Relationships of DNA Methylation, Methyl-Donor Rich Diet, and Physical Activity with Pubertal Timing and Tempo among Mexican Adolescents

by

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Table of Contents

List of Tables

List of Figures

Abstract

Variations in pubertal timing and tempo have implications for the risk of adult diseases. Influences on the timing of pubertal onset and pace of pubertal progression have been widely discussed, but the underlying biological mechanisms remain unclear. Epigenetic modifications, the study of heritable changes in gene expression that does not involve changes to the underlying DNA sequence, are known to regulate developmental processes; thus, puberty, known to be one developmentally plastic phase, could be potentially affected.

Given that gene expression changes mediated by DNA methylation, may play a role in pubertal tempo regulation, availability of methyl donor nutrients also could affect these pathways. Additionally, few studies have examined the associations of physical activity with DNA methylation, pubertal status and reproductive hormones among free-living adolescents. Since adolescence is the life stage of significant change during which cognitive, psychosocial and lifestyle behaviors that persist into adulthood are formed, examination of epigenetic and lifestyle influences could inform future interventions. We conducted a population-based analysis using the Mexico City birth cohort, ELEMENT, to deepen our understanding of the link between developmental epigenetic processes and pubertal trajectory.

In Aim 1, we used a longitudinal design to investigate the association of peripubertal blood DNA methylation at LINE-1 repetitive elements, known as the marker of global methylation, and growth-related candidate loci *IGF2, H19, HSD11B2* with pubertal timing and tempo, and observed gene-specific, sex-specific results. Among boys, a percent DNA methylation increase of *HSD11B2* was associated with earlier pubarche (onset of pubic hair). Increases in *IGF2* DNA

methylation were associated with later onset but faster progression of genital development in boys. Among girls, percent increase in DNA methylation of *H19* was associated with later onset of breast development.

In Aim 2, we examined first trimester maternal and adolescent diet patterns that may be associated with DNA methylation at LINE-1 repetitive elements in adolescence. We used LASSO to calculate an Epigenetics-Associated Diet Score (EADS) for each pattern; then tested the associations of these scores with pubertal status. We observed associations between maternal EADS and pubertal onset, but not pubertal progression. Each standard deviation (SD) higher maternal EADS was associated with higher odds of later onset of menarche. In contrast, we observed associations between adolescent EADS and pubertal progression, but not pubertal onset. For each SD higher adolescent EADS, there was increased odds of slower genital progression, and slower testicular development in boys.

In Aim 3, we used an isotemporal substitution paradigm and regression models to examine the association of different physical activity intensities with reproductive hormones (testosterone, cortisol, progesterone, and androstenedione concentrations), DNA methylation (LINE-1 repetitive elements and the genes *H19*, *HSD11B2*, and *PPARA*), and Tanner stages in both boys and girls. Results suggested that substituting 30 minutes of sedentary behavior for moderate physical activity per day was associated with lower testosterone levels in boys, while a 30-minute increase in sedentary behavior was associated with higher progesterone levels in girls. Substituting 30 minutes of sedentary behavior for vigorous physical activity per day was associated with higher percent of *HSD11B2* DNA methylation in boys, while a 30-minute increase in sedentary behavior was associated with lower percent of *HSD11B2* DNA methylation.

Findings from this dissertation suggested that modifications in peripubertal DNA methylation may influence pubertal outcomes. Epigenetic-associated diet and accelerometer-measured physical activity may also influence reproductive hormones and pubertal status in a sex-, timing- and intensity-specific manner. Limitations of this dissertation include 1) we did not genotype the ELEMENT population though our subjects come from a mixed ancestry population, 2) self-reported menarche and dietary information may not be accurate due to recall bias, and 3) we have only collected blood leukocytes to analyze DNA methylation level, and 4) growth-related candidate genes we included in the analysis are not directly linked to pubertal status. Nevertheless, this dissertation has numerous number of strengths. This is the first and only population-based study so far that conducted a full examination of global and gene-specific DNA methylation with pubertal onset and progression in both sexes. We also used validated food frequency questionnaires (FFQs) and accelerometers to estimate subjects' daily diet and physical activity patterns. In order to examine whether the findings are generalizable, future studies should consider recruiting a larger sample size with longitudinal design and repeated measurements.

Chapter 1 Introduction

Puberty and adolescence mark the metamorphosis of the child into the adult. This dynamic period of development is signaled by rapid, sexually dimorphic changes in body size, composition, and function (Rogol et al., 2002). Somatic growth and maturation are influenced by various factors that act independently or in concert to modify an individual's phenotypic plasticity, which could be pivotal to the future aging process (Rogol et al., 2002; Cutler & Mattson, 2006). From an endocrine perspective, puberty is the process by which and the period during which sexual maturation occurs and reproductive capacity is attained (Golub et al., 2008). Biologically, these maturational events are driven by reproductive hormonal changes and are continua that begin during intrauterine life and extend through the life cycle (Sisk & Foster, 2004). There are two seemingly related but hormonally distinct processes that mark the timing of change: 1) the maturation of the hypothalamic-pituitary-adrenal androgenesis system, or adrenarche (prepuberty), and 2) the maturation of the hypothalamic-pituitary-gonadal (HPG) system, or gonadarche (puberty) (Golub et al., 2008). Adrenarche typically begins at 6-8 years of age and involves increased production of adrenal androgens. Gonadarche usually begins several years later and involves increased production of reproductive hormones that are responsible for physical and sexual maturation. Appearance of pubic hair is considered a physical manifestation of adrenarche in boys and girls. The development of genital and testicles in boys and of breasts in girls are considered a manifestations of gonadarche (Wan et al., 2013). Tanner (1962) described five stages of puberty, ranging from 1 (no development) to 5 (adult development), that

classify visible secondary sexual characteristics and have been applied in clinical and research as the gold standard for assessing pubertal status (Shirtcliff, 2009).

Previous studies have shown that pubertal timing (onset of pubertal development) and tempo (pace of pubertal progression) are not synonymous and should be considered individually in puberty-related research (Negriff et al., 2015; Mendle et al., 2010; Marceau et al., 2011). There is empirical evidence of significant correlations between pubertal timing and tempo (Mendle et al., 2010; Biro et al., 2001), although the directions of the correlations are debated (Huang et al., 2009; Marceau et al., 2011). Nevertheless, it is well established that the timing of onset and tempo of pubertal development are important and unique risk factors for numerous health and behavior problems (Negriff et al., 2015; Negriff et al., 2010; Golub et al., 2008). The timing of puberty has a wide physiological variation in different racial/ethnic groups. Girls exhibiting pubertal change before age 6 among African Americans, age 7 among Caucasians in the United States or age 8 in other parts of the world or boys with pubertal findings before age 9 are considered to have precocious puberty (Herman-Giddens et al., 1997; Kaplowitz, 1999). Delayed puberty, on the other hand, is defined as the lack of pubertal onset by an age ≥2 SD above the population mean and can occur as late as age 13 in girls and age 14 in boys (Zhu et al., 2017).

Studies have implicated changes in pubertal timing and tempo as risk factors for multiple adult diseases, including polycystic ovary syndrome (PCOS), obesity, type 2 diabetes, and cardiovascular disease (Golub et al., 2008). In addition, adolescents with early pubertal timing and faster pubertal tempo are at a higher risk of accelerated skeletal maturation and short adult height, depression, eating disorders, substance use, potential sexual abuse, and delinquency (Marceau et al., 2011; Golub et al., 2008; Mendle et al., 2010; Stice et al., 2001; Ge et al., 2001;

Negriff et al., 2015). As reproductive hormones advance puberty, altered pubertal timing is a concern for the development of reproductive tract cancers later in life. Previous research has shown that an early age of menarche is a risk factor of breast cancer among girls; while a low age at male puberty is associated with an increased risk for testicular and prostate cancer (Golub et al., 2008).

Over recent decades, the risk factors for precocious or delayed puberty have been discussed, including chemical exposures, unbalanced diet, abnormal hormone levels caused by diseases, high intensity exercise and psychological stress (Cesario & Hughes, 2007; Jansen et al., 2017). However, the results are inconsistent across different human studies and underlying biological mechanisms that may lead to early or delayed pubertal onset and faster or slower pubertal tempo remain unclear.

With advances in molecular biology, we are beginning to understand that the aging process is characterized by a host of changes at the cellular and molecular levels, which include senescence, telomere shortening, and changes in gene expression (Jones et al., 2015). Given that epigenetic patterns established during development also change over the lifespan, epigenetic mark changes may constitute an important component of the aging process (Jones et al., 2015). Epigenetics is the study of heritable changes in gene expression that does not involve changes to the underlying DNA sequence (genotype), but can still affect how cells read the genes (phenotype) (Maccani & Marsit, 2009). At least three modes of epigenetic regulation, including DNA methylation, histone modification and non-coding RNA (ncRNA) associated gene silencing, are currently known to initiate and sustain gene regulation (Maccani & Marsit, 2009).

DNA methylation is one of the mechanisms of epigenetic regulation that is heritable through cell division (Bollati, 2008). In mammalian cells, DNA methylation usually occurs at the

C-5 position of cytosine within the CpG dinucleotide (Bollati, 2008). The process is first catalyzed by DNA methyltransferases (DNMTs) that transfer methyl groups from *S*-adenosylmethionine (SAM) to cytosine (Jones, 2001). There are three major DNMTs in mammals, including the maintenance DNMT1, and *de no* DNMT3a, and DNMT3b (Jones, 2001). The methylated cytosines then, bind to a family of methyl cytosine-binding proteins (MeCP1, MeCP2, MBD1, MBD2, MBD3 and MBD4) located in the promoter region of genes, thus inhibiting transcription factors from binding to that promoter (Jones, 2001).

Information on DNA methylation for this dissertation was analyzed from Long Interspersed Nuclear Element (LINE-1), and growth-related genes Insulin Like Growth Factor 2 (*IGF2*), *H19*, Hydroxysteroid 11-Beta Dehydrogenase 2 (*HSD11B2*), and Peroxisom Proliferator Activated Receptor Alpha (*PPARA*). LINE-1 are transposable elements and comprise approximately 17% of the human genome (Lander et al., 2001). *IGF2* and *H19* genes are imprinted in mammals. *IGF2* is a paternal imprinted growth factor that promotes both fetal and placental growth and also nutrient transfer from mother to offspring via the placenta (Nordin et al, 2012). *H19* gene is a maternal imprinted gene for a long noncoding DNA, playing a role in the negative regulation of body weight and cell proliferation (Gabory et al., 2009). *HSD11B2* catalyzes the glucocorticoid cortisol to the inactive metabolite cortisone, thus preventing illicit activation of the mineralocorticoid receptor (National Institutes of Health: Genetics Home Reference). *PPARA* is a transcription factor and a major regulator of lipid metabolism in the liver. Studies indicate that PPARA plays an important role in the management of energy stores during fasting, too (Kersten et al., 1999).

Recent work has highlighted the significance of DNA methylation in both DNA repair and genome stability, which is essential for cell development, the aging process,

carcinogenesis, etc. (Jones, 2001). The result of epigenetic changes during aging is altered local accessibility to the genetic material, leading to aberrant gene expression, reactivation of transposable elements, and genomic instability (Pal & Tyler, 2016). Until recently, one of the most promising new aging clocks that serves as a measurable biomarker of epigenetic age is DNA methylation (DNAm) age (Horvath, 2013). There is a strong correlation between reproductive aging and organismic aging; age of pubertal onset and concentrations of gonadal steroids are considered as potential predictors of aging (Nelson, 1988). The Developmental Origins of Health and Disease (DOHaD) theory includes puberty/adolescence period as one of the specific sensitive windows of developmental plasticity when environmental exposures and stressors could lead to time- and tissue- specific effects, via influencing the epigenome (Heindel & Vandenberg, 2015). Previous animal and population studies have found some evidence supporting the association of DNA methylation levels with pubertal brain development and pubertal aging (Almstrup et al., 2016; Morrison et al., 2014). Therefore, it is plausible to make the assumption that epigenetic modifications may be associated with the timing of pubertal onset and the pace of pubertal progression.

Epigenetic modification is a regular and natural occurrence that can be influenced by factors such as age, environmental exposure, lifestyle, and disease state (Maccani, 2009). Moreover, since adolescence is also the life stage of significant change during which cognitive, psychosocial and lifestyle behaviors that persist into adulthood are formed, examination of epigenetic and lifestyle influences could inform future interventions (Sata F, Springer, 2019; Nelson et al., 2005; Videon & Manning, 2003; Summerbell et al., 1996). Studies have shown poor diet and sedentary behavior patterns, such as high TV/video during adolescence are associated with more risk behaviors and higher risk of metabolic syndrome later in life (Nelson

& Gordon-Larsen, 2005; Pan & Pratt, 2008). Therefore, other than examining the association between epigenetic changes and puberty, we also aim to explore whether improving dietary quality and increasing physical activity would modify the associations.

DNA methylation depends upon the availability of methyl groups from *S*-adenosyl methionine, which is derived from methionine. Thus, methyl donor nutrients, including folate, choline/betaine, methionine, riboflavin (B_2 vitamin), pyridoxine (B_6 vitamin) and cobalamin (B_{12} vitamin) are essential of the one-carbon metabolism cycle and transmethylation process (Anderson et al., 2012). Animal studies showed that rats fed diets deficient in methyl donors have hypomethylated DNA methylation in their liver tissues, in both global methylation and gene-specific methylation patterns, including c-myc, c-Ha-ras, c-fos, and EGF-receptor (Tsujiuchi et al., 1999; Wainfan & Poirier, 1992). Moreover, methyl donor supplementation during weaning was able to prevent intergenerational amplification of obesity in mice models (Waterland et al., 2008; Cordero et al., 2013). Thus, we hypothesize that methyl donor nutrients may affect pubertal timing and tempo via modifying DNA methylation.

We are also interested in learning more about the association of physical activity patterns with reproductive hormones, epigenetic and puberty among healthy adolescents. Previous studies have suggested an association between physical activity and pubertal timing. One systematic review of 154 papers summarized that exercises or intense physical activities (e.g., ballet, soccer, figure skating) could lead to, on average, a 1-year delay in age at menarche in Caucasians (Yermachenko, 2014). The association between physical activity and reproductive hormones, which advance pubertal timing and progression, is also well-reported. A systematic review with 40 randomized controlled trials of healthy adult women concluded that there was an inverse correlation of physical activity with total estradiol and free estradiol concentrations, and

a positive correlation with SHBG concentrations. No significant association was observed with other estrogens and total testosterone concentrations (Ennour-Idrissi et al., 2015). Furthermore, previous evidence has shown current and cumulative reproductive hormone exposures were associated with change of LINE-1 and Alu DNA methylation levels among healthy postmenopausal women (Boyne et al., 2017). Nevertheless, to our knowledge, no study has gone one step further and examined the association between physical activity, DNA methylation, reproductive hormones and pubertal status among healthy adolescents. By testing the association, we hope to better understand the mechanism of how physical activity may regulate pubertal timing and tempo.

Developmental influences have lifelong effects on cardiovascular, metabolic function and aging process, and these heritable traits and susceptibilities can be transmitted across generations by non-genetic means (Hanson et al, 2011). Puberty, one of growth milestones with high developmental plasticity (Hanson et al., 2011), should be considered as a fitting timeframe for intervention. However, few population-based studies have targeted this period and so much remain unclear, and we hope this dissertation will address this research gap. Through examining the association of blood DNA methylation of LINE-1 repetitive elements and candidate genes with pubertal outcomes, as well as exploring the effect of diets rich in methyl donor nutrients and physical activity levels on these associations, we expect to deepen our understanding on the link between developmental epigenetic processes and pubertal trajectories. We also hope findings of this dissertation can provide some evidence to support behavior interventions during adolescence, one of environmentally vulnerable phases along life course.

Bibliography

Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *J Nutr Biochem*. 2012. doi:10.1016/j.jnutbio.2012.03.003.

Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, Baccarelli A. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech Ageing Dev*. 2009. doi:10.1016/j.mad.2008.12.003.

Boyne DJ, Friedenreich CM, McIntyre JB, Stanczyk FZ, Courneya KS, King WD. Endogenous sex hormone exposure and repetitive element DNA methylation in healthy postmenopausal women. *Cancer Causes Control*. 2017. doi:10.1007/s10552-017-0958-z.

Cesario SK, Hughes LA. Precocious puberty: A comprehensive review of literature. *J Obstet Gynecol Neonatal Nurs*. 2007. doi:10.1111/j.1552-6909.2007.00145.x.

Cordero P, Gomez-Uriz AM, Campion J, Milagro FI, Martinez JA. Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. *Genes Nutr*. 2013. doi:10.1007/s12263-012-0300-z.

Cutler RG, Mattson MP. The adversities of aging. Ageing Res Rev. 2006. doi:10.1016/j.arr.2006.05.002.

Ennour-Idrissi K, Maunsell E, Diorio C. Effect of physical activity on sex hormones in women: A systematic review and meta-analysis of randomized controlled trials. *Breast Cancer Res*. 2015. doi:10.1186/s13058-015-0647-3.

Gabory A, Ripoche M-A, Le Digarcher A, Watrin F, Ziyyat A, Forne T, Jammes H, Ainscough JFX, Surani MA, Journot L, et al. *H19* acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development*. 2009. doi:10.1242/dev.036061.

Ge X, Conger RD, Elder GH. Pubertal transition, stressful life events, and the emergence of gender differences in adolescent depressive symptoms. *Dev Psychol*. 2001. doi:10.1037/0012-1649.37.3.404.

Golub MS, Collman GW, Foster PMD, et al. Public Health Implications of Altered Puberty Timing. *Pediatrics*. 2008. doi:10.1542/peds.2007-1813G.
Hanson M, Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD. Developmental plasticity and developmental origins of non-communicable disease: Theoretical considerations and epigenetic mechanisms. *Prog Biophys Mol Biol*. 2011.doi:10.1016/j.pbiomolbio.2010.12.

Heindel JJ, Vandenberg LN. Developmental origins of health and disease: A paradigm for understanding disease cause and prevention. *Curr Opin Pediatr*. 2015. doi:10.1097/MOP.0000000000000191.

Herman-Giddens ME, Slora EJ, Wasserman RC, et al. Secondary sexual characteristics and menses in young girls seen in office practice: A study from the pediatric research in office settings network. *Pediatrics*. 1997. doi:10.1542/peds.99.4.505.

Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol*. 2013. doi:10.1186/gb-2013-14-10-r115.

Jansen EC, Miller AL, Lumeng JC, Kaciroti N, Brophy Herb HE, Horodynski MA, Contreras D, Peterson KE. Externalizing behavior is prospectively associated with intake of added sugar and sodium among low socioeconomic status preschoolers in a sex-specific manner. *Int J Behav Nutr Phys Act*. 2017. doi:10.1186/s12966-017-0591-y.

Jones MJ, Goodman SJ, Kobor MS. DNA methylation and healthy human aging. *Aging Cell*. 2015. doi:10.1111/acel.12349.

Jones PA. The role of DNA methylation in mammalian epigenetics. *Science*. 2001. doi:10.1126/science.1063852.

Kaplowitz P. Clinical characteristics of 104 children referred for evaluation of precocious puberty. *J Clin Endocrinol Metab*. 2004. doi:10.1210/jc.2003-031532.

Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting. *J Clin Invest*. 1999. doi:10.1172/JCI6223.

Kurdyukov S, Bullock M. DNA methylation analysis: Choosing the right method. *Biology*. 2016. doi:10.3390/biology5010003.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, Fitzhugh W, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001. doi:10.1038/35057062.

Maccani MA, Marsit CJ. Epigenetics in the placenta. *Am J Reprod Immunol*. 2009. doi:10.1111/j.1600-0897.2009.00716.x.
Marceau K, Ram N, Houts RM, Grimm KJ, Susman EJ. Individual differences in boys' and girls' timing and tempo of puberty: modeling development with nonlinear growth models. *Dev Psychol*. 2011. doi:10.1037/a0023838.

Mendle J, Harden KP, Brooks-Gunn J, Graber JA. Development's tortoise and hare: pubertal timing, pubertal tempo, and depressive symptoms in boys and girls. *Dev Psychol*. 2010. doi:10.1037/a0020205.

National Institutes of Health, Genetics Home Reference, *HSD11B2* gene, Published: March 19, 2019, https://ghr.nlm.nih.gov/gene/HSD11B2.

Negriff S, Blankson AN, Trickett PK. Pubertal timing and tempo: associations with childhood maltreatment. *J Res Adolesc*. 2015. doi:10.1111/jora.12128.

Negriff S, Susman EJ, Trickett PK. The Developmental pathway from pubertal timing to delinquency and sexual activity from early to late adolescence. *J Youth Adolesc*. 2011.

Nelson JF. Puberty, gonadal steroids and fertility: Potential reproductive markers of aging. *Exp Gerontol*. 1988. doi:10.1016/0531-5565(88)90038-1.

Nelson MC, Gordon-Larsen P, Adair LS, Popkin BM. Adolescent physical activity and sedentary behavior: Patterning and long-term maintenance. *Am J Prev Med*. 2005. doi:10.1016/j.amepre.2004.12.006.

Nordin M, Bergman D, Halje M, Engström W, Ward A. Epigenetic regulation of the Igf2/H19 gene cluster. *Cell Prolif*. 2014. doi:10.1111/cpr.12106.

Pal S, Tyler JK. Epigenetics and aging. *Sci Adv*. 2016. doi:10.1126/sciadv.1600584.

Pan Y, Pratt CA. Metabolic syndrome and its association with diet and physical activity in US adolescents. *J Am Diet Assoc*. 2008. doi:10.1016/j.jada.2007.10.049.

Rogol AD, Roemmich JN, Clark PA. Growth at puberty. *J Adolesc Heal*. 2002. doi:10.1016/S1054-139X(02)00485-8.

Sata F. "Developmental Origins of Health and Disease (DOHaD) cohorts and interventions: status and perspective". In: Pre-emptive Medicine: Public Health Aspects of Developmental Origins of Health and Disease. Ed. Sata F, Fukuoka H, Hanson M. Springer, 2019. 53-70.

Shirtcliff EA, Dahl RE, Pollak SD. Pubertal development: Correspondence between hormonal and physical development. *Child Dev*. 2009. doi:10.1111/j.1467-8624.2009.01263.x.

Sisk CL, Foster DL. The neural basis of puberty and adolescence. *Nat Neurosci*. 2004. doi:10.1038/nn1326.

Stice E, Presnell K, Bearman SK. Relation of early menarche to depression, eating disorders, substance abuse, and comorbid psychopathology among adolescent girls. *Dev Psychol*. 2001. doi:10.1037/0012-1649.37.5.608.

Summerbell CD, Moody RC, Shanks J, Stock MJ, Geissler C. Relationship between feeding pattern and body mass index in 220 free-living people in four age groups. *Eur J Clin Nutr*. 1996.

Suzuki R, Rylander-Rudqvist T, Saji S, Bergkvist L, Adlercreutz H, Wolk A. Dietary lignans and postmenopausal breast cancer risk by oestrogen receptor status: A prospective cohort study of Swedish women. *Br J Cancer*. 2008. doi:10.1038/sj.bjc.6604175.

Tsujiuchi T, Shimizu K, Onishi M, Sugata E, Fujii H, Mori T, Honoki K, Fukushima N. Involvement of aberrant DNA methylation on reduced expression of lysophosphatidic acid receptor-1 gene in rat tumor cell lines. *Biochem Biophys Res Commun*. 2006. doi:10.1016/j.bbrc.2006.08.159.

Videon TM, Manning CK. Influences on adolescent eating patterns: The importance of family meals. *J Adolesc Heal*. 2003. doi:10.1016/S1054-139X(02)00711-5.

Wainfan E, Poirier LA. Methyl groups in carcinogenesis: Effects on DNA methylation and gene expression. In: *Cancer Research*. ; 1992.

Wan W, Deng X, Archer KJ, Sun SS. Pubertal pathways and the relationship to anthropometric changes in childhood: The Fels longitudinal study. *Open J Pediatr*. 2012. doi:10.4236/ojped.2012.22020.

Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S. Methyl donor supplementation prevents transgenerational amplification of obesity. *Int J Obes*. 2008. doi:10.1038/ijo.2008.100.

Yermachenko A, Dvornyk V. Nongenetic determinants of age at menarche: a systematic review. *Biomed Res Int*. 2014. doi:10.1155/2014/371583.

Zhu J, Chan Y-M. Adult consequences of self-limited delayed puberty. *Pediatrics*. 2017. doi:10.1542/peds.2016-3177.

Chapter 2 Association of Blood Leukocyte DNA Methylation at LINE-1 and Growth-related Candidate Genes with Pubertal Timing and Progression

INTRODUCTION

Puberty is the process by which and during which sexual maturation occurs and reproductive capacity is attained (Golub et al., 2008). The timing of puberty ranges widely. Early onset of puberty has been documented, with U.S girls exhibiting pubertal change before age 6 among African Americans and age 7 among Caucasians, age 8 for girls in other parts of the world, and age 9 for Caucasian boys (Niculescu, 2008; Herman-Giddens et al., 1997; Herman-Giddens et al., 2012; Kaplowitz et al., 1999). Delayed puberty, on the other hand, is defined as the lack of pubertal onset by an age ≥2 SD above the population mean and can occur as late as age 13 in girls and age 14 in boys (Zhu & Chan, 2017). These variations in pubertal timing have implications for the risk of later adult diseases, including polycystic ovary syndrome (PCOS), obesity, type 2 diabetes, cardiovascular disease and reproductive tract cancers (Golub et al., 2008; Zhu & Chan, 2017). Moreover, they may negatively affect adult psychosocial functioning, educational achievement, height, and bone mineral density (Golub et al., 2008; Zhu & Chan, 2017). Over recent decades, the risk factors of earlier or later puberty, including chemical exposures, unbalanced diet, and abnormal hormone levels caused by diseases and psychological stress, have been discussed (Cesario et al., 2007; Jansen et al., 2017). However, the underlying biological mechanisms that lead to early or delayed puberty remain unclear. Moreover, studies in racially and ethnically diverse groups are few and have methodologic limitations (for instance, cross-sectional study design).

Epigenetic modification is a biological mechanism that may underline pubertal timing and progression, since it is known to regulate development processes and is responsive to environmental factors. The epigenome consists of heritable, yet potentially reversible, modifications including DNA methylation, posttranslational histone tail modifications and non-coding RNA (ncRNA)-associated gene silencing which regulate gene expression but do not alter the DNA sequence (Rozek et al., 2014). Epigenetic modifications including DNA methylation are known to regulate developmental processes (Turan et al., 2012; Banister et al., 2011; Relton et al., 2012). Given that sexual maturation is continual from the time it is initiated in intrauterine life through the life cycle (Golub et al., 2008), epigenetic programming may contribute to the timing of puberty. Several reports have examined the role of epigenetics in regulating changes in body composition and growth milestones, which typically occur at the same time as puberty (Relton et al., 2012; Chen et al., 2016). Epigenetic age, an estimate of biological age based on changes in DNA methylation at particular locations along the genome (Jones, 2001), was associated with longitudinal changes in weight, BMI, height and fat mass during childhood and adolescence in a sample of 1018 children (Simpkin et al., 2017). Other studies found the levels of cord blood DNA methylation were related to development of adiposity later in life (Relton et al., 2012; Perng et al., 2013). However, few studies have examined the specific effect of epigenetics on pubertal onset and progression. A rat study revealed epigenetic control of *Kiss1* is important for pubertal timing in females, and an epidemiological study linked repetitive element DNA hypomethylation at long interspersed nucleotide elements (LINE-1) to increased odds of menarche by age 12 among girls and lower luteinizing hormone levels at age 9 years among boys (Lomniczi et al., 2013; Huen et al., 2016).

Based upon previous biological evidence, there are two reasons to hypothesize an association between altered DNA methylation and changes in pubertal timing. Firstly, a growing body of literature has demonstrated that increased body mass index (BMI) is associated with altered methylation at multiple genes (Dick et al., 2014; Milagro et al., 2012; Soubry et al., 2013). Moreover, evidence from both animal and human studies suggest that pre-pubertal obesity might be causally related to earlier puberty (Freedman et al., 2002; Kaplowitz et al., 2008). Secondly, reproductive hormones, primarily testosterone and dehydroepiandrosterone (DHEA), two androgens that facilitate masculine development, and estradiol, an estrogen that facilitates feminine development (Shirtcliff et al., 2009), advance puberty. DNA methylation levels could potentially modify reproductive hormone levels or sensitivity/expression of hormone receptors (Huen et al., 2016; Ulrich et al., 2012; Takahashi et al., 2002; Kumar & Thakur, 2004). These two proposed mechanisms are possibly intertwined, given that feedback from reproductive hormones fat mass might stimulate the central pulsatile gonadotrophin secretion and trigger the onset of puberty (Ahima et al., 1997; Chehab et al., 1996). However, in spite of evidence to support the possible association between DNA methylation, BMI and sexual maturation outcomes, no research has examined relationships among all three in adolescent children.

