A Study of the Impact of Environmental Chemicals and Micronutrients on Pubertal Development in A Mexican Population

by

Yun Liu

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Doctoral Committee:

Professor Karen E. Peterson, Chair
Assistant Professor Andrew Jones
Professor Angeles E. Martínez-Mier, Indiana University
Associate Professor Brisa N. Sánchez

Yun Liu
yunliu@umich.edu
ORCID iD: 0000-0002-2979-9552

DEDICATION

This dissertation is dedicated to my parents, Hui Li and Xiumin Liu, for all of their love and support

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List of Acronyms

ARC – Arcuate nucleus neuron

BMI – Body mass index

BPA – Bisphenol A

DDE – Dichlorodiphenyldichloroethylene

DHEA – Dehydroepiandrosterone

DHEAS – Dehydroepiandrosterone sulfate

Dyn – Dynorphin

EDC – Endocrine-disrupting compound

FSH – Follicle-stimulating hormone

GnRH – Gonadotropin-releasing hormone

HPA – Hypothalamic-pituitary-adrenal axis

HPG – Hypothalamic-pituitary-gonadal axis

IGF-1– Insulin-like growth factor 1

LH– Luteinizing hormone

MKRN3 – Makorin RING finger protein 3

NKB – Neurokinin B

%BF – Percentage of body fat

PCB – Polychlorinated biphenyls

SHBG – Sex hormone binding globulin

T2DM –Type 2 diabetes mellitus

WC – Waist circumference

Abstract

Puberty is one of the most important developmental milestones in life, involving complex physical and psychological changes. A trend towards earlier puberty has been well documented since the early 20th century. The decline in the pubertal age requires special attention due to its implications for long-term health outcomes with increased risks of reproductive cancers, type-2 diabetes, cardiovascular diseases, and psychological sequelae, and hence is a major public health concern. Increasing evidence suggests that environmental chemicals and nutrition may contribute to the trend towards earlier sexual maturation.

Exposures that occur at susceptible developmental periods may impact the prenatal growth trajectory and development of the reproductive axis and potentially have long-term effects on the tempo of maturation later in life. Therefore, it is of public health importance to examine these modifiable elements at early life to improve the understanding of pubertal disorders and their link to adverse health conditions.

The objectives of this dissertation were to determine whether exposures to environmental chemicals such as fluoride, lead and nutritional factors such as micronutrients at multiple life stages including *in utero*, early childhood and peripuberty are potential determinants of sexual maturation by using mother-offspring pairs from the Early Life Exposure in Mexico to

ENvironmental Toxicants (ELEMENT) cohorts. We found that prenatal fluoride exposure during pregnancy was not associated with development of secondary sex characteristics directly, but associated with reduced peripubertal serum testosterone in boys and with increased peripubertal serum IGF-1 (insulin-like growth factor 1) in girls. Increased maternal consumption of selenium and zinc during pregnancy was related to advanced pubic hair growth, and increased maternal consumption of phosphorus and riboflavin during pregnancy was related to advanced genital development in boys. A significant negative association of prenatal and early childhood lead exposure with pubertal development was observed in girls.

Our findings suggest that fluoride, lead, and intakes of micronutrients during early life may have long-term impacts on development of sexual maturation in adolescents, particularly in a sex-specific fashion. This work highlights the need for more research to examine associations between environmental factors during sensitive periods of development and sexual maturation to better understand pubertal disorders and their long-term consequences.

