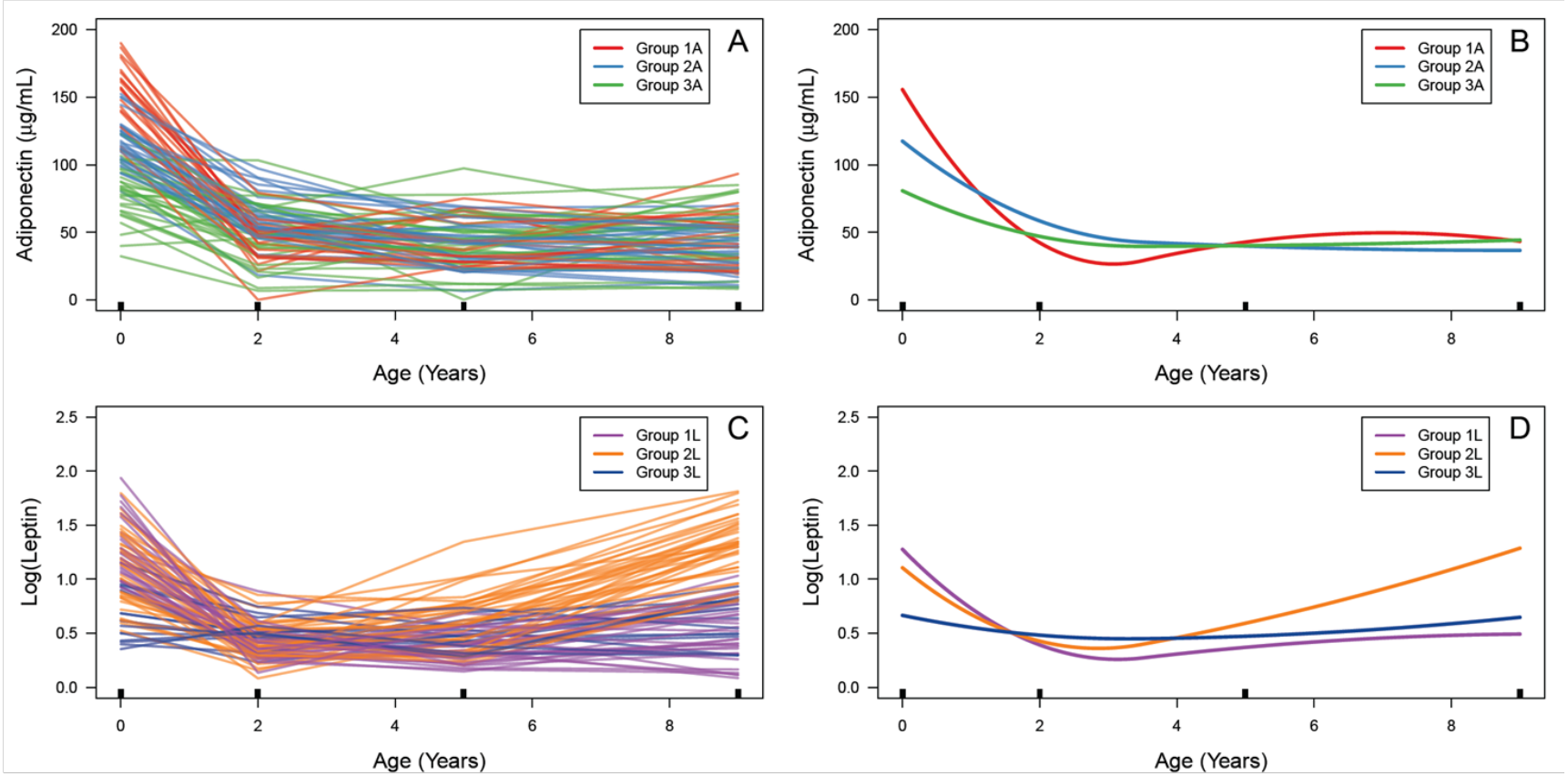
[1]Linear regression coefficient.

[2]Pearson's correlation coefficient.

Description of adipokine trajectory groups

Mixture modeling analysis identified three distinct trajectory patterns for both adiponectin and leptin development over the birth – 9-year period (Figure 1). Adiponectin and leptin levels for these groups across birth, 2, 5 and 9 years are described in Table 5. P-values for differences across groups were not generated as mixture modeling approaches estimate clustering through use of posterior probabilities, assuming some amount of uncertainty in group assignment. For adiponectin, groups were most separated over the birth – 2-year period, with group 1A (N=19) having the steepest decline in levels, group 2A (N=31) showing a more moderate decline and group 3A (N=30) showing the slowest decline.

Figure 1: Adiponectin and leptin development and trajectory groups from birth to 9 years in children (N=80).



(A) Individual child adiponectin trajectories. Black rectangles indicate age at which adiponectin was measured. Orange, blue, and green trajectories comprise groups 1A, 2A, and 3A, respectively, as identified by vertically-shifted mixture modeling (VSMM). (B) VSMM-defined adiponectin trajectory groups. (C) Individual child leptin trajectories. Purple, orange, and dark blue trajectories comprise groups 1L, 2L, and 3L. (D) VSMM-defined leptin trajectory groups.

At birth, mean±SD adiponectin levels for 1A, 2A, and 3A were 155.7±22.7 µg/ml, 117.5±17.3 µg/ml and 80.8±21.0 µg/ml, respectively. These differences did not persist into older age, with groups 1A, 2A, and 3A settling at a similar level over the 2 – 9-year period. At 9 years, mean adiponectin levels for 1A, 2A, and 3A were relatively close, at 44.2±20.6 µg/ml, 37.5±15.8 µg/ml and 46.7±21.9 µg/ml, respectively. BMI and waist circumference at 9 years were similar for 1A, 2A, and 3A groups.

For leptin, groups 1L (N=27) and 2L (N=37) had similar rates of decline from birth to 2 years (Table 5). However, from 2 to 9 years, group 1L grew slowly while group 2L showed a rapid increase in leptin levels. Group 3L (N=16) showed minimal changes in leptin levels over the birth – 9-year period. Mean±SD log(leptin) at birth for 1L, 2L, and 3L was 1.28±0.28, 1.11±0.34 and 0.67±0.24, respectively. At 9 years, leptin levels had decreased to 0.48±0.26 for 1L, increased to 1.29±0.29 for 2L, and stayed relatively constant at 0.63±0.22 for 3L. Compared to 1L and 3L, both 9-year BMI and waist circumference were highest for the rapidly-rising 2L group. We found only limited overlap in group membership comparing adiponectin to leptin groups.

Table 5. Child adipokine levels and size by cluster.

	Adiponectin Group		
	1A (N=19)	**2A (N=31)**	**3A (N=30)**
	mean (SD)	**mean (SD)**	**mean (SD)**
A_0 (µg/mL)	155.7 (22.7)	117.5 (17.3)	80.8 (21.0)
A_2 (µg/mL)	42.4 (16.8)	59.2 (17.5)	48.8 (22.8)
A_5 (µg/mL)	43.1 (17.5)	40.5 (15.9)	42.7 (21.8)
A_9 (µg/mL)	44.2 (20.6)	37.5 (15.8)	46.7 (21.9)
BMI_9 (kg/m^2)	20.4 (3.6)	20.9 (4.3)	19.7 (4.1)
$Waist_9$ (cm)	73.4 (10.0)	74.8 (12.1)	71.8 (11.5)
	Leptin Group		
	1L (N=27)	**2L (N=37)**	**3L (N=16)**
	mean (SD)	**mean (SD)**	**mean (SD)**
L_0	1.28 (0.28)	1.11 (0.34)	0.67 (0.24)
L_2	0.39 (0.15)	0.43 (0.17)	0.48 (0.14)
L_5	0.36 (0.14)	0.59 (0.26)	0.48 (0.13)
L_9	0.48 (0.26)	1.29 (0.29)	0.63 (0.22)
BMI_9 (kg/m^2)	17.3 (1.9)	23.3 (3.7)	18.5 (2.5)
$Waist_9$ (cm)	64.9 (5.4)	81.4 (9.6)	68.9 (9.7)

A - adiponectin (µg/ml), L - $\log_{10}$(leptin).

BMI - body mass index. Waist - waist circumference.

A_0,L_0 - at birth, A_2,L_2 - at 2 years, A_5,L_5 - at 5 years, A_9,L_9 - at 9 years.

BMI_9, $Waist_9$ - at 9 years.

Associations between early life factors and adipokine groups

Our analysis examined whether candidate perinatal factors may be associated with adipokine group membership, including sociodemographics at pregnancy (maternal age, years in US, education and family poverty category), child sex, gestational age, birth weight, birth length, maternal pre-pregnancy BMI, gestational weight gain, and sugar-sweetened beverage consumption during pregnancy (Table 6). Children with greater birth weight had increased odds of belonging to the rapidly-rising 2L compared to the reference 3L group (OR=1.21 95%CI 1.03, 1.43, per 100 gram increase). Additionally, children of mothers that consumed more sugar-sweetened beverages during pregnancy had increased odds of being in the steep-dropping and rebounding 1A compared to the reference 3A group (OR=1.08 95%CI 1.01, 1.17, per 1 drink/week increase). No other significant ORs were found between the early life characteristics examined and adipokine groups.

Table 6: Relationships between early life factors and adipokine group membership.

			Adiponectin Group		Leptin Group	
			1A[1]	2A	1L	2L
Early life characteristic	**mean (SD)**	**N**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Gestational age (weeks)	38.8 (1.2)	80	1.14 (0.67, 1.96)	1.39 (0.83, 2.34)	1.48 (0.79, 2.79)	1.18 (0.66, 2.12)
Birth weight (100g)	34.5 (4.4)	80	1.02 (0.86, 1.2)	1.01 (0.87, 1.18)	1.35 (0.88, 2.08)	1.21 (1.03, 1.43)
Birth length (cm)	50.6 (2.5)	80	1.07 (0.82, 1.4)	1.03 (0.84, 1.27)	1.2 (0.89, 1.6)	1.21 (0.89, 1.64)
Maternal pre-pregnancy BMI (kg/m^2)	26.5 (4.9)	80	1.1 (0.96, 1.27)	1.08 (0.84, 1.37)	0.98 (0.84, 1.14)	1.15 (0.96, 1.38)
Maternal gestational weight gain (kg)	14.3 (5.4)	80	0.99 (0.88, 1.13)	1.06 (0.93, 1.22)	1.08 (0.93, 1.26)	1.05 (0.9, 1.22)
Maternal SSB consumption (drinks/week)	14.3 (10.9)	79	1.08 (1.01, 1.17)	1.04 (0.97, 1.12)	0.89 (0.77, 1.03)	0.97 (0.92, 1.03)

OR - odds ratio, SD - standard deviation, BMI - body mass index, SSB - sugar-sweetened beverage.

[1]Odds ratios are calculated in reference to group 3 of either adiponectin (3A) or leptin (3L).

Discussion

In this study, we examined adiponectin and leptin development in Mexican American children at high risk of obesity. To our knowledge, this is the first time that adipokine trajectories have been analyzed over the entire birth to 9-year period. While leptin levels closely and positively reflected child body size at all ages, adiponectin had inverse and weaker associations with BMI at 2, 5, and 9 years. We identified three trajectory clusters for both leptin (1L, 2L, and 3L) and adiponectin (1A, 2A, and 3A). Of the perinatal factors examined, children with greater birth weight had increased odds of belonging to the rapidly-rising group 2L and those whose mothers consumed more sugar-sweetened beverages during pregnancy were at risk of being in the steep-dropping group 1A. Additionally, we found that correlations increased comparing the birth – 2, 2 – 5, and 5 – 9-year periods for both adiponectin and leptin. Taken together, our findings highlight the different roles that adiponectin and leptin have during obesity development in children and add evidence that perinatal factors may have long lasting effects on metabolic health.