To address these research gaps, this longitudinal observational study in Mexico City tests the hypothesis that peripubertal blood leukocyte DNA methylation at LINE-1 and specific genes (*HSD11B2*, as well as imprinted genes *IGF2* and *H19*) will be associated with pubertal onset and progression assessed at two time periods, adjusted for BMI, age and household socioeconomic status (SES). Regions were selected for DNA methylation analysis based on demonstrated variability across age and/or by various environmental factors in previous studies (Goodrich et al., 2016; Goodrich et al., 2015; Talens et al., 2012; Li et al., 2016; King et al.,

2015; Perkins et al., 2012). In addition, LINE-1 repetitive elements have been associated with pubertal status and hormone levels in Mexican Americans. *H19*, *IGF2*, and *HSD11B2* are linked to early life growth but their implications for adolescent health, specifically with regards to pubertal onset and tempo, have not yet been studied.

METHODS

3.1 Study Population

The study population comprised a subset of participants from the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) project, a longitudinal epidemiological study consisting of three sequentially-enrolled birth cohorts. As originally designed, ELEMENT focused primarily on lead exposure and its impact on cognitive performance, and analysis of other metals, chemicals and epigenetics have been incorporated overtime (Cantoral et al., 2015; Watkins et al., 2017). Participants were recruited at three maternity hospitals representing low- to moderate-income populations (Mexican Social Security Institute, Manuel Gea Gonzalez Hospital, and the National Institute of Perinatology) in Mexico City from 1994 to 2005. Mothers provided written consent upon enrollment in the study, and children also provided assent at peri-adolescent study visits. The research protocol was approved by the Human Subjects Committee of the National Institute of Public Health of Mexico, participant hospitals, and the Internal Review Board at all participating institutions including the University of Michigan. The subjects in this project were a subset of mother-child pairs from the second and third birth cohorts (n=646 pairs at baseline). At the clinic visit after the child was born, mothers provided household and demographic information, including age, education, and previous numbers of pregnancies. Their offsprings were followed from birth until 4 years of age. Starting in 2008, we re-contacted a subset of the offspring (n=250; henceforth referred to as the early-teen visit) based on availability

of prenatal and neonatal biospecimens (Cantoral et al., 2015). One more peri-pubertal visit (late-teen visit) was completed approximately five years later (549, with 223 having also participated in the 2010 visit). Fasting blood, pubertal status and anthropometry were collected at both teen visits (Perng et al., 2017).

3.2 Laboratory Measurements and Outcomes

DNA Methylation

Blood samples were obtained at the early teen visit and collected in PAXGene tubes by trained staff following standard protocols. High-molecular-weight DNA was extracted from blood leukocytes with the PAXgene Blood DNA kit (PreAnalytix, Switzerland). DNA samples were treated with sodium bisulfite using kits from Zymo or Qiagen (Li & Tollesfsbol, 2011). Percent of methylated cells was then quantitatively analyzed in well-characterized differentially methylated regions (DMRs) of two imprinted genes (the DMR upstream of the *H19* paternally imprinted, maternally expressed transcript (non-coding), *H19*, which is within the imprinting control region (Hoyo et al., 2011); and the DMR within exon 3 of the *IGF2AS* transcript of the maternally imprinted, paternally expressed insulin-like growth factor, *IGF2* (Heijmans et al., 2007), the promoter region of a non-imprinted gene (hydroxysteroid (11-beta) dehydrogenase 2, *HSD11B2*), and a conserved sequence found in the promoter region of LINE-1 repetitive elements of all subfamilies (sequence: 5'- CTCGTGGTGCGCCGTTTCTTAAGCCG). DNA methylation was quantified via pyrosequencing at 4 (*H19*, LINE-1) or 5 (*HSD11B2*) CpG sites. The Sequenom EpiTYPER was used to quantify DNA methylation at 5 units of *IGF2* representing a total of 7 CpG sites due to the resolution capabilities of EpiTYPER. Quality control includes running >10% of samples in duplicate and including human DNA controls of known methylation status (i.e. 0%, 100%) on each batch. Full details on primers, quality control,

and analysis methods have been previously published (Goodrich et al., 2016). Primer sequences and loci of CpG sites can be found in Supplemental Table 4. LINE-1, *HSD11B2*, and *H19* data exhibited batch effects and as such were standardized to controls included on experimental plates as previously described (Goodrich et al., 2016). For example, the value of 0% methylation controls on each plate of samples amplified and pyrosequenced together (a batch) for *HSD11B2* was subtracted from the raw DNA methylation values generated for each sample in the same batch.

Pubertal Outcomes

Pubertal outcomes were obtained at both early-teen and late-teen visits. Tanner stages of breast and pubic hair growth in girls as well as Tanner stages of genitalia and pubic hair growth in boys were examined and collected by trained physicians (Chavarro et al., 2017). Outcomes were recorded with a range from stage 1 indicating pre-puberty to stage 5 indicating full maturation (Marshall & Tanner, 1970). Testicular volumes were measured by trained physicians using orchidometers (range from 1 to 25 ml). Occurrence and age of menarche were gathered from a self-reported questionnaire (Chavarro et al., 2017; Cooper et al., 2006).

Covariates

Based on *a priori* knowledge and preliminary correlation tests between predictors and potential confounders, covariates included in the final model were SES and BMI of the child, obtained at the early-teen visit. The socioeconomic status (SES) information was collected at baseline during the pregnancy visits, using a validated questionnaire consisting of thirteen questions on housing quality, services, material goods and education of the head of household by AMAI (Asociación Mexicana de Agencias de Investigación de Mercados y Opinión Pública, version 13x6). With the use of fourteen hierarchical trees this scale classified households into six

SES categories (A/B, C+, C, D+, D, E; with A/B being the highest category) (Jansen et al., 2017; Cantoral et al., 2016). This scale was validated using the results of the National Survey of Household Income and Expenditure 2005, Mexico (ENIGH, Encuesta Nacional de Ingresos y Gastos de los Hogares 2004), using a point based system (Jansen et al., 2017; Cantoral et al., 2016). Weight and height of the child were measured by trained nurses, following standardized protocols we have previously described (Nuttall, 2015); BMI was calculated as weight over height squared (kg/m^2) (Nuttall, 2015). Children's age was recorded at each visit.

3.3 Statistical Methods

We examined the distribution of Tanner stages among individuals who attended only the early-teen visit and among those who attended both early- and late teen visits, and compared the distributions across categories of background characteristics using $\chi 2$ tests.

Some participants had missing CpG site values for *HSD11B2* (n of missing=14 at site 5), *IGF2* (n of missing= 20 at site 1, 30 at site 2, 15 at site 3, 47 at site 4, 14 at site 5), SES (n of missing=48). Thus, we performed multiple imputation (Rubin, 1988) including all covariates; five imputed datasets were obtained. In order to test the assumption that the gene variables were missing at random, we examined the DNA methylation distribution (means ± SD) of CpG sites in all genes before and after imputation. The final sample size for the DNA methylation dataset was 114 boys and 129 girls. Values were not imputed for 7 subjects that were missing 3 out of 4 genes.

We used interval censored regression models to analyze time to pubertal onset at each visit separately. We created binomial Tanner stage outcomes using > 1 as the pubertal onset cut-off point (Marshall & Tanner, 1970; Marceau et al., 2011) for pubic hair, genital development and breast development characteristics. Testicular volume ≤ 3 mL indicated pre-pubertal stage;

testicular volume > 3mL but ≤ 11mL indicates pubertal onset; and > 11mL indicated sexual maturity (Marshall & Tanner, 1970; Marceau et al., 2011). Age from early-teen visit was used as the time to follow-up here.

We used the following ordinal regression model:

$$Logit\left(Y_{ij}\right) = \beta_0 + \beta_1 \times (age) + \beta_2 \times (time\ difference) + \beta_3 \times (methylation) + \beta_4 \times (methylation \times time\ difference) + \beta_5 \times (age \times time\ difference) + \beta_6 \times (covariates)$$

to analyze pubertal progression between early- and late-teen visits in boys and girls separately where methylation is the percent methylation at a given CpG site in one of the foaur genes, age is the age at the early teen visit, and time difference is the time between the early and late teen visits.

We selected confounders based on *a priori* knowledge and variables that were significantly associated with DNA methylation and pubertal stage. We included BMI and SES in adjusted interval censored regression models. BMI, SES and age from early-teen visit as well as the time difference between these two visits in the adjusted ordinal regression model. We used a cutoff value of $p < 0.05$ to define statistical significance. However, since we conducted a large number of tests, we also considered significance after adjusting for multiple testing. A Bonferroni correction for multiple testing would be overly conservative given that correlations among the 18 CpG sites can be large (e.g. several CpG sites within LINE-1 (Pearson r>0.7) or within H19 (r>0.85) are highly correlated within assay). Moreover, pubertal outcomes are also correlated. Thus, we corrected for multiple testing by using a cutoff point of 0.0028, obtained as 0.05/18, which considers that for each outcome we tested 18 CpG sites. All analyses were conducted using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The cohort included 250 subjects who attended the early-teen visit (boys: 118 (47.2%), girls: 132 (52.8%), and 222 subjects who attended both visits (boys: 108 (48.6%), girls: 114 (51.4%)). After eliminating 7 individuals with major missing predictors, the analytical sample included 243 subjects (boys: 114 (46.9%), girls: 129 (53.1%)). The mean age for the early-teen visit was 10.4 years in boys and 10.3 years in girls; the mean was 13.7 years in boys and 13.5 for girls for the late-teen visit. We observed children moving to more advanced pubertal stages from the early- to late-teen visits. Among boys, 79.7% and 48.3% were at Tanner stage 1 for pubic hair and genital development in the early-teen visit; and the number dropped down to 25.0% and 6.5% in the second visit. In terms of testicular volume, the percentage of boys in the pre-pubertal stage dropped from 15.3% to 0%. Among girls, 74.2% of them had Tanner stage 1 for pubic hair and 65.9% for breast development in the early-teen visit; and the percentage dropped to 7.9% and 4.4% later (**Table 1**).

LINE-1 methylation was higher among boys compared to girls, both before (**Table 2**) and after (**Supplemental Table 1**) imputation of missing values. No statistically significant sex differences of DNA methylation levels at *HSD11B2*, *H19* or *IGF2* were observed.

Among boys, we observed associations between early-teen DNA methylation and pubertal outcomes cross-sectionally at the early teen visit as well as prospectively at the late teen visit, and with the progression between the two. In the cross-sectional adjusted analysis (**Table 3**), we found for each percent increase of DNA methylation at *H19* CpG sites 2 and 3 (equivalent to 0.31 and 0.29 SD increase of DNA methylation, respectively; they were obtained as 1/SD in % (sex-specific and site-specific) from **Supplemental Table 1**), there were 36% and 26% increased odds of later pubarche (p=0.025 and 0.039). However, the associations did not remain

statistically significant in the prospective analysis (**Table 3**). We also found for each percent methylation increase of *HSD11B2* site 4 (0.62 SD), there was a 60% increased odds of earlier pubarche (p=0.030), and a 67% increased odds in earlier onset of genital development (p=0.003), in cross-sectional analysis. Prospectively, for each percent methylation increase of *HSD11B2* site 4, there was 20% increased odds of earlier pubarche (p=0.034). *HSD11B2* site 4 was also associated with 17% increased odds of slower genital development progression (p=0.016, **Table 4**). For each percent methylation increase of *IGF2* site 3 (0.13 SD), there was 7% increased odds of later onset of genital development, in both cross-sectional and prospective analyses (p=0.010 and 0.005). *IGF2* methylation was also associated with faster genital development progression (p=0.036) (**Table 4**).

Among girls, we found for each percent increase in methylation of LINE-1 CpG sites 3 and 4 (0.36 and 0.44 SD equivalents, respectively), there were 11% and 17% increased odds of later onset of breast development (p=0.008 and <0.001) in the cross sectional analysis. For each percent increase in methylation of *H19* sites 1 and 4 (0.11 SD of each), there were 5% increased odds in the earlier onset of breast development (both p values <0.001). However, the associations mentioned above were not statistically significant in prospective analyses. In terms of *HSD11B2*, we found for each percent increase in methylation of site 1 and 3 (0.51 and 0.45 SD), there were 20% and 13% increased odds of later onset of breast development (p<0.001 and 0.02); for each percent increase in methylation of site 4 (0.53 SD), there were 25% increased odds in the expected earlier onset of breast development (p<0.001). In addition, with one percent increase methylation of *IGF2* site 5 (0.18 SD), there was 7% increased odds of earlier pubarche (p=0.020) (**Table 3**). DNA methylation was not found to be associated with pubertal progression among girls (**Table 4**).

After corrections for multiple testing, associations of LINE-1, *H19* and *HSD11B2* with breast onset among girls, as well as the association of *HSD11B2* with genital onset among boys remained significant with *p* values < 0.0028 (**Table 3**). None of the pubertal progression results maintained statistical significance, however, after correction for multiple testing (**Table 4**).

DISCUSSION

There have been a limited number of population-based longitudinal studies examining the potential association between epigenetics and puberty. The goal of this sex-specific analysis was to investigate the potential effects of peri-pubertal DNA methylation on pubertal status and progression. We found that DNA methylation of *H19, IGF2,* and *HSD11B2* were associated with pubic hair and genital onset in boys, while methylation of LINE-1, *H19, IGF2* and *HSD11B2* were associated with pubic hair and breast development among girls. These findings suggest that DNA methylation at genes known to influence early-life growth and development may also influence pubertal outcomes, though the mechanism (direct or indirect) remains to be elucidated.

While it is well established that pubertal timing and progression is controlled by many genes (Seminara et al., 2004; Abreu et al., 2013; Silveira et al., 2010), the epigenetic regulation involved in this process is less understood. One study found rats treated with 5-azacytidine (Aza), a DNA methylation inhibitor had delayed vaginal opening, failed to reach puberty, as assessed by the lack of ovulation, and showed no estrous cyclicity (Lomniczi et al., 2013). In our gene-specific analysis, we found decreased DNA methylation levels in *H19* and *IGF2* were linked to later onset of breast development and menarche in girls. Among boys, decreased DNA methylation levels in *HSD11B2* were associated with delayed pubic hair onset and genitalia onset.

Our findings build on prior studies in other important ways, with both consistent and contrasting results. For example, in a longitudinal study, Huen et al examined the relationship of Alu and LINE-1 repetitive element DNA methylation measured in umbilical cord and 9-year old child blood samples with puberty status in Mexican-American participants of the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort. They found no association between child LINE-1 methylation and odds of genital or pubic hair development in boys, but found a significant association with later onset of menarche in girls (Huen et al., 2016). We also did not observe any association between peri-pubertal LINE-1 methylation and male sexual characteristics in either cross-sectional or longitudinal analysis. However, cross-sectional analysis showed that elevated LINE-1 methylation was suggestively associated with later breast onset, but not menarche in girls. The findings were consistent in terms of directionality. As both breast development and menarche are advanced by estrogens (Karapanou & Papadimitriou, 2010), the different findings may be due to age differences between the two cohorts. Moreover, based on observational studies, the estimated mean age at menarche is different in Mexico City (11.40 years (Marván et al., 2016)), versus among Mexican Americans (12.25 years (Chumlea et al., 2003)), and timing differed between the two studies as children were only followed through age 12 in the CHAMACOS study.

To our knowledge, although no population-based studies have examined the associations between *H19* and *IGF2* methylation and pubertal outcomes specifically, several articles support the effect of methylation on imprinted genes with growth, growth-related hormone concentrations, adiposity and birth weight. For instance, Huang et al found greater peripheral blood *H19/IGF2* methylation was associate with elevated subcutaneous fat measures in 315 young adults (Gallou-Kabani et al., 2010). In addition, Deodati et al observed that

elevated *IGF2* methylation levels from blood lymphocytes were associated with higher levels of triglycerides, triglyceride/HDL-cholesterol ratio and C-peptide concentrations among overweight and obese adolescents (Deodati et al., 2013). Future studies are needed to explore the sexually differentiated mechanisms behind DNA methylation and pubertal status.

In the post-hoc analysis examining the pubertal progression pattern, our findings that may provide statistical evidence to support the "catch-up growth" and "compensatory growth" theory in pubertal development. Based on previous studies (Apter & Vihko, 1985), we had hypothesized that individuals with early pubertal onset have faster pubertal progression, and those with later onset will have slower pubertal progression. However, we found older age of onset of puberty was associated with shorter duration (tempo) of puberty, and vice versa (Marceau et al., 2011). *IGF2* site 3 was associated with later onset of genital development in both cross-sectional and prospective analyses (**Table 3**). Correspondingly, we observed 20% increased odds of faster genital development progression with *IGF2* methylation, though the association was not statistically significant. Similarly, *HSD11B2* site 4 was associated with earlier onset of genital development but slower tempo among boys. Much research on catch-up growth has been published since 1963 (Ashworth & Millward, 1986; Wi & Boersma, 2002; Wilmott, 2013). Most studies observed that among infants and children whose growth had been slowed by illness or starvation, there was a rapid and longer phase of growth until the children reached their pre-illness growth curve (Ashworth & Millward, 1986; Wi & Boersma, 2002). However, catch-up in pubertal characteristics during adolescence is less well-defined. Consistent with the interpretations from our results, pubertal timing and tempo adjustments exist among Mexican boys. Considering pre-adult periods of adaptive plasticity from juvenility to adolescence establishes longevity and the age of reproduction and fecundity (Hochberg et al.,

2011), our results indicated modified DNA methylation levels may affect this timing and progression. Some significant associations seen at pubertal onset were attenuated in the progression model, which may be due in underpowered statistics part to less sensitivity of the ordinal regression model compared to the Cox proportional hazard model (Jeon, 2015).

This analysis had some limitations, including a moderate sample size with approximately 20% missing rates of *IGF2* DNA methylation at some of the CpG sites. Though we used multiple regression imputation to increase the number of predictors, this method can underestimate standard error, which might result in inflated p-values (Soley-bori, 2013). While pubertal status was based on a highly trained physicians' observation, age of menarche was self-reported and may not be accurate due to recall bias. Our sample comes from a mixed ancestry population but we did not genotype this population in order to estimate ancestry. As such, it is possible that ancestry differences could influence the relationships we are observing between DNA methylation and pubertal timing. Since the epigenome and transcriptome vary by cell and tissue type, analyzing DNA methylation in blood leukocytes which consist of multiple cell types is a limitation, though recent studies have identified blood leukocyte differentially methylated genes associated with BMI or adiposity in adults that replicated in adipose or skeletal muscle, biologically relevant tissues (Perng et al., 2013; Day et al., 2017; Demerath et al., 2015). The number of genes studied was also a limitation, and we recommend epigenome-wide studies in this area to identify key genes regulating puberty. In addition, we performed both unadjusted and adjusted model analyses of onset and progression and observed that including BMI did not significantly attenuate the associations. Nevertheless, we cannot rule out the possibility that DNA methylation is a mediator between BMI and sexual maturation, if BMI was measured at an earlier time point.

In conclusion, this is the first study to evaluate the effect of DNA methylation of H19, IGF2, and HSD11B2 on pubertal onset and progression among boys and girls. Unlike previous puberty- related studies that primarily used menarche as the only pubertal indicator (Demetriou et al., 2013; He et al., 2009), we applied multiple secondary sexual characteristics (pubic hair, genital development and testicular volumes in boys, as well as pubic hair, breast development and menarche in girls). We found suggestive evidence of associations of DNA methylation with pubertal onset among adolescents. By using ordinal regression models and repeated measurements of Tanner stages, we observed the effect of DNA methylation on pubertal progression in boys only. The findings also raise the possibility of influencing pubertal timing by regulating DNA methylation levels. Future work in this field should consider epigenetic regulation in a larger panel of genes that may directly or indirectly influence puberty.

Bibliography

Abreu AP, Dauber A, Macedo DB, Noel SD, Brito VN, Gill JC, Cukier P, Thompson IR, Navarro VM, Gagliardi PC, et al. Central precocious puberty caused by mutations in the imprinted gene MKRN3. *N Engl J Med*. 2013. doi:10.1056/NEJMoa1302160.

Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS. Leptin accelerates the onset of puberty in normal female mice. *J Clin Invest*. 1997. doi:10.1172/JCI119172.

Apter D, Vihko R. Premenarcheal endocrine changes in relation to age at menarche. *Clin Endocrinol (Oxf)*. 1985. doi:10.1111/j.1365-2265.1985.tb00165.x.

Ashworth A, Millward DJ. Catch-up growth in children. *Nutr Rev*. 1986. doi:10.1111/j.1753-4887.1986.tb07613.x.

Banister CE, Koestler DC, Maccani MA, Padbury JF, Andres Houseman E, Marsit CJ. Infant growth restriction is associated with distinct patterns of DNA methylation in human placentas. *Epigenetics*. 2011. doi:10.4161/epi.6.7.16079.

Cantoral A, Téllez-Rojo MM, Ettinger AS, Hu H, Hernández-Ávila M, Peterson K. Early introduction and cumulative consumption of sugar-sweetened beverages during the pre-school period and risk of obesity at 8-14 years of age. *Pediatr Obes*. 2016. doi:10.1111/ijpo.12023.

Cantoral A, Téllez-Rojo MM, Levy TS, Hernández-Ávila M, Schnaas L, Hu H, Peterson KE, Ettinger AS. Differential association of lead on length by zinc status in two-year old Mexican children. *Environ Health*. 2015. doi:10.1186/s12940-015-0086-8.

Cesario SK, Hughes L. Precocious puberty: a comprehensive review of literature. *J Obstet Gynecol Neonatal Nurs*. 2007. doi:10.1111/j.1552-6909.2007.00145.x.

Chavarro JE, Watkins DJ, Afeiche MC, Zhang Z, Sánchez BN, Cantonwine D, Mercado-García A, Blank-Goldenberg C, Meeker JD, Téllez-Rojo MM, et al. Validity of self-assessed sexual maturation against physician assessments and hormone levels. *J Pediatr*. 2017. doi:10.1016/j.jpeds.2017.03.050.

Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet*. 1996. doi:10.1038/ng0396-318.

Chen BH, Marioni RE, Colicino E, Peters MJ, Ward-Caviness CK, Tsai PC, Roetker NS, Just AC, Demerath EW, Guan W, et al. DNA methylation-based measures of biological age: Meta-analysis predicting time to death. *Aging*. 2016. doi:10.18632/aging.101020.

Chumlea WC, Schubert CM, Roche AF, Kulin HE, Lee P, Himes JH, Sun SS. Age at menarche and racial comparisons in US girls. *Pediatrics*. 2003. doi:10.1542/peds.111.1.110.

Cooper R, Blell M, Hardy R, Black S, Pollard TM, Wadsworth MEJ, Pearce MS, Kuh D. Validity of age at menarche self-reported in adulthood. *J Epidemiol Community Health*. 2006. doi:10.1136/jech.2005.043182.

Day SE, Coletta RL, Kim JY, Garcia LA, Campbell LE, Benjamin TR, Roust LR, De Filippis EA, Mandarino LJ, Coletta DK. Potential epigenetic biomarkers of obesity-related insulin resistance in human whole-blood. *Epigenetics*. 2017. doi:10.1080/15592294.2017.1281501.

Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou YH, Hedman ÅK, Sandling JK, Li LA, Irvin MR, et al. Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. *Hum Mol Genet*. 2015. doi:10.1093/hmg/ddv161.

Demetriou CA, Chen J, Polidoro S, Van Veldhoven K, Cuenin C, Campanella G, Brennan K, Clavel-Chapelon F, Dossus L, Kvaskoff M, et al. Methylome analysis and epigenetic changes associated with menarcheal age. *PLoS One*. 2013. doi:10.1371/journal.pone.0079391.

Deodati A, Inzaghi E, Liguori A, Puglianiello A, Germani D, Brufani C, Fintini D, Cappa M, Barbetti F, Cianfarani S. IGF2 methylation is associated with lipid profile in obese children. *Horm Res Paediatr*. 2013. doi:10.1159/000351707.

Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, Meduri E, Morange PE, Gagnon F, Grallert H, et al. DNA methylation and body-mass index: A genome-wide analysis. *Lancet*. 2014. doi:10.1016/S0140-6736(13)62674-4.

Freedman DS, Khan LK, Serdula MK, Dietz WH, Srinivasan SR, Berenson GS. Relation of age at menarche to race, time period, and anthropometric dimensions: the Bogalusa Heart Study. *Pediatrics*. 2002. doi:10.1542/peds.110.4.e43.

Gallou-Kabani C, Gabory A, Tost J, Karimi M, Mayeur S, Lesage J, Boudadi E, Gross MS, Taurelle J, Vigé A, et al. Sex- and diet-specific changes of imprinted gene expression and dna methylation in mouse placenta under a high-fat diet. *PLoS One*. 2010. doi:10.1371/journal.pone.0014398.

Golub MS, Collman GW, Foster PMD, Kimmel CA, Rajpert-De Meyts E, Reiter EO, Sharpe RM, Skakkebaek NE, Toppari J. Public health implications of altered puberty timing. *Pediatrics*. 2008. doi:10.1542/peds.2007-1813G.

Goodrich JM, Dolinoy DC, Sánchez BN, Zhang Z, Meeker JD, Mercado-Garcia A, Solano-González M, Hu H, Téllez-Rojo MM, Peterson KE. Adolescent epigenetic profiles and environmental exposures from early life through peri-adolescence. Environ Epigenetics. 2016. doi:10.1093/eep/dvw018.

Goodrich JM, Sánchez BN, Dolinoy DC, Zhang Z, Hernández-Ávila M, Hu H, Peterson KE, Téllez-Rojo MM. Quality control and statistical modeling for environmental epigenetics: A study on in Utero lead exposure and DNA methylation at birth. *Epigenetics*. 2015. doi:10.4161/15592294.2014.989077.

He C, Kraft P, Chen C, Buring JE, Paré G, Hankinson SE, Chanock SJ, Ridker PM, Hunter DJ, Chasman DI. Genome-wide association studies identify loci associated with age at menarche and age at natural menopause. *Nat Genet*. 2009. doi:10.1038/ng.385

Heijmans BT, Kremer D, Tobi EW, Boomsma DI, Slagboom PE. Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus. *Hum Mol Genet*. 2007. doi:10.1093/hmg/ddm010.

Herman-Giddens ME, Steffes J, Harris D, Slora E, Hussey M, Dowshen SA, Wasserman R, Serwint JR, Smitherman L, Reiter EO. Secondary sexual characteristics in boys: data from the pediatric research in office settings network. *Pediatrics*. 2012. doi:10.1542/peds.2011-3291.

Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Carel JC, Boileau P, Le Bouc Y, Deal CL, Lillycrop K, et al. Child health, developmental plasticity, and epigenetic programming. *Endocr Rev*. 2011;32(2):159-224. doi:10.1210/er.2009-0039.

Hoyo C, Murtha AP, Schildkraut JM, Jirtle R, Demark-Wahnefried W, Forman MR, Iversen ES, Kurtzberg J, Overcash F, Huang Z, et al. Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before and during pregnancy. *Epigenetics*. 2011. doi:10.4161/epi.6.7.16263.

Huen K, Harley K, Kogut K, Rauch S, Eskenazi B, Holland N. DNA methylation of LINE-1 and Alu repetitive elements in relation to sex hormones and pubertal timing in Mexican-American children. *Pediatr Res*. 2016. doi:10.1038/pr.2016.31.

Jansen EC, Miller AL, Lumeng JC, Kaciroti N, Brophy Herb HE, Horodynski MA, Contreras D, Peterson KE. Externalizing behavior is prospectively associated with intake of added sugar and sodium among low socioeconomic status preschoolers in a sex-specific manner. *Int J Behav Nutr Phys Act*. 2017. doi:10.1186/s12966-017-0591-y.

Jansen EC, Miller AL, Lumeng JC, Kaciroti N, Brophy Herb HE, Horodynski MA, Contreras D, Peterson KE. Externalizing behavior is prospectively associated with intake of added sugar and sodium among low socioeconomic status preschoolers in a sex-specific manner. *Int J Behav Nutr Phys Act*. 2017. doi:10.1186/s12966-017-0591-y.

Jeon J. The strengths and limitations of the statistical modeling of complex social phenomenon: Focusing on SEM, path analysis, or multiple regression models. *Int J Soc Behav Educ Econ Bus Ind Eng*. 2015. doi:10.5281/zenodo.1105869.

Jones PA. The Role of DNA Methylation in Mammalian Epigenetics. *Science*. 2001. doi:10.1126/science.1063852.

Kaplowitz PB, Oberfield SE. reexamination of the age limit for defining when puberty is precocious in girls in the United Staes: implication for evaluation and treatment. *Int J Confl Manag*. 1999. doi:10.1086/250095.

Kaplowitz PB. Link between body fat and the timing of puberty. *Pediatrics*. 2008. doi:10.1542/peds.2007-1813F.

Karapanou O, Papadimitriou A. Determinants of menarche. *Reprod Biol Endocrinol*. 2010. doi:10.1186/1477-7827-8-115.