CHAPTER 1

Introduction

1.1 Secular trends in pubertal development

A significant shift in the timing of puberty has been described by several studies across the world (Figure 1.1) (Parent et al. 2015). European historical reports have revealed a remarkable decline in the age at menarche between the early 1800s and the mid-1900s, showing that the average menarcheal age decreased from 15-17 years to 13-13.5 years (Tanner 1973). Previous data from large U.S. studies have shown a similar trend towards an earlier age at menarche from 1877 to 1970 (Wyshak and Frisch 1982). Furthermore, the continuation of this trend has been suggested by subsequent studies from both U.S. (Anderson et al. 2003) and Europe (Aksglaede et al. 2009; Rubin et al. 2009), though to a lesser extent. During similar periods, a downward trend towards earlier menarcheal age has also been found in several other countries such as Canada (Al-Sahab et al. 2010; Harris et al. 2008), Mexico (Marvan et al. 2016), Korea (Cho et al. 2010; Lee et al. 2016), China (Lyu et al. 2014), India (Pathak et al. 2014), and Israel (Flash-Luzzatti et al. 2014). In addition to decreased age at menarche, a significant decline in the age at thelarche (the onset of breast growth) has been reported in both U.S. and European studies (Sorensen et al. 2012). Age at thelarche decreased markedly from approximately 11 years (Euling et al. 2008) before 1980 to 9.5-10.4 years from 1988 to 1994 in the U.S. (Sun et al. 2002) and to around 10 years from 1990 to 2006 in Europe (Aksglaede et al. 2009; Papadimitriou et al. 2008; Semiz et al. 2008).

In contrast to the relatively high number of large-scale studies examining the pubertal timing in girls, only a few studies have been performed in boys. In Europe, a few studies of boys examined both genital development and volume of testis by using orchidometry and found no significant downward trend towards an earlier age at pubertal onset from the mid-1960s to the late 1990s (Juul et al. 2006; Lee and Styne 2013; Mul et al. 2001). However, a study polished in 2010 reported a significant reduction in age at onset of genitalia and having testicular volume>3mL by 3 months between 1991–1993 and 2006-2008 among participants from The Copenhagen Puberty Study (Figure 1.2) (Sorensen et al. 2010). A study from The multisite Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Study of Early Child Care and Youth Development used historical data to show that age at pubertal onset of genital development decreased from 11 years to 10.4 years in white participants and 9.6 years in African American participants (Susman et al. 2010). Comparable results were reported by another cross-sectional study from the American Academy of Pediatrics' Pediatric Research in Office Settings (PROS) network. The average age at pubertal onset of genitalia was found to have dropped to 10.1 years in whites and 9.1 years in African Americans (Herman-Giddens et al. 2012). Taken together, there is a decreasing trend towards pubertal timing in boys to a lesser extent compared to the trend we have observed in girls.

1.2 Long-term health consequences of early puberty

Although downward trends toward pubertal timing in adolescents have been observed, but does it really matter? Previous research has shown that early puberty is associated with increased risk of long-term health effects including reproductive cancers, type 2 diabetes, obesity, cardiovascular diseases, and psychological functions (Walvoord 2010). For example, a study using data from

Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial cohort (PLCO) reported that women who initiated menses before age 12 had a higher risk of breast cancer compared with those who initiated menses after age 14 (Lacey et al. 2009), which was confirmed by a report from Nurses' Health Study (NHS) (Kotsopoulos et al. 2010). From a North Carolina case-control study (1999–2008), the risk of ovarian cancer was significantly increased with younger age at menarche (<12 years), but only in white women (Moorman et al. 2009). Two studies in Europe showed that earlier puberty, defined as the age at appearance of hair under the arms and in the groin or at voice broke, was associated with increased risk of testicular cancer (Group 1994; Moller and Skakkebaek 1996).

Moreover, a few cohort studies have revealed that early puberty is associated with increased risk of type 2 diabetes mellitus (T2DM) and obesity. For instance, investigators using data from the NHS cohort found that young age at menarche was associated with increased risk of T2DM in adulthood, even after adjusting for body mass index (BMI) (He et al. 2010). In a meta-analysis, menarcheal age before 12 years was associated with an increased risk for obesity in adulthood compared with those with normal or later age at menarche (Prentice and Viner 2013). One investigation conducted among women who enrolled in the Midlife Women's Health Study in Baltimore, MD reported that with one year increase in the menarcheal age, the odds of obesity decreased by 31% (Gallicchio et al. 2016).