Our mixture modeling analyses identified key differences in adiponectin compared to leptin development over time. For adiponectin, we observed a large decrease in levels over the 0 – 2-year period and suggest that this drop drives the heterogeneity in child trajectories. On the other hand, for leptin, children tended to be most separated over the 2 – 9-year period, with group 2L children displaying a steady rise and increased BMI and waist circumference at 9 years. We offer several reasons for these observed differences in adipokine trajectories.

In our study, leptin levels were related closely to child birth weight and BMI (Table 3) and, as a result, leptin trajectories are likely to reflect child growth. In fact, the leptin groups described here match closely in shape the three BMI trajectories identified by Pryor et al, who reported on low-stable, moderate and rapidly-rising BMI groups, with the latter two indistinguishable over the 5-month to 2.5-year period (Pryor et al. 2011).

In contrast, although adiponectin was inversely associated with BMI at 2, 5, and 9 years, these correlations were weaker compared to those of leptin. Other factors that may affect adiponectin levels over infancy remain poorly understood. Adiponectin is known to play a critical role in insulin sensitivity, regulating glucose, triglyceride and free fatty acid metabolism (Kadowaki et al. 2005; Dadson et al. 2011). Given the significant differences in pre and post-partum energy regulating mechanisms, increasing energy requirements and changing body composition during infant development, adiponectin levels may more closely reflect these changes rather than of body size itself (Ong et al. 1999; Veldhuis et al. 2005; Ay et al. 2009). Further supporting this interpretation, we and others have previously shown that adiponectin is significantly related to triglyceride and lipoprotein levels, independently of BMI in 9-year-old children (Butte et al. 2005; Volberg et al. 2013).

We did observe that group 1A had the steepest drop in adiponectin levels and a slight rebound to higher values. Further, children whose mothers had higher sugar-sweetened beverage consumption had higher odds of belonging to this group. Mechanisms responsible for this association remain unclear but greater sugar-sweetened beverage consumption during pregnancy may lead to increased glucose transfer to the fetus, resulting in insulin-mediated effects on offspring adiponectin levels (Dabelea 2007; Lustig 2011). Importantly, it remains unclear whether there are long-term health consequences for children in group 1A. We had lipid profile data only on a small subset (N=35) of the 80 children in this study and our data showed that group 1A children tended to have higher mean triglycerides (123.2 vs. 87.7 mg/dL; N=9 and 12

respectively), very low-density lipoproteins (24.7 vs. 17.6 mg/dL) and lower high-density lipoproteins (46.4 vs. 55.1 mg/dL) compared to group 3A. Despite the limited sample size, we suggest that children with rapid adiponectin decreases over infancy may develop adverse lipid levels and emphasize the need to further examine possible metabolic consequences of early life changes in adiponectin.

Additionally, we did observe that children with greater birth weight had increased odds of being in the rapidly-rising 2L group. This result is in line with several reports showing an association between increased birth weight and membership to more rapidly-rising BMI groups (Li et al. 2007; Carter et al. 2012). Previous studies have shown that children born at either end of the birth weight spectrum are at higher risk of obesity and metabolic disorders in adulthood (Hediger et al. 1999; Ong et al. 2000; Ibanez et al. 2006; Giapros et al. 2007). In this cohort, the majority of children were appropriate for gestational age (N=70) and our results further emphasize the important role of birth weight in determining later life risk of obesity.

Finally, we also report that correlations increased comparing the birth – 2, 2 – 5, and 5 – 9-year periods for both adiponectin and leptin. Only one study has previously examined these relationships for adipokines, reporting no association for adiponectin and finding a significant and positive association for leptin, comparing child levels at birth and 3 years (Mantzoros et al. 2009). Our results are in agreement with those from several other reports that focused on BMI, showing that child BMI predicts adult obesity and this association strengthens with child age (Whitaker et al. 1997; Freedman et al. 2005). Taken together, these data may suggest greater plasticity during the early infancy period and progressive constrainment to specific metabolic health with age.

Results of this study should be interpreted taking into account several limitations. Our analyses were performed on a relatively modest sample size of 80 children with complete adipokine data at birth, 2, 5, and 9 years. Importantly, other than BMI and waist circumference at 9 years, we had only limited data on children's lipid profile and no other measures of metabolic health. As a result, consequences of the differing adiponectin trajectories remain unclear. Finally, there may be fluctuations in adipokine levels between the 0, 2, 5, and 9-year time points not assessed by our analyses.

Strengths of this study include its extensive data collection, with availability of biological samples, anthropometric measures and questionnaire-based health assessments at a variety of time points. Further, examining adipokine trajectories is particularly relevant to the CHAMACOS population, given its high prevalence of child obesity. It is also important to mention that the novel mixture-modeling approach applied here helps improve our understanding of adipokine development over childhood and obesity etiology in general.

CHAPTER 6

Maternal bisphenol A exposure during pregnancy and its association with adipokines in Mexican-American children.

ABSTRACT

Objectives: To determine whether prenatal or concurrent urinary BPA concentrations are associated with key metabolism-related hormones, adiponectin and leptin (adipokines), in 9-year-old children.

Methods: For this analysis, we used 188 mother-child pairs from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) prospective study. BPA was measured in urinary spot samples during early (12.6±3.9 weeks gestation) and late (26.3±2.5 weeks gestation) pregnancy and in 9-year-old children.

Results: We found that BPA concentrations during late pregnancy were associated with increased plasma leptin in boys (β=0.06, P=0.01), controlling for maternal pre-pregnancy body mass index (BMI), pregnancy soda consumption and smoking, years in U.S. prior to pregnancy, maternal education, household poverty status, child BMI and child soda, fast food and sweet snack consumption at 9 years. Additionally, we found that BPA concentrations during early pregnancy are directly associated with plasma adiponectin levels in girls (β=3.71, P=0.03). However, we did not find any significant relationships between concurrent BPA concentrations and 9-year child adiponectin or leptin.

Conclusion: Overall, our data suggest that prenatal BPA concentrations may influence adipokine levels in 9-year-old children.

Introduction

Bisphenol A (BPA) is used to make epoxy resins and polycarbonate plastics – present in household applications such as water bottles, food containers, canned food, dental fillings and household electronics (Vandenberg et al. 2007). In the United States (U.S.), BPA ranks among the highest volume production chemicals and National Health and Nutrition Examination Survey (NHANES) data show that urinary BPA is detectable in 93% of the U.S. population (Calafat et al. 2008). There is some evidence that BPA can cross the placenta and is present in fetal circulation and amniotic fluid (Balakrishnan et al. 2010).

Increasing data argue that BPA may act as an 'environmental obesogen' – a term used to describe chemicals that can alter energy balance, promoting adipogenesis and lipid accumulation (Grun et al. 2006) - and several reports suggest that BPA affects levels of important metabolism-related hormones, adiponectin and leptin – also known as adipokines (Miyawaki et al. 2007; Hugo et al. 2008; Ben-Jonathan et al. 2009; Somm et al. 2009). Adiponectin, a protein hormone secreted by adipose tissue, targets muscle and liver to increase uptake and catabolism of fatty acids and carbohydrates, promoting insulin sensitivity (Kadowaki et al. 2005). In children, lower adiponectin levels are associated with both metabolic syndrome and type 2 diabetes (Cruz et al. 2004; Shaibi et al. 2007). Leptin, a hormone synthesized mainly by adipose tissue but also by the stomach, skeletal muscle and liver, acts on the hypothalamus to convey satiety and regulate long-term energy balance (Margetic et al. 2002; Mantzoros et al. 2011). Plasma leptin levels correlate positively with adiposity, however, obese individuals commonly develop 'leptin resistance' or tolerance – a hyperleptinemic state with a lack of leptin's regulatory effects (Considine et al. 1996; Ong et al. 2006).

BPA exposure may be linked to changes in metabolic health in a sex-dependent manner. Using a mouse model, Miyawaki et al (2007) showed that perinatal exposure to BPA resulted in sex-specific increases in body and adipose tissue weight and changes in leptin levels in the offspring (Miyawaki et al. 2007). Additionally, Somm et al (2009) showed that although perinatal exposure to BPA resulted in greater weight of both male and female Sprague-Dawley rats on postnatal day 1, this difference persisted only in females at postnatal day 21 (Somm et al. 2009). In *in vitro* cell models, BPA has been shown to promote differentiation of 3T3-L1 fibroblast cells into adipocytes (Masuno et al. 2005) and inhibit adiponectin release from human adipocytes (Hugo et al. 2008).

Few data are available on associations between prenatal BPA exposure and children's metabolic health. Chou et al (2011) reported that mothers in the highest quartile of BPA blood concentrations at delivery had boys with increased odds of low adiponectin and high leptin at birth (Chou et al. 2011). However, this study assessed BPA concentrations in participants' blood – a measure known to have severe limitations due to high risk of contamination and blood collection occurred near time of birth where medical interventions in hospitals can dramatically increase BPA exposures (Calafat et al. 2009; Vandentorren et al. 2011; Duty et al. 2013). Data from the National Health and Nutrition Examination Survey (NHANES) and other cross-sectional studies show that increased body mass index (BMI) is associated with greater urinary BPA concentrations (Trasande et al. 2012; Wang et al. 2012). Nevertheless, whether higher BPA concentrations actually precede the onset of obesity remains uncertain. (Becker et al. 2009; Lakind et al. 2011; Trasande et al. 2012; Wang et al. 2012)

In the first longitudinal study of BPA and obesity, we did not observe associations of prenatal BPA urinary concentrations and increased body weight in children participating in the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort. In

fact, prenatal BPA urinary concentrations were negatively associated with BMI Z-score in girls (Harley et al. 2013). Thus, whether and how prenatal or childhood BPA exposure leads to altered metabolic health remains unclear.

In this study, we assessed whether maternal urinary BPA concentrations during the first and second halves of pregnancy are associated with plasma adiponectin and leptin levels in children when they reach 9 years of age. Additionally, we investigated whether concurrent BPA concentrations are associated with adipokine levels in children at 9 years. Given that many previous studies report different responses to BPA exposure by sex, we tested for interaction by sex in our models. Analyses were performed using data from the CHAMACOS study, a Mexican-American cohort with a high prevalence of childhood obesity.

Methods

Subjects and study design

The CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of environmental exposures on growth and development in children living in the Salinas Valley, CA (Eskenazi et al. 2004; Eskenazi et al. 2005). Mothers were enrolled during pregnancy between October 1999 and October 2000, with 537 mother-child pairs in the study at delivery and 327 pairs remaining at the 9-year visit. Eligible women were ≥18 years of age, <20 weeks gestation at enrollment, English or Spanish speaking, eligible for low income health insurance (Medicaid), and planning to deliver at the county hospital. Women were interviewed at ~13 and ~26 weeks gestation and anthropometric measures of the children were obtained at birth, 6 months, and 1, 2, 3½, 5, 7 and 9 years of age. Adiponectin and leptin were measured on a convenience sub-sample of 188 children having blood samples at 9 years and complete anthropometric and demographic data. Of these children, 131 had maternal urinary BPA measures for the first half of pregnancy, 179 for the second half and 172 had 9-year urinary BPA data. No differences were observed comparing maternal and child demographic and anthropometric measures included in Table 1 between this sub-sample and the overall CHAMACOS cohort. All study activities were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley and the Centers for Disease Control and Prevention (CDC).