King K, Murphy S, Hoyo C. Epigenetic regulation of newborns' imprinted genes related to gestational growth: Patterning by parental race/ethnicity and maternal socioeconomic status. *J Epidemiol Community Health*. 2015. doi:10.1136/jech-2014-204781.

Kumar RC, Thakur MK. Androgen receptor mRNA is inversely regulated by testosterone and estradiol in adult mouse brain. *Neurobiol Aging*. 2004. doi:10.1016/j.neurobiolaging.2003.10.011.

Li Y, Tollefsbol TO. DNA methylation detection: Bisulfite genomic sequencing analysis. *Methods Mol Biol*. 2011. doi:10.1007/978-1-61779-316-5_2.

Li Y, Xie C, Murphy SK, Skaar D, Nye M, Vidal AC, Cecil KM, Dietrich KN, Puga A, Jirtle RL, et al. Lead exposure during early human development and DNA methylation of imprinted gene regulatory elements in adulthood. *Environ Health Perspect*. 2016. doi:10.1289/ehp.1408577.

Lomniczi A, Loche A, Castellano JM, Ronnekleiv OK, Bosch M, Kaidar G, Knoll JG, Wright H, Pfeifer GP, Ojeda SR. Epigenetic control of female puberty. *Nat Neurosci*. 2013. doi:10.1038/nn.3319.

Lomniczi A, Loche A, Castellano JM, Ronnekleiv OK, Bosch M, Kaidar G, Knoll JG, Wright H, Pfeifer GP, Ojeda SR. Epigenetic control of female puberty. *Nat Neurosci*. 2013. doi:10.1038/nn.3319.

Marceau K, Ram N, Houts RM, Grimm KJ, Susman EJ. Individual differences in boys' and girls' timing and tempo of puberty: modeling development with nonlinear growth models. *Dev Psychol*. 2011. doi:10.1037/a0023838.

Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. Arch Dis Child. 1970. doi:10.1136/adc.45.239.13.

Marván ML, Catillo-López RL, Alcalá-Herrera V, Callejo D del. The decreasing age at menarche in Mexico. *J Pediatr Adolesc Gynecol*. 2016. doi:10.1016/j.jpag.2016.02.006.

Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M. CLOCK, PER2 and BMAL1 DNA methylation: Association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiol Int*. 2012. doi:10.3109/07420528.2012.719967.

Niculescu D. Puberty: Ontogeny, Neuroendocrinology, Physiology, and Disorders. In: Williams Textbook of Endocrinology. Vol 4. ; 2008. doi:10.4183/aeb.2008.127.

Nuttall FQ. Body mass index: Obesity, BMI, and health: A critical review. *Nutr Today*. 2015. doi:10.1097/NT.0000000000000092.

Perkins E, Murphy SK, Murtha AP, Schildkraut J, Jirtle RL, Demark-Wahnefried W, Forman MR, Kurtzberg J, Overcash F, Huang Z, et al. Insulin-like growth factor 2/H19 methylation at birth and risk of overweight and obesity in children. *J Pediatr*. 2012. doi:10.1016/j.jpeds.2012.01.015.

Perng W, Hector EC, Song PXK, Tellez Rojo MM, Raskind S, Kachman M, Cantoral A, Burant CF, Peterson KE. Metabolomic determinants of metabolic risk in Mexican adolescents. *Obesity*. 2017. doi:10.1002/oby.21926.

Perng W, Mora-Plazas M, Marín C, Rozek LS, Baylin A, Villamor E. A Prospective study of LINE-1 DNA methylation and development of adiposity in school-age children. *PLoS One*. 2013. doi:10.1371/journal.pone.0062587.

Relton CL, Groom A, St. Pourcain B, Sayers AE, Swan DC, Embleton ND, Pearce MS, Ring SM, Northstone K, Tobias JH, et al. DNA methylation patterns in cord blood DNA and body size in childhood. *PLoS One*. 2012. doi:10.1371/journal.pone.0031821.

Rozek LS, Dolinoy DC, Sartor MA, Omenn GS. Epigenetics: Relevance and implications for public health. *Annu Rev Public Health*. 2014. doi:10.1146/annurev-publhealth-032013-182513.

Rubin DB. An overview of multiple imputation. *Proc Surv Res methods Sect Am Stat Assoc*. 1988:79-84.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, et al. The GPR54 gene as a regulator of puberty. *Obstet Gynecol Surv*. 2004. doi:10.1097/00006254-200405000-00020.

Shirtcliff EA, Dahl RE, Pollak SD. Pubertal development: Correspondence between hormonal and physical development. *Child Dev*. 2009. doi:10.1111/j.1467-8624.2009.01263.x.

Silveira LFG, Trarbach EB, Latronico AC. Genetics basis for GnRH-dependent pubertal disorders in humans. *Mol Cell Endocrinol*. 2010. doi:10.1016/j.mce.2010.02.023.

Simpkin AJ, Howe LD, Tilling K, Gaunt TR, Lyttleton O, McArdle WL, Ring SM, Horvath S, Smith GD, Relton CL. The epigenetic clock and physical development during childhood and

adolescence: longitudinal analysis from a UK birth cohort. *Int J Epidemiol*. 2017:dyw307. doi:10.1093/ije/dyw307.

Soley-bori M. Dealing with missing data: Key assumptions and methods for applied analysis. *PM931 Dir Study Heal Policy Manag*. 2013;(4):20.

Soubry A, Schildkraut JM, Murtha A, Wang F, Huang Z, Bernal A, Kurtzberg J, Jirtle RL, Murphy SK, Hoyo C. Paternal obesity is associated with IGF2 hypomethylation in newborns: Results from a Newborn Epigenetics Study (NEST) cohort. *BMC Med*. 2013. doi:10.1186/1741-7015-11-29.

Takahashi S, Inaguma S, Sakakibara M, Cho Y-M, Suzuki S, Ikeda Y, Cui L, Shirai T. DNA methylation in the androgen receptor gene promoter region in rat prostate cancers. *Prostate*. 2002. doi:10.1002/pros.10099.

Talens RP, Christensen K, Putter H, Willemsen G, Christiansen L, Kremer D, Suchiman HED, Slagboom PE, Boomsma DI, Heijmans BT. Epigenetic variation during the adult lifespan: Cross-sectional and longitudinal data on monozygotic twin pairs. *Aging Cell*. 2012. doi:10.1111/j.1474-9726.2012.00835.x.

Turan N, Ghalwash MF, Katari S, Coutifaris C, Obradovic Z, Sapienza C. DNA methylation differences at growth related genes correlate with birth weight: A molecular signature linked to developmental origins of adult disease? *BMC Med Genomics*. 2012. doi:10.1186/1755-8794-5-10.

Ulrich CM, Toriola AT, Koepl LM, Sandifer T, Poole EM, Duggan C, McTiernan A, Issa JPJ. Metabolic, hormonal and immunological associations with global DNA methylation among postmenopausal women. *Epigenetics*. 2012. doi:10.4161/epi.21464.

Watkins DJ, Sánchez BN, Téllez-Rojo MM, Lee JM, Mercado-García A, Blank-Goldenberg C, Peterson KE, Meeker JD. Impact of phthalate and BPA exposure during in utero windows of susceptibility on reproductive hormones and sexual maturation in peripubertal males. *Environ Health*. 2017. doi:10.1186/s12940-017-0278-5.

Wi J-M, Boersma B. Catch-up growth: definition, mechanisms, and models. *J Pediatr Endocrinol Metab*. 2002; 15 Suppl 5:1229-1241.

Wilmott RW. Patterns of catch-up growth. *J Pediatr*. 2013;162(2):220. doi:10.1016/j.jpeds.2012.12.021.

Zhu J, Chan Y-M. Adult Consequences of Self-Limited Delayed Puberty. *Pediatrics*. 2017. doi:10.1542/peds.2016-3177.

Table 2.1 Distributions of Tanner stages and other covariates among 250 ELEMENT children at the early-teen visit (Visit 1) and at the late-teen visit (Visit 2) for 222 children who continued follow-up.

Boys	**Visit 1 (N=118)**		**Visit 2 (N=108)**	
	N	**%**	**N**	**%**
Pubic Hair: Tanner Stage				
Refused Observation/Missing	3	2.54	3	2.78
1	94	79.66	27	25.00
2	17	14.41	16	14.81
3	3	2.54	30	27.78
4	1	0.85	18	16.67
5	0	0.00	14	12.96
Genital Development: Tanner Stage				
Refused Observation/Missing	3	2.54	3	2.78
1	57	48.31	7	6.48
2	43	36.44	17	15.74
3	10	8.47	26	24.07
4	5	4.24	37	34.26
5	0	0.00	18	16.67
Testicular Development (L)				
Refused Observation/Missing	3	2.54	3	2.78
1-3 ml	18	15.25	0	0.00
3-11 ml	75	63.56	16	14.81
>11 ml	22	18.65	89	82.41
Testicular Development (R)				
Refused Observation/Missing	4	3.39	3	2.78
1-3 ml	18	15.25	0	0.00
3-11 ml	76	64.41	16	14.81
>11 ml	20	16.95	89	82.41
Age (years)	10.35 ± 1.61		13.72 ± 1.75	

BMI	19.06 ± 3.14	20.43 ± 3.68

Household SES: Quartile	**N=100**	
1	24	24.00
2	27	27.00
3	24	24.00
4	25	25.00

Girls	**Visit 1 (N=132)**		**Visit 2 (N=114)**	
	N	**%**	**N**	**%**
Pubic Hair: Tanner Stage				
Refused Observation/Missing	0	0.00	2	1.75
1	98	74.24	9	7.90
2	22	16.67	39	34.21
3	9	6.82	29	25.44
4	2	1.52	21	18.42
5	1	0.76	14	12.28
Breast Development: Tanner Stage				
Refused Observation/Missing	0	0.00	2	1.75
1	87	65.90	5	4.39
2	20	15.15	12	10.53
3	18	13.63	46	40.35
4	7	5.30	31	27.19
5	0	0.00	18	15.79
Menarche				
Refused Observation/Missing	0	0.00	1	0.88
Yes	30	22.73	90	78.95
No	102	77.27	23	20.17
Age (years)	10.30 ± 1.72		13.54 ± 1.75	

BMI	19.66 ± 3.95	21.61 ± 4.07	
Household SES: Quartile	**N=102**		
1	25	24.51	
2	29	28.43	
3	24	23.53	
4	24	23.53	

Table 2.2: DNA Methylation at LINE-1, H19, HSD11B2 and IGF2 among all individuals and stratified by sex[1].

	Entire Cohort		Boys		Girls		*P* value[2]
	N	Mean % methylation (SD)	N	Mean % methylation (SD)	N	Mean % methylation (SD)	
LINE-1 methylation							
Site 1	243	79.88 (3.87)	113	80.48 (4.13)	130	79.37 (3.57)	**0.026**
Site 2	243	81.89 (1.99)	113	82.20 (2.15)	130	81.62 (1.80)	**0.022**
Site 3	243	78.64 (2.93)	113	79.26 (2.99)	130	78.10 (2.77)	**0.002**
Site 4	243	73.54 (2.14)	113	73.96 (1.96)	130	73.18 (2.22)	**0.004**
***H19* methylation**							
Site 1	245	59.08 (8.24)	115	58.46 (7.57)	130	59.63 (8.79)	0.266
Site 2	245	58.23 (4.83)	115	58.23 (3.32)	130	58.22 (5.87)	0.990
Site 3	245	59.26 (3.78)	115	59.05 (3.54)	130	59.45 (3.98)	0.412
Site 4	245	56.66 (8.51)	115	55.83 (7.74)	130	57.39 (9.09)	0.153
***HSD11B2* methylation**							
Site 1	246	-1.54 (2.14)	115	-1.57 (2.35)	131	-1.51 (1.94)	0.844
Site 2	246	0.08 (0.92)	115	-0.04 (0.92)	131	0.19 (0.92)	0.057
Site 3	245	-2.20 (2.25)	115	-2.23 (2.33)	130	-2.18 (2.19)	0.856
Site 4	244	-0.76 (1.75)	115	-0.70 (1.58)	129	-0.81 (1.89)	0.601
Site 5	229	0.24 (4.46)	108	0.20 (4.33)	121	0.28 (4.58)	0.898
***IGF2* methylation**							
Site 1	223	35.15 (12.23)	101	34.71 (11.98)	122	35.51 (12.46)	0.630
Site 2	213	44.85 (14.06)	94	42.99 (14.56)	119	46.31 (13.54)	0.087
Site 3	228	53.79 (6.40)	102	53.57 (6.92)	126	53.97 (5.97)	0.644
Site 4	196	37.87 (4.26)	88	37.69 (4.65)	108	38.01 (3.94)	0.607
Site 5	229	52.94 (6.51)	103	52.90 (6.21)	126	52.97 (6.76)	0.932

[1] LINE-1, *HSD11B2*, and *H19* data exhibited batch effects and as such were standardized to controls included on experimental plates as previously described (Goodrich et al., 2016), while the *IGF2* data exhibited no batch effect.

[2] *P*-value of 2-sample t test comparing boys and girls.

Table 2.3: DNA Methylation at LINE-1, H19, HSD11B2 and IGF2 among all individuals and stratified by sex after imputation of missing values[1,2].

	Entire Cohort	**Boys**	**Girls**	***P* value[3]**
	Mean % methylation (SD)	Mean % methylation (SD)	Mean % methylation (SD)	
***LINE-1* methylation**	N = 243	N = 114	N = 129	
Site 1	79.99 (4.11)	80.56 (4.37)	79.49 (3.80)	**0.033**
Site 2	81.92 (2.03)	82.28 (2.17)	81.61 (1.83)	**0.015**
Site 3	78.65 (3.00)	79.35 (3.06)	78.03 (2.80)	**<0.001**
Site 4	73.56 (2.17)	73.99 (2.00)	73.18 (2.25)	**0.006**
***H19* methylation**	N= 243	N= 114	N=129	
Site 1	58.95 (8.38)	58.22 (7.70)	59.60 (8.90)	0.148
Site 2	58.20 (4.86)	58.27 (3.27)	58.14 (5.92)	0.755
Site 3	59.26 (3.74)	59.06 (3.41)	59.44 (4.00)	0.443
Site 4	56.58 (8.66)	55.71 (7.76)	57.35 (9.31)	0.069
***HSD11B2* methylation**	N=243	N=114	N=129	
Site 1	-1.56 (2.16)	-1.55 (2.38)	-1.57 (1.95)	0.841
Site 2	0.09 (0.93)	-0.02 (0.92)	0.20 (0.93)	0.087
Site 3	-2.21 (2.29)	-2.17 (2.37)	-2.25 (2.22)	0.736
Site 4	-0.72 (1.77)	-0.66 (1.62)	-0.78 (1.89)	0.636
Site 5	0.18 (4.48)	0.31 (4.25)	0.07 (4.68)	0.556
***IGF2* methylation**	N=243	N=114	N=129	
Site 1	34.91 (12.79)	34.35 (13.04)	35.42 (12.54)	0.319
Site 2	44.28 (15.42)	42.59 (16.80)	45.79 (13.93)	0.065
Site 3	53.56 (6.71)	53.25 (7.49)	53.83 (5.93)	0.650
Site 4	37.00 (4.75)	36.31 (5.52)	37.61 (3.86)	**0.006**
Site 5	53.02 (6.14)	53.16 (6.60)	52.90 (5.70)	0.888

[1] Regression imputation method was applied.

[2] LINE-1, *HSD11B2*, and *H19* data exhibited batch effects and as such were standardized to controls included on experimental plates as previously described (Goodrich et al., 2016), while the *IGF2* data exhibited no batch effect.

[3] ***P*-value of 2-sample t test comparing boys and girls.**

Table 2.4: Associations between site-specific visit 1 (early-teen) DNA methylation and visit 1 (early-teen) and visit 2 (late-teen) pubertal onset, in adjusted Cox survival models[1,2].

Boys (N=114)		**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
		Visit 1	Visit 2	Visit 1	Visit2	Visit 1	Visit 2	Visit 1	Visit 2
	Site	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted
LINE1	1	0.95	1.03	0.97	1.00	1.01	1.01	0.99	1.03
		(0.84, 1.08)	(0.89, 1.03)	(0.89, 1.05)	(0.90, 1.03)	(0.92, 1.10)	(0.92, 1.10)	(0.91, 1.08)	(0.91, 1.08)
	2	0.81	0.96	0.88	0.96	1.05	1.08	1.03	1.07
		(0.59, 1.11)	(0.83, 1.10)	(0.73, 1.07)	(0.83, 1.08)	(0.90, 1.22)	(0.89, 1.21)	(0.89, 1.19)	(0.88, 1.18)
	3	0.93	0.97	0.97	0.95	1.04	1.10	1.02	1.08
		(0.77, 1,12)	(0.89, 1.10)	(0.86, 1.10)	(0.90, 1.08)	(0.93, 1.15)	(0.92, 1.14)	(0.91, 1.13)	(0.91, 1.11)
	4	1.02	1.02	0.92	1.00	1.04	1.01	1.06	1.03
		(0.76, 1.37)	(0.91, 1.31)	(0.77, 1.10)	(0.88, 1.15)	(0.88, 1.22)	(0.87, 1.18)	(0.91, 1.23)	(0.90, 1.19)
H19	1	1.01	1.03	1.03	1.00	1.01	1.01	1.03	1.03
		(0.93, 1.09)	(0.99, 1.07)	(0.99, 1.08)	(0.97, 1.04)	(0.96, 1.06)	(0.97, 1.06)	(0.98, 1.08)	(0.98, 1.08)
	2	**0.64**	0.96	0.93	0.96	1.08	1.08	1.07	1.07
		(0.43, 0.94)	(0.87, 1.05)	(0.84, 1.03)	(0.90, 1.03)	(0.97, 1.21)	(0.97, 1.20)	(0.97, 1.18)	(0.97, 1.18)
	3	**0.74**	0.97	0.93	0.95	1.10	1.10	1.08	1.08
		(0.55, 0.99)	(0.88, 1.06)	(0.84, 1.03)	(0.89, 1.02)	(0.98, 1.24)	(0.98, 1.24)	(0.97, 1.19)	(0.98, 1.19)
	4	1.02	1.02	1.03	1.00	1.00	1.01	1.02	1.03
		(0.94, 1.10)	(0.98, 1.06)	(0.98, 1.07)	(0.97, 1.03)	(0.96, 1.05)	(0.97, 1.06)	(0.98, 1.07)	(0.98, 1.07)
HSD11B2	1	0.85	0.89	0.89	0.97	1.01	1.00	0.98	0.97
		(0.69, 1.03)	(0.78, 1.02)	(0.76, 1.04)	(0.87, 1.08)	(0.86, 1.19)	(0.85, 1.18)	(0.85, 1.14)	(0.84, 1.13)
	2	1.07	0.96	0.96	1.09	0.66	0.66	0.67	0.67
		(0.62, 1.85)	(0.68, 1.34)	(0.67, 1.37)	(0.81, 1.46)	(0.40, 1.08)	(0.41, 1.06)	(0.43, 1.06)	(0.43, 1.04)
	3	0.86	0.95	0.93	0.90	1.01	1.00	0.99	0.98
		(0.68, 1.08)	(0.84, 1.06)	(0.80, 1.08)	(0.81, 1.01)	(0.87, 1.16)	(0.87, 1.15)	(0.87, 1.13)	(0.86, 1.13)
	4	**1.60**	**1.20**	**1.67**	1.01	0.99	1.01	1.03	1.05
		(1.05, 2.44)	**(1.01, 1.43)**	**(1.19, 2.33)**	(0.88, 1.16)	(0.78, 1.27)	(0.78, 1.30)	(0.83, 1.29)	(0.84, 1.31)
	5	1.06	0.99	0.96	0.99	0.99	0.98	0.98	0.97
		(0.91, 1.23)	(0.92, 1.07)	(0.89, 1.05)	(0.93, 1.05)	(0.91, 1.07)	(0.90, 1.07)	(0.90, 1.06)	(0.90, 1.06)

IGF2	1	1.01 (0.97, 1.05)	1.00 (0.97, 1.03)	0.99 (0.96, 1.01)	0.99 (0.96, 1.01)	1.01 (0.98, 1.04)	1.01 (0.97, 1.04)	1.01 (0.98, 1.04)	1.01 (0.98, 1.04)
	2	1.01 (0.98, 1.05)	1.01 (0.99, 1.03)	1.02 (1.00, 1.05)	1.01 (0.99, 1.03)	1.01 (0.98, 1.03)	1.01 (0.98, 1.03)	1.00 (0.98, 1.02)	1.00 (0.98, 1.02)
	3	0.95 (0.89, 1.01)	0.96 (0.92, 1.01)	**0.93 (0.88, 0.99)**	**0.93 (0.89, 0.98)**	1.03 (0.97, 1.10)	1.03 (0.96, 1.10)	1.01 (0.96, 1.07)	1.01 (0.95, 1.07)
	4	0.95 (0.87, 1.03)	1.00 (0.94, 1.07)	0.95 (0.88, 1.02)	0.97 (0.92, 1.03)	1.05 (0.97, 1.13)	1.04 (0.97, 1.11)	1.04 (0.97, 1.11)	1.03 (0.96, 1.10)
	5	0.99 (0.89, 1.10)	1.02 (0.96, 1.08)	0.98 (0.92, 1.05)	0.99 (0.94, 1.03)	1.07 (0.99, 1.15)	1.06 (0.98, 1.14)	1.05 (0.99, 1.11)	1.05 (0.98, 1.11)

Girls (N=129)		**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**		**Menarche Age**	
		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Site	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted	Adjusted
LINE1	1	0.96 (0.82, 1.12)	1.00 (0.91, 1.09)	0.99 (0.92, 1.06)	0.96 (0.89, 1.05)	0.97 (0.86, 1.09)	0.96 (0.89, 1.04)	0.97 (0.89, 1.07)	0.97 (0.92, 1.03)
	2	1.05 (0.73, 1.50)	0.95 (0.76, 1.20)	0.92 (0.81, 1.04)	0.88 (0.73, 1.06)	0.81 (0.58, 1.14)	0.89 (0.76, 1.05)	0.88 (0.71, 1.10)	0.97 (0.86, 1.10)
	3	1.07 (0.86, 1.34)	0.95 (0.84, 1.08)	**0.89 (0.82, 0.97)**	0.97 (0.87, 1.09)	0.83 (0.67, 1.03)	0.98 (0.89, 1.08)	0.87 (0.72, 1.06)	0.94 (0.92, 1.10)
	4	1.01 (0.78, 1.31)	0.92 (0.79, 1.07)	**0.83 (0.75, 0.93)**	0.88 (0.76, 1.02)	0.85 (0.66, 1.09)	0.88 (0.77, 1.01)	0.89 (0.74, 1.06)	1.01 (0.84, 1.04)
H19	1	1.03 (0.95, 1.12)	1.02 (0.98, 1.07)	**1.05 (1.02, 1.07)**	1.02 (0.99, 1.06)	0.99 (0.94, 1.06)	1.02 (0.99, 1.06)	1.01 (0.95, 1.06)	1.01 (0.99, 1.04)
	2	1.21 (1.00, 1.47)	1.02 (0.96, 1.07)	1.02 (0.96, 1.09)	**0.93 (0.88, 0.99)**	0.98 (0.88, 1.09)	0.99 (0.94, 1.04)	0.96 (0.85, 1.09)	1.00 (0.96, 1.04)
	3	**1.22 (1.01, 1.49)**	1.07 (0.99, 1.17)	1.06 (0.99, 1.13)	0.99 (0.91, 1.07)	0.99 (0.87, 1.13)	1.01 (0.93, 1.10)	0.97 (0.86, 1.09)	1.01 (0.95, 1.08)
	4	1.03 (0.95, 1.13)	1.03 (0.98, 1.08)	**1.05 (1.03, 1.08)**	1.03 (0.99, 1.06)	1.00 (0.94, 1.06)	1.03 (1.00, 1.06)	1.01 (0.95, 1.06)	1.01 (0.99, 1.04)
HSD11B2	1	0.98 (0.79, 1.22)	0.97 (0.84, 1.11)	**0.80 (0.71, 0.91)**	0.86 (0.72, 1.02)	0.93 (0.76, 1.14)	0.95 (0.80, 1.12)	0.92 (0.77, 1.10)	0.98 (0.88, 1.09)

	2	0.87 (0.56, 1.34)	0.89 (0.58, 1.36)	0.91 (0.75, 1.11)	0.88 (0.66, 1.18)	0.88 (0.58, 1.32)	0.73 (0.50, 1.08)	0.93 (0.66, 1.32)	0.84 (0.63, 1.11)
	3	1.07 (0.87, 1.33)	1.04 (0.90, 1.20)	**0.87 (0.77, 0.98)**	0.92 (0.79, 1.07)	0.89 (0.72, 1.11)	0.91 (0.79, 1.06)	0.91 (0.77, 1.08)	0.96 (0.87, 1.06)
	4	1.32 (0.91, 1.90)	1.06 (0.93, 1.20)	**1.25 (1.13, 1.38)**	1.08 (0.95, 1.24)	1.18 (0.87, 1.60)	0.93 (0.76, 1.13)	1.21 (0.94, 1.57)	0.90 (0.76, 1.07)
	5	0.97 (0.87, 1.09)	0.95 (0.87, 1.04)	0.96 (0.91, 1.01)	0.99 (0.93, 1.05)	0.96 (0.87, 1.06)	0.97 (0.92, 1.03)	0.99 (0.91, 1.07)	1.01 (0.96, 1.05)
IGF2	1	0.99 (0.95, 1.04)	1.00 (0.97, 1.02)	1.00 (0.98, 1.02)	1.00 (0.98, 1.03)	1.03 (0.99, 1.08)	1.01 (0.99, 1.03)	1.02 (0.99, 1.05)	1.01 (0.99, 1.02)
	2	1.02 (0.98, 1.06)	1.00 (0.98, 1.03)	1.01 (1.00, 1.03)	1.01 (0.99, 1.04)	0.99 (0.96, 1.03)	0.99 (0.97, 1.01)	1.00 (0.97, 1.03)	0.99 (0.98, 1.01)
	3	0.96 (0.88, 1.04)	0.98 (0.90, 1.07)	1.00 (0.95, 1.05)	0.97 (0.91, 1.04)	**1.12 (1.02, 1.22)**	0.99 (0.94, 1.04)	1.03 (0.98, 1.09)	0.98 (0.94, 1.01)
	4	0.99 (0.89, 1.10)	1.00 (0.93, 1.08)	0.97 (0.91, 1.04)	0.94 (0.86, 1.03)	1.08 (0.95, 1.23)	0.94 (0.87, 1.03)	1.03 (0.93, 1.15)	1.00 (0.93, 1.06)
	5	1.05 (0.94, 1.17)	**1.07 (1.01, 1.14)**	1.01 (0.96, 1.07)	1.02 (0.96, 1.09)	1.12 (1.00, 1.26)	1.00 (0.95, 1.06)	1.02 (0.96, 1.09)	0.99 (0.95, 1.03)

[1] Adjusted for age, BMI and SES status at visit 1 (early-teen).

[2] Bolded value indicates the association is significant with a *P* value < 0.05.

Table 2.5: Associations between site-specific visit 1 (early-teen) DNA methylation and pubertal progression from visit 1 (early-teen) to visit 2 (late-teen), in adjusted multivariate regression models[1,2,3].