Early pubertal timing has also been linked to cardiovascular diseases. A population-based prospective study among women aged 40–79 years living in Norfolk, United Kingdom concluded that early menarcheal age was associated with increased risk of cardiovascular disease events and

cardiovascular disease mortality (Lakshman et al. 2009), which was confirmed by another large UK Biobank study (Day et al. 2015). Reports from China and Korea also provide evidence to support the associations between pubertal timing and risk of cardiovascular conditions. In the Korean report, investigators found that the risk of coronary heart disease was 51% lower in women who started menses after 17 years compared with those who started menses before 17 years (Chang et al. 2011). Similar results were found in a cross-sectional study conducted in Fujian, China among 6242 women aged 21 to 92 years (Qiu et al. 2013).

Additionally, puberty may play an important role in the etiology of psychological problems. Multiple lines of evidence reported that early pubertal timing may be positively associated with a range of mental health problems in adolescents, such as depression, bulimia nervosa, psychosomatic symptoms and anxiety (Black and Klein 2012; Culpin et al. 2015; Ge et al. 2001; Lien et al. 2010).

1.3 Environmental influences on early puberty

Although the exact causes of the decreasing pubertal timing are not completely understood, multiple genetic, metabolic and environmental factors are largely accepted to be important regulators of pubertal development. Various studies have focused on the impacts of environmental chemicals on the timing of puberty, particularly, endocrine-disruptor chemicals (EDC) (Jacobson-Dickman and Lee 2009). For instance, exposure to polychlorinated biphenyls (PCB), phthalates and dichlorodiphenyldichloroethylene (DDE) is associated with advanced breast development and/or menarche (Toppari and Juul 2010). In contrast, exposure to dioxin-like compounds is associated with delayed breast development (Den Hond et al. 2002). A numbers of animal studies

have suggested that EDCs may interrupt pubertal timing by mimicking or blocking hormone signaling, or by modifying the synthesis or metabolism of sex hormones (Jacobson-Dickman and Lee 2009).

In addition to environmental chemicals, increasing evidence suggests that nutrition may contribute to the timing of pubertal development. To date, a number of epidemiological studies have examined the association between pubertal timing and nutritional factors during childhood. According to an old nested case-control study of 666 Canadian girls at age 9.5–12.5 years, higher energy intake was associated with earlier menarcheal age (Moisan et al. 1990b). Several studies have linked fat intake to the earlier age at menarche. For example, a cohort study of 213 girls in Massachusetts found that intake of omega-3 fatty acids at age 10 years was associated with earlier menarche (Maclure et al. 1991). Based on results generated from a prospective study among 261 German girls, energy-adjusted fat intake was associated with increased risk of onset of menarche at age 7–14 years (Merzenich et al. 1993). Similar results were also observed in 3298 British girls enrolled in the Avon Longitudinal Study of Parents and Children cohort (ALSPAC) that higher intakes of polyunsaturated fatty acids (PUFA) at 3 and 7 years were associated with increased risk of early occurrence of menarche (Rogers et al. 2010). Additionally, protein intake is a potential contributor to the pubertal development in both boys and girls. Several reports have suggested positive associations between animal protein intake and earlier pubertal development. Thus, two prospective analyses showed that higher intakes of animal protein were positively associated with an earlier age at menarche in 67 US girls at 3-5 years (Berkey et al. 2000), in 112 German girls at 5-6 years (Gunther et al. 2010), and in 3298 British girls at 3 and 7 years (Rogers et al. 2010).

In summary, the potential influence of environmental factors on sexual maturation has been studied with a focus on the EDCs such as several persistent organic pollutants, dietary intake of energy, and macronutrients such as fat and protein. Studies of the impacts of environmental chemicals such as fluoride, lead and intakes of micronutrients, which have been suspected to play a role in changing the timing of puberty are rare. Most of these studies assessed the influence on pubertal timing by using single pubertal indicator, mostly age at menarche. Furthermore, only a few studies have evaluated the environmental influence on the pubertal development in boys. Most of the current available investigations have assessed the effects on the timing of puberty from early childhood to prepubescence, while studies examining these effects during the prenatal period are rare.