Questionnaire data

Mothers were interviewed at the two prenatal visits using structured questionnaires administered in Spanish or English by bilingual, bicultural trained interviewers. We collected sociodemographic information, including maternal age at pregnancy, years of living in the U.S. prior to pregnancy, education, household poverty category, and smoking during pregnancy. During the second prenatal interview, we used a previously validated food frequency questionnaire (FFQ) to assess dietary intake, including maternal soda consumption, during pregnancy (Block et al. 1990; Harley et al. 2005) and asked additional questions at the 9-year interview about child soda, fast food and sweet snack consumption at age 9. Maternal pre-pregnancy BMI was calculated using the mother's self-reported pre-pregnancy weight and measured height. Infant birth weight, length, and gestational age were obtained from delivery medical records abstracted by a registered nurse. Children were categorized as 'small for gestational age' if their birth weight was <10th percentile for gestational age based on their

ethnicity, maternal parity, and infant sex (Overpeck et al. 1999). Children were considered as 'at term' if they were born at or after 37 weeks of gestation.

Anthropometric measurements

Children's weight and height were measured at the 9-year visit using a bioimpedance electronic scale (Tanita TBF-300A Body Composition Analyzer.) and wall-mounted stadiometer (Seca 222), respectively. Child height was measured in triplicate and the average of measurements was used. BMI was calculated as mass in kilograms divided by height in meters squared. Children were categorized as normal weight, overweight, or obese using the sex and age-specific BMI cut-offs (85^{th} and 95^{th} percentile, respectively) provided by the 2000 CDC child growth data. Mothers were categorized as normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2) or obese ($\geq$30 kg/m^2) using the standard CDC BMI cutoffs for adults.

Plasma adiponectin and leptin measurements

Blood samples were collected from the children at the 9-year visit and stored at -80ºC. Adiponectin and leptin were measured in plasma using enzyme-linked immunoassay (ELISA) RayBio Human Adiponectin and Human Leptin kits (Norcross, GA) as previously described (Volberg et al. 2013). The minimum detectable concentrations for adiponectin and leptin were 10 pg/mL and 6 pg/mL respectively. All samples were run in duplicate and the values were averaged. The intra- and inter-plate coefficients of variance (CV) were 4% and 12%, respectively, for adiponectin and 3% and 15%, respectively, for leptin.

Maternal and child urinary BPA measurement

Urinary spot samples were collected in sterile, polypropylene urine cups during the first (12.6±3.9 weeks gestation) and second (26.3±2.5 weeks gestation) half of pregnancy and from children at 9 years of age (9.4±0.4 years). Samples were stored at -80^0C and shipped to the CDC (Atlanta, GA) for analysis using online solid-phase extraction coupled with isotope-dilution high-performance liquid chromatography tandem mass spectrometry with peak focusing (Ye et al. 2005). Total urinary concentration of BPA (free and conjugated) was measured with a limit of detection (LOD) at 0.4 µg/L. For concentrations below the LOD, we used the instrument-reported values. When no signal was detected, we imputed values at random based on a log-normal probability distribution whose parameters were determined by maximum likelihood estimation (Lubin et al. 2004). Blanks, low (~2.8 µg/L) and high (~10 µg/L) quality control materials were included in all runs. To account for urinary dilution, we corrected maternal BPA concentrations using specific gravity, assessed using a hand-held refractometer (National Instrument Company, Baltimore, MD). Child 9-year BPA concentrations were corrected using urinary creatinine determined by the Vitros CREA assay (Ortho Clinical Diagnostics, NJ).

Statistical analyses

Child adiponectin levels were approximately normally distributed, but leptin levels were right-skewed. Thus, all leptin-based analyses use $\log_{10}$-transformed values. Urinary BPA concentrations during early and late pregnancy and at 9 years were also right-skewed and were $\log_2$-transformed to reduce the influence of outliers. Using multivariate linear regression, we examined separately effects of BPA exposure during first and second halves of pregnancy and at age 9 on 9-year child adiponectin and leptin. Our models included an interaction term between child sex and BPA concentrations and BPA effect coefficients for each sex were estimated using

the 'lincom' post-regression command in Stata. Potential confounders were identified *a priori* using a directed acyclic graph and included maternal pre-pregnancy BMI, soda consumption and smoking during pregnancy, years of residence in U.S., maternal education, household poverty status, and child soda, fast food and sweet snack consumption at 9 years. Many of these covariates were chosen based on previous literature showing that several risk factors for obesity, including consumption of soda, school lunches and meals prepared outside of the home, are also related to increased urinary BPA concentrations in the general U.S. population of children (Lakind et al. 2011). Importantly, previous mechanistic evidence has shown that BPA may have direct effects on adipokines (Hugo et al. 2008; Ben-Jonathan et al. 2009). Thus, models presented in this study control for child BMI to examine the BPA – adipokine association, independent of BMI's effect on adipokines. All statistical analyses were conducted using STATA 12 (College Station, TX) for Windows. We set statistical significance at $p<0.05$ for main effects and $p<0.10$ for interaction terms.

Results

Child and maternal characteristics

Of the 188 children in this study, 87 (46%) were boys and 101 (54%) were girls (Table 1). At time of pregnancy, mothers tended to be young (mean: 26.3±5.2 years), to have resided in the US for 10 years or less (75%), and to not have completed high school (80%). Almost half of mothers reported consuming 1 or more sodas per week (46%). During the pregnancy, the majority of families (62%) were living at or below the federal threshold for poverty (U.S. Census Bureau 2000). Most mothers were overweight (38%) or obese (26%) according to their pre-pregnancy BMI. Children were mainly delivered at term (≥37 weeks, N=178, 95%). Approximately 5% of the children were born preterm and 4% were small for gestational age. Mothers reported that the majority of children consumed soda and fast food at least weekly (58% and 57%, respectively) and 22% consumed sweet snacks daily. At 9 years, 17% of children were overweight and 39% were obese.

Table 1: Demographic characteristics of mothers and children from the CHAMACOS Study, Salinas Valley, CA (N=188).

Characteristic	N	(%)
Child sex		
Boy	87	(46.3)
Girl	101	(53.7)
Maternal age at pregnancy		
18-24	82	(43.6)
25-29	59	(31.4)
30-34	30	(16.0)
35-45	17	(9.0)
Maternal years in US at pregnancy		
<1	37	(19.7)
1-10	103	(54.9)
>10	48	(25.4)
Maternal education at pregnancy		
≤6th Grade	86	(45.7)
7-12 Grade	64	(34.0)
≥High School	38	(20.2)
Maternal soda consumption during pregnancy		
< 1 drink/week	102	(54.3)
1-6 drinks/week	70	(37.2)
7+ drinks/week	16	(8.5)
Household poverty category at pregnancy		
≤Poverty threshold	117	(62.2)
>Poverty level but <200% poverty level	65	(34.6)
≥200% Poverty level	6	(3.2)
Maternal pre-pregnancy BMI		
Normal (18.5–24.9 kg/m^2)	68	(36.1)
Overweight (25–29.9 kg/m^2)	71	(37.8)
Obese (≥ 30 kg/m^2)	49	(26.1)
Child gestational age at birth		
34 - 36 Weeks	10	(5.3)
≥37 Weeks	178	(94.7)
Child birth size		
Small for gestational age (<10th percentile)	8	(4.3)
Appropriate for gestational age	161	(85.6)
Large for gestational age (>90th percentile)	19	(10.1)
Child soda consumption at 9 years		
< 1 time/week	78	(41.5)
1-6 times/week	90	(47.9)
7+ times/week	20	(10.6)
Child fast food consumption at 9 years		
< 1 time/week	82	(43.6)
1-2 times/week	99	(52.7)
3+ times/week	7	(3.7)
Child sweet snack consumption at 9 years		
< 1 time/day	166	(88.3)
1-2 times/day	19	(10.1)
3+ times/day	3	(1.6)
Child BMI[1] at 9 years		
Normal (≤85th percentile)	83	(44.2)
Overweight (>85th, <95th percentile)	32	(17.0)
Obese (≥95th percentile)	73	(38.8)

[1]Child's weight status was determined using age and sex adjusted body mass index cut offs for 85th and 95th percentiles from CDC child growth charts.

Child adiponectin and leptin plasma levels

At 9 years of age, average child adiponectin plasma levels were 42.6±18.6 µg/mL (Table 2). No statistically significant difference was found between levels in boys and girls. Adiponectin levels were significantly and inversely correlated with 9-year child BMI (r=-0.38, p<0.001). Average child plasma leptin levels were 8.2±1.1 ng/mL. Boys (7.1±1.0 ng/mL) tended to have slightly lower leptin levels compared to girls (9.3±1.2 ng/mL; p=0.08). Leptin levels were significantly and positively correlated with 9-year child BMI (r=0.82, p<0.001).

Table 2: Leptin and adiponectin levels in 9-year-old CHAMACOS children.

				T-test[1] by sex
Adipokine	**N**		**Mean(SD)**	**P-value**
Leptin[2] (ng/mL)				
Boys	87		7.1 (1.0)	
Girls	101		9.3 (1.2)	0.08
Total	188		8.2 (1.1)	
Adiponectin (µg/mL)				
Boys	87		42.1 (19.7)	
Girls	101		43.0 (17.6)	0.75
Total	188		42.6 (18.6)	

[1]T-test for difference by sex

[2]Geometric means and standard deviations presented for leptin

Maternal and concurrent child urinary BPA levels

Table 3 shows urinary BPA concentrations (both uncorrected and corrected for urinary dilution) for mothers during pregnancy and for their children at 9 years, stratified by child sex. Geometric mean (GM) concentrations presented here (0.9 µg/L and 1.1 µg/L for early and late pregnancy, respectively) are lower than those previously reported for pregnant women from NHANES 2003-2004 (GM=2.53 µg/L) (Woodruff et al. 2011). Additionally, 9-year-old CHAMACOS children tended to have lower urinary BPA concentrations (GM=1.6 µg/L) than those reported for NHANES children aged 6-11 (GM=3.6 µg/L) (Calafat et al. 2008). No statistically significant difference was found in 9-year BPA concentrations between boys and girls (p=0.74).

Table 3: Early and late pregnancy and 9-year child BPA concentrations.

			Uncorrected (µg/L)	Corrected for urinary dilution (µg/L[1], µg/g[2])
Timing	**N**	**%>LOD[4]**	**GM (GSD)**	**GM (GSD)**
Early pregnancy[1]				
Boys	66	81	0.9 (0.9)	1.1 (0.8)
Girls	65	78	0.9 (1.1)	1.2 (1.0)
Total	131	79	0.9 (1.0)	1.1 (0.9)
Late pregnancy[1]				
Boys	81	81	1.0 (0.9)	1.2(0.8)
Girls	98	85	1.1 (0.9)	1.2(0.8)
Total	179	83	1.1 (0.9)	1.2 (0.8)
9 year child[2,3]				
Boys	77	96	1.7 (1.0)	1.6 (0.8)
Girls	95	87	1.5(1.0)	1.7 (0.8)
Total	172	91	1.6 (1.0)	1.7 (0.8)

[1]Specific gravity corrected

[2]Creatinine corrected (µg/g-creatinine)

[3]T-test by sex P-value = 0.74

[4]LOD = 0.4 µg/L

Prenatal and concurrent urinary BPA concentration and 9-year child plasma adipokines.

Table 4 shows associations between prenatal and concurrent urinary BPA measures and child adiponectin and leptin plasma levels at 9 years, controlling for maternal pre-pregnancy BMI, pregnancy soda consumption and smoking, years in U.S. prior to pregnancy, maternal education, household poverty status, child 9-year BMI, and child soda, fast food and sweet snack consumption at 9 years. We observed significant effect modification by sex for associations between late pregnancy BPA and leptin (interaction p=0.01) and between early pregnancy BPA and adiponectin (interaction p=0.1).

Focusing on the sex-specific associations, we found that among boys, late pregnancy urinary BPA concentrations were positively associated with 9-year leptin levels (β=0.06, p=0.01). Additionally, among girls, we found that early pregnancy BPA concentrations were positively associated with 9-year adiponectin levels (β=3.71, p=0.03). Concurrent measures of urinary BPA were not associated with adipokine levels in 9-year-old children after controlling for BMI.

Table 4. Prenatal and concurrent associations between log_2 BPA and adiponectin and leptin in 9-year-old children.