Boys (N=114)		**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Site	Main Effect	Site by Time	Main Effect	Site by Time	Main Effect	Site by Time	Main Effect	Site by Time
LINE1	1	1.03	0.97	1.01	0.97	1.05	0.96	1.10	0.94
		(0.76, 1.39)	(0.89, 1.06)	(0.86, 1.20)	(0.93, 1.01)	(0.82, 1.36)	(0.89, 1.05)	(0.83, 1.47)	(0.87, 1.03)
	2	1.00	0.97	0.96	0.99	1.12	0.94	1.22	0.90
		(0.52, 1.93)	(0.79, 1.19)	(0.70, 1.31)	(0.92, 1.07)	(0.67, 1.87)	(0.79, 1.11)	(0.66, 2.24)	(0.76, 1.08)
	3	1.03	0.98	0.99	1.00	1.05	0.95	1.11	0.94
		(0.64, 1.66)	(0.85, 1.12)	(0.80, 1.22)	(0.95, 1.06)	(0.71, 1.54)	(0.86, 1.06)	(0.73, 1.67)	(0.84, 1.04)
	4	1.09	0.98	0.90	1.04	1.11	0.96	1.18	0.97
		(0.38, 3.12)	(0.73, 1.33)	(0.63, 1.27)	(0.96, 1.14)	(0.59, 2.08)	(0.81, 1.13)	(0.64, 2.16)	(0.82, 1.13)
H19	1	0.99	1.02	1.04	0.99	1.00	1.01	1.00	1.01
		(0.80, 1.23)	(0.96, 1.08)	(0.95, 1.14)	(0.98, 1.01)	(0.85, 1.16)	(0.97, 1.05)	(0.87, 0.97)	(0.97, 1.04)
	2	0.82	1.06	0.92	1.05	1.07	0.99	1.05	0.98
		(0.31, 2.14)	(0.82, 1.37)	(0.73, 1.14)	(0.99, 1.12)	(0.74, 1.54)	(0.89, 1.09)	(0.77, 1.43)	(0.90, 1.07)
	3	0.86	1.06	0.95	1.02	1.09	0.98	1.07	0.98
		(0.36, 2.10)	(0.83, 1.35)	(0.77, 1.16)	(0.97, 1.06)	(0.74, 1.61)	(0.89, 1.09)	(0.77, 1.48)	(0.90, 1.07)
	4	1.00	1.01	1.04	0.99	1.01	1.01	1.01	1.01
		(0.79, 1.25)	(0.95, 1.08)	(0.95, 1.13)	(0.97, 1.01)	(0.87, 1.17)	(0.97, 1.05)	(0.88, 1.15)	(0.97, 1.04)
HSD11B2	1	0.84	1.04	0.77	**1.12**	0.94	1.03	0.96	1.03
		(0.42, 1.68)	(0.90, 1.20)	(0.53, 1.12)	**(1.01, 1.24)**	(0.60, 1.50)	(0.92, 1.15)	(0.64, 1.45)	(0.93, 1.14)
	2	0.94	1.00	0.70	1.16	0.52	1.41	0.63	1.39
		(0.25, 3.46)	(0.73, 1.37)	(0.33, 1.50)	(0.94, 1.42)	(0.09, 2.97)	(0.87, 2.28)	(0.17, 2.33)	(0.99, 1.95)
	3	0.87	1.03	0.82	1.07	0.96	0.99	0.98	1.00
		(0.41, 1.87)	(0.88, 1.21)	(0.53, 1.25)	(0.96, 1.19)	(0.61, 1.52)	(0.91, 1.08)	(0.65, 1.48)	(0.91, 1.09)
	4	1.36	0.97	1.59	**0.83**	1.08	1.01	1.14	1.00
		(0.47, 3.96)	(0.76, 1.23)	(0.92, 2.74)	**(0.72, 0.97)**	(0.49, 2.40)	(0.85, 1.19)	(0.55, 2.38)	(0.85, 1.18)
	5	1.03	0.99	0.97	1.01	1.00	0.94	0.97	0.96
		(0.74, 1.43)	(0.91, 1.07)	(0.83, 1.14)	(0.97, 1.05)	(0.78, 1.29)	(0.85, 1.04)	(0.78, 1.22)	(0.89, 1.04)
IGF2	1	1.00	1.01	0.99	1.01	0.98	1.01	1.00	1.00
		(0.90, 1.10)	(0.98, 1.03)	(0.94, 1.04)	(1.00, 1.02)	(0.90, 1.07)	(0.99, 1.03)	(0.92, 1.09)	(0.98, 1.02)

	2	1.00	1.00	1.01	0.99	1.02	1.00	1.01	1.00
		(0.92, 1.10)	(0.97, 1.02)	(0.97, 1.06)	(0.98, 1.01)	(0.95, 1.10)	(0.98, 1.02)	(0.94, 1.08)	(0.98, 1.02)
	3	0.94	1.02	0.94	1.02	0.96	1.01	0.97	1.02
		(0.79, 1.13)	(0.98, 1.07)	(0.85, 1.04)	(0.99, 1.04)	(0.82, 1.12)	(0.98, 1.05)	(0.85, 1.12)	(0.98, 1.06)
	4	0.95	1.02	0.93	**1.04**	1.04	1.01	1.04	1.01
		(0.78, 1.15)	(0.98, 1.06)	(0.82, 1.05)	**(1.00, 1.07)**	(0.85, 1.27)	(0.96, 1.06)	(0.87, 1.25)	(0.96, 1.06)
	5	0.96	1.03	1.00	1.01	1.03	1.00	1.01	0.99
		(0.80, 1.15)	(0.99, 1.07)	(0.90, 1.11)	(0.98, 1.03)	(0.86, 1.22)	(0.95, 1.04)	(0.86, 1.18)	(0.94, 1.04)

Girls (N=129)		**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**	
		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Site	Main effect	Site by Time	Main effect	Site by Time	Main effect	Site by Time
LINE1	1	1.00	1.01	0.96	0.97	1.06	0.98
		(0.73, 1.38)	(0.94, 1.08)	(0.76, 1.22)	(0.91, 1.03)	(0.75, 1.51)	(0.89, 1.08)
	2	1.03	1.01	0.93	0.94	1.19	0.99
		(0.44, 2.41)	(0.82, 1.24)	(0.57, 1.50)	(0.83, 1.06)	(0.49, 2.90)	(0.78, 1.26)
	3	1.04	1.00	0.95	0.95	1.17	1.06
		(0.48, 2.25)	(0.82, 1.22)	(0.69, 1.30)	(0.87, 1.04)	(0.71, 1.94)	(0.92, 1.22)
	4	1.06	1.02	0.93	0.97	1.20	1.03
		(0.41, 2.71)	(0.79, 1.32)	(0.63, 1.38)	(0.89, 1.06)	(0.59, 2.42)	(0.81, 1.31)
H19	1	1.03	1.01	1.01	1.01	0.98	1.01
		(0.86, 1.23)	(0.97, 1.05)	(0.90, 1.13)	(0.98, 1.04)	(0.82, 1.16)	(0.94, 1.07)
	2	1.08	1.03	0.94	0.98	1.01	1.00
		(0.64, 1.82)	(0.90, 1.20)	(0.81, 1.09)	(0.94, 1.02)	(0.76, 1.34)	(0.93, 1.08)
	3	1.11	1.04	0.95	0.98	0.99	0.99
		(0.64, 1.93)	(0.90, 1.20)	(0.69, 1.31)	(0.90, 1.07)	(0.66, 1.48)	(0.89, 1.11)
	4	1.02	1.00	1.01	1.01	0.98	1.01
		(0.84, 1.24)	(0.96, 1.05)	(0.91, 1.12)	(0.99, 1.03)	(0.82, 1.16)	(0.94, 1.07)
HSD11B2	1	0.92	0.95	0.93	0.95	1.17	1.09
		(0.46, 1.85)	(0.80, 1.13)	(0.60, 1.43)	(0.86, 1.06)	(0.64, 2.14)	(0.92, 1.30)
	2	0.74	0.99	0.77	0.93	1.29	1.02
		(0.24, 2.25)	(0.78, 1.26)	(0.32, 1.86)	(0.76, 1.15)	(0.38, 4.46)	(0.62, 1.65)
	3	1.00	1.02	0.91	0.99	1.21	1.04

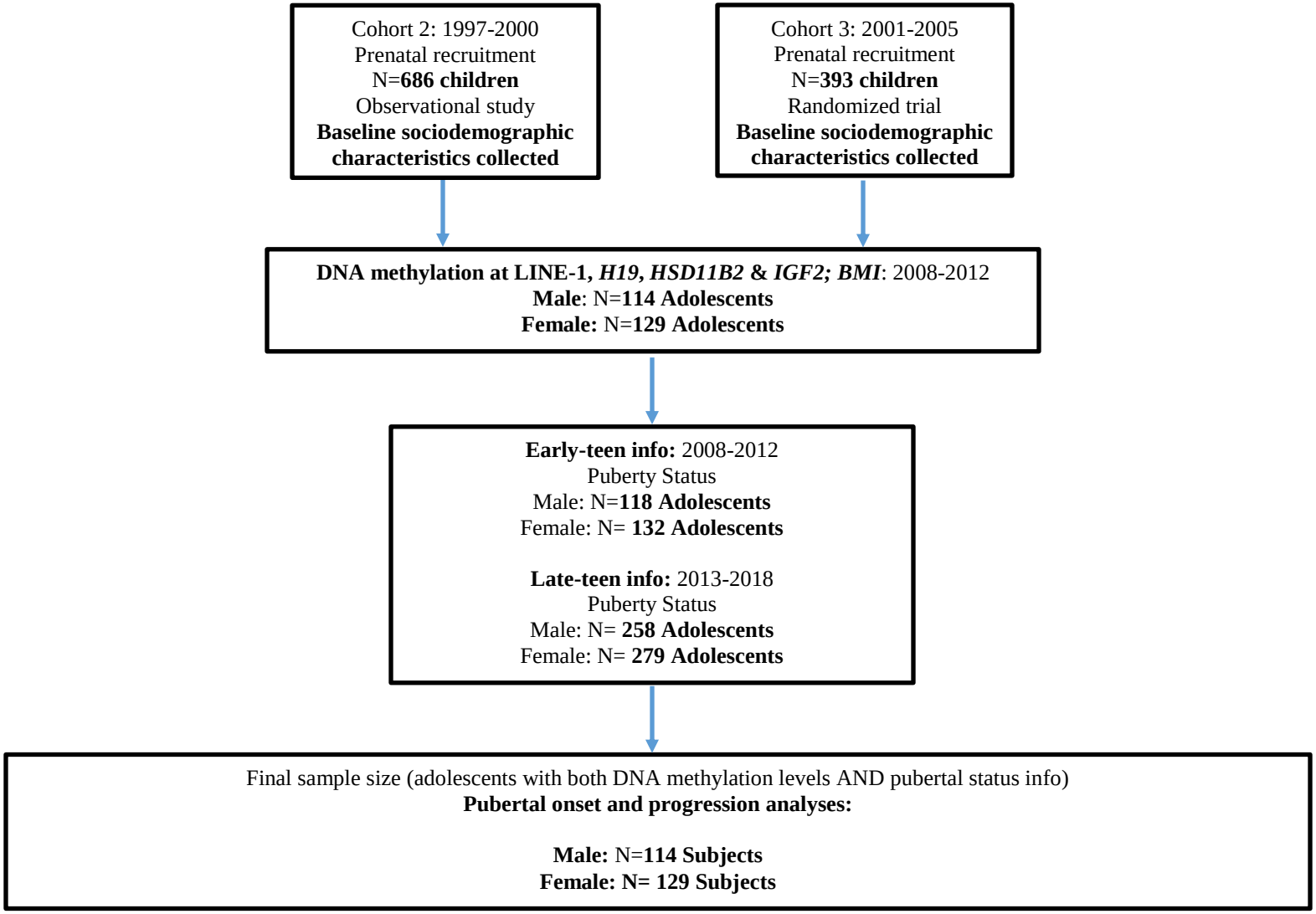
		(0.44, 2.27)	(0.80, 1.29)	(0.62, 1.32)	(0.90, 1.08)	(0.65, 2.24)	(0.84, 1.30)
	4	1.24	1.13	1.24	1.15	0.82	0.86
		(0.63, 2.47)	(0.93, 1.37)	(0.80, 1.93)	(0.97, 1.37)	(0.42, 1.64)	(0.74, 1.00)
	5	0.97	1.02	0.98	1.01	1.05	1.02
		(0.74, 1.27)	(0.96, 1.08)	(0.82, 1.17)	(0.97, 1.04)	(0.80, 1.39)	(0.95, 1.10)
IGF2	1	0.99	1.00	0.99	1.00	0.95	0.98
		(0.89, 1.10)	(0.97, 1.02)	(0.92, 1.07)	(0.98, 1.02)	(0.84, 1.06)	(0.95, 1.02)
	2	1.02	1.01	1.02	1.00	1.01	0.99
		(0.92, 1.13)	(0.98, 1.03)	(0.96, 1.08)	(0.99, 1.01)	(0.92, 1.11)	(0.96, 1.02)
	3	0.97	1.00	1.01	1.00	0.89	0.93
		(0.80, 1.17)	(0.96, 1.04)	(0.87, 1.17)	(0.97, 1.03)	(0.69, 1.16)	(0.84, 1.04)
	4	1.01	1.02	0.87	0.98	0.90	0.95
		(0.72, 1.41)	(0.93, 1.12)	(0.65, 1.18)	(0.91, 1.06)	(0.65, 1.25)	(0.84, 1.07)
	5	1.04	1.01	0.99	1.00	0.87	0.96
		(0.84, 1.29)	(0.97, 1.06)	(0.86, 1.16)	(0.96, 1.03)	(0.62, 1.23)	(0.88, 1.04)

[1] Adjusted for age, BMI and SES status at visit 1 (early-teen).

[2] Bolded value indicates the association is significant with a *P* value < 0.05.

[3] Model: logit (Y ij) = β0 + β1*age + β2*time_difference + β3*CpG_Site + β4*CpG_Site*time_difference +β5*age*time_difference

Figure 2.1: Selection of ELEMENT subjects for the study[1].



[1] **Offspring from enrollment cohorts 2 and 3 were re-contacted and re-enrolled based on availability of prenatal and neonatal biospecimens. We did not re-contact Cohort 1 participants, originally recruited in 1994-96, because the majority had advanced stages or had completed pubertal development.**

Chapter 3 Dietary Exposures, Epigenetics, and Pubertal Tempo

INTRODUCTION

Early or late age at pubertal onset is an established risk factor for a number of reproductive tract cancers, insulin resistance, and adiposity in adulthood, as well as all-cause mortality (Golub et al., 2007; Niculescu, 2008; Zhu & Chan, 2016; Jacobson & Heuch, 2007). Over recent decades, the risk factors for earlier or later puberty, including chemical exposures, unbalanced diet, and abnormal hormone levels caused by diseases and psychological stress, have been widely studied (Cesario et al., 2007; Jansen et al., 2016; Mills et al., 1986; Marceau et al., 2012).

Patterns of health, illness and disease risks are influenced and "programmed" at different stages of the life course by a combination of genetic, epigenetic and environmental factors, as articulated by the "Development Origins of Health and Disease (DOHaD)" concept (El-Heis & Godfrey, 2015; Li, 2002). Epigenetics is the study of mitotically heritable yet potentially reversible molecular modifications to DNA and chromatin without alteration to the underlying DNA sequence (Li, 2002; Reik et al., 2001). The influence of epigenetic regulation, which includes DNA methylation, on pubertal onset has been considered in animal models and global changes in specific epigenetic factors appear to be key players in the regulation of the onset of puberty (Lomniczi et al., 2013; Tomikawa et al., 2012; Rzeczkowska et al., 2014). However, potential associations of DNA methylation with pubertal tempo (e.g. pubertal onset and progression through stages of maturation) in both sexes has not been considered in human or animal studies. In female rats, interference of DNA methylation was associated with delayed

vaginal opening and compromised fecundity by inhibiting *Kiss1* gene, whose product is involved in controlling expression of GnRH (Lomniczi et al., 2013). Another study found that elevated expression of neurokinin B (Nkb) and *Kiss1* amplified gonadotropin hormone-releasing hormone (GnRH) secretion, triggering the onset of puberty in the mice (Gill et al., 2012).

The impact of diet on pubertal tempo has also been addressed in several epidemiological studies (Berkey et al., 2000; De Ridder et al., 1991; Merzenich et al., 1993; Li et al., 2012) They observed that high total energy intake, as well as high animal (red meat) versus vegetable protein ratio, is associated with early menarche (Berkey et al., 2000; De Ridder et al., 1991; Merzenich et al., 1993; Li et al., 2012). However, these studies focused primarily on macronutrients and relied on menarche as the sole puberty indicator (Wolff et al., 2008; Kaput, 2004). Previous animal and population-based studies provide strong evidence of diet and gene interactions (Ross, 2003; Mehedint et al., 2010; Ba et al., 2011; Amaral et al., 2011; Anderson et al., 2012). Yet, our understanding of the impact of micronutrients on puberty as well as the underlying mechanisms is limited, especially, but not only, in boys.

Methyl donor nutrients -- including folate, choline/betaine, methionine, riboflavin (B_2 vitamin), pyridoxine (B_6 vitamin) and cobalamin (B_{12} vitamin) -- play essential roles in the one-carbon metabolism cycle (Mentch & Locasale, 2016). DNA methylation, the most extensively studied epigenetic modification (De Araújo et al., 2015) provides a link to the one-carbon metabolism cycle through the generation of methyl donor, S-adenosylmethionine (SAM) (Mentch & Locasale, 2016). DNA methyltransferases (DNMTs) methylate the carbon-5 position of cytosine bases to methylated DNA using SAM (Mentch & Locasale, 2016). Therefore, the functioning of the cycle and subsequent availability of SAM is important for the establishment

and maintenance of DNA methylation and is in part dependent on the availability of methyl donor micronutrients.

To our knowledge, no previous study has examined the potential association between methyl-donor rich diet, DNA methylation and puberty tempo. To address these research gaps, this study examines the effect of maternal and adolescent diet on puberty tempo, and in particular, how foods rich in methyl donor nutrients influence puberty through epigenetic regulation. We hypothesize that methyl donor-rich diet may have an impact on pubertal tempo via altering DNA methylation. We utilized data from an ongoing cohort study in Mexico City to 1) examine which methyl donor-rich foods from maternal first trimester and adolescent diets are associated with methylation of the surrogate marker for global methylation, long interspersed nucleotide (LINE-1) repeats, collected during adolescence (8-14 years of age), and calculate a DNA methylation-associated dietary scores from both maternal first trimester and adolescence, and 2) examine the association of these scores with pubertal onset and progression in adolescent boys and girls.

MATERIALS AND METHODS

3.1 Study Population

The study population comprised a subset of participants from the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) project, a longitudinal epidemiological study consisting of three sequentially enrolled birth cohorts (Cantoral et al., 2015). The mother-child pairs were recruited at three maternity hospitals representing low- to moderate-income populations in Mexico City from 1997 to 2005. The subjects in this project were a subset of children from the second and third birth cohorts (n=646 pairs at baseline). At the research visit after the child was born, mothers provided household and demographic

information, including age, education, and previous numbers of pregnancies. Their newborns were followed from birth until 4 years of age. Starting in 2010, a subset of offspring were re-contacted (n=250; henceforth referred to as *Visit 1*) based on availability of prenatal and neonatal biospecimens (Perng et al., 2017). One more peri-pubertal visit (*Visit 2*) was completed approximately five years later (n=549, with 223 having also participated in the 2010 *Visit 1*). Fasting blood, pubertal status, anthropometry and household socioeconomic status were collected at both teen visits (Perng et al., 2017).

3.2 Laboratory Measurements and Outcomes

DNA methylation

Blood samples were obtained at *Visit 1* and collected in PAXGene tubes by trained staff following standard protocols. High-molecular-weight DNA was extracted from blood leukocytes with the PAXgene Blood DNA kit (PreAnalytix, Switzerland). DNA samples were treated with sodium bisulfite using kits from Zymo or Qiagen (Li & Tollefsbol, 2011). Percent of methylated cells was then quantitatively analyzed in a consensus region of repetitive elements from the LINE-1 family. DNA methylation was quantified via pyrosequencing using a PyroMark MD at 4 CpG sites. Full details on primers, quality control (which included running unmethylated and fully methylated human DNA controls with each batch and duplicating > 10% of the samples), and analysis methods have been previously published (Goodrich et al., 2016). DNA methylation levels exhibited batch effects and as such were standardized to controls included on each experimental batch (96-well plate), as previously described (Goodrich et al., 2016).

Dietary intake

Diets of pregnant women were assessed during the first trimester using an interviewer-administered semi-quantitative food frequency questionnaire (FFQ) designed to allow recall of dietary intake over the previous month (Willett, 2009). The list of 104 food items was built from the items that proved most representative of local consumption based on the 1983 Dietary Survey of the Mexican National Institute of Nutrition (Hernández-Avila et al., 1998)

Usual dietary intake of adolescents over the past week was collected using a 116-item interviewer-administered semi-quantitative FFQ adapted from the 2006 Mexican Health and Nutrition Survey (Villalpando et al., 2003) at *Visit 1*. The questionnaire asked participants to recall how often they typically consumed one serving of a standard portion size of each food item (in g or ml); response options ranged from never to ≥6 times per day.

Pubertal outcomes

Pubertal outcomes were obtained at both *Visit 1* and *Visit 2*. Trained physicians assessed Tanner stages of breast and pubic hair growth in girls as well as Tanner stages of genitalia and pubic hair growth in boys using standardized methods at both visits (Chavarro et al., 2017; Marshall & Tanner, 1970). Outcomes were recorded with a range from stage 1 indicating pre-puberty to stage 5 indicating full maturation (Marshall & Tanner, 1970; Biro et al., 1995). Testicular volumes were measured by trained physicians using orchidometers (range from 1 to 25 ml). Occurrence and age of menarche were gathered from a self-reported questionnaire (Marshall & Tanner, 1970; Biro et al., 1995; Jansen et al., 2017).

Covariates

Based on *a priori* knowledge and preliminary correlation tests of predictors, outcomes and potential confounders, covariates included in the final models were household SES and BMI of the child, obtained at *Visit 1*. The SES measure included material wellbeing (for

instance, number of bedrooms in the home) and education level of the head of the household. Combined information was ranked from A to E based on the scale developed by Mexican Association of Market Research and Public Opinion (AMAI) (Amaya et al., 2013). Weight and height of the child were measured by trained nurses, following standardized protocols, as previously described [41]; BMI was calculated as weight over height squared (kg/m^2) (Nuttall, 2015). Children's age was collected at both visits.

3.3 Statistical analysis

Descriptive statistics were obtained for all variables. We created an 'Epigenetic-Associated Diet Score' (EADS) as a weighted average of methyl-donor rich food items for maternal and adolescent diets, respectively, with weights based on the association of the food items with the mean of methylation of 4 CpG sites of LINE-1. The weights were obtained by using the LASSO regression with LINE-1 methylation as the dependent variable, and intake of methyl-donor rich food items as the predictors. The LASSO method is a powerful method in feature selection, which can efficiently reduce the number of variables included in the model. LASSO applies a shrinking process that penalizes the coefficients of the regression variables shrinking some to zero, while variables that still have a non-zero coefficient after the shrinking are selected to be part of the model (Zhang & Huang, 2008). Methyl donor rich food items used as input for the LASSO regression were those that are known to have high content of at least one of the following six major micronutrients: folate, choline/betaine, methionine, B2/riboflavin, B6/pyridoxine, and B12/cobalamin. To examine the food items used in the regression we identified, for each of these six "methyl donor" nutrients, up to 20 food items with highest specific nutrient content per serving based on information gathered from National Institute of Health (NIH) Dietary Supplement Fact Sheets datasets (National Institutes of Health: Office of

Dietary Supplements. Dietary Supplemental Fact Sheets. https://ods.od.nih.gov/factsheets (31 October 2018, date last accessed). We cross-referenced the up to 20 items for each micronutrient, with food items included on the FFQ used in the study. For the ELEMENT study, some food items have been grouped into categories based on nutritional similarity and cultural relevance (Jansen et al., 2017). Because many food items are rich in more than one of the six micronutrients, in total, there were a possible of 21 unique food groups that served as predictors in the LASSO regression model (Supplemental Table 1).

We retained seven food items (high-fat dairy, yogurt, beef, potato, refined grain, chicken and whole grain) from the maternal diet and eight food items (tomato, yogurt, fish, egg, cruciferous vegetables, leafy greens, pork and other vegetables) from the adolescent diet based on cross-validated results from LASSO selections. Based on the output from the LASSO algorithm, we calculated predicted LINE-1 methylation values and named them EADS. EADS was calculated separately for maternal diet and adolescent diet. From these regression models, we then calculated residuals by taking the observed LINE-1 methylation value and subtracting the EADS. We term these residuals as "methylation residuals" (MR), and note that they represent DNA methylation variation not explained by the dietary score (Spearman correlation between EADS and MR = -0.206). To improve interpretation of model results, these scores were transformed into z-scores; i.e., coefficients from outcome models (below) can be interpreted as differences in outcome per one SD higher EADS.

Secondly, we examined means ± SD for the selected food groups according to categories of maternal and adolescent characteristics to identify potential confounders. We conducted a linear trend test for ordinal characteristics (age, BMI) and a type-III Wald test for nominal characteristics (SES).

To examine the association between EADS and onset of each pubertal outcome, we performed time-to-event analysis using interval-censored regression models. Within the interval censored time to event model, age at *Visit 1* was the 'time in follow-up' and attainment of each of the following Tanner stages and menarche was the 'event'. Children were classified as having experienced the 'pubertal onset event' of interest if Tanner stages were > 1 (Chavarro et al., 2017; Marshall & Tanner, 1970; Biro et al., 1995) for pubic hair, genital development, breast development characteristics or answered "Yes" on self-reported menarche questionnaire at the visit time. Testicular volume ≤ 3 mL indicates pre-pubertal stage; testicular volume > 3mL but ≤ 11mL indicates pubertal onset; and > 11mL indicates sexual maturity (Chavarro et al., 2017; Marshall & Tanner, 1970; Biro et al., 1995). Models were adjusted for BMI and SES measured at *Visit 1*. Then, we repeated the model further adjusted for the MR term to test the association while controlling the effect of methylation not due to diet on puberty.

Next we assessed the association between EADS and pubertal progression, using the following ordinal regression model: logit $(Y_{ij}) = \beta_0 + \beta_1 * Age_i + \beta_2 * Time_{j-i} + \beta_3 * EADS + \beta_4 * EADS * Time_{j-i} + \beta_5 * MR + \beta_6 * MR * Time_{j-i} + \beta_7 * Age_i * Time_{j-i} + \beta_8 *$covariates to analyze the potential, gender-specific associations between EADS and pubertal progression between *Visit 1* and *Visit 2*. Age_i represents the age at *Visit 1*. Time represents the difference between visits.

RESULTS

The analytical sample included 118 boys and 132 girls who attended the *Visit 1*, of whom 108 boys and 114 girls remained at *Visit 2*. The mean age for *Visit 1* was 10.4 years in boys and 10.3 years in girls; the mean age was 13.7 years in boys and 13.5 for girls at *Visit 2*. We observed fewer children remaining at lower pubertal stages in *Visit 2*. Among boys, 79.7% and 48.3% were at Tanner stage 1 for pubic hair and genital development in *Visit 1*; and the number

dropped to 25.0% and 6.5% in *Visit 2*. In terms of testicular volume, the percentage of boys in the pre-pubertal stage dropped from 15.3% to 0%. Among girls, 74.2% were classified Tanner stage 1 for pubic hair and 65.9% for breast development in *Visit 1*; and the percentage dropped to 7.9% and 4.4%, respectively, at the later visit (**Table 1**). Since some mothers were not recruited until the child was born, the study sample with maternal diet information was smaller (85 boys, 92 girls). Nevertheless, we observed similar changes in pubertal stages from *Visit 1* to *Visit 2* (**Table 1**).

As analyzed in our previous published results, LINE-1 methylation was higher among boys compared to girls in our study cohort (Averaged LINE-1 methylation across 4 CpG sites (mean/SD): 78.98/2.39 in boys, 78.07/2.15 in girls). Unpaired t-test suggested a significant sex-difference of LINE-1 methylation (*P* value = 0.002) while actual methylation differences were minimum. The food groups that contributed to the EADS were different for maternal diet and adolescent diet (**Table 2**). LASSO-selected items that contributed to the maternal EADS included: high-protein, high-fat and high-carbohydrate food items, such as high-fat dairy, yogurt, beef, chicken, potato, refined grains and whole grains. The contributions from some items were positive while others negative. High-protein and high-fat food items were negatively associated with LINE-1 methylation, and thus diets with a high frequency of these items resulted in lower maternal EADS. Overall, whole grains were positively associated with LINE-1 methylation levels, and thus diets rich in these diets had higher EADS.

The adolescent EADS comprised fresh high-fiber vegetables, including tomato, cruciferous vegetables, leafy greens, other vegetables, as well as lean protein sources, such as yogurt, fish, egg and pork. Similarly, both positive and negative directions were observed between adolescent food items and LINE-1 methylation. Fresh vegetables were positively

associated with LINE-1 methylation level, while lean protein food items were negatively associated, and thus resulted in higher or lower adolescent EADS, respectively (**Table 2**).

Maternal and adolescent EADS had different strengths of associations with pubertal onset and progression. We observed significant associations between maternal, but not adolescent EADS, on pubertal onset in girls (**Table 3**). In the adjusted analysis, each SD increase of the maternal EADS was associated with a 76% lower probability of having menarche at Visit 1 (p=0.059), and 33% lower probability of having menarche at Visit 2 ((p=0.028). Each SD increase of the maternal EADS was associated with approximately half a year increase of the age at menarche (p=0.031). However, statistically significant associations were not observed between adolescent EADS and most indicators of pubertal onset.

In terms of pubertal progression, we observed statistically significant associations between adolescent EADS, but not for the associations with maternal EADS (**Table 4**). Among boys, for each standard deviation higher in the adolescent EADS, there was 13% increased odds of slower genital progression (p=0.050), as well as 26% and 27% increased odds of slower testicular developments (Left and right: p=0.001 and 0.001). Among girls, each standard deviation higher in the adolescent EADS score was associated with 16% increased odds of faster pubic hair progression (p=0.082) only.