1.4 Regulation of pubertal development

Puberty is a key period of transition, which leads to the development of secondary sex characteristics and the eventual achievement of reproductive capacity, under the control of a complex series of coordinated neuroendocrine mechanisms. Changes in puberty involve the maturation and full activation of the hypothalamic-pituitary-gonadal (HPG) axis and hypothalamic-pituitary-adrenal (HPA) axis, which are responsible for the complete development of gonads and secondary sex characteristics (Buck Louis et al. 2008). There are three major players involved in these neuro-hormonal systems. The first step in the HPG axis is the hypothalamus, where gonadotropin-releasing hormone (GnRH) is produced and secreted in a pulsatile fashion. Secondly, gonadotropins such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are secreted by the anterior pituitary. Finally, the gonads produce gametes and release sex hormones (Tena-Sempere 2013).

The HPG axis is active in mid-gestation, neonatal, and early infancy and then becomes quiescent in childhood. The first traces of GnRH neurons are found in the fetus at 6 weeks of gestation. By 12 weeks of gestation, the pituitary FSH and LH present in significant concentration in the fetal serum. Starting at mid-gestation, the activity of HPG axis surges and the concentrations of LH and FSH rise to the amount that is contributing to development of ovaries by 20-24 weeks of gestation (Mueller 2013). However, the LH and FSH concentrations decrease by the late gestation through the negative feedback of placental estrogen to HPG axis. This also explains why the LH and FSH levels are low at birth. From 4 weeks to 1 year of life, due to the removal of placental estrogens, HPG axis is activated again. Also, FSH and LH increase to measurable level and reach the peak concentration around the first year of life. During this period, sex hormones such as testosterone and estradiol reach levels that are comparable to the levels at early-middle puberty, which is defined as "Mini-puberty of infancy" (Quigley 2002). From approximately 2-7 years of age, HPG axis remains silent leading to low levels of FSH and LH and absence of sex hormones, which is called "Quiescence of childhood".

From 8 years to adolescence, an unknown event triggers the beginning of puberty by reactivating the HPG axis manifested by increased GnRH. The rise in GnRH stimulates the secretion of gonadotropins such as luteinizing LH and FSH from the anterior pituitary, which in turn act on the gonads to promote the production of gametes and sex hormones. Increased release of sex hormones subsequently initiates the appearance of secondary sex characteristics (Abreu and Kaiser 2016). The HPG axis is under the control of feedforward and feedback loops, as illustrated in Figure 1.3 (Buck Louis et al. 2008). In girls, the activation and maturation of HPG axis ultimately lead to the initiation of menses and production of sex hormones such as androgens, estradiol and

progesterone, which are responsible for the development of pubic and auxiliary hair, breast, and ovaries and uterus. In boys, LH stimulates the secretion of androgens such as testosterone and androstenedione, which are responsible for the development of genitalia and pubic hair (pubarche).

Another important event associated with puberty in both sexes is adrenarche, which is defined as an early stage of sexual maturation that typically occurs at around 6 to 8 years of age. During adrenarche, the activation and maturation of HPA axis acts on the adrenal glands to produce androgens in parallel to the production of estradiol through ovaries. Secretion of adrenal androgens such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) is largely responsible for the development of armpit hair, acne and pubarche in both boys and girls (Figure 1.3) (Buck Louis et al. 2008).

Although the triggering event(s) of initiation of puberty remain unknown, some regulatory systems have been identified and proposed to control the onset of puberty and reproductive functions, such as kisspeptin, neurokinin B (NKB), and dynorphin (Dyn) as well as a new pathway involving makorin RING finger protein 3 (MKRN3) gene. Several human studies have reported that kisspeptin stimulates the release of GnRH, which in turn acts on the pituitary to induce the release of gonadotropins (Tena-Sempere 2013). Researchers have also found that kisspeptin, NKB, and Dyn are co-expressed in arcuate nucleus (ARC) neurons called KNDy neurons. Lines of evidence have reported that NKB stimulates the release of LH release via increasing the secretion of kisspeptin, which induces the production of GnRH subsequently (Soliman et al. 2014), while Dyn may inhibit the release of kisspeptin (Tena-Sempere 2013). MKRN3 was first identified in 2013 as an additional player that regulates the initiation of puberty. Animal studies have found that the

level of MKRN3 is high at pre-pubertal period, then decreases before the onset of puberty, and subsequently reaches a very low level in adulthood. Previous findings have demonstrated that the beginning of reduction in expression of MKRN3 may lead to an earlier puberty by exerting an inhibitory effect on the secretion of GnRH (Hughes 2013).