		Boys			Girls			Sex-interaction
	N	β	(95% CI)	P-value	β	(95% CI)	P-value	P-value
Leptin								
Early pregnancy[1]	131	0.03	(-0.03, 0.09)	0.27	0.02	(-0.03, 0.06)	0.49	0.67
Late pregnancy[1]	179	0.06	(0.01, 0.11)*	0.01	-0.03	(-0.07, 0.02)	0.26	0.01
9 year child[2]	172	-0.04	(-0.23, 0.14)	0.64	-0.11	(-0.27, 0.04)	0.15	0.58
Adiponectin								
Early pregnancy[1]	131	-0.67	(-4.77, 3.43)	0.75	3.71	(0.38, 7.04)*	0.03	0.10
Late pregnancy[1]	179	2.42	(-0.94, 5.78)	0.16	1.72	(-1.33, 4.77)	0.27	0.76
9 year child[2]	172	2.69	(-9.65, 15.04)	0.67	9.44	(-0.76, 19.6)	0.07	0.41

Adjusted for: maternal pre-pregnancy BMI, Years in the US, poverty status, maternal education, smoking during pregnancy, soda consumption during pregnancy, child 9-year BMI, and child soda, fast food and sweet snack consumption at 9 years.

[1]Specific gravity adjusted

[2]Creatinine adjusted

*P-value < 0.05

Discussion

In this cohort of Mexican-American children, we observed that the BPA–adipokine association may vary by child sex. We found that late pregnancy urinary BPA concentrations were positively associated with leptin levels in 9-year-old boys, adjusting for covariates including child BMI. Additionally, early pregnancy BPA concentrations were positively related to adiponectin levels in 9-year-old girls. While the mechanism for this sexual dimorphism remains unclear, it may be related to BPA's ability to interfere with activity of endogenous estrogens (17β-estradiol), disrupting normal binding at either non-classical membrane or nuclear estrogen receptors (Gould et al. 1998; Kuiper et al. 1998; Alonso-Magdalena et al. 2005; Alonso-Magdalena et al. 2006; Wetherill et al. 2007). Previous studies indicate that biosynthesis and function of 17β-estradiol, along with tissue distribution of estrogen receptors may vary by sex (Simpson et al. 1999; Nilsson et al. 2001; Gillies et al. 2010). We speculate that this may account for some of the sex-specific responses observed with respect to BPA exposure.

Our finding of a positive association between prenatal BPA and leptin in boys is consistent with several animal studies. Wei et al (2011) showed increased leptin levels in male rats and Miyawaki et al (2007) found increased body and adipose tissue weight in male mice as a result of perinatal BPA exposures (Miyawaki et al. 2007; Wei et al. 2011). Although we did not observe an association of prenatal BPA with BMI in boys in this cohort (Harley et al. 2013), we now report an association with increased leptin independent of BMI. BMI may not be as sensitive an endpoint and it is plausible that BPA may directly alter leptin secretion from adipocytes given that it has been shown to do so with respect to adiponectin (Hugo et al. 2008). However, additional mechanistic data are needed to examine the BPA-leptin relationship.

Previous data on CHAMACOS children suggest that prenatal BPA concentrations are negatively associated with BMI z-score in girls only (Harley et al. 2013). Here, adjusting for child BMI, we found that prenatal BPA was positively associated with adiponectin in girls. It is important to note that while many previous studies have shown perinatal BPA exposure to be positively associated with offspring obesity (Rubin et al. 2001; Miyawaki et al. 2007; Somm et

al. 2009; Hiyama et al. 2011), and, in an *in vitro* model, with adiponectin suppression (Hugo et al. 2008), others have found no or reverse associations (Honma et al. 2002; Negishi et al. 2003; Ryan et al. 2010; Nakamura et al. 2012). A recent study by Anderson et al (2013) indicated that perinatally exposed male and female mice exhibited increased energy expenditure and activity and, further, females had marginally lower body weight and improved adiponectin and leptin levels throughout their lives (Anderson et al. 2013). Overall, the reasons for differences in reported associations in animal and human studies remain unclear. Both timing and dose of BPA exposure do vary for the animal studies mentioned and this may contribute to the inconsistencies in results.

Only one other study has provided data on prenatal BPA and adiponectin and leptin in children. Chou et al (2011) examined associations between prenatal BPA and adiponectin and leptin in children at birth (Chou et al. 2011). They reported a positive association between BPA blood concentrations during pregnancy and leptin in boys at birth. They also found an inverse relationship between prenatal BPA and adiponectin in girls, which we did not. Reasons for this discrepancy remain unclear, but it is important to note that the Chou study measured BPA in maternal plasma collected at birth. There are several limitations to measurement of BPA in blood compared to urine, including higher proportion of non-detects and greater risk of cross-contamination yielding incorrect results (Calafat 2011). Additionally, studies have shown that exposures to medical devices in the hospital near the time of birth can dramatically increase BPA levels (Calafat et al. 2009; Vandentorren et al. 2011; Duty et al. 2013).

We did not find significant associations between concurrent BPA exposure and child adipokines at 9 years, controlling for BMI. To our knowledge, no such data on adiponectin and leptin are available from other reports. Previously, we found positive associations between concurrent urinary BPA concentrations and child BMI (Harley et al. 2013). Several other studies have also reported a direct association between concurrent BPA and BMI in children (Trasande et al. 2012; Wang et al. 2012). Using NHANES 2003-2008, Trasande et al (2012) found a positive association between urinary BPA concentrations and BMI in children aged 6-19 (Trasande et al. 2012). Such data are limited by their cross-sectional nature and cannot address whether BPA exposure preceded development of obesity or is merely a reflection of it. Overall, our findings suggest that prenatal, and not concurrent, BPA exposure is more relevant in determining child adipokine levels. Future work should focus on further exploring potential metabolic health consequences of BPA exposure during this critical developmental period.

One of the limitations of this study is the short-term nature of the BPA exposure measurement. Urinary BPA concentrations vary widely throughout the day and the spot urine samples used in this analysis reflect only recent exposure. Ye et al (2011) observed large within-day variance for spot collections (70%) (Ye et al. 2011). However, we expect this misclassification to be non-differential, biasing our results towards the null. Additionally results may not be generalizable to other populations as this study was conducted on a cohort of largely first generation, immigrant and relatively low socioeconomic status Mexican-American families from an agricultural community. A major strength of this study is its prospective nature, providing data on associations between prenatal BPA exposure and child adipokine levels 9 years later. Further, these results are directly relevant to the CHAMACOS population, given its high prevalence of obesity.

CHAPTER 7

Methylation across the adipogenic PPARγ gene and its relationship with child body size at birth and 9 years.

ABSTRACT

Objectives: To examine methylation of the peroxisome proliferator-activated receptor γ (*PPARγ*) gene, its relationship with perinatal factors, including *in utero* Bisphenol A exposure, and its associations with child size, adiponectin and leptin levels in children at birth and 9 years.

Methods: We measured *PPARγ* methylation across 23 CpG sites using the Infinium Illumina 450K Array for two sets of children from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort at birth and 9 years (N=117 at birth, N=108 at 9 years for discovery set, N=116 at birth, N=131 at 9 years for validation set).

Results: Structure of *PPARγ* methylation across the 23 sites was highly conserved comparing birth to 9-year time points in both the discovery and validation sets. Furthermore, we found high inter-CpG correlations between the north shore sites 1, 2 and 3 in both sets. We also report that methylation in site 1 was significantly and negatively associated with child size at birth (β=-2.5, P=0.04) and at 9 years (β=-4.8, P<0.001) in the discovery set, and these relationships were replicated in the validation set. However, no significant associations were found between BPA prenatal exposure and other the perinatal factors examined and *PPARγ* site 1 methylation.

Conclusion: *PPARγ* methylation may play an important role in obesity development in children, reflecting child birth weight and BMI.

Introduction:

While accumulating evidence argues for an important contribution of the prenatal environment in the etiology of obesity, critical questions remain about potential underlying molecular mechanisms (Lustig 2011). A key proposed pathway suggests that features of the prenatal environment may cause lasting epigenetic changes during child development, leading to adverse health outcomes in later life (Waterland et al. 2007; Lustig 2011). Epigenetics refers to heritable changes to deoxyribonucleic acid (DNA) that do not affect the DNA code but may affect gene expression. Perhaps the best understood and most widespread epigenetic mark is that of DNA methylation (Robertson et al. 2000; Robertson 2005; Bell et al. 2011; Bell et al. 2012). This phenomenon involves attachment of a methyl group to the cytosine base within cytosine-guanine dinucleotides, also known as 'CpG methylation'. Higher CpG methylation (hypermethylation) within the promoter region of a gene is thought to reduce gene expression (Jaenisch et al. 2003).

Scientific understanding of the structure of DNA methylation is rapidly evolving and current literature outlines a classification system for CpG sites, highlighting that their function may be intimately related to their location in the genome (Irizarry et al. 2009; Baccarelli et al. 2010). For example, approximately 70% of gene promoter regions are thought to contain a CpG island - DNA region with densely concentrated CpGs that typically have low levels of methylation (Saxonov et al. 2006). CpG sites flanking the island are located in regions termed north shore (upstream, towards 5' end) and south shore (downstream, towards 3' end) and are thought to be particularly important in regulating protein expression (Irizarry et al. 2009; Bell et al. 2012).

There has been an effort to explore the environmental and differential CpG methylation interaction and its contribution to obesity programming from an early age. Animal studies have focused on the *Agouti* mouse, where hypomethylation of the intracisternal A particle (IAP) increases expression of the *Agouti* gene, which, in turn, results in the yellow coat color, obese phenotype. Using this model, Waterland et al (2008) showed that inheritance of the *Agouti* gene was associated with trans-generational amplification of obesity and that maternal methyl-donor supplementation could prevent this effect (Waterland et al. 2008). With special relevance to environmental exposures, Dolinoy et al (2007) showed that treating pregnant *Agouti* mice with Bisphenol A (BPA) lead to hypomethylation of the IAP and increased yellow coat color in the offspring (Dolinoy et al. 2007).

While the *Agouti* mouse model represents an ideal epigenetic biosensor (Dolinoy et al. 2007) for connecting environmental factors with obesity development through changes in DNA methylation, human data examining such relationships are less consistent and no epigenetic marks have displayed strong signals or have been replicated across different studies. For example, Wang et al (2010) screened 27,000 CpG sites for associations with BMI, identifying CpG sites in only two genes that withstood replication and multiple testing adjustment (Franks et al. 2010; Wang et al. 2010).

An alternative approach, one that helps avoid multiple testing issues, involves an *a priori* selection of candidate genes. With respect to obesity development, the gene peroxisome proliferator-activated receptor γ *(PPARγ*) is thought to play a critical role, functioning as the only gene that is both necessary and sufficient for fat cell production (Vidal-Puig et al. 1996; Tontonoz et al. 2008). *PPARγ* upregulation has been linked to improvement of critical metabolism-related hormones (increased adiponectin and decreased leptin) and increased insulin

sensitivity at the expense of greater body weight in adults (Maeda et al. 2001) and animals (Kubota et al. 1999; Larsen et al. 2003; Toruner et al. 2004). Importantly, while methylation has been shown to affect *PPARγ* expression in animal and *in vitro* studies, no human data on *PPARγ* methylation, its relationship with obesity and/or with perinatal factors are available (Noer et al. 2006; Fujiki et al. 2009). Additionally, given past issues with replication in genome-wide analyses, it is particularly important to focus on reproducibility when considering study design.

To address these data gaps, we: 1) examined plasma *PPARγ* methylation in a discovery (N=117 at birth, N=108 at 9 years) and validation (N=116 at birth, N=131 at 9 years) sets; 2) determined relationships between *PPARγ* methylation and child adiponectin, leptin, birth weight and body mass index (BMI); and 3) characterized associations between perinatal factors, including *in utero* BPA concentrations, and *PPARγ* methylation at birth and at 9 years. Analyses were performed using data from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study, an ongoing prospective study with a high prevalence of childhood obesity.