DISCUSSION

Among participants in this Mexico City cohort, we observed different epigenetic-associated diet patterns in mothers and offspring. Most food items in the maternal diet selected in the LASSO model were rich in fat and protein, and were negatively associated with LINE-1 DNA methylation. However, selected components of the adolescent diet included more high fiber vegetables that in turn had a positive association with DNA methylation levels. We also

found evidence suggesting time- and gender-specific associations between EADS and pubertal tempo. Specifically, we observed significant associations between maternal EADS and pubertal onset in girls only, and significant associations between adolescent EADS and pubertal progression predominantly in boys.

The environment during development is emerging as a strong predictor of phenotype and disease in later life. Major environmental influences on developmental plasticity, including nutrition, behavior, stress, and toxicants, can act though different mechanisms and result in an array of changes to the epigenome, including DNA methylation (Faulk & Dolinoy, 2011). Based on previous *in vivo* and *in vitro* studies, periods of DNA methylation lability occur in three waves: primordial germ cell methylation reprogramming, post-fertilization zygotic methylation reprogramming, and somatic cell differentiation methylation reprogramming of adults (Faulk & Dolinoy, 2011). Other than the perinatal period, growth and the hormonally active puberty as well as senescence are also vulnerable periods to a variety of external insults (Barouki et al., 2012). Previous population- and animal-based research has highlighted the importance of maternal methyl donor dietary intake on fetal development (Barouki et al., 2012; Zeisel, 2006; Waterland & Jirtle, 2003), childhood respiratory health (Håberg et al., 2009; Miller, 2008), high childhood cognition scores (Villamor et al., 2012), healthy weight status [Barouki et al., 2012; Waterland et al., 2008; Dolinoy et al., 2007; Carlin et al., 2013), and lower cancer risks (Ly et al., 2011). However, its impact on timing and stages of puberty is not well understood. This study expands our understanding of both maternal first trimester diet and adolescent diet on LINE-1 DNA methylation and subsequently pubertal tempo, as well as the change in the strength of the associations with timing of the dietary exposure.

Our results showed a stronger effect of maternal diet with pubertal onset, while a statistically significant association was observed between adolescent diet and pubertal progression. The findings suggest a long-term effect of maternal diet and a short-term health impact of concurrent diet on pubertal tempo. Previous animal-based studies found evidence that maternal high-fat (Aagaard-Tillery et al., 2008) diet or high-methyl donor (Cordero et al., 2013) diet, or undernutrition (Jousse et al., 2011) would alter the epigenomic profile, including mRNA expression and fatty acid synthase (FASN) promoter methylation of the developing offspring and result in alterations in fetal gene expression. In terms of the effect of adolescent diet, Tomizawa et al. found that a 3-week folate-, methionine- and choline-deficient during the developmental phase was associated with decreased glutamate receptor 1 (*Gria1*) gene expression in the mouse hippocampus, affecting learning and memory (Tomizawa et al., 2015). Previous studies have suggested that the need for methyl donors might be much greater in pregnancy when DNA methylation patterns in the developing zygote are reprogrammed (Faulk & Dolinoy, 2011). However, methyl donors in adolescence may contribute to the maintenance and the stability of DNA methylation, which may potentially withstand more fluctuations in availability of these nutrients.

We also observed sex-specific differences between the effects of EADS and pubertal tempo. For instance, high maternal EADS was statistically associated with later menarche onset among girls, while higher adolescent EADS was associated with slower progression of genital and testicular development among boys. These were potential interesting findings since they highlighted the differences in the female and male reproductive system development. According to biological evidence, females are considered the "fundamental sex", in which without chemical prompting, all fertilized eggs would develop into females (Haley, 2012). Gonadal differentiation

occurs before the end of the embryonic period, approximately the 7th week of gestation; both the reproductive ducts, external genitalia and sex differentiates occur around the 10th week of gestation (Haley, 2012). After birth, maternal and placental estrogens no longer suppress the hypogonadal production of GnRH and pituitary gonadotropin. This results in the second major surge of hormone production in female and male development. During puberty, initiated by hormonal signals from the brain to the gonads (the ovaries in a girl, the testes in a boy), transformation of the nervous, muscular and reproductive systems are promoted, height and weight growth are accelerated. However, studies have found sexual dimorphism in the growth of the hippocampus in adolescence, which was associated with a more pronounced pubertal growth in males (Suzuki et al., 2005; Lenroot et al., 2007). These and previous studies suggest that the embryonic period is more sensitive for the female reproductive system, while adolescence may be a more efficient time for the male reproductive system to mature. Such differences may make children more prone to the impact of diet at specific developmental periods, which was supported by our EADS findings.

It is possible that the associations between LINE-1 methylation and diet were not induced solely by methyl-donor nutrients. Previous studies showed that chronic maternal high-fat diet could modify gene expression through epigenetic changes (Vucetic et al., 2011; Masuyama et al., 2016; Dudley et al., 2011). In terms of health impact, animals that were exposed to high-fat diet *in utero* had been associated with higher susceptibility to type 2 diabetes, overweight/obesity and non-alcoholic fatty liver disease (Williams et al., 2013). Animal studies also provided evidence on the causal association between maternal protein restriction and alterations in DNA methylation (Goyal et al., 2010; Lillycrop et al., 2005). Among

micronutrients, Gaedicket et al. found that vitamin E deficiency resulted in reduced expression on microRNA (Gaedicke et al., 2008).

This analysis has some limitations, including a relatively small sample size. Considering the sparsity and bias of the LASSO selection in high-dimensional linear regression, we applied cross-validation to estimate prediction error (Zhang & Huang, 2008). However, every method of statistical inference depends on a complex set of assumptions, so interpretation of these analyses should be done cautiously. In terms of data collection, maternal diet, adolescent diet and menarche information were self-reported and may not be accurate due to recall bias. We would argue that this systematic within-person error applies to all subjects equally since methyl donor-rich dietary patterns are not known in the general population. Thus, it should not distort measures of EADS and the associations between EADS and pubertal tempo (Rosner et al., 1989). Moreover, since the epigenetic programming varies by genomic loci and by cell and tissue type, we need to consider the limitation of including the LINE-1 DNA methylation from blood leukocytes as the sole indicator for individual's global methylation status.

To our knowledge, this is the first longitudinal study examining the association between methyl-donor rich diet, DNA methylation and pubertal tempo. Our findings suggested timing- and sex-specific differences between the effects of methyl donor associated diets and pubertal tempo. Our observations may suggest the potential to develop dietary recommendations for mothers in the first trimester or for adolescents that may influence pubertal tempo. Future work in this field should consider examining other "developmentally plastic" phases and include epigenome-wide DNA methylation to consider whether the same findings apply.

Bibliography

Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, Lane RH. Developmental origins of disease and determinants of chromatin structure: Maternal diet modifies the primate fetal epigenome. *J Mol Endocrinol*. 2008. doi:10.1677/JME-08-0025.

Amaral CL Do, Bueno RDBEL, Burim RV, Queiroz RHC, Bianchi MDLP, Antunes LMG. The effects of dietary supplementation of methionine on genomic stability and p53 gene promoter methylation in rats. *Mutat Res*. 2011. doi:10.1016/j.mrgentox.2011.03.006.

Amaya AA, Salgado A, Martínez J, Chablé S. Perfil Familiar , Socioeconómico Y Demográfico De Jóvenes De Un Tutelar De Menores. *Rev Electron Psicol Iztacala*. 2013.

Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *J Nutr Biochem*. 2012. doi:10.1016/j.jnutbio.2012.03.003.

Ba Y, Yu H, Liu F, Geng X, Zhu C, Zhu Q, Zheng T, Ma S, Wang G, Li Z, et al. Relationship of folate, vitamin B12 and methylation of insulin-like growth factor-II in maternal and cord blood. *Eur J Clin Nutr*. 2011. doi:10.1038/ejcn.2010.294.

Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ. Developmental origins of non-communicable disease: Implications for research and public health. *Environ Heal A Glob Access Sci Source*. 2012. doi:10.1186/1476-069X-11-42.

Berkey CS, Gardner JD, Lindsay Frazier A, Colditz GA. Relation of childhood diet and body size to menarche and adolescent growth in girls. *Am J Epidemiol*. 2000. doi:10.1093/aje/152.5.446.

Biro FM, Lucky AW, Huster GA, Morrison JA. Pubertal staging in boys. *J Pediatr*. 1995. doi:10.1016/S0022-3476(95)70265-2.

Cantoral A, Téllez-Rojo MM, Levy TS, Hernández-Ávila M, Schnaas L, Hu H, Peterson KE, Ettinger AS. Differential association of lead on length by zinc status in two-year old Mexican children. *Environ Heal A Glob Access Sci Source*. 2015. doi:10.1186/s12940-015-0086-8.

Carlin JL, George R, Reyes TM. Methyl donor supplementation blocks the adverse effects of maternal high fat diet on offspring physiology. *PLoS One*. 2013. doi:10.1371/journal.pone.0063549.

Cesario SK, Hughes L a. Precocious puberty: a comprehensive review of literature. *J Obstet Gynecol Neonatal Nurs*. 2007. doi:10.1111/j.1552-6909.2007.00145.x.

Chavarro JE, Watkins DJ, Afeiche MC, Zhang Z, Sánchez BN, Cantonwine D, Mercado-García A, Blank-Goldenberg C, Meeker JD, Téllez-Rojo MM, et al. Validity of self-assessed sexual maturation against physician assessments and hormone levels. *J Pediatr*. 2017. doi:10.1016/j.jpeds.2017.03.050.

Cordero P, Gomez-Uriz AM, Campion J, Milagro FI, Martinez JA. Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. *Genes Nutr*. 2013. doi:10.1007/s12263-012-0300-z.

De Araújo ÉSS, Kashiwabara AY, Achatz MIW, Moredo LF, De Sá BCS, Duprat JP, Rosenberg C, Carraro DM, Krepischi ACV. LINE-1 hypermethylation in peripheral blood of cutaneous melanoma patients is associated with metastasis. *Melanoma Res*. 2015. doi:10.1097/CMR.0000000000000141.

De Ridder CM, Thijssen JHH, Van T Veer P, Van Duuren R, Bruning PF, Zonderland ML, Erich WBM. Dietary habits, sexual maturation, and plasma hormones in pubertal girls: A longitudinal study. *Am J Clin Nutr*. 1991. doi:10.1093/ajcn/54.5.805.

Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci*. 2007. doi:10.1073/pnas.0703739104.

Dudley KJ, Sloboda DM, Connor KL, Beltrand J, Vickers MH. Offspring of mothers fed a high fat diet display hepatic cell cycle inhibition and associated changes in gene expression and DNA methylation. *PLoS One*. 2011. doi:10.1371/journal.pone.0021662.

El-Heis S, Godfrey K. Developmental origins of health and disease. *Obstet Gynaecol Reprod Med*. 2015. doi:10.1016/j.ogrm.

Faulk C, Dolinoy DC. Timing is everything: The when and how of environmentally induced changes in the epigenome of animals. *Epigenetics*. 2011. doi:10.4161/epi.6.7.16209.

Gaedicke S, Zhang X, Schmelzer C, Lou Y, Doering F, Frank J, Rimbach G. Vitamin E dependent microRNA regulation in rat liver. *FEBS Lett*. 2008. doi:10.1016/j.febslet.

Gill JC, Navarro VM, Kwong C, Noel SD, Martin C, Xu S, Clifton DK, Carroll RS, Steiner RA, Kaiser UB. Increased neurokinin B (Tac2) expression in the mouse arcuate nucleus is an early marker of pubertal onset with differential sensitivity to sex steroid-negative feedback than Kiss1. *Endocrinology*. 2012. doi:10.1210/en.

Golub MS, Collman GW, Foster PMD, Kimmel CA, Rajpert-De Meyts E, Reiter EO, Sharpe RM, Skakkebaek NE, Toppari J. Public health implications of altered puberty timing. *Pediatrics*. 2008. doi:10.1542/peds.

Goodrich JM, Dolinoy DC, Sánchez BN, Zhang Z, Meeker JD, Mercado-Garcia A, Solano-González M, Hu H, Téllez-Rojo MM, Peterson KE. Adolescent epigenetic profiles and environmental exposures from early life through peri-adolescence. *Environ Epigenetics*. 2016. doi:10.1093/eep/dvw018.

Goyal R, Goyal D, Leitzke A, Gheorghe CP, Longo LD. Brain renin-angiotensin system: Fetal epigenetic programming by maternal protein restriction during pregnancy. *Reprod Sci*. 2010. doi:10.1177/1933719109351935.

Håberg SE, London SJ, Stigum H, Nafstad P, Nystad W. Folic acid supplements in pregnancy and early childhood respiratory health. *Arch Dis Child*. 2009. doi:10.1136/adc.2008.142448.

Haley A. Embryology of the Female Reproductive Tract. In: Springer. ; 2012. doi:10.1007/174.

Hernández-Avila M, Romieu I, Parra S, Hernández-Avila J, Madrigal H, Willett W. Validity and reproducibility of a food frequency questionnaire to assess dietary intake of women living in Mexico City. *Salud Publica Mex*. 1998. doi:10.1590/S0036-36341998000200005.

Jacobsen BK, Heuch I, Kvåle G. Association of low age at menarche with increased all-cause mortality: A 37-year follow-up of 61,319 Norwegian women. *Am J Epidemiol*. 2007. doi:10.1093/aje/kwm237.

Jansen EC, Marin C, Mora-Plazas M, Villamor E. Higher childhood red meat intake frequency is associated with earlier age at menarche. *J Nutr*. 2016. doi:10.3945/jn.115.226456.

Jansen EC, Miller AL, Lumeng JC, Kaciroti N, Brophy Herb HE, Horodynski MA, Contreras D, Peterson KE. Externalizing behavior is prospectively associated with intake of added sugar and sodium among low socioeconomic status preschoolers in a sex-specific manner. *Int J Behav Nutr Phys Act*. 2017. doi:10.1186/s12966-017-0591-y.

Jousse C, Parry L, Lambert-Langlais S, Maurin A-C, Averous J, Bruhat A, Carraro V, Tost J, Letteron P, Chen P, et al. Perinatal undernutrition affects the methylation and expression of the leptin gene in adults: implication for the understanding of metabolic syndrome. *FASEB J*. 2011. doi:10.1096/fj.11-18179.

Kaput J. Diet-disease gene interactions. *Nutrition*. 2004. doi:10.1016/j.nut.

Lenroot RK, Gogtay N, Greenstein DK, Wells EM, Wallace GL, Clasen LS, Blumenthal JD, Lerch J, Zijdenbos AP, Evans AC, et al. Sexual dimorphism of brain developmental trajectories during childhood and adolescence. *Neuroimage*. 2007. doi:10.1016/j.neuroimage.

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet*. 2002. doi:10.1038/nrg887.

Li XF, Lin YS, Kinsey-Jones JS, O'Byrne KT. High-fat diet increases LH pulse frequency and kisspeptin-neurokinin B expression in puberty-advanced female rats. *Endocrinology*. 2012. doi:10.1210/en.

Li Y, Tollefsbol TO. DNA methylation detection: Bisulfite genomic sequencing analysis. *Methods Mol Biol*. 2011. doi:10.1007/978-1-61779-316-5_2.

Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr*. 2005. doi:135/6/1382 [pii].

Lomniczi A, Loche A, Castellano JM, Ronnekleiv OK, Bosch M, Kaidar G, Knoll JG, Wright H, Pfeifer GP, Ojeda SR. Epigenetic control of female puberty. *Nat Neurosci*. 2013. doi:10.1038/nn.3319.

Ly A, Lee H, Chen J, Sie KKY, Renlund R, Medline A, Sohn KJ, Croxford R, Thompson LU, Kim YI. Effect of maternal and postweaning folic acid supplementation on mammary tumor risk in the offspring. *Cancer Res*. 2011. doi:10.1158/0008-5472.

Marceau K, Dorn LD, Susman EJ. Stress and puberty-related hormone reactivity, negative emotionality, and parent-adolescent relationships. *Psychoneuroendocrinology*. 2012. doi:10.1016/j.psyneuen.

Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. *Arch Dis Child*. 1970. doi:10.1136/adc.45.239.13.

Masuyama H, Mitsui T, Eguchi T, Tamada S, Hiramatsu Y. The effects of paternal high-fat diet exposure on offspring metabolism with epigenetic changes in the mouse *adiponectin* and *leptin* gene promoters. *Am J Physiol - Endocrinol Metab*. 2016. doi:10.1152/ajpendo.

Mehedint MG, Craciunescu CN, Zeisel SH. Maternal dietary choline deficiency alters angiogenesis in fetal mouse hippocampus. *Proc Natl Acad Sci*. 2010. doi:10.1073/pnas.0914328107.

Mentch SJ, Locasale JW. One-carbon metabolism and epigenetics: Understanding the specificity. *Ann N Y Acad Sci*. 2016. doi:10.1111/nyas.12956.

Merzenich H, Boeing H, Wahrendorf J. Dietary fat and sports activity as determinants for age at menarche. *Am J Epidemiol*. 1993. doi:10.1093/oxfordjournals.aje.a116850.

Miller RL. Prenatal maternal diet affects asthma risk in offspring. *J Clin Invest*. 2008. doi:10.1172/JCI37171.

Mills JL, Shiono PH, Shapiro LR, Crawford PB, Rhoads GG. Early growth predicts timing of puberty in boys: Results of a 14-year nutrition and growth study. *J Pediatr*. 1986. doi:10.1016/S0022-3476(86)80141-X.

National Institutes of Health: Office of Dietary Supplements. Dietary Supplemental Fact Sheets. https://ods.od.nih.gov/factsheets (31 October 2018, date last accessed).

Niculescu D. Puberty: Ontogeny, Neuroendocrinology, Physiology, and Disorders. In: Williams Textbook of Endocrinology. ; 2008. doi:10.4183/aeb.

Nuttall FQ. Body mass index: Obesity, BMI, and health: A critical review. *Nutr Today*. 2015;50(3):117-128. doi:10.1097/NT.0000000000000092.

Perng W, Hector EC, Song PXK, Tellez Rojo MM, Raskind S, Kachman M, Cantoral A, Burant CF, Peterson KE. Metabolomic determinants of metabolic risk in Mexican adolescents. *Obesity*. 2017. doi:10.1002/oby.21926.

Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science*. 2001. doi:10.1126/science.1063443.

Rosner B, Willett WC, Spiegelman D. Correction of logistic regression relative risk estimates and confidence intervals for systematic within-person measurement error. *Stat Med*. 1989. doi:10.1002/sim.4780080905.

Ross SA. Diet and DNA methylation interactions in cancer prevention. In: *Annals of the New York Academy of Sciences*. ; 2003. doi:10.1111/j.1749-6632.2003.tb05974.x.

Rzeczkowska PA, Hou H, Wilson MD, Palmert MR. Epigenetics: A new player in the regulation of mammalian puberty. *Neuroendocrinology*. 2014. doi:10.1159/000362559.

Suzuki M, Hagino H, Nohara S, Zhou S-Y, Kawasaki Y, Takahashi T, Matsui M, Seto H, Ono T, Kurachi M. Male-specific volume expansion of the human hippocampus during adolescence. *Cereb Cortex*. 2005. doi:10.1093/cercor/bhh121.

Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, et al. Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc Natl Acad Sci*. 2012. doi:10.1073/pnas.1114245109.

Tomizawa H, Matsuzawa D, Ishii D, Matsuda S, Kawai K, Mashimo Y, Sutoh C, Shimizu E. Methyl-donor deficiency in adolescence affects memory and epigenetic status in the mouse hippocampus. *Genes, Brain Behav*. 2015. doi:10.1111/gbb.12207.

Villalpando S, Shamah-Levy T, Ramírez-Silva CI, Mejía-Rodríguez F, Rivera J a. Prevalence of anemia in children 1 to 12 years of age: results from a nationwide probabilistic survey in Mexico. *Salud Publica Mex*. 2003. doi:10.1590/S0036-36342003001000005.

Villamor E, Rifas-Shiman SL, Gillman MW, Oken E. Maternal intake of methyl-donor nutrients and child cognition at 3 years of age. *Paediatr Perinat Epidemiol*. 2012. doi:10.1111/j.1365-3016.2012.01264.x.

Vucetic Z, Kimmel J, Reyes TM. Chronic high-fat diet drives postnatal epigenetic regulation of-opioid receptor in the brain. *Neuropsychopharmacology*. 2011. doi:10.1038/npp.

Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol*. 2003. doi:10.1128/MCB.23.15.5293.

Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S. Methyl donor supplementation prevents transgenerational amplification of obesity. *Int J Obes*. 2008. doi:10.1038/ijo.2008.100.

Willett WC. *Nutritional Epidemiology*.; 2009. doi:10.1093/acprof:oso/9780195122978.001.0001.

Williams L, Seki Y, Vuguin PM, Charron MJ. Animal models of in utero exposure to a high fat diet: A review. *Biochim Biophys Acta - Mol Basis Dis*. 2014. doi:10.1016/j.bbadis.

Wolff MS, Britton JA, Boguski L, Hochman S, Maloney N, Serra N, Liu Z, Berkowitz G, Larson S, Forman J. Environmental exposures and puberty in inner-city girls. *Environ Res*. 2008. doi:10.1016/j.envres.

Zeisel SH. Choline: Critical role during fetal development and dietary requirements in adults. Annu Rev Nutr. 2006. doi:10.1146/annurev.nutr.26.061505.111156.

Zhang CH, Huang J. The sparsity and bias of the lasso selection in high-dimensional linear regression. *Ann Stat*. 2008. doi:10.1214/07-AOS520.

Zhu J, Chan Y-M. Adult consequences of self-limited delayed puberty. *Pediatrics*. 2017. doi:10.1542/peds.2016-3177.

Table 3.1: Distributions of Tanner stages and other covariates among ELEMENT children at the early-teen visit (Visit 1) and at the late-teen visit (Visit 2) for children who continued follow-up, in both adolescent and maternal diet analysis samples.

	Adolescent Diet Sample				Maternal Diet Sample			
Boys	**Visit 1 (N=118)**		**Visit 2 (N=108)**		**Visit 1 (N=102)**		**Visit 2 (N=94)**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Pubic Hair[1]								
1	94	79.66	27	25.00	83	81.37	27	28.72
2	17	14.41	16	14.81	13	12.75	16	17.02
3	3	2.54	30	27.78	2	1.96	25	26.60
4	1	0.85	18	16.67	1	0.98	11	11.70
5	0	0.00	14	12.96	0	0.00	12	12.77
Missing	3	2.54	3	2.78	3	2.94	3	3.19
Genital Development								
1	57	48.31	7	6.48	53	51.96	7	7.45
2	43	36.44	17	15.74	35	34.31	17	18.09
3	10	8.47	26	24.07	8	7.84	22	23.40
4	5	4.24	37	34.26	3	2.94	31	32.98
5	0	0.00	18	16.67	0	0.00	14	14.89
Missing	3	2.54	3	2.78	3	2.94	3	3.19
Testicular Development (L)								
1-3 ml	18	15.25	0	0.00	17	16.66	0	0.00
3-11 ml	75	63.56	16	14.81	65	63.74	16	17.02
>11 ml	22	18.65	89	82.41	17	16.66	75	79.79
Missing	3	2.54	3	2.78	3	2.94	3	3.19
Testicular Development (R)								
1-3 ml	18	15.25	0	0.00	17	16.66	0	0.00
3-11 ml	76	64.41	16	14.81	65	63.74	16	17.02
>11 ml	20	16.95	89	82.41	16	15.68	75	79.79
Missing	4	3.39	3	2.78	4	3.92	3	3.19

Age (years)	10.35 ± 1.61		13.72 ± 1.75		10.24 ± 1.60		13.51 ± 1.73	
BMI	19.06 ± 3.14		20.43 ± 3.68		18.96 ± 3.19		20.33 ± 3.88	
Household SES: Quartile	**N=100**				**N=98**		**N=90**	
1	24	24.00			23	23.47	21	23.33
2	27	27.00			26	26.53	24	26.67
3	24	24.00			22	22.45	22	24.44
4	25	25.00			27	27.55	23	25.56
Girls	**Visit 1 (N=132)**		**Visit 2 (N=114)**		**Visit 1 (N=117)**		**Visit 2 (N=103)**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Pubic Hair								
1	98	74.24	9	7.90	92	78.63	9	8.74
2	22	16.67	39	34.21	15	12.82	38	36.89
3	9	6.82	29	25.44	8	6.84	26	25.24
4	2	1.52	21	18.42	1	0.85	17	16.50
5	1	0.76	14	12.28	1	0.85	12	11.65
Missing	0	0.0	2	1.75	0	0.00	1	0.97
Breast Development								
1	87	65.90	5	4.39	82	70.09	5	4.85
2	20	15.15	12	10.53	18	15.38	12	11.65
3	18	13.63	46	40.35	13	11.11	45	43.69
4	7	5.30	31	27.19	4	3.42	24	23.30
5	0	0.00	18	15.79	0	0.00	16	15.53
Missing	0	0.0	2	1.75	0	0.00	1	0.97
Menarche								
Yes	30	22.73	90	78.95	22	18.80	79	76.70
No	102	77.27	23	20.17	95	81.20	23	22.33
Missing	0	0.00	1	0.88	0	0.00	1	0.88
Age (years)	10.30 ± 1.72		13.54 ± 1.75		10.12 ± 1.66		13.37 ± 1.72	

BMI	19.66 ± 3.95		21.61 ± 4.07	19.63 ± 3.87		21.80 ± 4.16
Household SES: Quartile	**N=102**			**N=108**		**N=94**
1	25	24.51		30	27.78	23	24.47
2	29	28.43		28	25.93	26	27.66
3	24	23.53		26	24.07	24	25.53
4	24	23.53		24	22.22	21	22.34

[1] **Distributions were listed across different Tanner Stages. Also applies for Genital Development and Breast Development.**

Table 3.2: Diet patterns related to child's LINE-1 methylation at Visit 1 using LASSO feature selection.

Maternal Diet Pattern		
Food Item	**Estimate[1]**	**Average Intake (Serving/day) in Study Sample: Mean (± SD)**
High-fat dairy	-22.46	1.00 ± 0.76
Yogurt	-18.88	0.45 ± 0.44
Beef	-37.73	0.26 ± 0.20
Potato	30.12	0.31 ± 0.23
Refined grain	-2.41	2.42 ± 1.15
Chicken	-9.41	0.35 ± 0.25
Whole grain	2.77	0.37 ± 0.61
Adolescent Diet Pattern		
Food Item	**Estimate**	**Average Intake (g/day) in Study Sample: Mean (± SD)**
Tomato	1.55	6.34 ± 12.12
Yogurt	0.20	91.04 ± 73.97
Fish	-0.78	10.35 ± 10.57
Egg	-0.33	30.49 ± 24.76
Cruciferous vegetables	0.55	5.44 ± 7.27
Leafy greens	0.05	42.58 ± 49.32
Pork	0.11	7.95 ± 11.82
Other vegetables	-0.04	84.40 ± 68.41

[1] **Estimate = β * 100**

Table 3.3: List of top ranked food sources of 6 methyl donor nutrients based on National Institutes of Health (NIH) Dietary Supplement Fact Sheets[1,2].