It has been acknowledged that the early life environment can have long-lasting effects on human growth and development, potentially through epigenetic changes, which is known as "the Developmental Origins of Health and Disease" (Godfrey et al. 2016). Due to their high plasticity, developmental stages of prenatal and early postnatal life in parallel to the periods of HPG activation are considered as sensitive and critical periods for development of puberty, and thus susceptibility to factors that are associated with growth and sexual maturation (Roth and DiVall 2016). Several studies have proposed that the regulation of reproduction is responsive to the GnRH neuroendocrine systems in hypothalamus, which expresses a degree of plasticity throughout life, particularly the time of fetal development (Evans et al. 2016).

1.5 Study population

The participants consist of individuals from sequentially enrolled epidemiologic birth cohorts (Cohort I to III), which are part of The Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) project. Cohorts I, II and III 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 starting from year 1994 to 2006. Fluoride exposures in Mexico City are diverse and comparable to those in the U.S. All the data to be analyzed for specific aim 1 will be achieved from a follow-up of children from the

combined birth cohorts. Archived data collected from cohort III will be analyzed for specific aim 2 and 3.

The recruitment of Cohort I ran from year 1994 to 1996, and children were born in year 1994 and 1995. This cohort was initially recruited as a postpartum, randomized trial of calcium supplementation during lactation among 616 women randomized to calcium or placebo group and a follow-up of their infants for neurobehavioral performance. Subjects were given calcium (or placebo) in the postpartum period from birth to 7 months. Exclusion criteria included factors that could interfere with maternal calcium metabolism, medical conditions that could cause low birth weight, logistic reasons that would interfere with data collection (e.g. households living outside the metropolitan area), delivering a premature neonate (<37 weeks) or an infant with an Apgar score at 5 minutes of 6 or under, conditions requiring placement in a neonatal intensive care unit, a physician's diagnosis of multiple fetuses, intention not to breastfeed, preeclampsia, psychiatric, kidney, or cardiac diseases, gestational diabetes, history of repeated urinary infections, family or personal history of kidney stone formation, seizure disorder requiring daily medication, ingestion of corticosteroids or blood pressure >140mmHg systolic or >90 mmHg diastolic.

Two subgroups constitute Cohort II. One subgroup of cohort II was recruited during year 1997-2000 and children were born in year 1997-1999. This subgroup was initially recruited for studying the relationships among lead biomarkers during pregnancy and lactation and a follow-up of infants and children to examine neurodevelopment outcomes at ages 3, 6, 12, 18, 24, 36, 48, and 60 months old. In this subgroup, 771 women were willing to participate and 327 were recruited during the 1st trimester and followed to term delivery. Exclusion criteria included: having plans to leave the area

in the following 5 years; having a psychiatric disorder; daily consumption of alcoholic beverages; addiction to illegal drugs; continuous use of prescription drugs; diagnosis of high-risk pregnancy, preeclampsia, renal or circulatory disease including hypertension, or gestational diabetes; suffering from seizures that required medical treatment; and being pregnant with > 14 weeks of gestation. In the second subgroup, study recruitment was performed during year 1999-2000 and children were born during year 1999-2001. This subgroup was initially recruited for postpartum predictors of infant development at age in 3, 6, 12, 18, 24,36, 48, and 60 months old. The total number of participants is 463 in this subgroup.

Cohort III subjects were recruited from 2000 to 2006. Children were born from 2001 to 2004. This cohort was recruited as a randomized trial of calcium supplementation during pregnancy and lactation. 670 women were assigned randomly in their first trimester of pregnancy to receive1,200 mg daily calcium supplement (n = 334) or placebo (n = 336). Subjects were given calcium supplementation (or placebo) from 1st trimester all the way through 12 months postpartum.