Methods

Subject and study design

The CHAMACOS study is a longitudinal birth cohort designed to assess the health effects of pesticides and other environmental exposures on growth and development of primarily Mexican-American children living in Salinas Valley, CA (Eskenazi et al. 2004; Eskenazi et al. 2005). Mothers were enrolled during pregnancy between October 1999 and October 2000, with 537 mother-child pairs in the study at delivery and 327 pairs remaining at the 9-year visit. Eligible women were ≥18 years of age, <20 weeks gestation at enrollment, English or Spanish speaking, Medi-Cal eligible and planning to deliver at the county hospital. Women were interviewed twice during pregnancy, shortly after delivery, and when their children were 6 months, and 1, 2, 3½, 5, 7, and 9 years of age.

Our analyses of DNA methylation used two sets of CHAMACOS children, which we term 'discovery' and 'validation' sets. The discovery set contained 117 children at birth and 108 9-year-old children, with 27 children having methylation measures at both time points for a total of 198 children. The validation set had 116 children at birth and 131 children at 9 years, with 71 children having methylation measures at both time points for a total of 176 children. There was no overlap in children between the discovery and validation sets.

Questionnaire data

Interviews were conducted in Spanish or English by bilingual, bicultural trained interviewers. Maternal age was assessed during the first prenatal interview at 14±5 weeks gestation. Maternal pre-pregnancy BMI was calculated using the mother's self-reported pre-pregnancy weight and measured height. During the second prenatal interview at 26±3 weeks gestation, we used a previously validated food frequency questionnaire (FFQ) to assess maternal sugar-sweetened beverage consumption (Block et al. 1990; Harley et al. 2005). In brief, participant mothers were asked to report number of drinks in the last 3 months of 100% orange, grapefruit, apple, grape, or other real 100% fruit juice; fruit drinks (Tampico, Sunny D, lemonade, Kool-Aid); or non-diet soda (recoded as drinks per week). This FFQ is based on the Spanish-language Block 98 Questionnaire and was modified for use for the CHAMACOS Mexican-American population (Block et al. 1990). Additional details on the FFQ used here are

available in Harley et al (Harley et al. 2005). Data on infant birth weight and gestational age were obtained from delivery medical records abstracted by a registered nurse.

Anthropometric measurements

An electronic scale (Tanita Mother-Baby Scale Model 1582, Tanita Corp.) was used to measure recumbent infant weight at the 6-month visit and child and mother weight at the 9-year visit. Child 9-year height was measured in triplicate using a stadiometer (Seca 222) and the average of measurements was used. BMI was calculated as mass in kilograms divided by height in meters squared. Children were categorized as normal weight, overweight or obese using the sex and age-specific BMI cut-offs (85^{th} and 95^{th} percentile, respectively) provided by the 2000 Centers for Disease Control and Prevention (CDC) child growth data. Monthly rate of weight gain during the first 6 months of life was calculated as weight at the 6-month visit minus birth weight divided by exact age in months at the 6-month visit and reported in 100 grams/month. This approach to examining infancy weight gain has been previously used and validated by Stettler et al (2002) (Stettler et al. 2002).

Plasma adiponectin and leptin measurements

Adiponectin and leptin were measured in banked blood plasma samples stored at -80ºC using enzyme-linked immunoassay (ELISA) RayBio Human Adiponectin and Human Leptin kits (Norcross, GA). The minimum detectable concentrations for adiponectin and leptin ELISAs were 10pg/ml and 6pg/ml respectively. All samples were run in duplicate and the values were averaged. The intra- and inter-plate coefficients of variance (CV) were 3% and 12%, respectively, for adiponectin and 3% and 13%, respectively, for leptin.

Maternal urinary BPA measurement

Urinary spot samples were collected in sterile, polypropylene urine cups during the first (12.3±3.8 weeks gestation) and second (26.3±2.2 weeks gestation) half of pregnancy. Samples were stored at -80^0C and shipped to the CDC (Atlanta, GA) for analysis using online solid-phase extraction coupled with isotope-dilution high-performance liquid chromatography tandem mass spectrometry with peak focusing (Ye et al. 2005). Total urinary concentration of BPA (free and conjugated) was measured with a limit of detection (LOD) at 0.4 µg/L. For concentrations below the LOD, we used the instrument-reported values. When no signal was detected, we imputed values at random based on a log-normal probability distribution whose parameters were determined by maximum likelihood estimation (Lubin et al. 2004). Blanks, low (~2.8 µg/L) and high (~10 µg/L) quality control materials were included in all runs. To account for urinary dilution, we corrected maternal BPA concentrations using specific gravity, assessed using a hand-held refractometer (National Instrument Company, Baltimore, MD).

Child DNA Methylation Measurement

DNA was isolated from blood clots previously collected and stored at -80C using the QIAamp DNA blood maxi kit (Qiagen, CA). To measure DNA methylation, we used the Infinium Illumina 450K array, which is based on multiplexed genotyping of bisulfite converted genomic DNA. This technology is currently considered as a leading method to measure genome-wide methylation, providing both broad and dense coverage, in total interrogating 485,577 CpG sites over 99% of RefSeq genes. The workflow involves bisulfite conversion of DNA, performed using Zymo Bisulfite Conversion Kits (Zymo Research, Orange, CA). Subsequently, each

sample is whole-genome amplified, enzymatically fragmented, purified and applied to the BeadChips according to the Illumina methylation protocol. BeadChips were processed with robotics and analyzed using the Illumina Hi-Scan system at the Genomics Core. The relative methylation beta (β, %methylation) for each CpG site is calculated as the ratio of methylated-probe signal to total (methylated + unmethylated) fluorescent signal intensity. For the *PPARγ* site, the intra- and inter-chip CVs were 8% and 12%, respectively.

Statistical Analyses

Adiponectin levels were approximately normally distributed, but leptin levels were right-skewed at birth and at 9 years. Thus, all leptin-based analyses use base-ten log-transformed values. Previous analyses show that beta values (methylation %) suffer from heteroscedasticity for highly methylated or unmethylated CpG sites (Du et al. 2010). Thus methylation is reported in M-values, using the formula: M-value = $\log_2$(beta/(1-beta)). All methylation data was background subtracted and normalized in accordance with Illumina software protocols.

The Illumina 450K array measures methylation in 23 sites across the *PPARγ* promoter. To analyze *PPARγ* methylation structure across these sites, we first plotted methylation levels by site and examined inter-site correlations at birth and 9 years in both sets. Next, we averaged M-values for CpG sites in the same region (north/south shore, island) for the birth and 9-year time points, assessing their changes over time.

We used linear regression to determine site-specific associations between methylation and child adipokines and size within the birth and 9-year time points in the discovery set. Sites significantly associated with adipokines and/or size were then re-examined in the validation set. Further, we combined data from both sets and used Bonferroni adjustment for multiple testing (23 tests per outcome) to determine whether these associations remained significant. In addition to these within time point analyses, we also explored whether site-specific *PPARγ* methylation at birth is associated with BMI and adipokine levels at 9 years. Lastly, we examined whether perinatal factors could have long lasting influence on epigenetic marks, focusing on methylation levels at 9 years. Statistical analyses were conducted using STATA 12 (College Station, TX) for Windows and R statistical software (R Foundation for Statistical Computing, Vienna, Austria).

Results

Maternal and Child Characteristics of Discovery and Validation Sets

Table 1 shows maternal and child characteristics by discovery and validation sets.

Table 1: CHAMACOS Maternal and child characteristics by discovery (117 at birth, 108 at 9 years) and validation (116 at birth, 131 at 9 years) sets.

	Discovery Set			Validation Set			
Maternal Characteristics	**N**	**μ**	**95% CI**	**N**	**μ**	**95% CI**	**P-value[1]**
Age (years)	198	25.4	24.7, 26.1	176	26.3	25.6, 27.1	0.07
Pre-pregnancy BMI[1] (kg/m^2)	198	26.8	26.1, 27.5	176	27.6	26.8, 28.4	0.12
Pregnancy SSB consumption (drinks/wk)	197	16.2	14.7, 17.6	170	15	13.3, 16.7	0.32
BPA during first half of pregnancy[2]	124	0.21	-0.02, 0.44	128	0.19	-0.02, 0.39	0.88
BPA during second half of pregnancy[2]	185	0.26	0.08, 0.43	158	0.28	0.1, 0.46	0.84
Child Characteristics							
Gestational age (wks)	198	39.1	38.9, 39.3	176	38.9	38.7, 39.1	0.09
Birth weight (grams)	198	3538	3476, 3600	176	3460	3396, 3524	0.09
Weight gain in first 6 months (100g/m)	163	7.24	7.0, 7.48	148	7.39	7.13, 7.64	0.43
BMI at 9 years (kg/m2)	120	20.6	19.7, 21.4	150	21.2	20.5, 22.0	0.25
Adiponectin at birth (μg/mL)	68	106.7	98.7, 114.8	93	107.1	100.6, 113.4	0.95
Adiponectin at 9 years (μg/mL)	88	45.0	41.3, 48.6	93	41.8	37.7, 45.8	0.24
$\log_{10}$(Leptin) at birth	68	1.0	0.9, 1.1	93	1.1	1.0, 1.2	0.53
$\log_{10}$(Leptin) at 9 years	88	0.8	0.7, 0.9	93	1.0	0.9, 1.1	0.01

[1]T-test comparing characteristics by set.

[2]Urinary BPA measures are log 2 transformed and specific gravity adjusted.

BMI - Body Mass Index, SSB - Sugar-Sweetened Beverage Consumption, BPA - Bisphenol A.

At time of pregnancy, mothers tended to be slightly older in the validation set (μ=26.3 years, 95% CI 25.6, 27.1) compared to mothers in the discovery set (μ=25.4 years, 95% CI 24.7, 26.1) and this difference reached borderline statistical significance (P=0.07). Additionally, children in the validation set had statistically higher 9 year leptin (μ=1.0, 95% CI 0.9, 1.1) compared to those in the discovery set (μ=0.8, 95% CI 0.7, 0.9). Further, we found that children in the

validation set tended to have lower gestational age and birth weight, however these differences did not reach statistical significance (P=0.09 for both). Children were similar for all other anthropometric and adipokine values between the two sets. No differences were found for maternal urinary BPA concentrations between the two sets for first half (μ=0.21, 95% CI -0.02, 0.44 vs. μ=0.19, 95% CI -0.02, 0.39, P=0.88) and second half measures (μ=0.26, 95% CI 0.08, 0.43 vs. μ=0.28, 95% CI 0.1, 0.46, P=0.84).