Top Ranked Food Items	Folate	ELEMENT Food Group Available	Choline/Betaine	ELEMENT Food Group Available
1	Beef liver	Organ meat	Beef liver	Organ meat
2	Spinach	Leafy vegetables	Whole egg	Eggs
3	Black peas	N/A	Beef, lean	Beef
4	Fortified breakfast cereal	Refined grains	Soybeans	N/A
5	White rice	Refined grains	Chicken breast	Chicken
6	Asparagus	Other vegetables	Ground beef	Beef
7	Spaghetti	Refined grains	Cod fish	Fish
8	Brussels spouts	Cruciferous vegetables	Shiitake mushrooms	N/A
9	Romaine lettuce	Leafy vegetables	Potatoes	Potato
10	Avocado	Avocado	Wheat germ	N/A
11	Broccoli	Cruciferous vegetables	Kidney beans	Legumes
12	Mustard greens	N/A	Quinoa	N/A
13	Green peas	N/A	1% fat milk	Milk
14	Kidney beans	Legumes	Yogurt	Yogurt
15	White bread	Legumes	Brussels sprouts	Cruciferous vegetables
16	Dry roasted peanuts	Legumes	Broccoli	Cruciferous vegetables
17	Wheat germ	N/A	Cottage cheese	High fat dairy
18	Canned tomato juice	N/A	Tuna fish	Fish
19	Dungeness crab	Fish	Dry roasted peanuts	Legumes
20	Orange juice	Fruits	Cauliflower	Cruciferous vegetables
21	Frozen turnip greens	N/A	Green peas	N/A
22	Fresh orange	Fruits	Sunflower seeds	Legumes
23	Raw papaya	Fruits	Brown rice	Whole grain
24	Banana	Fruits	Pita bread	Refined grain
25	Whole egg	Eggs	Cabbage	Cruciferous vegetable

Top Ranked Food Items	Methionine	ELEMENT Food Group Available	B2 (Riboflavin)	ELEMENT Food Group Available

1	Whole egg	Eggs	Beef liver	Organ meat
2	Chicken	Chicken	Fortified breakfast cereal	Refined grains
3	Canned tuna	Fish	Oats/Oatmeal	N/A
4	Turkey	N/A	Yogurt	Yogurt
5	Pork	Pork	Milk	Milk
6	Cheese	High fat dairy	Beef, steak	Beef
7	Beef	Beef	Clams	N/A
8	Salmon	Fish	Mushrooms	N/A
9	Lamb	N/A	Almonds	N/A
10	Soybeans	N/A	Cheese	High fat dairy
11	Yogurt	Yogurt	Rotisserie chicken	Chicken
12	White beans	Legumes	Whole egg	Eggs
13			Quinoa	N/A
14			Bagel	N/A
15			Pink salmon	Fish
16			Spinach	Leafy greens
17			Apple, with skin	Fruits
18			Kidney beans	Legumes
19			Macaroni	N/A
20			Whole Wheat Bread	Whole grains
21			Codfish	Fish
22			Sunflower seeds	N/A
23			Tomatoes	Tomato
24			White rice	Refined grains
25			Brown rice	Whole grains

Top Ranked Food Items	B6 (Pyridoxine)	ELEMENT Food Group Available	B12 (Cobalamin)	ELEMENT Food Group Available
1	Chickpeas	Other vegetables	Clams	N/A
2	Beef liver	Organ meat	Beef liver	Organ meat
3	Fresh tuna	Fish	Fortified breakfast cereals	Refined grains
4	Salmon	Fish	Trout	N/A
5	Chicken breast	Chicken	Salmon	Fish
6	Fortified breakfast cereal	Refined grains	Canned tuna fish	Fish
7	Potatoes	Potato	Cheeseburger	Processed meat
8	Turkey	N/A	Haddock	N/A
9	Banana	Fruits	Beef	Beef
10	Marinara sauce	N/A	Milk	Milk
11	Ground beef	Beef	Yogurt	Yogurt
12	Waffles	N/A	Cheese	High fat dairy
13	Bulgur	N/A	Beef taco	N/A
14	Cheese	High fat dairy	Cured ham	Processed meat
15	Winter squash	Other vegetables	Whole egg	Eggs
16	White rice	Refined grains	Chicken	Chicken
17	Mixed nuts	Legumes		
18	Raisins	N/A		
19	Onions	N/A		
20	Spinach	Leafy greens		
21	Tofu	N/A		
22	Watermelon	Fruits		
23				
24				
25				

[1] Yellow highlighted food items were those collected in FFQ from ELEMENT study cohort.

[2] 1: most content rich; 25: least content rich .

Table 3.4: Associations between predicted maternal and adolescent epigenetic-associated diet score and visit 1 (early-teen) and visit 2 (late-teen) pubertal onset, accounting for methylation residuals[1,2].

Maternal Diet:								
Boys (N=85)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	0.92	0.91	0.83	1.17	1.19	1.28	1.14	1.20
	(0.30. 2.85)	(0.63, 1.31)	(0.54, 1.27)	(0.88, 1.56)	(0.82, 1.72)	(0.86, 1.91)	(0.81, 1.62)	(0.84, 1.73)
Girls (N=92)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**		**Menarche Age**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	1.17	0.76	1.42	1.24	**0.24**	**0.67**	**0.48**	0.85
	(0.47, 2.96)	(0.50, 1.15)	(0.81, 2.49)	(0.84, 1.84)	**(0.06, 1.06)**	**(0.47, 0.96)**	**(0.25, 0.93)**	(0.68, 1.06)
Adolescent Diet:								
Boys (N=118)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	0.53	0.88	0.99	0.80	1.31	1.32	1.45	1.46
	(0.21. 1.33)	(0.64, 1.21)	(0.70, 1.38)	(0.61, 1.06)	(0.82, 2.07)	(0.80, 2.15)	(0.89, 2.35)	(0.86, 2.47)
Girls (N=132)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**		**Menarche Age**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	1.17	**0.71**	0.95	0.78	0.97	0.88	0.98	0.93
	(0.67, 2.05)	**(0.47, 1.07)**	(0.61, 1.49)	(0.53, 1.15)	(0.65, 1.43)	(0.66, 1.19)	(0.71, 1.36)	(0.74, 1.16)

[1] **Adjusted for age, BMI and SES status at visit 1 (early-teen).**

[2] **Bolded value indicates the association is significant with a *P* value < 0.1.**

[3] **Model 2: $\beta_0 + \beta_1$*Epigenetic-Associated Diet Score + β_2*Methylation Residual + β_3*Covariates**

Table 3.5: Associations between predicted **maternal** and **adolescent** epigenetic-associated diet score and visit 1 (early-teen) as well as visit 2 (late-teen) pubertal onset[1,2].

Maternal Diet:								
Boys (N=85)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	0.96 (0.32, 2.91)	0.92 (0.65, 1.30)	0.83 (0.54, 1.26)	1.22 (0.92, 1.60)	1.18 (0.83, 1.67)	1.24 (0.85, 1.80)	1.15 (0.82, 1.61)	1.19 (0.84, 1.69)
Girls (N=90)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**		**Menarche Age**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	1.16 (0.50, 2.68)	0.82 (0.56, 1.22)	1.58 (0.91, 2.76)	1.36 (0.94, 1.97)	**0.48 (0.21, 1.09)**	0.77 (0.56, 1.07)	0.58 (0.33, 1.03)	0.86 (0.69, 1.08)
Adolescent Diet:								
Boys (N=118)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	0.52 (0.21, 1.31)	0.89 (0.65, 1.22)	1.01 (0.73, 1.38)	0.80 (0.62, 1.04)	1.25 (0.81, 1.93)	1.26 (0.80, 1.99)	1.45 (0.90, 2.33)	1.41 (0.87, 2.28)
Girls (N=132)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**		**Menarche Age**	
	Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)		Hazard Ratio (CI)	
	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted	Visit 1 Adjusted	Visit 2 Adjusted
	1.24 (0.75, 2.07)	0.74 (0.51, 1.10)	1.00 (0.66, 1.52)	0.83 (0.58, 1.20)	1.05 (0.72, 1.52)	0.91 (0.68, 1.21)	1.02 (0.74, 1.40)	0.93 (0.74, 1.17)

[1] **Adjusted for age, BMI and SES status at visit 1 (early-teen).**

[2] **Bolded value indicates the association is significant with a *P* value < 0.1.**

[3] **Model 1: $\beta_0 + \beta_1$*Epigenetic-Associated Diet Score + β_2*Covariates**

Table 3.6: Associations between predicted **maternal** and **adolescent** epigenetic-associated diet score and pubertal progression from visit 1 (early-teen) to visit 2 (late-teen), in adjusted multivariate regression models[1,2,3].

Maternal Diet:								
Boys (N=85)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time
	0.83	0.98	0.77	1.17	1.28	0.90	1.27	0.99
	(0.38, 1.79)	(0.74, 1.28)	(0.46, 1.28)	(0.91, 1.51)	(0.72, 2.27)	(0.67, 1.21)	(0.76, 2.14)	(0.74, 1.31)
Girls (N=92)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**			
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)			
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time		
	0.64	0.86	1.08	0.92	**2.59**	1.19		
	(0.32, 1.26)	(0.67, 1.10)	(0.68, 1.71)	(0.77, 1.10)	**(1.05, 6.35)**	(0.87, 1.62)		
Adolescent Diet:								
Boys (N=118)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time
	0.89	0.93	1.03	**0.87**	**1.94**	**0.74**	**2.15**	**0.73**
	(0.57, 1.39)	(0.79, 1.10)	(0.77, 1.38)	**(0.75, 1.00)**	**(1.15, 3.27)**	**(0.61, 0.88)**	**(1.32, 3.51)**	**(0.61, 0.87)**
Girls (N=132)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**			
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)			
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time		
	1.28	**1.16**	0.97	0.98	1.10	0.88		
	(0.88, 1.85)	**(0.98, 1.36)**	(0.62, 1.51)	(0.83, 1.14)	(0.55, 2.17)	(0.69, 1.12)		

[1] Adjusted for age, BMI and SES status at visit 1 (early-teen).

[2] Bolded value indicates the association is significant with a *P* value < 0.1.

[3] Model: logit (Y ij) = : logit (Y_{ij}) = $\beta_0 + \beta_1$* $Age_i + \beta_2$*$Time_{j-i} + \beta_3$* EADS + β_4*EADS*$Time_{j-i} + \beta_5$*MR + β_6*MR*$Time_{j-i} + \beta_7$*Age_i*$Time_{j-i}$ + β_8*covariates

Table 3.7: Associations between predicted **maternal** and **adolescent** epigenetic-associated diet score and pubertal progression from visit 1 (early-teen) to visit 2 (late-teen), in adjusted multivariate regression models[1,2,3].

Maternal Diet:								
Boys (N=85)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time
	0.82	0.99	0.77	1.19	1.24	0.94	1.19	1.03
	(0.38, 1.74)	(0.76, 1.30)	(0.47, 1.25)	(0.94, 1.51)	(0.72, 2.14)	(0.73, 1.22)	(0.73, 1.93)	(0.80, 1.33)
Girls (N=92)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**			
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)			
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time		
	0.64	0.85	1.18	0.96	**2.19**	1.19		
	(0.33, 1.24)	(0.67, 1.08)	(0.74, 1.87)	(0.80, 1.15)	**(0.89, 5.36)**	(0.88, 1.63)		
Adolescent Diet:								
Boys (N=118)	**Pubic Hair**		**Genital Development**		**Testicular Volume (L)**		**Testicular Volume (R)**	
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)	
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time
	0.84	0.98	1.07	**0.87**	**1.71**	**0.80**	**1.94**	**0.80**
	(0.55, 1.28)	(0.83, 1.16)	(0.80, 1.42)	**(0.76, 1.00)**	**(1.07, 2.75)**	**(0.68, 0.95)**	**(1.15, 3.27)**	**(0.67, 0.95)**
Girls (N=132)	**Pubic Hair**		**Breast Development**		**Menarche (Y/N)**			
	Odds Ratio (CI)		Odds Ratio (CI)		Odds Ratio (CI)			
	Main Effect	Score by Time	Main Effect	Score by Time	Main Effect	Score by Time		
	1.28	**1.16**	1.01	1.01	0.97	0.85		
	(0.89, 1.83)	**(0.99, 1.35)**	(0.66, 1.57)	(0.86, 1.18)	(0.52, 1.81)	(0.68, 1.06)		

[1] Adjusted for age, BMI and SES status at visit 1 (early-teen).

[2] Bolded value indicates the association is significant with a *P* value < 0.1.

[3] Model: logit (Y ij) = β0 + β1*baseline age + β2*time_difference + β3*EADS + β4*EADS *time_difference +β5*baseline age*time_difference

Table 3.8: Pyrosequencing assays with primer sequences and details of CpG sites assessed.

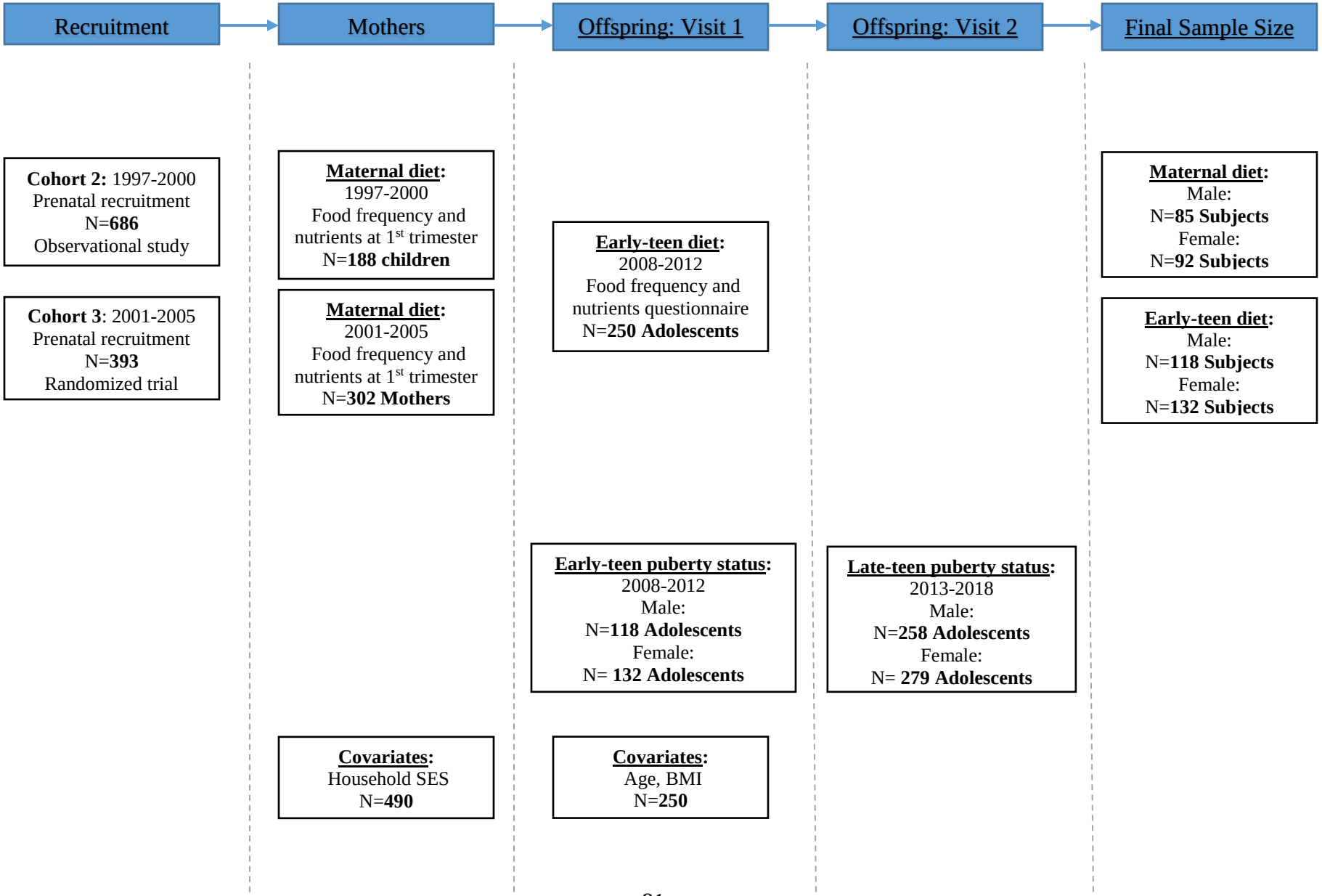
				Primer Sequences			
Gene or Element Name	**# of CpG Sites Assessed**	**Loci of CpG Sites***	**DNA Methylation Analysis Method**	**Forward**	**Reverse§**	**Sequencing**	**Locus of Amplified Region**
LINE-1	4	Various$	Pyrosequencing	TTGAGTTAGGTGTGGGATATAGTT	CAAAAAATCAAAAAATTCCCTTTCC	AGGTGTGGATATAGT	Various

*** Loci are based off of genome build CRCh38/hg38.**

§ All reverse primers for pyrosequencing are 5' biotinylated.

$ A sequence in the promoter region of nearly all LINE-1s is amplified and sequenced here. The specific sequence is as follows 5'-CTCGTGGTGCGCCGTTTCTTAAGCCG.

Figure 3.1: Timeline and selection of ELEMENT subjects for the study.



Chapter 4 Accelerometer-measured Physical Activity is Associated with Reproductive Hormones and DNA Methylation in the ELEMENT Cohort

INTRODUCTION

The health benefits of physical activity for school-aged children and adolescents are well documented (Janseen, 2007; Twisk, 2001; Tolfrey et al., 2000). Physical activity has beneficial effects on adiposity, musculoskeletal health and fitness, and several components of cardiovascular health, includinginflammatory markers, endothelial function, and heart rate variability (Janseen, 2007). Adolescents, however, experience unfavorable shifts in activity patterns from early to late adolescence, including decreases in moderate to vigorous physical activity, coupled with secular increases in leisure-time computer use (Nelson et al., 2006; Biddle et al., 2004). The Developmental Origins of Health and Disease (DOHaD) theory includes adolescence period as one of the specific sensitive windows of developmental plasticity (Heindel & Vandenberg, 2015). Adolescence is also a life stage of adjustments in healthy lifestyle behaviors that may persist into adulthood (Sata, 2019), highlighting the potential importance of behavioral interventions during this period.

Early or late age at pubertal maturation is an established risk factor for a number of reproductive tract cancers, insulin resistance, and higher adiposity in adulthood, as well as all-cause mortality (Golub et al., 2007; Niculescu, 2008; Zhu & Chan, 2017; Jacobsen et al., 2007). The risk factors for earlier or later puberty, including chemical exposures, unbalanced diet, and abnormal hormone levels caused by diseases and psychological stress, have been widely studied (Cesario & Hughes, 2007; Jansen et al., 2016; Mills et al., 1986; Marceau et al., 2012). Studies

also have suggested an association between high intensity exercise and sexual maturation, documenting delayed pubertal growth among the elite male and female rhythmic gymnasts, wrestlers and dancers (Georgepoulos et al., 1999; Rogol et al., 2000). Few studies, however, have examined the association between the overall physical activity and sexual maturation in healthy, free-living adolescents. Methodologically, it can be difficult to monitor usual daily physical activities, which are defined as any bodily movement produced by skeletal muscles that result in energy expenditure (Caspersen et al., 1984). Yet, the period and intensity of the exercise that elite athletes perform are easier to track since given training schedules and competition records. In addition, physical activity questionnaires used in population-based research are subject to self-reported bias compared to accelerometry.

Some evidence supports an association between physical activity and reproductive hormone levels, which are known to advance sexual maturation. Schmitz et al. observed an inverse association between physical activity level and estradiol and testosterone concentrations among adult breast cancer survivors (Schmitz et al., 2007). No association was found between physical activity levels and luteinizing hormone, follicle-stimulating hormone or dehydroisoandrosterone (DHEA) sulfate (Schmitz et al., 2007). Another study found that total physical activity was negatively associated with concentrations of estrogen, estradiol and androstenedione in postmenopausal women (McTiernan et al., 2006). Our work in animal models also suggested that physical activity can elicit effects on estrogen receptor alpha (*ESR1*) DNA methylation in female mouse blood, suggesting that increase activity may partially abrogate the effects of high fat diet on the aging epigenome (Kochmanski et al., 2017).

To our knowledge, no previous study has examined potential mechanisms that may explain the effect of physical activity levels on sexual maturation and reproductive hormones,

and we hypothesize that epigenetic gene regulation may mediate this pathway. Previously, we showed an association between DNA methylation, a mitotically inherited epigenetic mark, and pubertal status in free-living adolescents (Wu et al., 2018). Other reports documented an association between DNA methylation and reproductive hormones. For example, Suzuki et al. observed a significant correlation between 0N promotor region of DNA hypermethylation and the loss of estrogen receptor genes ERβ1, ERβ2, and ERβ4 mRNA expression in human epithelial ovarian carcinoma (Suzuki et al., 2008). A cross-sectional study showed current and cumulative estrogen exposure was associated with positive repetitive element DNA methylation LINE-1 and Alu in a group of healthy postmenopausal women (Boyne et al., 2017). Based on available information on the associations of 1) physical activity and sexual maturation, as well as reproductive hormones, 2) physical activity and DNA methylation, and 3) DNA methylation and sexual maturation, as well as reproductive hormones, it is plausible that DNA methylation could be a potential mediator of the associations between physical activity and hormones or sexual maturation outcomes.

To address these research gaps, we utilized data from an ongoing cohort study in Mexico City to examine 1) the association of accelerometer-based physical activity on levels of reproductive hormones and sexual maturation status among free-living, healthy adolescents, and 2) the associations of physical activity and DNA methylation of long interspersed nucleotide (LINE-1) repeats, and the genes *H19*, *HSD11B2*, and *PPARA*.

METHODS

3.1 Study Population

The study population comprised a subset of participants from the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) project, a longitudinal epidemiological

study consisting of three sequentially enrolled birth cohorts. As originally designed, ELEMENT focused primarily on lead exposure and its impact on cognitive performance, and analysis of other metals, chemicals and epigenetics have been incorporated overtime (Cantoral et al., 2015; Watkins et al., 2017). Participants were recruited at three maternity hospitals representing low- to moderate-income populations (Mexican Social Security Institute, Manuel Gea Gonzalez Hospital, and the National Institute of Perinatology) in Mexico City from 1994 to 2005. Mothers provided written consent upon enrollment in the study, and children provided assent at peri-adolescent study visits. The research protocol was approved by the Human Subjects Committee of the National Institute of Public Health of Mexico, participant hospitals, and the Internal Review Board at all participating institutions including the University of Michigan. The subjects in this project were a subset of mother-child pairs from the second and third birth cohorts (n=646 pairs at baseline). At the first study visit after the child was born, mothers provided household and demographic information, including age, education, and previous numbers of pregnancies. Their offspring were followed from birth until 4 years of age. Starting in 2015, we re-contacted a subset of the offspring for a teenage visit (n=549). Accelerometer-based physical activity levels, fasting blood, pubertal status, and anthropometry were collected at this visit.

3.2 Measurements and Outcomes

Physical Activity Levels

Participants wore the ActiGraph GT3X+ accelerometer on the non-dominant wrist fastened with a wrist strap throughout the 7-day duration of study. They were asked to wear the accelerometer each day for 24 hours; since accelerometer would have to be cut off by trained staff at the end of the study, intermittent removal of the device was unlikely.

Data were imported in ActiLife (ActiGraph LLC. 2009, Version 6.13.3) and the duration of the physical activity was estimated with the use of a fused lasso-based calculator package developed in R. Participants with at least four days of accelerometer data, including at least one weekend day, were retained for further analysis. Days with fewer than 10 hours of accelerometer activity data were removed. To estimate the duration of physical activity in different intensity categories, sleep time estimated from the participant's activity log was first removed. We then used Chandler Vector Magnitude cutoffs (Chandler et al., 2016) to categorize awake time for each day into sedentary behavior, light, moderate and vigorous activity levels. Minutes per day for each individual participant were summed as minutes of that week spent across different physical activity intensity categories. The weekly value was then divided by the number of valid days the individual wore ActiGraph, to yield as average minutes spent at that type of physical activity intensity per day.

Reproductive Hormones

Testosterone, cortisol, progesterone and androstenedione serum concentrations were measured using LC-MS/MS. Detailed materials and methods information has been previously reported (Rege et al., 2018).

DNA Methylation

Blood samples were obtained among 369 adolescents (boys: 174; girls: 195) at the teenage visit and collected in tubes with EDTA-preservative (BD Vacutainer) by trained staff following standard protocols. High-molecular-weight DNA was extracted from blood leukocytes with the Flexigene kit (Qiagen). DNA samples were treated with sodium bisulfite using kits from Zymo Research (Irvine, CA) (Li & Tollefsbol, 2011). Percent of methylated cells was then quantitatively analyzed in a differentially methylated region (DMR) of the imprinted gene, *H19*;

in the promoter of *PPARA*; the promoter region of *HSD11B2*; and a conserved sequence found in LINE-1 repetitive elements of all subfamilies (sequence: 5'- CTCGTGGTGCGCCGTTTCTTAAGCCG). DNA methylation was quantified via pyrosequencing at 2 (*PPARA*), 4 (*H19* & LINE-1), or 5 (*HSD11B2*) CpG sites (**Supplemental Table 1**). For all regions, the target sequence was amplified with HotStarTaq Master Mix (Qiagen) from approximately 50 ng bisulfite-converted DNA. Positive controls of known methylation status (0%, 25%, 50%, 75%, and 100%) and negative controls were included in all PCR plates (batches). Samples were randomized across batches. The percentage of methylated cells was quantified by a PyroMark ID Pyrosequencer (Qiagen) (Montrose et al., 2018). Pyro Q-CpG Software computes percent methylation and performs internal quality control checks (e.g., completed bisulfite conversion, signal vs. background). A random subset of samples (>10% of samples and all controls) were run in duplicate, and in this case duplicate reads were averaged.

Pubertal Outcomes

Tanner stages of breast and pubic hair growth in girls as well as Tanner stages of genitalia and pubic hair growth in boys were examined and collected by trained physicians using standard methods (Chavarro et al., 2017; Marshall & Tanner, 1970). Stages were recorded with a range from stage 1 indicating pre-puberty to stage 5 indicating full maturation (Marshall & Tanner, 1970). Physicians used orchidometers (range from 1 to 25 ml) to measure testicular volumes in boys.

Covariates

Based on *a priori* knowledge and preliminary correlation tests between predictors and potential confounders, household socioeconomic status (SES), and BMI and age of the participant all obtained at the teenage visit were included in the final simple linear regression and

linear logistic regression models. Household SES was collected using a validated questionnaire consisting of thirteen questions on housing quality, services, material goods and education of the head of household by AMAI (Asociación Mexicana de Agencias de Investigación de Mercados y Opinión Pública, version 13x6). This scale classifies households into six SES categories (A/B, C+, C, D+, D, E; with A/B being the highest category), and was validated using the results of National Survey of Household Income and Expenditure 2005, Mexico (ENIGH, Encuesta Nacional de Ingresos y Gastos de los Hogares 2004) (Jansen et al., 2017). Weight and height of the child were measured by trained nurses, following standardized protocols we have previously described [34]; BMI was calculated as weight divided by height squared (kg/m^2) (Nuttall, 2015).

3.3 Statistical Methods

We examined the distribution of Tanner stages, physical activity levels, reproductive hormone levels and demographic information among individuals who did not have DNA methylation data collected and among those who had DNA methylation collected.

We used the isotemporal substitution method to estimate the effect of replacing 30 minutes of sedentary behavior with light, moderate or vigorous intensity physical activity for the same amount of time (Melary et al., 2009). In analyses including DNA methylation data, we averaged values for all CpG sites within a region (LINE-1, *H19*, and *HSD11B2*) due to the similarity across sites. For *PPARA*, we ran separate models for the two CpG sites. The association between physical activity and reproductive hormones as well as the association between physical activity and DNA methylation were examined in simple linear regression models. The association between physical activity and Tanner stages were examined in ordinal logistic regression models. We included household SES, participant's BMI and age as

confounders in adjusted models. The analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

The analytical sample included 248 boys and 271 girls who attended the late teen visit, of whom 174 boys and 195 girls had their blood leukocyte DNA methylation quantified. The mean age was 14.5 years for both boys (SD: 2.05 years) and girls (SD: 2.14 years) at the visit. We did not observe significant differences in physical activity, Tanner stage distributions, or reproductive hormone levels between the full analytical sample and sub-sample (**Table 1**). According to unpaired t-test results, there were significant sex differences for LINE-1 and *H19* DNA methylation levels (**Table 2**).

The substitution models showed that substituting 30 minutes sedentary behavior for 30 minutes moderate physical activity per day was associated with lower testosterone levels in boys, while holding other activity types constant (Unadjusted: -30.62 ng/dL (-46.50 ng/dL, -14.74 ng/dL); Adjusted: -22.06 ng/dL (-36.99 ng/dL, -7.14 ng/dL)). Among girls, a 30-minutes increase in sedentary behavior was associated with higher progesterone concentrations (Unadjusted: 7.35 ng/dL (2.25 ng/dL, 12.46 ng/dL); Adjusted 6.58 ng/dL (1.13 ng/dL, 12.03 ng/dL)) (**Table 3**).

In terms of the associations between physical activity and DNA methylation, our results suggested that substituting 30 minutes sedentary behavior for 30 minutes vigorous physical activity per day was associated with higher percent of *HSD11B2* methylation in boys, holding other activity type constant (Adjusted: 2.47 (0.05, 4.90)). However, among girls, a 30-minutes increase in sedentary behavior was associated with lower percent of *HSD11B2* methylation (Adjusted: -0.27 (-0.54, -0.01)) (**Table 4**).

After adjusting for household SES, participant's BMI and age, no statistically significant associations were observed between different intensities of physical activity and Tanner stages in the substitution models (**Table 5**).

DISCUSSION

In this cross-sectional analysis of a Mexico City cohort, we observed associations between physical activity levels and reproductive hormones, but not with Tanner stages of sexual maturation. We found a statistically significant, inverse association between moderate physical activity and testosterone in boys, and there was a statistically significant positive association between sedentary behavior and progesterone concentrations in girls. Increased minutes spent in vigorous physical activity was associated with higher percent DNA methylation of *HSD11B2* in boys, while increased minutes spent in sedentary behavior was associated with lower percent DNA methylation of *HSD11B2* in girls. In preliminary analyses, we found no evidence for DNA methylation as a mediator of the associations between physical activity and reproductive hormones or sexual maturation in our study.

Previous studies have offered potential explanations of the effect of physical activity on reproductive hormones by elucidating the synergy between physical activity and adiposity in their association with reproductive hormones (McTiernan et al., 2006). Among postmenopausal women, subjects who were the heaviest and the most sedentary had the lowest levels of sex hormone-biding globulin (SHGB), while subjects who had the lowest BMI and highest physical activity had the highest levels of SHGB. In addition, there was an inverse correlation between progesterone concentration and SHBG (Dalton, 1984). Our results, similarly, suggested that grater duration of sedentary behavior were associated with higher progesterone and lower levels SHBG. Healthy male participants are usually underrepresented in studies of health behaviors and

reproductive hormones. We, however, found some evidence to support the inverse association between physical activity and testosterone levels among boys from our cohort. Physical activity might lower testosterone levels by decreasing adiposity, or possibly by increasing SHBG levels (and decreasing the bioavailability of testosterone) given decreased blood insulin levels (Kaaks, 1996; Kaaks, 2010; Pugeat et al., 1991; Lynch et al., 2011).

Sexual maturation is advanced by changes in levels of the reproductive hormones (Sisk & Zehr, 2005; Forbes & Dahl, 2010). Based on the significant results between physical activity and reproductive hormones, it is plausible to deduce that physical activity may have an impact on sexual maturation. However, despite the biological plausibility, we did not observe any evidence supporting an association between physical activity and Tanner stages, after adjusting for household SES, age and BMI. Other than the limitation of being a cross-sectional study, two possible explanations are 1) Tanner stages in this study was collected at the teenage visit, at which most participants had reached advanced stages in pubertal development; and 2) Moderate physical activity daily might be associated with subclinical physiological changes, e.g. reproductive hormone and DNA methylation modifications, but it might not be intense enough to induce actual changes in Tanner stages. Future research should incorporate a longitudinal study design and larger sample size with more frequent measures to closely monitor physiological changes of sexual maturation stages.

Previous studies have examined the association between daily physical activity and DNA methylation levels among various population groups (van Roekel et al., 2018; King-Himmelreich et al., 2016; Barrès R et al., 2012), but not in healthy, free-living adolescents specifically. Barrès et al. observed that genomic DNA methylation quantified by LUminometric Methylation Assay (LUMA) decreased in skeletal muscle biopsies obtained from 14 healthy

sedentary men and women after acute exercise. They also found that exercise could induce a dose-dependent expression of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*PGC-1α*), pyruvate dehydrogenase kinase 4 (*PDK4*) and peroxisome proliferator-activated receptor delta (*PPAR-σ*) (Barrès R et al., 2012). Another study among 106 patients examined physical activity and DNA methylation at 6 tumor-related genes, including homeobox transcription factor (*CDX2*), bone morphogenetic protein 2 (*BMP-2*), *p16* (*INK4A*), calcium channel-related (*CACNA2D3*), *GATA-5* transcription factor and estrogen receptor (*ER*) among gastric cancer patients. These authors found that more physical activity was correlated with a lower methylation frequency of *CACNA2D3* (Yuasa et al., 2009). Zhang et al. showed that among 161 cancer-free participants aged 45-75 years, individuals with physical activity 26-30 min/day had a significantly higher levels of LINE-1 DNA methylation from peripheral blood compared to those with physical activity ≤ 10 min/day. However, the association was attenuated and became statistically insignificant after adjusting for age, gender, race/ethnicity, education, body composition, cigarette smoking, alcohol drinking and dietary folate intake (Zhang et al., 2011). Similarly, we did not observe any effect of physical activity on LINE-1 DNA methylation in our healthy adolescent cohort after adjustment. Our results did suggest a statistically significant association between vigorous physical activity and *HSD11B2* DNA methylation levels, adjusted for confounders. The results are biologically coherent since *HSD11B* genes regulate many metabolic processes, assist in regenerating active glucocorticoids from circulating inert 11-keto forms in specific tissues and catalyze the interconversion of cortisol and corticosterone (Kotelevtsev et al., 1997). Given that intense exercise tends to increase stress- and immunity-related outcomes (Cieslak et al., 2003), we hypothesize that *HSD11B2* DNA

methylation might be also involved in stress or insulin metabolism pathways. However, future longitudinal studies are needed to validate the hypothesis.

This analysis has some limitations. First, as a cross-sectional study, predictors and outcomes of interest were simultaneously collected and assessed, thus we cannot assess temporality of the associations. Secondly, when measuring energy expenditure, the hip or waist is the most common site to wear an accelerometer (Murphy, 2009). Since accelerometers record acceleration in different axes or planes of movement, a monitor worn on the wrist could introduce "noise" into collected data, for example, moving hands when speaking, whereas a waist device is less sensitive to such movement. Thirdly, since the epigenetic programming varies by genomic loci and by cell and tissue type, we need to consider the limitation of quantifying DNA methylation in blood leukocytes as the sole indicator, though blood is a biologically available surrogate tissue. If possible, future studies should consider including other bio-indicators that may be able to capture minor changes, for instance, liver or muscle tissue, to further examine the mechanisms. Fourthly, we cannot exclude the possibility that BMI (or adiposity) is a mediator, instead of a confounder in the associations between physical activity and hormones or sexual maturation status.

We believe that this is the first study that shows the associations between accelerometer-measured daily physical activity and reproductive hormones, as well as its association with pubertal development among healthy, free-living adolescents. We also examined whether the physiological molecular marker, DNA methylation, was associated with physical activity. Our findings suggested that moderate and vigorous physical activity are associated with reproductive hormones and DNA methylation levels. Future studies should

consider examination of these questions using a longitudinal study design with repeated measures at multiple time points.

Bibliography

Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, Caidahl K, Krook A, O'Gorman DJ, Zierath JR. Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab*. 2012. doi:10.1016/j.cmet.

Biddle SJH, Gorely T, Stensel DJ. Health-enhancing physical activity and sedentary behaviour in children and adolescents. *J Sports Sci*. 2004. doi:10.1080/02640410410001712412.

Boyne DJ, Friedenreich CM, McIntyre JB, Stanczyk FZ, Courneya KS, King WD. Endogenous sex hormone exposure and repetitive element DNA methylation in healthy postmenopausal women. *Cancer Causes Control*. 2017. doi:10.1007/s10552-017-0958-z.

Cantoral A, Téllez-Rojo MM, Levy TS, Hernández-Ávila M, Schnaas L, Hu H, Peterson KE, Ettinger AS. Differential association of lead on length by zinc status in two-year old Mexican children. *Environ Health*. 2015. doi:10.1186/s12940-015-0086-8.

Caspersen CJ, Powell KE, Christenson GM. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. *Public Health Rep*. 1984. doi:10.2307/20056429.

Cesario SK, Hughes L a. Precocious puberty: a comprehensive review of literature. *J Obstet Gynecol Neonatal Nurs*. 2007. doi:10.1111/j.1552-6909.2007.00145.x.

Chandler JL, Brazendale K, Beets MW, Mealing BA. Classification of physical activity intensities using a wrist-worn accelerometer in 8-12-year-old children. *Pediatr Obes*. 2016. doi:10.1111/ijpo.12033.

Chavarro JE, Watkins DJ, Afeiche MC, Zhang Z, Sánchez BN, Cantonwine D, Mercado-García A, Blank-Goldenberg C, Meeker JD, Téllez-Rojo MM, et al. Validity of Self-Assessed Sexual Maturation Against Physician Assessments and Hormone Levels. *J Pediatr*. 2017. doi:10.1016/j.jpeds.

Cieslak TJ, Frost G, Klentrou P. Effects of physical activity, body fat, and salivary cortisol on mucosal immunity in children. *J Appl Physiol*. 2003. doi:10.1152/japplphysiol.00400.

Dalton ME. The effect of progesterone administration on sex hormone binding globulin binding capacity in women with severe premenstrual syndrome. *J Steroid Biochem*. 1984. doi:10.1016/0022-4731(84)90249-8.

Forbes EE, Dahl RE. Pubertal development and behavior: Hormonal activation of social and motivational tendencies. *Brain Cogn*. 2010. doi:10.1016/j.bandc.

Georgopoulos N, Markou K, Theodoropoulou A, Paraskevopoulou P, Varaki L, Kazantzi Z, Leglise M, Vagenakis AG. Growth and pubertal development in elite female rhythmic gymnasts. *J Clin Endocrinol Metab*. 1999. doi:10.1210/jcem.84.12.6177.

Golub MS, Collman GW, Foster PMD, Kimmel CA, Rajpert-De Meyts E, Reiter EO, Sharpe RM, Skakkebaek NE, Toppari J. Public health implications of altered puberty timing. *Pediatrics*. 2008. doi:10.1542/peds.

Heindel JJ, Vandenberg LN. Developmental origins of health and disease: A paradigm for understanding disease cause and prevention. *Curr Opin Pediatr*. 2015. doi:10.1097/MOP.0000000000000191.

Jacobsen BK, Heuch I, Kvåle G. Association of low age at menarche with increased all-cause mortality: A 37-year follow-up of 61,319 Norwegian women. *Am J Epidemiol*. 2007. doi:10.1093/aje/kwm237.

Jansen EC, Marin C, Mora-Plazas M, Villamor E. Higher childhood red meat intake frequency is associated with earlier age at menarche. *J Nutr*. 2016. doi:10.3945/jn.115.226456.

Jansen EC, Miller AL, Lumeng JC, Kaciroti N, Brophy Herb HE, Horodynski MA, Contreras D, Peterson KE. Externalizing behavior is prospectively associated with intake of added sugar and sodium among low socioeconomic status preschoolers in a sex-specific manner. *Int J Behav Nutr Phys Act*. 2017. doi:10.1186/s12966-017-0591-y.

Janssen I. Physical activity guidelines for children and youth. *Canadian Journal of Public Health / Revue Canadienne de Santé Publique*. 2007. doi:10.2139/ssrn.2308560.

Kaaks R. Nutrition, hormones, and breast cancer: Is insulin the missing link? *Cancer Causes Control*. 1996. doi:10.1007/BF00051703.

Kaaks R. Nutrition, physical activity and cancer risks: The role of insulin and insulin-like growth factor-1. *Endocr Abstr*. 2010.

King-Himmelreich TS, Schramm S, Wolters MC, Schmetzer J, Möser C V., Knothe C, Resch E, Peil J, Geisslinger G, Niederberger E. The impact of endurance exercise on global and AMPK gene-specific DNA methylation. *Biochem Biophys Res Commun*. 2016. doi:10.1016/j.bbrc.2016.04.078.

Kochmanski J, Marchlewicz EH, Savidge M, Montrose L, Faulk C, Dolinoy DC. Longitudinal effects of developmental bisphenol A and variable diet exposures on epigenetic drift in mice. *Reprod Toxicol*. 2017. doi:10.1016/j.reprotox.

Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CRW, Seckl JR, et al. 11 -Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci*. 1997. doi:10.1073/pnas.94.26.14924.

Li Y, Tollefsbol TO. DNA methylation detection: Bisulfite genomic sequencing analysis. *Methods Mol Biol*. 2011. doi:10.1007/978-1-61779-316-5_2.

Lynch BM, Neilson HK, Friedenreich CM. Physical activity and breast cancer prevention. *Recent Results Cancer Res*. 2011. doi:10.1007/978-3-642-04231-7_2.

Marceau K, Dorn LD, Susman EJ. Stress and puberty-related hormone reactivity, negative emotionality, and parent-adolescent relationships. *Psychoneuroendocrinology*. 2012. doi:10.1016/j.psyneuen.

Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. *Arch Dis Child*. 1970. doi:10.1136/adc.45.239.13.

McTiernan A, Wu LL, Chen C, Chlebowski R, Mossavar-Rahmani Y, Modugno F, Perri MG, Stanczyk FZ, Van Horn L, Wang CY. Relation of BMI and physical activity to sex hormones in postmenopausal women. *Obesity*. 2006. doi:10.1038/oby.

Mekary RA, Willett WC, Hu FB, Ding EL. Isotemporal substitution paradigm for physical activity epidemiology and weight change. *Am J Epidemiol*. 2009. doi:10.1093/aje/kwp163.

Mills JL, Shiono PH, Shapiro LR, Crawford PB, Rhoads GG. Early growth predicts timing of puberty in boys: Results of a 14-year nutrition and growth study. *J Pediatr*. 1986. doi:10.1016/S0022-3476(86)80141-x.

Montrose L, Padmanabhan V, Goodrich JM, Domino SE, Treadwell MC, Meeker JD, Watkins DJ, Dolinoy DC. Maternal levels of endocrine disrupting chemicals in the first trimester of pregnancy are associated with infant cord blood DNA methylation. *Epigenetics*. 2018. doi:10.1080/15592294.2018.1448680.

Murphy SL. Review of physical activity measurement using accelerometers in older adults: Considerations for research design and conduct. *Prev Med*. 2009. doi:10.1016/j.ypmed.2008.12.001.

Nelson MC, Neumark-Stzainer D, Hannan PJ, Sirard JR, Story M. Longitudinal and secular trends in physical activity and sedentary behavior during adolescence. *Pediatrics*. 2006. doi:10.1542/peds.

Niculescu D. Puberty: Ontogeny, Neuroendocrinology, Physiology, and Disorders. In: Williams Textbook of Endocrinology. ; 2008. doi:10.4183/aeb.

Nuttall F. Body mass index: Obesity, BMI, and health: A critical review. *Nutr Today*. 2015. doi:10.1097/NT.0000000000000092.

Pugeat M, Crave JC, Elmidani M, Nicolas MH, Garoscio-Cholet M, Lejeune H, Déchaud H, Tourniaire J. Pathophysiology of sex hormone binding globulin (SHBG): Relation to insulin. *J Steroid Biochem Mol Biol*. 1991. doi:10.1016/0960-0760(91)90310-2.

Rege J, Turcu AF, Kasa-Vubu JZ, Lerario AM, Auchus GC, Auchus RJ, Smith JM, White PC, Rainey WE. 11-Ketotestosterone is the dominant circulating bioactive androgen during normal and premature adrenarche. *J Clin Endocrinol Metab*. 2018. doi:10.1210/jc.2018-00736.

Rogol AD, Clark PA, Roemmich JN. Growth and pubertal development in children and adolescents: Effects of diet and physical activity. In: *American Journal of Clinical Nutrition*. ; 2000. doi:10.1093/ajcn/72.2.521S.

Sata F. "Development Origins of Health and Disease (DOHaD) Cohorts and Interventions: Status and Perspective". In: Pre-emptive Medicine: Public Health Aspects of Developmental Origins of Health and Disease. Ed. Sata F, Fukuoka H, Hanson M. Springer, 2019. 53-70.

Schmitz KH, Lin H, Sammel MD, Gracia CR, Nelson DB, Kapoor S, DeBlasis TL, Freeman EW. Association of physical activity with reproductive hormones: The Penn ovarian aging study. *Cancer Epidemiol Biomarkers Prev*. 2007. doi:10.1158/1055-9965.EPI-07-0061.

Sisk CL, Zehr JL. Pubertal hormones organize the adolescent brain and behavior. *Front Neuroendocrinol*. 2005. doi:10.1016/j.yfrne.

Suzuki F, Akahira JI, Miura I, Suzuki T, Ito K, Hayashi SI, Sasano H, Yaegashi N. Loss of estrogen receptor β isoform expression and its correlation with aberrant DNA methylation of the 5′-untranslated region in human epithelial ovarian carcinoma. *Cancer Sci*. 2008. doi:10.1111/j.1349-7006.

Tolfrey K, Jones AM, Campbell IG. The effect of aerobic exercise training on the lipid-lipoprotein profile of children and adolescents. *Sport Med*. 2000. doi:10.2165/00007256-200029020-00003.

Twisk JWR. Physical activity guidelines for children and adolescents a critical review. *Sport Med*. 2001. doi:10.2165/00007256-200131080-00006.

van Roekel EH, Dugué P-A, Jung C-H, Joo JE, Makalic E, Wong EM, English DR, Southey MC, Giles GG, Lynch BM, et al. Physical activity, television viewing time, and DNA methylation in peripheral blood. *Med Sci Sport Exerc*. 2018. doi:10.1249/MSS.0000000000001827.

Watkins DJ, Sánchez BN, Téllez-Rojo MM, Lee JM, Mercado-García A, Blank-Goldenberg C, Peterson KE, Meeker JD. Impact of phthalate and BPA exposure during in utero windows of susceptibility on reproductive hormones and sexual maturation in peripubertal males. *Environ Health*. 2017. doi:10.1186/s12940-017-0278-5.

Wu Y, Peterson KE, Sánchez BN, Dolinoy DC, Mercado-Garcia A, Téllez-Rojo MM, Goodrich JM (in press). Association of blood leukocyte DNA methylation at LINE-1 and growth-related candidate genes with pubertal onset and progression. *Epigenetics*. 2018. doi: 10.1080/15592294.2018.1556198.

Yuasa Y, Nagasaki H, Akiyama Y, Hashimoto Y, Takizawa T, Kojima K, Kawano T, Sugihara K, Imai K, Nakachi K. DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients. *Int J Cancer*. 2009. doi:10.1002/ijc.24231.

Zhang FF, Cardarelli R, Carroll J, Zhang S, Fulda KG, Gonzalez K, Vishwanatha JK, Morabia A, Santella RM. Physical activity and global genomic DNA methylation in a cancer-free population. *Epigenetics*. 2011. doi:10.4161/epi.6.3.14378.

Zhu J, Chan Y-M. Adult consequences of self-limited delayed puberty. *Pediatrics*. 2017. doi:10.1542/peds.

Table 4.1: Distributions of Tanner stages and other covariates among ELEMENT children at the teenage visit, without and with DNA methylation data.

Boys:	**Sample without DNA Methylation Collected**		**Sample with DNA Methylation Collected**	
Pubic Hair: Tanner Stage	**N= 248**	**%**	**N =174**	**%**
1	45	18.15	37	21.26
2	32	12.90	25	14.37
3	59	23.79	43	24.71
4	52	20.97	36	20.69
5	48	19.35	26	14.94
Missing	12	4.84	7	4.02
Genital Development: Tanner Stage				
1	12	4.84	9	5.17
2	32	12.90	25	14.37
3	44	17.74	32	18.39
4	97	39.11	70	40.23
5	51	20.56	31	17.82
Missing	12	4.84	7	4.02
Testicular Development (L)				
1-3 ml	0	0	0	0
3-11 ml	23	9.27	19	10.92
>11 ml	213	85.89	148	85.06
Missing	12	4.84	7	4.02
Testicular Development (R)				
1-3 ml	1	0.40	1	0.57
3-11 ml	24	9.68	19	10.92
>11 ml	210	84.68	147	84.48
Missing	13	5.24	7	4.02
Accelerometer-based Physical Activity Levels (min/day)	**N=248**		**N=174**	
Sedentary (< 1.1 METs) [1]	596.68 ± 76.98		594.13 ± 74.57	

Light (1.1 - 3.0 METs)	252.56 ± 47.11	252.81 ± 46.89
Moderate (< 3.0 - 6.0 METs)	71.44 ± 26.24	73.03 ± 26.94
Vigorous (> 6.0 METs)	5.19 ± 5.49	5.74 ± 5.81
Age (years)	14.51 ± 2.05	14.22 ± 1.99
BMI	21.17 ± 3.89	20.95 ± 3.88
Household SES: Quartile		
1	58	23.39
2	56	22.58
3	70	28.23
4	62	25.00
Missing	2	0.81
Reproductive Hormone Levels (ng/dl)	**N=186**	**N=158**
Testosterone	2789.46 ± 1961.95	2783.79 ± 2004.60
Cortisol	97124.73 ± 46750.60	98549.07 ± 46334.16
Progesterone	171.77 ± 1249.76	190.16 ± 1355.60
Androstenedione	561.66 ± 290.93	574.58 ± 300.26

Girls:	**Sample without DNA Methylation Collected**		**Sample with DNA Methylation Collected**	
Pubic Hair: Tanner Stage	**N=271**	**%**	**N=195**	**%**
1	18	6.64	15	7.69
2	62	22.88	56	28.72
3	61	22.51	43	22.05
4	69	25.46	50	25.64
5	53	19.56	26	13.33
Missing	8	2.95	5	2.56
Breast Development: Tanner Stage				
1	11	4.06	9	4.62
2	26	9.59	23	11.79
3	66	24.35	57	29.23

4	97	35.79	64	32.82
5	63	23.25	37	18.97
Missing	8	2.95	5	2.56
Menarche				
Yes	227	83.76	157	80.51
No	41	15.13	36	18.46
Missing	3	1.11	2	1.03
Accelerometer-based Physical Activity Levels (min/day)	**N= 271**		**N=195**	
Sedentary (< 1.1 METs)	582.17 ± 73.96		576.82 ± 70.78	
Light (1.1 – 3.0 METs)	255.80 ± 39.60		258.51 ± 39.42	
Moderate (< 3.0 – 6.0 METs)	77.85 ± 25.00		79.32 ± 25.47	
Vigorous (> 6.0 METs)	2.56 ± 3.02		2.78 ± 3.12	
Age (years)	14.47 ± 2.14		13.95 ± 2.04	
BMI	22.07 ± 4.39		21.82 ± 4.30	
Household SES: Quartile				
1	78	28.78		
2	69	25.46		
3	59	21.77		
4	64	23.62		
Missing	1	0.37		
Reproductive Hormone Levels (ng/dl)	**N= 193**		**N= 173**	
Testosterone	425.77 ± 877.99		438.87 ± 917.32	
Cortisol	95677.62 ± 57032.18		93700.95 ± 54898.50	
Progesterone	794.21 ± 1886.37		803.62 ± 1960.11	
Androstenedione	900.01 ± 447.08		889.68 ± 436.13	

[1] **METs are metabolic equivalent. One MET is defined as the energy it takes to sit quietly.**

Table 4.2: Percent DNA methylation at LINE-1, as well as *H19*, *HSD11B2* and *PPARA* among all individuals and stratified by sex[1].

	Entire Cohort		Boys		Girls		*P* value[2]
	N	Mean % methylation (SD)	N	Mean % methylation (SD)	N	Mean % methylation (SD)	
LINE-1 methylation							
Site 1	330	72.83 (6.19)	166	73.47 (6.15)	164	72.17 (6.19)	0.051
Site 2	329	75.09 (3.73)	166	75.69 (3.37)	163	74.49 (3.99)	**0.003**
Site 3	329	71.24 (5.95)	166	71.87 (5.26)	163	70.61 (6.54)	0.050
Site 4	322	81.56 (4.39)	162	81.85 (4.32)	160	81.27 (4.45)	0.220
Average value across 4 sites	319	75.17 (4.41)	161	75.71 (4.17)	159	74.62 (4.59)	**0.029**
***H19* methylation**							
Site 1	346	50.10 (4.51)	167	50.79 (4.89)	179	49.45 (4.04)	**0.004**
Site 2	344	48.87 (3.47)	167	49.09 (3.82)	177	48.65 (3.10)	0.276
Site 3	345	47.90 (3.92)	167	48.19 (4.17)	178	47.62 (3.66)	0.137
Site 4	343	50.05 (3.27)	166	50.36 (3.67)	177	49.76 (2.82)	0.099
Average value across 4 sites	341	49.24 (3.17)	165	49.64 (3.46)	176	48.87 (2.84)	**0.025**
***HSD11B2* methylation**							
Site 1	291	3.58 (4.98)	141	3.67 (5.06)	150	3.50 (4.92)	0.778
Site 2	290	1.34 (2.70)	140	1.44 (2.77)	150	1.25 (2.65)	0.548
Site 3	285	2.21 (4.40)	138	2.45 (4.71)	147	1.99 (4.10)	0.376
Site 4	282	0.69 (1.53)	137	0.80 (1.51)	145	0.59 (1.56)	0.264
Site 5	274	3.02 (4.06)	132	2.86 (2.82)	142	3.16 (4.94)	0.545
Average value across at least 4 sites	251	1.95 (2.25)	129	2.04 (2.03)	145	1.87 (2.44)	0.540
***PPAR-alpha* methylation**							
Site 1	362	3.92 (1.80)	173	4.04 (1.74)	189	3.81 (1.85)	0.228
Site 2	355	17.64 (2.61)	169	17.47 (2.38)	186	17.79 (2.81)	0.246

Table 4.3: Isotemporal substitution of sedentary activities, per 30-minute/day increase, and selected reproductive hormones among Mexican boys and girls [1,2].

Boys (N=180)	**Testosterone (ng/dl)**		**Cortisol (ng/dl)**	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	0.38	5.21	-23.32	21.50
	(-8.45, 7.69)	(-2.48, 12.90)	(-231.87, 185.23)	(-199.20, 242.21)
Moderate PA	**-30.62**	**-22.06**	94.62	187.08
	(-46.50, -14.74)	**(-36.99, -7.14)**	(-315.96, 505.19)	(-241.32, 615.48)
Vigorous PA	21.56	18.85	-829.38	-661.76
	(-36.06, 79.18)	(-34.36, 72.06)	(-2318.92, 660.15	(-2189.18, 865.66)
Total PA	2.02	0.14	-13.80	-42.54
	(-3.69, 7.72)	(-5.09, 5.37)	(-161.41, 133.80)	(-192.78, 107.69)
BMI		**-80.72**		368.30
		(-145.69, -15.76)		(-1496.38, 2232.99)
SES		-25.53		-119.22
		(-232.45, 181.39		(-6058.65, 5820.21)
Age		**484.61**		**6393.84**
		(339.78, 629.44)		**(2236.60, 10551.07)**
	Progesterone (ng/dl)		**Androstenedione (ng/dl)**	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	-1.36	-2.03	-0.69	0.05
	(-6.95, 4.22)	(-8.14, 4.09)	(-1.96, 0.58)	(-1.08, 1.18)
Moderate PA	2.38	1.66	-1.13	0.69
	(-8.62, 13.38)	(-10.21, 13.53)	(-3.63, 1.37)	(-1.49, 2.87)
Vigorous PA	-1.26	-3.16	-2.73	-0.63
	(-41.17, 38.65)	(-45.48, 39.16)	(-11.87, 6.40)	(-8.44, 7.19)
Total PA	-0.50	-0.11	0.53	0.23
	(-4.46, 3.45)	(-4.27, 4.06)	(-0.37, 1.43)	(-0.53, 0.99)
BMI		-7.74		-1.06
		(-59.40, 43.93)		(-10.54, 8.41)
SES		-33.51		-14.37
		(-198.07, 131.05)		(-44.65, 15.91)
Age		-56.74		**92.65**

	(-171.93, 58.44)	**(71.50, 113.80)**

Table 4.3 (Continued)

Girls (N=187)	Testosterone (ng/dl)		Cortisol (ng/dl)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	-1.21	-1.05	-159.40	-35.04
	(-5.52, 3.10)	(-5.42, 3.32)	(-436.69, 117.89)	(-317.72, 247.64)
Moderate PA	-0.02	-1.05	395.29	316.49
	(-7.07, 7.03)	(-7.90, 5.79)	(-45.72, 836.30)	(-114.31, 747.30)
Vigorous PA	-2.46	20.22	-1330.97	484.80
	(-52.01, 47.08)	(-29.25, 69.70)	(-4471.65, 1809.70	(-2668.03, 3637.63)
Total PA	-1.87	-2.14	105.55	81.48
	(-4.32, 0.59)	(-4.64, 0.35)	(-50.72, 261.83)	(-77.42, 240.37)
BMI		12.15		202.00
		(-18.01, 42.30)		(-1720.93, 2124.92)
SES		-63.99		-468.93
		(-166.10, 38.12)		(-6924.85, 5986.99)
Age		60.04		**7322.74**
		(-13.09, 133.17)		**(2755.29, 11890.18)**
	Progesterone (ng/dl)		**Androstenedione (ng/dl)**	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	-6.94	-3.03	-0.72	0.45
	(-15.99, 2.12)	(-12.72, 6.66)	(-2.91, 1.46)	(-1.78, 2.68)
Moderate PA	4.74	0.92	1.34	0.17
	(-9.67, 19.14)	(-13.85, 15.68)	(-2.14, 4.81)	(-3.23, 3.57)
Vigorous PA	-23.07	11.23	-3.96	12.61
	(-125.65, 79.50)	(-96.84, 119.30)	(-28.72, 20.80)	(-12.28, 37.50)
Total PA	**7.35**	**6.58**	0.87	0.47
	(2.25, 12.46)	**(1.13, 12.03)**	(-0.36, 2.11)	(-0.78, 1.73)
BMI		-3.71		4.10
		(-69.62, 62.20)		(-11.08, 19.28)
SES		114.36		-4.67
		(-106.92, 335.65)		(-55.64, 46.29)
Age		**171.41**		**77.08**
		(14.86, 327.97)		**(41.03, 113.14)**

[1] **Bolded value indicates the association is significant with a *P* value < 0.05.**

[2] **All models were adjusted for late-teen age, BMI and household SES.**

Table 4.4: Isotemporal substitution of activities, per 30-minute/day increase, and percent DNA methylation (%)[1, 2,3].