PPARγ Methylation Structure

Figure 1: PPARγ CpG Sites

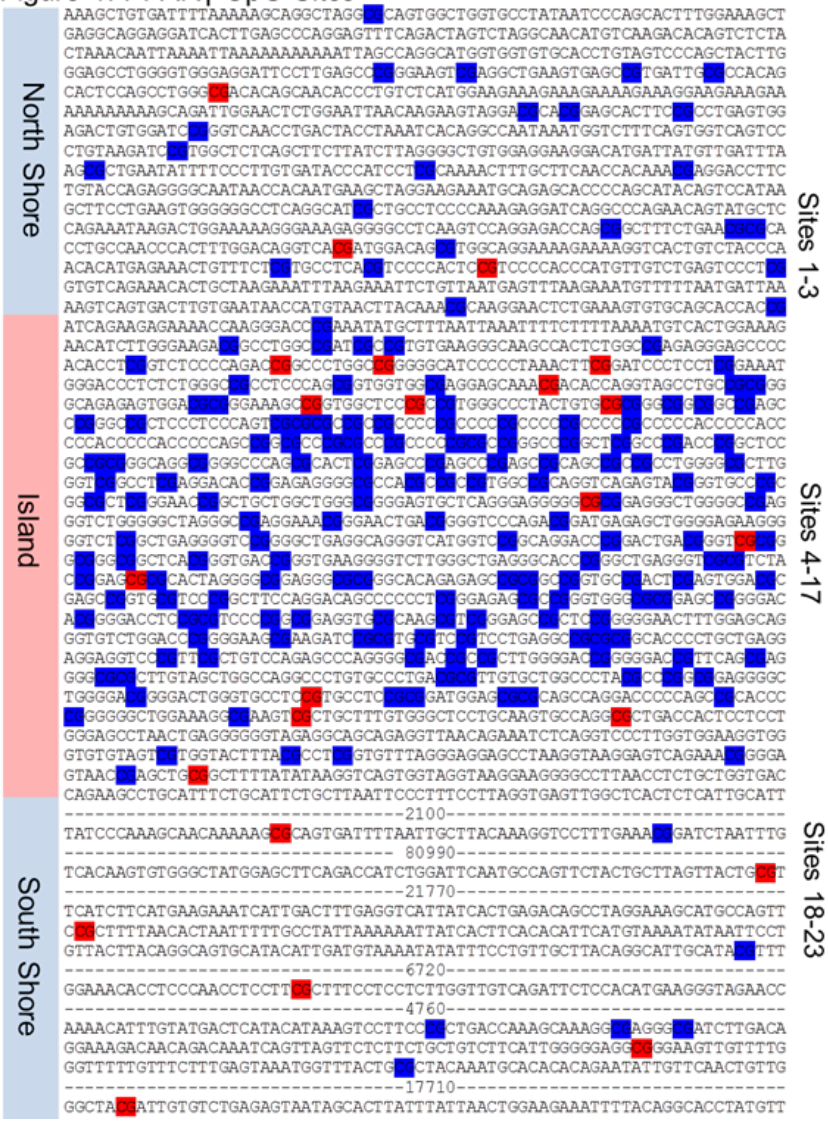
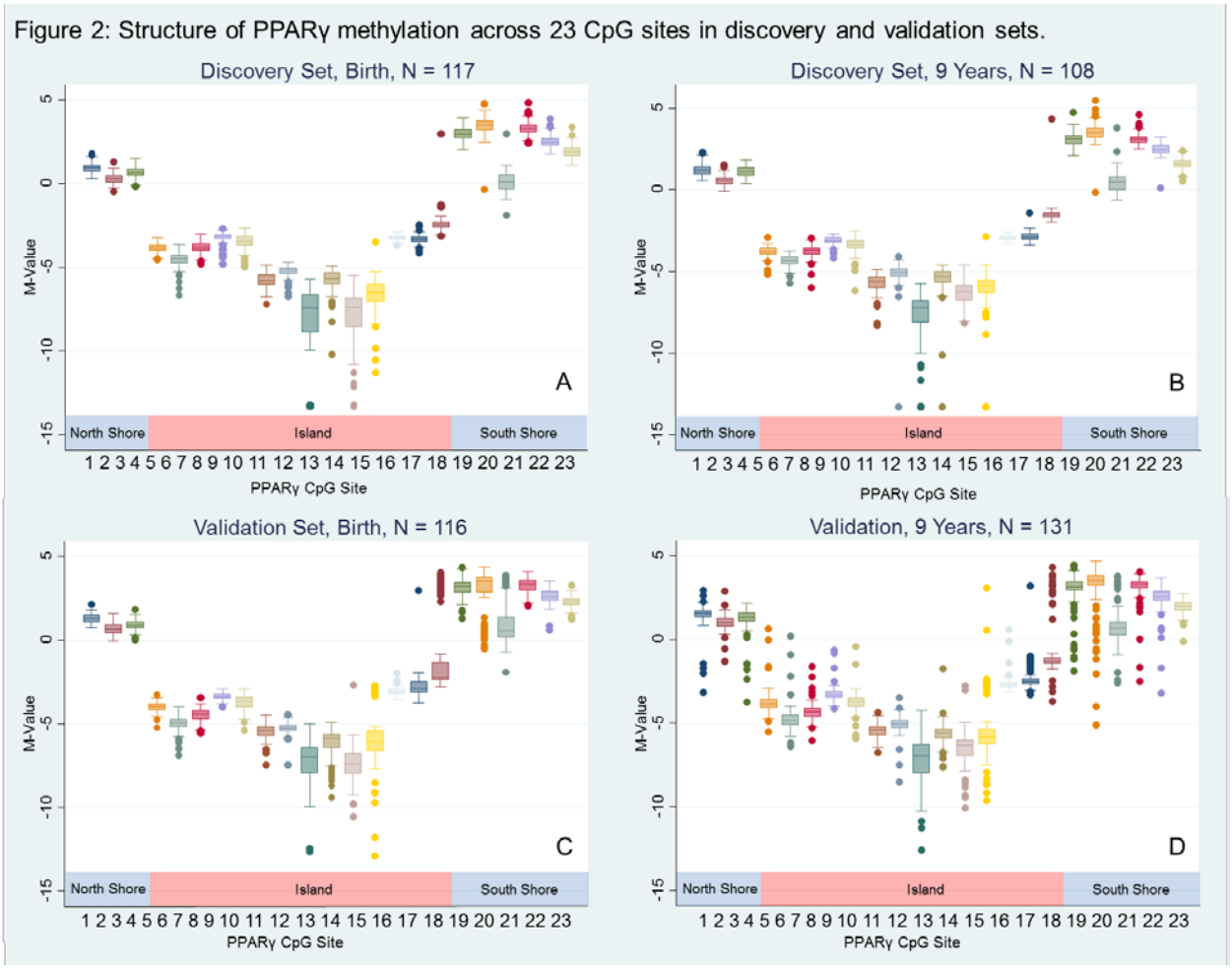


Figure 1 shows the distribution of 23 CpG sites (red squares) measured by the Illumina Methylation 450K Array. Blue squares indicate all other CpG sites (~200). Light blue shade represents north shore sites 1-3 and south shore sites 18-23 and orange shade indicates island sites 4-17.

Figure 2 shows methylation M-Values across the 23 *PPARγ* CpG sites.



We observed the same pattern of methylation across the sites in the discovery set at time of birth (A), at 9 years (B) and in the validation set at time of birth (C) and at 9 years (D). As expected, CpG sites in both the north and south shore (blue shade) tended to be highly methylated, island sites (orange shade) have significantly lower methylation. Interestingly, the methylation patterns are closely replicated between the different ages and sets. For example, site 20 in the south shore has significantly lower methylation compared to neighboring sites in all 4 groups. Additionally, we provide average M-values by site location (i.e. M-values were averaged for sites 1-3 to get the average north shore methylation) and age for both sets in table 2. Average methylation increased from the birth to 9-year time points for north shore sites (ΔM-value=0.35, 0.25 for discovery and validation sets, respectively; P<0.001 for both). A suggestive trend of increased methylation with age was found for island sites (P=0.17, 0.03 for discovery and validation sites) and no increases were found for south shore sites (P=0.95, 0.38 for both sets).

Table 2: Average M-values across 23 PPARγ sites for discovery and validation sets, by age and site location.											
	North Shore, Sites 1-3				Island, Sites 4-17			South Shore, Sites 18-23		
	N	Average M-Value	Std.Dev	P-value[1]	Average M-Value	Std.Dev	P-value	Average M-Value	Std.Dev	P-value
Discovery, Birth	117	0.63	0.41		-4.84	2.01		2.38	1.22	
Discovery, 9 Years	108	0.98	0.42	<0.001	-4.48	1.92	0.17	2.39	1.15	0.95
Validatin, Birth	116	0.96	0.39		-4.9	1.98		2.57	1.16	
Validation, 9 Years	131	1.23	0.69	<0.001	-4.37	1.88	0.03	2.43	1.32	0.38

[1]T-test for difference over age within set.

We also examined inter-CpG site correlations in both discovery and validation sets. North shore sites 1-3 had closest correlations with each other compared to all other sites. These pairwise correlations are summarized in table 3:

Table 3: Inter-CpG Correlation									
	Discovery Set					Validation Set			
Birth	N = 117				N = 116				
Site	1	P-Value	2	P-Value	Site	1	P-Value	2	P-Value
1					1				
2	0.63	<0.001			2	0.56	<0.001		
3	0.4	<0.001	0.73	<0.001	3	0.35	<0.001	0.71	<0.001
9 Years	N = 108				N = 131				
Site	1	P-Value	2	P-Value	Site	1	P-Value	2	P-Value
1					1				
2	0.54	<0.001			2	0.45	<0.001		
3	0.52	<0.001	0.78	<0.001	3	0.41	<0.001	0.57	<0.001

Strongest correlations were observed between sites 2 and 3 within all 4 groups. We did assess correlations across all 23 sites (correlation matrix in supplementary figure). Sites within the north and south shores had high inter-correlation while island sites had no observable pattern.

Relationship between PPARγ Methylation and size (birth weight and BMI) and child adipokines

Table 4 summarizes our results of associations between *PPARγ* site methylation and child size and adiponectin and leptin levels. Using the discovery set, we found that methylation at birth in sites 1 and 18 was significantly and inversely associated with birth weight. Methylation at 9 years in sites 1, 20, and 22 was significantly and inversely associated with 9-year BMI. Additionally, within the 9-year time point, methylation in sites 1 and 20 was significantly and positively associated with adiponectin and negatively associated with leptin.

Table 4: Relationships between PPARγ methylation, child size and adipokines.