Boys:				**LINE-1 (N=154)**					**_H19_ (N=156)**	
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**	**Sedentary**	**Light**	**Moderate**	**Vigorous**	**Total**
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**	**β_0 (95% CI)**	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**
Dropped	-0.19	0.73	-0.77	0.27	Dropped	-0.27	0.37	-1.83	0.25
	(-0.82, 0.44)	(-0.53, 1.99)	(-5.28, 3.74)	(-0.22, 0.76)		(-0.86, 0.31)	(-0.69, 1.44)	(-5.74, 2.07)	(-0.15, 0.64)
			HSD11B2 **(N=129)**					***PPAR-alpha*** **Site 1 (N=160)**	
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**	**Sedentary**	**Light**	**Moderate**	**Vigorous**	**Total**
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**	**β_0 (95% CI)**	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**
Dropped	0.12	-0.37	**2.47**	0.25	Dropped	0.14	-0.48	1.68	0.03
	(-0.28, 0.52)	(-1.07, 0.33)	**(0.05, 4.90)**	(-0.02, 0.52)		(-0.13, 0.41)	(-1.01, 0.04)	(-0.20, 3.56)	(-0.15, 0.22)
			PPAR-alpha **Site 2 (N=157)**						
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**					
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**					
Dropped	0.12	-0.17	1.21	0.01					
	(-0.26, 0.51)	(-0.92, 0.57)	(-1.55, 3.96)	(-0.25, 0.27)					
Girls:				**LINE-1 (N=159)**					**_H19_ (N=174)**	
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**	**Sedentary**	**Light**	**Moderate**	**Vigorous**	**Total**
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**	**β_0 (95% CI)**	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**
Dropped	-0.57	0.12	0.16	0.04	Dropped	0.07	0.18	-3.15	-0.20
	(-1.39, 0.24)	(-1.19, 1.42)	(-9.15, 9.48)	(-0.41, 0.48)		(-0.38, 0.52)	(-0.54, 0.91)	(-8.40, 2.10)	(-0.46, 0.07)
			HSD11B2 **(N=145)**					***PPAR-alpha*** **Site 1 (N=184)**	
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**	**Sedentary**	**Light**	**Moderate**	**Vigorous**	**Total**
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**	**β_0 (95% CI)**	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**
Dropped	0.25	-0.25	-1.24	**-0.27**	Dropped	-0.04	0.10	0.43	-0.02
	(-0.24, 0.75)	(-1.00, 0.51)	(-8.10, 5.62)	**(-0.54, -0.01)**		(-0.33, 0.25)	(-0.35, 0.55)	(-3.00, 3.86)	(-0.18, 0.15)
			PPAR-alpha **Site 2 (N=181)**						
Sedentary	**Light**	**Moderate**	**Vigorous**	**Total**					
β_0 (95% CI)	**β_1 (95% CI)**	**β_2 (95% CI)**	**β_3 (95% CI)**	**β_4 (95% CI)**					
Dropped	-0.03	0.27	2.71	0.17					
	(-0.46, 0.40)	(-0.39, 0.93)	(-2.34, 7.76)	(-0.08, 0.42)					

[1] Bolded value indicates the association is significant with a *P* value < 0.05.

[2] All models were adjusted for late-teen age, BMI, and household SES.

[3]Sedentary physical activities were dropped because they were used as the reference group in all models.

Table 4.5: Isotemporal substitution of sedentary activities, per 30-minute/day increase, and odds of pubertal maturation stages[1].

Boys (N=224)	**Pubic Hair**		**Genital Development**	
	Odds Ratio (CI)		Odds Ratio (CI)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	0.79 (0.59, 1.07)	0.82 (0.57, 1.17)	0.75 (0.55, 1.01)	0.90 (0.63, 1.28)
Moderate PA	**0.47 (0.26, 0.86)**	0.65 (0.33, 1.27)	0.58 (0.32, 1.06)	0.82 (0.42, 1.59)
Vigorous PA	0.76 (0.08, 7.63)	1.06 (0.10, 10.90)	0.94 (0.11, 8.44)	0.69 (0.07, 6.85)
Total PA	**1.29 (1.01, 1.65)**	1.08 (0.83, 1.40)	1.17 (0.92, 1.48)	0.93 (0.71, 1.21)
BMI		1.09 (0.98, 1.21)		0.99 (0.89, 1.10)
SES		1.10 (0.80, 1.52)		1.40 (1.00, 1.96)
Age		**3.27 (2.43, 4.14)**		**2.50 (1.88, 3.32)**
	Testicular Volume (L)		**Testicular Volume (R)**	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	0.82 (0.56, 1.19)	0.93 (0.58, 1.49)	0.82 (0.56, 1.20)	0.95 (0.59, 1.52)
Moderate PA	0.65 (0.32, 1.28)	0.89 (0.38, 2.07)	0.53 (0.26, 1.05)	0.70 (0.31, 1.61)
Vigorous PA	0.38 (0.03, 5.27)	0.38 (0.01, 9.69)	0.66 (0.05, 9.18)	0.67 (0.03, 17.33)
Total PA	1.30 (0.97, 1.73)	1.07 (0.76, 1.49)	1.15 (0.87, 1.52)	0.92 (0.65, 1.30)
BMI		1.08 (0.94, 1.24)		1.07 (0.93, 1.22)
SES		1.14 (0.74, 1.76)		1.14 (0.74, 1.76)
Age		**1.91 (1.40, 2.61)**		**1.96 (1.43, 2.69)**

Girls (N=271)	Pubic Hair		Breast Development	
	Odds Ratio (CI)		Odds Ratio (CI)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
Light PA	**0.65 (0.46, 0.91)**	1.15 (0.79, 1.68)	**0.63 (0.45, 0.90)**	1.16 (0.78, 1.72)
Moderate PA	**1.77 (1.03, 3.04)**	0.91 (0.51, 1.62)	**1.78 (1.04, 3.06)**	0.98 (0.54, 1.76)
Vigorous PA	**0.00 (0.00, 0.02)**	0.11 (0.00, 15.29)	**0.00 (0.00, 0.01)**	0.03 (0.00, 3.54)
Total PA	**1.34 (1.12, 1.60)**	1.09 (0.90, 1.34)	**1.33 (1.11, 1.60)**	1.09 (0.89, 1.34)
BMI		1.05 (0.96, 1.14)		1.07 (0.98, 1.17)
SES		1.16 (0.89, 1.50)		1.24 (0.95, 1.63)
Age		**2.57 (1.99, 3.32)**		**2.82 (2.11, 3.77)**

[1] **Bolded value indicates the association is significant with a *P* value < 0.05.**

[2] **All models were adjusted for late-teen age, BMI, and household SES.**

Table 4.6: Primer sequences for pyrosequencing assays and genomic location of CpG sites assessed.

Gene or Element Name	# of CpG Sites Assessed	Loci of CpG Sites‡	DNA Methylation Analysis Method	Primer Sequences			Locus of Amplified Region
				Forward	Reverse†	Sequencing	
LINE-1	4	various*	pyrosequencing	TTGAGTTAGGTGTGGGATATAGTT	CAAAAAATCAAAAAATTCCCTTTCC	AGGTGTGGATATAGT	various*
H19	4	chr11: 2003031, 2003029, 2003027, and 2003024	pyrosequencing	TTTGTTGATTTTATTAAGGGAG	CTATAAATAAACCCCAACCAAAC	GTGTGGAATTAGAAGT	chr11: 2002966-2003111
PPARA	2	chr22: 46149160 and 46149179	pyrosequencing	GGAGGTTTTTATGAGGATGTAGTT	ACACATATTAACCAACAATAACTATCAT	GGATGTGGTTGTTTG	chr22: 46149046-46149244
HSD11B2	5	chr16: 67430541, 67430543, 67430562, 67430564, and 67430580	pyrosequencing	TTAAGTTTTGGAAGGAAAGGGAAAGA	ACATCCCCATACCCTTTACTAATC	AGTTTTTGTTTTAGGTAGG	chr16: 67430512-67430745

‡Loci are based off of genome build GRCh38/hg38

†All reverse primers for pyrosequencing are 5'biotinylated.

*A sequence in the promoter region of nearly all LINE-1s (located throughout the genome) is amplified and sequenced here. The specific sequence, with CpG sites in bold, is as follows: 5'-CT**CG**TGGTG**CG**C**CG**TTTCTTAAGC**CG**

Chapter 5 Conclusion

MAIN FINDINGS

This dissertation, to our knowledge, is the first population-based study demonstrating the associations of DNA methylation of repetitive element LINE-1, and candidate genes *H19*, *HSD11B2*, *IGF2* and *PPARA* with pubertal onset and progression among healthy, free-living Mexican adolescents. Findings of this dissertation also suggest methyl donor nutrients may modify the associations between DNA methylation and pubertal status. In addition, results suggest that physical activities are related to DNA methylation and reproductive hormone regulation. In general, directions and strengths of all these associations are observed in a sex-specific and timing-specific manner.

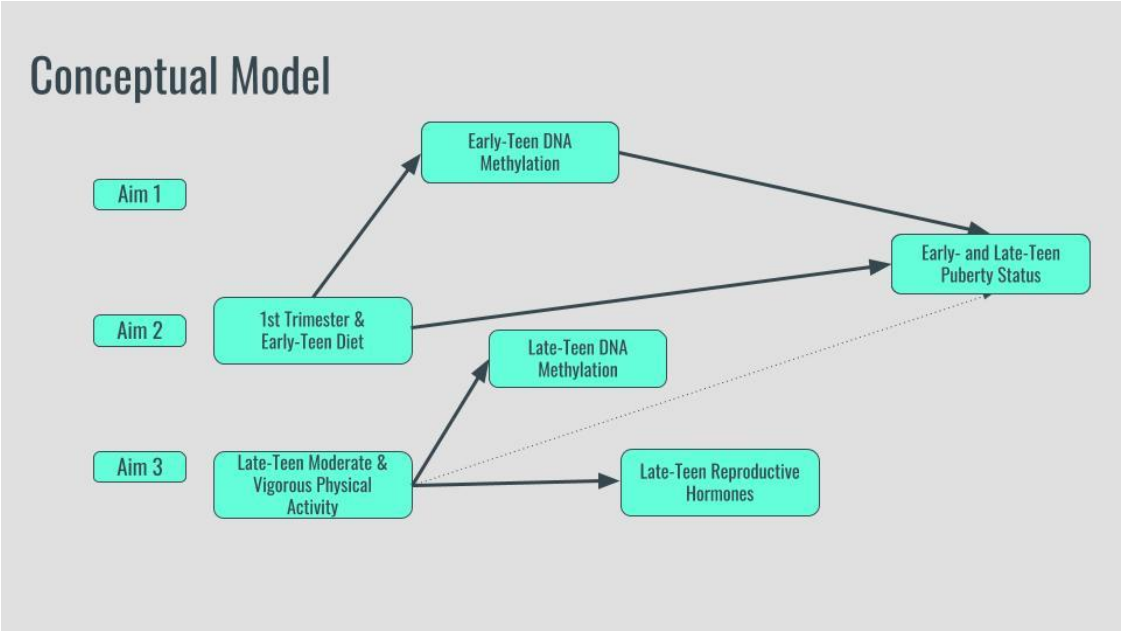


Figure 5.1: Conceptual model.

In the 2nd Chapter, we found evidence supporting the gene-specific, site-specific, and sex-specific associations of DNA methylation with pubertal timing and tempo. Longitudinally, among boys, a percent methylation increase of *HSD11B2* site 4 was associated with increased odds of earlier pubarche. Percent methylation increase of *IGF2* site 3 was associated with increased odds of later onset of genital development. In regard to pubertal tempo, increased methylation of *HSD11B2* site 1 was associated with increased odds of faster genital development progression, while site 4 was associated with increased odds of slower progression of genital development. Increased methylation of *IGF2* was associated with faster progression of genital development. Among girls, percent increase in methylation of *H19* site 2 was longitudinally associated with increased odds in the later onset of breast development. DNA methylation was not related to pubertal tempo among girls.

Results from Chapter 2 overall indicated that DNA methylation, one of the mechanisms of epigenetic modifications, was associated with age of pubertal onset and the pace of pubertal progression. First and foremost, the growth-related, maternally imprinted gene *IGF2* was associated with onset and development of puberty in boys, while the paternally imprinted gene *H19* was relevant to the onset of puberty in girls. Genomic imprinting is an epigenetic phenomenon in which only a single allele of a gene is expressed in a parent-of-origin development manner (Waterland and Jirtle, 2004). Most imprinted genes are found in clusters, and these imprinted domains are regulated in coordinate fashion via long-range mechanisms such as the antisense RNA interference and methylation-sensitive boundary elements (Waterland and Jirtle, 2004). For instance, *H19* gene is located 100 kb downstream of *IGF2* gene, and they shared the same regulatory elements (Waterland and Jirtle, 2004). The imposition of a functional haploid state at imprinted loci greatly increases their prominence in disease formation since a

single genetic mutation or lone epigenetic event can alter their function (Jirtle and Weidman, 2007; Das et al., 2009). The *conflict hypothesis* states that imprinting is the unintended results of a reproductive battle between the sexes (Jirtle & Weidman, 2007). Specifically, this hypothesis predicts that genes are only active on paternal chromosomes will promote prenatal growth to maximize the evolutionary fitness of the offspring, while genes that are only active when inherited from the mother suppress offspring growth in order to maximize the mother's reproductive success (Jirtle & Weidman, 2007). We still do not fully understand the mechanisms behind our sex-specific findings with imprinted genes, but they could be relevant to differences in methylation patterns (Gebert et al., 2009) or sensitivity toward reproductive hormone pathways (Berteaux et al., 2004) across sexes.

Given that elevated methylation of *HSD11B2* was associated with earlier onset of pubertal timing, we speculated the gene affected the outcome via working through the metabolism and inflammation pathways (Staab & Maser, 2009). Published bservational studies to some extent agree with these results, showing that increased adiposity might result in the higher estrogen levels that were linked to early breast development and menarche (Laitinen et al., 2001; Jasik & Lustig, 2008). We also found that increased methylation of *IGF2* was associated with increased odds of later onset of genital development, and faster genital development progression. Our observation illustrated the possibility of "catch-up growth/compensatory gain" of sexual characteristics during adolescence. The theory has been well characterized for height velocity (Prader et al., 1963); our results suggested the same concept might apply in puberty transition.

In the 3rd Chapter, we presented maternal and adolescent epigenetic-associated dietary patterns among Mexico City birth cohort, and observed that epigenetic-associated food

groups were associated with pubertal onset and tempo in an exposure timing-specific, sex-specific manner. Items that contributed to the maternal first trimester epigenetic-associated diet score (EADS) selected by LASSO included foods high in protein, fat and simple carbohydrate. High-protein and high-fat food items were negatively associated with LINE-1 methylation, and thus diets with a high frequency of these items resulted in lower maternal EADS. The findings make biological sense since food items high in fat and refined carbohydrates often lack methyl donor nutrients. Whole grains were positively associated with LINE-1 methylation levels, and thus diets rich in these foods had higher EADS. Compared to maternal EADS, adolescent EADS comprised fresh high-fiber vegetables and lean protein sources. Both positive and negative directions were observed between adolescent food items and LINE-1 methylation. Fresh vegetables, which are known with high content of methyl donor nutrients, were positively associated with LINE-1 methylation levels, while lean protein food items were negatively associated, and thus resulted in higher or lower adolescent EADS, respectively.

Maternal and adolescent EADS had different measures of associations with pubertal onset and progression. We recognized that there were statistically significant associations between maternal first trimester EADS, but not adolescent EADS, and pubertal onset in girls only. In terms of pubertal progression, we observed statistically significant associations with adolescent EADS, but not maternal first trimester EADS among boys only.

To our best knowledge, Chapter 3 is the first study that elaborated the associations of dietary patterns with a focus on methyl-donor nutrients in both pregnant women and healthy adolescents with adolescents' pubertal outcomes. It also examined the association between the EADS scores of the dietary patterns and pubertal events. First, our results suggested a long-term effect of maternal first trimester diet and a short-term health impact of concurrent diet on

pubertal timing and tempo. According to DOHaD theory, exposures from early fetal life are able to influence the expression of genes, with effects on later health and disease (Suzuki, 2018). The "fetal origins hypothesis/thrifty phenotype hypothesis" also posits that poor prenatal nutrition in early life can increase the adverse effects of an affluent diet in adulthood, leading to an increased risk of various non-communicable diseases (Suzuki, 2018). Our results were consistent with this theory by showing increased maternal first trimester EADS was associated with higher odds of later menarche timing, which was biologically probable considering that higher levels of LINE-1 methylation from white blood cells have been inversely related to body fat mass among health young individuals (Marques-Rocha et al., 2016). Secondly, we observed that different sex groups presented different associations with maternal first trimester and adolescent EADS. Specifically, maternal EADS had major influence on girls, while adolescent EADS, the concurrent diet, had predominant impact on boys. This finding might be related to the timing of sex dimorphism. Sexual differentiation begins early in the embryonic life and is reinforced and finished later in development (Berenbaum & Beltz, 2011). During early development the gonads of the fetus remain undifferentiated, meaning all fetal genitalia are the same and are phenotypically female (Wizemann & Pardue, 2001). The expression of a gene on the Y chromosome starts 6 to 7 weeks of gestation later and the production of testosterone does not begin until about 9 weeks of gestation (Wizemann & Pardue, 2001). We speculate that maternal EADS might be able to create biological environment for female reproductive organs while offspring is still in the womb. However, male reproductive organs may be more receptive of concurrent environmental exposures during the adolescent developmental phase.

In the 4th Chapter, we found evidence suggesting the effects of physical activity on hormone regulation in the cross-sectional analysis. Substituting 30 minutes sedentary behavior

for the same amount of moderate physical activity per day was associated with lower testosterone levels in boys, holding other activity types constant. Among girls, a 30-minute increase in sedentary behavior was associated with higher progesterone concentrations. However, no statistically significant associations were observed between intensities of physical activity and Tanner stages using substitution models, although biologically, reproductive hormones advance pubertal status.

In terms of the association between physical activity and DNA methylation, our results suggested that substituting 30 minutes sedentary behavior for 30 minutes in vigorous physical activity per day was associated with higher percent of *HSD11B2* methylation in boys, while holding other activity types constant. However, among girls, a 30-minute increase in sedentary behavior was associated with lower percent of *HSD11B2* methylation. Higher levels of physical activity have been associated with increased LINE-1 global DNA methylation in the peripheral blood among 647 non-Hispanic white woman with a family history of breast cancer (White et al., 2013). We did not, however, find any significant associations between physical activity intensities and LINE-1 DNA methylation. This inconsistency could be because LINE-1 hypomethylation is found to play a potential role as a prognostic biomarker of cancer risks (Baba et al., 2018), while less variation of LINE-1 DNA methylation is observed among healthy adolescents (Phokaew et al., 2008; Zhang et al., 2011). In addition, we observed a positive association between daily vigorous physical activity and percent of *HSD11B2* DNA methylation. This finding is biologically plausible because hypermethylated *HSD11B2* is associated with decreased transcriptional activity (Alikhani-Koopaei et al., 2004), indicating less glucocorticoid cortisol will be catalyzed to the inactive metabolite cortisone. This is in line with the established

association that moderate to high intensity exercise provokes increased in circulating cortisol levels in human blood (Hill et al., 2008).

Although results needed to be interpreted carefully since Chapter 4 was a cross-sectional analysis, findings can guide future research. Physical activity was associated with changes of reproductive hormone and DNA methylation levels, but not pubertal status. Other than limitations of sample size, we considered one of potential explanations that is daily physical activity might be able to induce subclinical physiological changes, but might not be consistent and intense enough to lead to actual changes in pubertal timing. Future research with more frequent physiological measures is needed to test this speculation. In addition, we have seen the association between adolescent physical activity and *HSD11B2* DNA methylation, suggesting it would be fruitful to examine whether maternal physical activity would have similar effects on DNA methylation later in life. Showing the long-term effects of regular physical activity would further motivate behavioral interventions.

STRENGTHS AND LIMITATIONS

Based on our conceptual model (**Figure 1**) and literature review, we have carefully chosen the timing of the variables, in order to examine the longitudinal effect of the exposures. Though the sample sizes are limited for this sex-specific analysis, we have cautiously selected confounders to account for potential external variables that might obscure the 'real' effect. However, there are some major limitations of this dissertation work that should be acknowledged. Common limitations across all three chapters centered on restrictions of our data collection methods. First, our sample comes from a mixed ancestry population but we did not genotype this population. It is possible that ancestry differences could influence the relationships we are observing between DNA methylation and pubertal timing. Second, while pubertal status

of pubic hair, breast, genital and testicular volume development was based on a highly trained physicians' observation, age of menarche was self-reported and may be subject to recall bias. Third, since the epigenome and transcriptome vary by cell and tissue type, analyzing DNA methylation in blood leukocytes which consist of multiple cell types is a limitation. Recent studies, however, have identified concordant methylation alterations in blood and other physiologically related tissue (Masliah et al., 2013; Reinius et al., 2012), suggesting that blood might be a reliable surrogate for some epigenetic analysis. Last but not the least, we have included participant's BMI in all adjusted models and observed that including BMI in models did not significantly attenuate the associations. Nevertheless, we cannot rule out the possibility that BMI was the mediator of the associations since some of association analyses are cross-sectional.

There also are some chapter distinct limitations. In Chapter 2, we used multiple regression imputation to increase the number of predictors. This method can underestimate standard error, which might result in inflated p-values (Soley-bori, 2013). In Chapter 3, though we have applied cross-validation to estimate prediction error when using LASSO selection, interpretations of the results may not be applicable in another cohort if those assumptions are not met. In Chapter 4, as a cross-sectional study, predictors and outcomes of interest were simultaneously collected and assessed, we cannot assess the temporality of the associations. Additionally, an accelerometer worn on the wrist could introduce more "noise" into collected date than a device worn on the waist.

The strengths of this dissertation deserve mention. This is the only population-based study that has conducted a full examination of global and gene-specific DNA methylation and pubertal status in both sexes. Since the differentiation of secondary sex characteristics might be related to different hormone or metabolism pathways, we analyzed the each association with

individual pubertal characteristic. Moreover, with Tanner stages gathered from two visits, we have examined not only the pubertal timing, but also the pubertal tempo, to understand the effect of DNA methylation on developmental trajectory better. In addition, we have used validated maternal and adolescent FFQs to assess participants' dietary intake, and accelerometers to estimate their daily physical activity patterns. Variations and errors in data collection are not completely avoidable, but with the help of these tools, we expect information to be as reflective of their everyday life as possible.

IMPLICATIONS, RECOMMENDATIONS AND SCOPE OF FUTURE RESEARCH

As suggested by limitations above, future research should consider improvements in some aspects of the study design and data collection methods. We have carefully considered the timing of exposure and made the effort to select the variables that make the most biological sense. However, limited by the timeframe of onsite visits, we have performed a few cross-sectional analyses to examine the associations. Thus, future studies should consider revisiting these research questions using a longitudinal study design with repeated measures of multiple time points. For instance, in Chapter 4, we did not find any significant evidence supporting the association between physical activity levels and pubertal status. One of the possible explanations was because accelerometer-based daily physical activity was collected at late-teen visit, at which most participants had advanced pubertal stages. Additionally, we would highly recommend future research to include more candidate genes that targeted on pubertal development pathways. Candidate genes included in this dissertation are growth-related, but they are not directly linked to pubertal status. Therefore, we think it is worth identifying key genes regulating puberty and re-examining the associations. Last but not least, we used food frequency questionnaire (FFQ) to assess usual dietary intake of methyl donor nutrients from our Mexican City cohort. However, it

would be reassuring if we could collect blood or urine micronutrient biomarkers to validate the findings.

CONCLUSIONS

This dissertation provides evidence supporting the association of DNA methylation with pubertal timing and tempo. It also finds that methyl donor rich diets affect epigenetics regulation, which in turn influence pubertal status. Furthermore, there is an association between physical activity and reproductive hormones, and DNA methylation levels. Findings indicate that epigenetic changes during developmentally plastic phases have the potential to reshape the timing of pubertal onset and trajectory of pubertal progression, which then might influence the aging process. It is gratifying to observe that diet and physical activity behaviors have the potential to regulate these processes.

Bibliography

Alikhani-Koopaei R, Fouladkou F, Frey FJ, Frey BM. Epigentic regulation of 11β-hydroxysteroid dehydrogenase type 2 expression. *J Clin Invest*. 2004. doi:10.1172/JCI21647.

Baba Y, Yagi T, Sawayama H, Hiyoshi Y, Ishimoto T, Iwatsuki M, Miyamoto Y, Yoshida N, Baba H. Long interspersed element-1 methylation level as a prognostic biomarker in gastrointestinal cancers. In: *Digestion*. ; 2018. doi:10.1159/000484104.

Berenbaum SA, Beltz AM. Sexual differentiation of human behavior: Effects of prenatal and pubertal organizational hormones. *Front Neuroendocrinol*. 2011. doi:10.1016/j.yfrne.2011.03.001.

Berteaux N, Lottin S, Adriaenssens E, Van Coppennolle F, Leroy X, Coll J, Dugimont T, Curgy JJ. Hormonal regulation of H19 gene expression in prostate epithelial cells. *J Endocrinol*. 2004. doi:10.1677/joe.1.05696.

Cutler RG, Mattson MP. The adversities of aging. *Ageing Res Rev*. 2006. doi:10.1016/j.arr.2006.05.002.

Das R, Hampton DD, Jirtle RL. Imprinting evolution and human health. *Mamm Genome*. 2009. doi:10.1007/s00335-009-9229-y.

Gebert C, Wrenzycki C, Herrmann D, Gröger D, Thiel J, Reinhardt R, Lehrach H, Hajkova P, Lucas-Hahn A, Carnwath JW, et al. DNA methylation in the IGF2 intragenic DMR is re-established in a sex-specific manner in bovine blastocysts after somatic cloning. *Genomics*. 2009. doi:10.1016/j.ygeno.

Hill EE, Zacki E, Battaglini C, Viru M, Viru A, Hackney AC. Exercise and circulating cortisol levels: The intensity threshold effect. *J Endocrinol Invest*. 2008. doi:10.1007/BF03345606.

Jasik CB, Lustig RH. Adolescent obesity and puberty: The "perfect storm." In: *Annals of the New York Academy of Sciences*. ; 2008. doi:10.1196/annals.1429.009.

Jirtle RL, Weidman JR. Imprinted and more equal. *Am Sci*. 2007. doi:10.1511/2007.64.143.

Laitinen J, Power C, Järvelin MR. Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity. *Am J Clin Nutr*. 2001. doi: 10.1093/ajcn/74.3.287.

Marques-Rocha JL, Milagro FI, Mansego ML, Zulet MA, Bressan J, Martínez JA. Expression of inflammation-related miRNAs in white blood cells from subjects with metabolic syndrome after 8 wk of following a Mediterranean diet-based weight loss program. *Nutrition*. 2016. doi:10.1016/j.nut.

Masliah E, Dumaop W, Galasko D, Desplats P. Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. *Epigenetics*. 2013. doi:10.4161/epi.25865.

Phokaew C, Kowudtitham S, Subbalekha K, Shuangshoti S, Mutirangura A. LINE-1 methylation patterns of different loci in normal and cancerous cells. *Nucleic Acids Res*. 2008. doi:10.1093/nar/gkn571.

Prader A, Tanner JM, von Harnack GA. Catch-up growth following illness or starvation. An example of developmental canalization in man. *J Pediatr*. 1963. doi:10.1016/S0022-3476(63)80035-9.

Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlén SE, Greco D, Söderhäll C, Scheynius A, Kere J. Differential DNA methylation in purified human blood cells: Implications for cell lineage and studies on disease susceptibility. *PLoS One*. 2012. doi:10.1371/journal.pone.0041361.

Soley-bori M. Dealing with missing data: Key assumptions and methods for applied analysis. *PM931 Dir Study Heal Policy Manag*. 2013.

Staab CA, Maser E. 11β-Hydroxysteroid dehydrogenase type 1 is an important regulator at the interface of obesity and inflammation. *J Steroid Biochem Mol Biol*. 2010. doi:10.1016/j.jsbmb.2009.12.013.

Suzuki K. The developing world of DOHaD. In: *Journal of Developmental Origins of Health and Disease*. ; 2018. doi:10.1017/S2040174417000691.

Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition*. 2004. doi:10.1016/j.nut.2003.09.011.

White AJ, Sandler DP, Bolick SCE, Xu Z, Taylor JA, Deroo LA. Recreational and household physical activity at different time points and DNA global methylation. *Eur J Cancer*. 2013. doi:10.1016/j.ejca.2013.02.013.

Wizemann TM, Pardue M. *Exploring the Biological Contributions to Human Health: Does Sex Matter?* Washington (DC): National Academies Press (US), 2011.

Zhang FF, Cardarelli R, Carroll J, Zhang S, Fulda KG, Gonzalez K, Vishwanatha JK, Morabia A, Santella RM. Physical activity and global genomic DNA methylation in a cancer-free population. *Epigenetics*. 2011. doi:10.4161/epi.6.3.14378.