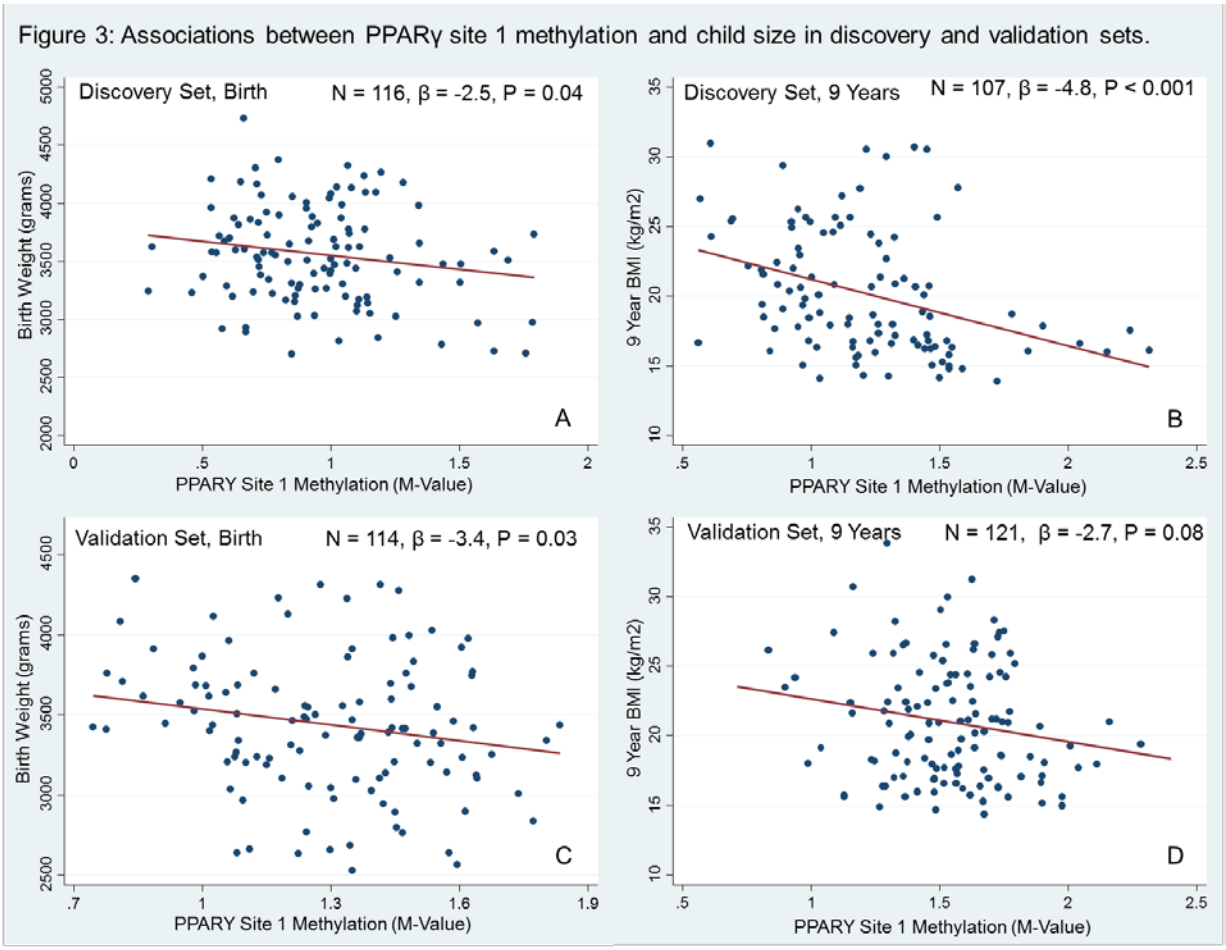
		Discovery								
Birth		Birth Weight[1]			Adiponectin			log_{10}(Leptin)		
		N	β[2]	P-Value	N	β	P-Value	N	β	P-Value
Site 1		116	-2.5	0.04	26	-12.3	0.59	26	-0.36	0.14
Site 18		117	-2.2	0.04	26	19	0.31	26	-0.17	0.4
9 Years		Body Mass Index			Adiponectin			log_{10}(Leptin)		
		N	β	P-Value	N	β	P-Value	N	β	P-Value
Site 1		107	-4.8	<0.001	88	12.2	0.02	88	-0.36	0.02
Site 20		108	-2.1	0.01	88	6.3	0.03	88	-0.17	0.04
Site 22		108	-2.8	0.02	88	10.1	0.12	88	0.01	0.99
		Validation								
Birth		Birth Weight			Adiponectin			log_{10}(Leptin)		
		N	β	P-Value	N	β	P-Value	N	β	P-Value
Site 1		114	-3.4	0.03	66	3.9	0.76	73	0.1	0.6
Site 18		116	-0.8	0.32	73	5.7	0.43	73	-0.01	0.93
9 Years		Body Mass Index			Adiponectin			log_{10}(Leptin)		
		N	β	P-Value	N	β	P-Value	N	β	P-Value
Site 1		121	-2.7	0.08	85	0.4	0.96	85	0.02	0.89
Site 20		116	1.1	0.12	87	-2.2	0.31	87	-0.01	0.86
Site 22		122	0.1	0.9	77	-2.1	0.77	82	0.06	0.65

[1]Used in analyses in kg units.

[2]Beta coefficient from linear regression.

When we re-examined these associations in the validation set, only methylation at site 1 was significantly associated with child size. Overall, *PPARγ* site 1 methylation at birth had statistically significant inverse associations with child birth weight (β=-2.5, P=0.04; β=-3.4, P=0.03) in both the discovery and validation sets. Additionally, site 1 methylation at 9 years was significantly and negatively associated with 9-year BMI in the discovery set (β=-4.8, P<0.001) and borderline significant in the validation set (β=-2.7, P=0.08) (Figure 3). These associations

were not replicated with respect to adipokines. Additionally, using pooled data from both batches, we found that *PPARγ* site 1 methylation remained significantly and inversely related to child size within the birth and 9-year time points, even after Bonferroni adjustment for multiple testing.



Subsequently, we examined whether methylation at birth was associated with BMI and adipokine levels at 9 years but found no significant relationships (Table 5).

Table 5: Associations between PPARγ site 1 methylation at birth and 9-year BMI, adiponectin and leptin.

	PPARγ site 1 methylation at birth					
9-Year Outcome	**Discovery Set**			**Validation Set**		
	N	**β[1]**	**P-Value**	**N**	**β[1]**	**P-Value**
Body Mass Index	39	-0.7	0.79	89	0.6	0.74
Adiponectin	24	-4.6	0.68	61	11.2	0.27
$\log_{10}$(Leptin)	24	0.03	0.91	61	0.11	0.59

[1]Beta coefficient from linear regression.

Associations between perinatal factors and PPARγ Methylation

Given our results showing that *PPARγ* methylation at site 1 may be closely related to child size, we next examined its associations with perinatal factors, including maternal age at pregnancy, pre-pregnancy BMI, maternal sugar-sweetened beverage consumption during pregnancy, maternal BPA concentrations during the first and second halves of pregnancy, child birth weight, gestational age and rate of growth in the first 6 months of life. To address the current data gap on whether perinatal factors have long-term influence on epigenetic changes, we focused our analyses associations with site 1 methylation levels at 9 years. Our results are summarized in table 6:

Table 6: Associations between perinatal factors and 9-year PPARγ site 1 methylation.

	Discovery Set			Validation Set		
Maternal Characteristics	**N**	**β[1]**	**P-Value**	**N**	**β**	**P-Value**
Age (years)	108	-0.01	0.49	131	0.01	0.54
Pre-pregnancy BMI (kg/m^2)	107	-0.01	0.13	126	-0.01	0.17
Pregnancy SSB consumption (drinks/wk)	108	-0.01	0.09	126	0.002	0.27
BPA during first half of pregnancy[2]	68	-0.04	0.18	97	-0.02	0.54
BPA during second half of pregnancy[2]	101	0.02	0.53	116	0.03	0.21
Child Characteristics						
Gestational age (wks)	108	0.01	0.62	127	-0.01	0.8
Birth weight (grams)	108	0.1	0.35	127	0.1	0.26
Weight gain in first 6 months (100g/m)	102	-0.17	0.42	119	0.06	0.76

[1]Beta coefficient from simple linear regression.

[2]Urinary BPA measures are log 2 transformed and specific gravity adjusted.

BMI - Body Mass Index, SSB - Sugar-Sweetened Beverage Consumption, BPA - Bisphenol A.

No significant associations were found between these perinatal factors and *PPARγ* site 1 methylation at 9 years.

Discussion

In this analysis, we aimed to address several gaps on 1) structure of *PPARγ* methylation, 2) associations between *PPARγ* site methylation and measures of child size (birth weight and BMI) and adipokine levels at birth and 9 years, and 3) relationships between perinatal factors and *PPARγ* methylation. We found that *PPARγ* methylation displays a highly conserved pattern at birth and 9 years, in both the discovery and validation sets. Further, we report that within the birth and 9-year time points, *PPARγ* site 1 methylation has a consistent and inverse association with child size in both sets. We did not find any statistically significant associations between the perinatal characteristics examined and *PPARγ* site 1 methylation. Overall, our analyses indicate that *PPARγ* methylation may play an important role in obesity development in children, reflecting child birth weight and BMI.

PPARγ CpG organization is typical of many other genes, with its promoter region containing a CpG island flanked by north and south shore sites. However general questions remain on relationships between sites within a given structural region and to what extent epigenetic marks are stable over time. Virtually no studies provide detailed analysis of CpG sites located near each other (<100 base pairs - bp). Eckhardt et al (2006) reported that correlations in CpG methylation were significant for distances < 1,000 bp and deteriorated rapidly for distances > 2,000 bp (Eckhardt et al. 2006). Adding to this, we found that sites within north or south shore were highly inter-correlated and this pattern was less observed for island sites. This result further emphasizes the idea that location of a particular CpG site may be functionally important.

With respect to changes in DNA methylation over time, although some reports indicate stable methylation patterns (Fraga et al. 2005; Wong et al. 2010) others do not (Talens et al. 2010). Fraga et al (2004) showed that while 3-year-old monozygotic (MZ) twins showed relatively few epigenetic differences, there was considerably larger variability in older twin pairs? (Fraga et al. 2005). Additionally, Wong et al (2010) found epigenetic differences in MZ twins as young as 5 years of age (Wong et al. 2010). On the other hand, analyses of blood samples from the Netherlands Twin Register showed that of 8 regions examined, 5 displayed stable methylation patterns for up to 20 years (Talens et al. 2010). Echoing this, our results indicate that *PPARγ* methylation is stable over the birth to 9-year period and that even minute differences between CpG sites are conserved.

Importantly, although the pattern of CpG sites remained similar over time (Figure 2), we found that north shore sites had slightly but significantly higher M-values (4% to 6% methylation) at 9 years compared to birth, in both discovery and validation sets. Previous literature has identified both hypo and hyper-methylation changes with age and taken together, these findings suggest that different genomic regions may have varying stability over time (Bell et al 2006) (Bollati et al. 2009; Maegawa et al. 2010; Teschendorff et al. 2010). Overall, our data show that *PPARγ* CpG methylation is carefully maintained, highlighting its potentially important role in regulating *PPARγ* function.

In addition to data gaps on methylation structure and organization, very little remains known about the epigenetic changes that accompany obesity development. Our report of an inverse relationship between *PPARγ* site 1 methylation and body size within both the birth and 9-year time points is consistent with the idea that higher methylation silences *PPARγ*, suppressing adipogenesis – as expected in individuals with smaller birth weight and/or BMI. However, we did not find that site 1 methylation at birth could predict BMI or adipokine levels at 9 years. Our analyses on these relationships over time were limited by relatively small numbers of children

and future work should address whether changes in *PPARγ* methylation precede obesity development.

We were able to detect a significant positive relationship with adiponectin and significant negative association with leptin (also in the expected direction) in only the discovery group at 9 years. Reasons why these relationships were not validated are unclear and further research into the possible epigenetic regulation of adipokines is warranted. Of note, site 1 was located in the north shore – further emphasizing the potentially critical role of north shore sites in regulating gene expression.

Given that we were interested in evaluating whether the perinatal environment can have long-lasting effects on methylation, we restricted our analysis, testing for associations between perinatal characteristics and only *PPARγ* site 1 methylation at 9 years. We found no significant associations within either the discovery or validation set. Additionally, given BPA's sexually dimorphic responses (Miyawaki et al. 2007; Wei et al. 2011), we tested for sex-specific associations, also finding no significant relationships. Whether *PPARγ* methylation is truly resilient against environmental influence remains unclear and further research in larger population samples is needed.

An important point to consider is that we measured methylation in DNA isolated from whole blood without adjusting for heterogeneity of its component cell types. As DNA methylation is thought to be, at least in part, cell-specific – there is ongoing research to determine validity of blood-based methylation measures (Adalsteinsson et al. 2012; Jacoby et al. 2012; Reinius et al. 2012). To date, some studies argue that it is important to consider the different blood cell subtypes, including neutrophils, lymphocytes and monocytes (Reinius et al. 2012), while others do not (Talens et al. 2010; Jacoby et al. 2012). Overall, these conflicting data suggest that methylation stability across cell types depends on the CpG site itself. Our data displayed a consistent pattern of CpG methylation over multiple blood samples suggesting that heterogeneity of blood cell types may not significantly affect *PPARγ* methylation. Nevertheless, whether *PPARγ* methylation in blood is a suitable marker for its activity in adipocytes remains unclear and establishing this relationship is an important future direction. Overall, while our research highlights a potentially important role of DNA methylation in regulating *PPARγ* activity, key questions remain on its relationship with adipokines and ability to affect future metabolic health.

Supplementary Figure, Correlation Matrix for PPARγ sites 1-23.
Discovery Set, Birth, N = 116.

North Shore

Island

South Shore

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1.00																						
2	0.62	1.00																					
3	0.39	0.72	1.00																				
4	-0.37	-0.32	-0.26	1.00																			
5	-0.29	-0.29	-0.21	0.25	1.00																		
6	-0.19	-0.30	-0.11	0.29	0.35	1.00																	
7	-0.53	-0.50	-0.27	0.41	0.44	0.49	1.00																
8	-0.37	-0.29	-0.08	0.36	0.14	0.50	0.47	1.00															
9	-0.26	-0.26	-0.30	0.32	0.12	0.23	0.30	0.32	1.00														
10	-0.46	-0.41	-0.21	0.35	0.38	0.43	0.56	0.43	0.22	1.00													
11	-0.16	-0.12	0.02	0.01	0.15	0.07	-0.06	0.08	-0.06	0.10	1.00												
12	-0.44	-0.44	-0.16	0.21	0.15	0.40	0.58	0.38	0.13	0.45	0.03	1.00											
13	-0.10	-0.12	-0.02	0.12	0.15	0.24	0.24	0.12	0.21	0.13	0.06	0.11	1.00										
14	0.08	0.09	0.06	0.13	0.11	0.35	0.05	0.28	0.28	0.03	-0.10	-0.05	-0.02	1.00									
15	0.06	0.10	0.19	0.14	0.07	0.17	0.22	0.12	0.09	0.19	-0.16	0.18	-0.09	0.08	1.00								
16	-0.19	0.00	0.16	0.20	0.14	0.22	0.38	0.14	0.12	0.38	-0.21	0.33	0.07	0.10	0.61	1.00							
17	-0.08	0.16	0.41	0.16	0.14	0.08	0.20	0.14	0.10	0.22	-0.14	0.16	0.04	0.09	0.58	0.65	1.00						
18	0.14	0.13	0.16	0.07	-0.08	0.02	0.04	-0.13	-0.05	0.01	-0.11	-0.11	0.00	-0.04	0.09	-0.02	0.12	1.00					
19	0.38	0.37	0.27	-0.14	-0.19	-0.17	-0.33	-0.11	-0.15	-0.12	-0.11	-0.23	-0.01	-0.03	0.11	-0.06	0.14	0.21	1.00				
20	0.67	0.24	0.09	-0.15	-0.12	-0.11	-0.30	-0.28	-0.14	-0.23	-0.09	-0.30	-0.04	0.08	0.06	-0.18	-0.23	0.01	0.14	1.00			
21	0.49	0.41	0.28	-0.34	-0.36	-0.43	-0.54	-0.37	-0.31	-0.30	-0.01	-0.43	-0.15	-0.20	-0.03	-0.24	-0.03	0.17	0.59	0.23	1.00		
22	0.31	0.17	0.09	-0.12	-0.09	-0.22	-0.28	-0.30	-0.10	-0.06	-0.07	-0.20	-0.09	-0.16	0.09	-0.04	0.05	0.24	0.51	0.22	0.48	1.00	
23	0.56	0.38	0.18	-0.15	-0.30	-0.29	-0.47	-0.35	-0.12	-0.30	-0.12	-0.49	-0.13	-0.06	-0.11	-0.30	-0.20	0.25	0.46	0.40	0.56	0.50	1.00

CHAPTER 8

CONCLUSION AND SYNTHESIS OF FINDINGS

Evidence from both molecular and epidemiological studies argues that the *in utero* environment may program children to be on a continuous path towards altered metabolic health in later life. To date, informative biomarkers of metabolic health are the protein hormones adiponectin and leptin, however no data are available on their development over the entire childhood period. Furthermore, a specific mechanism, tying the perinatal environment with later life outcomes may involve epigenetic changes in the offspring epigenome but human data are lacking.

In particular, this dissertation's specific aims were designed to address the following data gaps:

- While plasma adiponectin levels are inversely related to BMI and plasma leptin levels are directly related to BMI in older children, these relationships remain unclear at younger ages.

- The extent to which early life adiponectin/leptin levels predict future life adiponectin/leptin levels remains unclear but would help assess children's tendency to particular metabolic health over time.

- Although children display differing BMI trajectories from birth, little is known about the accompanying changes in adiponectin/leptin levels.

- Several features of the perinatal environment, specifically maternal BMI, diet, gestational weight gain, child birth weight, gestational age and infancy growth rate, are associated with changes in child body size, but their relationships with adipokines remain unclear.

- *In utero* exposure to the high-volume production chemical BPA affects metabolic health in animals and cross-sectional human data show that BPA is positively associated with obesity, however whether BPA exposure precedes adipokine disturbances remains unknown.

- Most currently published studies on DNA methylation patterns associated with obesity suffer from multiple testing issues and virtually no human data exist on methylation with the key adipogenic gene *PPARγ*.

- While *PPARγ* methylation has been shown to control adipogenesis in animals, no data are available on relationships between *PPARγ* methylation and child size or adiponectin/leptin levels.

To address these data gaps, we took advantage of the rich collection of questionnaire, anthropometric and biological data available in the CHAMACOS longitudinal birth cohort.

Our key findings are:

- Adjusting for 9-year BMI, adiponectin closely reflects children's lipid profile (including triglycerides, very low-density lipoproteins and high-density lipoproteins) while leptin does not. This result highlights the utility of adiponectin as a marker for overall metabolic health, energy use and insulin sensitivity. **Chapter 4.**

- Over the birth to 9-year period, leptin remains tightly and positively correlated with child weight (corr. = 0.46, 0.52, 0.73, 0.78 at birth, 2, 5, and 9 years, respectively) while adiponectin displayed a much weaker relationship (corr. = 0.08, -0.24, -0.28, -0.42 at birth, 2, 5, and 9 years, respectively). It is interesting to observe this strengthening association over time, and we speculate that this implies a gradual solidifying of the molecular mechanisms regulating adipokine levels. **Chapter 5.**

- Correlations over the 0 – 2, 2 – 5, and 5 – 9-year periods increased for both leptin (r=0.06, 0.31 and 0.62) and adiponectin (r=0.25, 0.41 and 0.46). These results suggest greater plasticity during the early infancy period and progressive constrainment to specific metabolic health with age. **Chapter 5.**

- Our mixture modeling analyses of leptin growth over childhood showed that leptin trajectories are heterogeneous over the 2 – 9-year period. In fact, the leptin groups described here match closely in shape the three BMI trajectories identified by Pryor et al (2011), who reported on low-stable, moderate and rapidly-rising BMI groups. In contrast, adiponectin trajectories were most different over the birth – 2-year period. Given the significant differences in pre and post-partum energy regulating mechanisms and changing body composition during infant development, adiponectin levels may more closely reflect these changes rather than of body size itself. **Chapter 5.**

- We did identify a steep-dropping adiponectin trajectory (1A) and rapidly-rising leptin trajectory (2L). 2L children are at increased risk of obesity at 9 years, however the long term consequences of being in 1A remain unclear. We had lipid profile data only on a small subset (N=35) of the 80 children in this study and our data showed that group 1A children tended to have higher mean triglycerides (123.2 vs. 87.7 mg/dL; N=9 and 12 respectively), very low-density lipoproteins (24.7 vs. 17.6 mg/dL) and lower high-density lipoproteins (46.4 vs. 55.1 mg/dL) compared to the references group 3A. **Chapter 5.**

- With respect to the perinatal environment - adipokine relationships, we found that children whose mothers had higher sugar-sweetened beverage (SSB) consumption during pregnancy had increased odds of being in the steep-dropping 1A group and children with higher birth weight had increased odds of being in the rapidly-rising 2L group. **Chapter 5.** Additionally, we found that children with increased rate of growth during the first 6 months of life had lower adiponectin levels at 9 years, controlling for their 9-year BMI.

Chapter 4. These results add evidence to the hypothesis that metabolic health may be programmed during early life development.

- We did observe that pre-natal BPA concentrations may affect adiponectin and leptin levels in 9-year-old children, however these associations were sexually dimorphic and some were in unexpected directions. Controlling for sociodemographic covariates and 9-year child BMI, we found that BPA concentrations during late pregnancy were associated with increased plasma leptin in boys (β=0.06, P=0.01). Surprisingly, we found that BPA concentrations during early pregnancy were directly associated with plasma adiponectin levels in girls (β=3.71, P=0.03). While there is some evidence that *in utero* BPA exposure may lead to a competing endpoint of hyperactivity, resulting in lower obesity and increased adiponectin, further data are needed to resolve these inconsistencies. **Chapter 6.**

- We found a stable methylation pattern across *PPARγ* CpG sites comparing birth to 9 years in both discovery and validation sets. This argues for a potentially important role of methylation in regulating *PPARγ* activity. **Chapter 7.**

- We identified that *PPARγ* site 1 methylation was inversely and significantly associated with child size at birth and at 9 years in the discovery set and replicated this relationship in the validation set. The direction of this association is consistent with the idea that higher methylation silences *PPARγ,* suppressing adipogenesis – as expected in individuals with smaller birth weight and/or BMI. Additionally, we point out that site 1 is located in the north shore – a region thought to be particularly important in gene regulation. **Chapter 7.**

- Our overarching hypothesis was that perinatal characteristics may affect child metabolic health through epigenetic changes. However, we found no significant associations with *PPARγ* site 1 methylation in our analyses of early life factors, including maternal age, pre-pregnancy BMI, SSB consumption, BPA levels during pregnancy or child gestational age, birth weight, and weight gain during first 6 months of life. Whether *PPARγ* methylation is truly robust against environmental influence remains unclear and further research is needed. **Chapter 7.**

Overall, we found evidence supporting the idea that children are on particular metabolic health trajectories from birth that become progressively more defined with time. We found several risk factors for altered adipokine levels, including increased maternal SSB consumption during pregnancy, increased child birth weight and increased rate of growth in first 6 months of life. Our longitudinal data on adiponectin and leptin highlight their different utilities, arguing that leptin remains tightly linked to body size while adiponectin is more reflective of underlying metabolic health over childhood. Finally, although epigenetic changes may play an important role in determine body size, their relationships with adipokines and *in utero* factors remain poorly understood. Future work on DNA methylation should focus on understanding its overall structure over the genome, with emphasis placed on reproducibility and validation.

CHAPTER 9

FUTURE DIRECTIONS

Key future directions include:

- Examining ability of early life adiponectin levels to predict development of metabolic syndrome or type 2 diabetes in later life.

- Further exploring determinants of adiponectin levels, particularly focusing on the early childhood period.

- Given emerging data that features of BMI growth curves, including infancy peak and adiposity rebound, can predict future child growth and metabolic health, it is important to understand changes in adipokines during these critical developmental points (Wen et al. 2012).

- Additional work is needed to examine mechanisms of sex differences in response to BPA exposure and, furthermore, the exact biological pathways in which BPA can affect leptin and adiponectin levels remain unknown.

- There remain large data gaps on the overall structure of DNA methylation and its relationship with obesity and adipokine levels. Future work should examine other adipogenic genes not limited to PPARγ, focusing on replication and validity within the large-scale multi-CpG study designs.

DISSERTATION PUBLICATIONS

1. Associations between perinatal factors and adiponectin and leptin in 9-year-old Mexican-American children. (Chapter 4)
Volberg V, Harley KG, Aguilar RS, Rosas LG, Huen K, Yousefi P, Davé V, Phan N, Lustig RH, Eskenazi B, Holland N.

Published in Pediatric Obesity. Jan 16, 2013.

2. Adiponectin and Leptin Trajectories in Mexican-American Children 1 from Birth to 9 Years of Age. (Chapter 5)
Volberg V, Heggeseth B, Harley K, Huen K, Yousefi P, Davé V, Tyler K, Vedar M, Eskenazi B, Holland N.

Submitted to PLOS-ONE. March 21, 2013

3. **Maternal bisphenol A exposure during pregnancy and its association with adipokines in Mexican-American children. (Chapter 6)**
Volberg V, Harley K, Calafat A, Davé V, McFadden J, Eskenazi B, Holland N.

Submitted to Environmental and Molecular Mutagenesis. April 20, 2013.

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