1.6 Dissertation overview

The objective of this dissertation was to improve our understanding of the impact of environmental chemicals and intake of micronutrients at different developmental stages on sexual maturation in children. This dissertation research was carried out among participants from Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study, which is a birth cohort study conducted in Mexico City, Mexico.

Chapter 2 is the first epidemiological study to investigate the association of exposure to fluoride prenatally and peripubertally on physical markers of pubertal onset and reproductive and growth

hormones related to sexual maturation in 240 children aged 8-15 years. We used single-trimester models to assess the impact of fluoride during pregnancy and whether certain stage of pregnancy is more sensitive to fluoride exposure. Multiple imputation was performed to estimate the missing values of fluoride biomarkers.

Chapter 3 assessed the effect of maternal consumption of micronutrient during each stage of pregnancy on physical markers of sexual maturation in 400 children aged 9-17 years. This is the first study to examine the association of intake of single micronutrients during early life with pubertal development in offspring. Micronutrients considered for inclusion in this analysis were based on the associations with IGF-1, leptin or body composition, which are predictors of pubertal development. For the final analysis, we selected micronutrients that influence IGF-1 levels such as selenium, zinc, phosphorus, thiamine, riboflavin and vitamin D; influence leptin such as vitamins A and C; and influence body composition such as vitamin B-6, vitamin B-12, vitamin C, vitamin D and iron.

Chapter 4 evaluated lead exposure at different life stages from *in utero*, early childhood to late childhood in relation to pubertal development. Using repeated measures, we were able to extend this investigation from understanding the associations with pubertal development to pubertal tempo in offspring. In addition, the potential mediation effect of body fat on lead-puberty was assessed.

Chapter 5 summarized the previous chapters of this dissertation, considered the public health implications, and provided recommendations and future directions based on our findings.

Figure 1.1: Secular trend in age at menarche

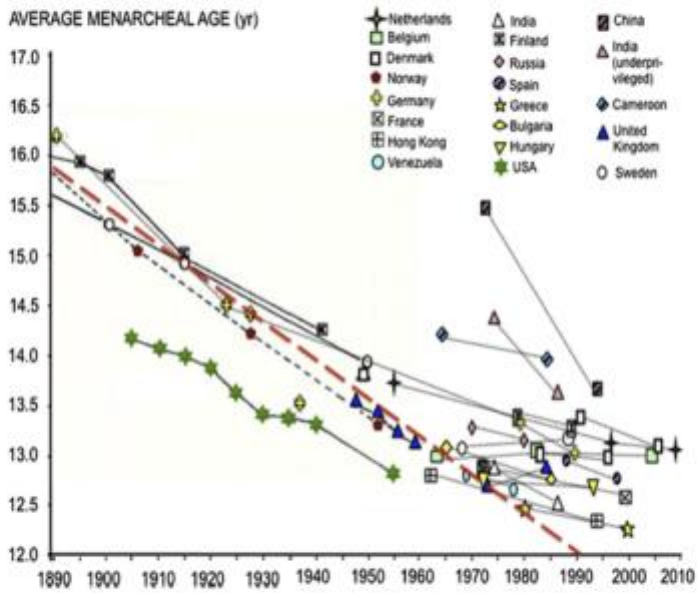


Fig. 1. Evolution of average menarcheal age (year) in the USA and Nordic countries between 1890 and 1960 (data compiled by Tanner (1962) and further, between 1960 and 2010, in different countries in Europe, USA and around the world (updated data compiled by Parent et al. (2003)). The broken red line represents the projected reduction after 1960, based on the former changes in Scandinavian countries as reported by Tanner: mean menarcheal age would have fallen down to below 12 yrs by the end of the 20th century. In fact, after 1960, average menarcheal age has leveled off in many countries while still progressing rapidly in countries such as India or China.

Figure taken from Parent et al., 2015

Figure 1.2: Pubertal development in boys from a European study.

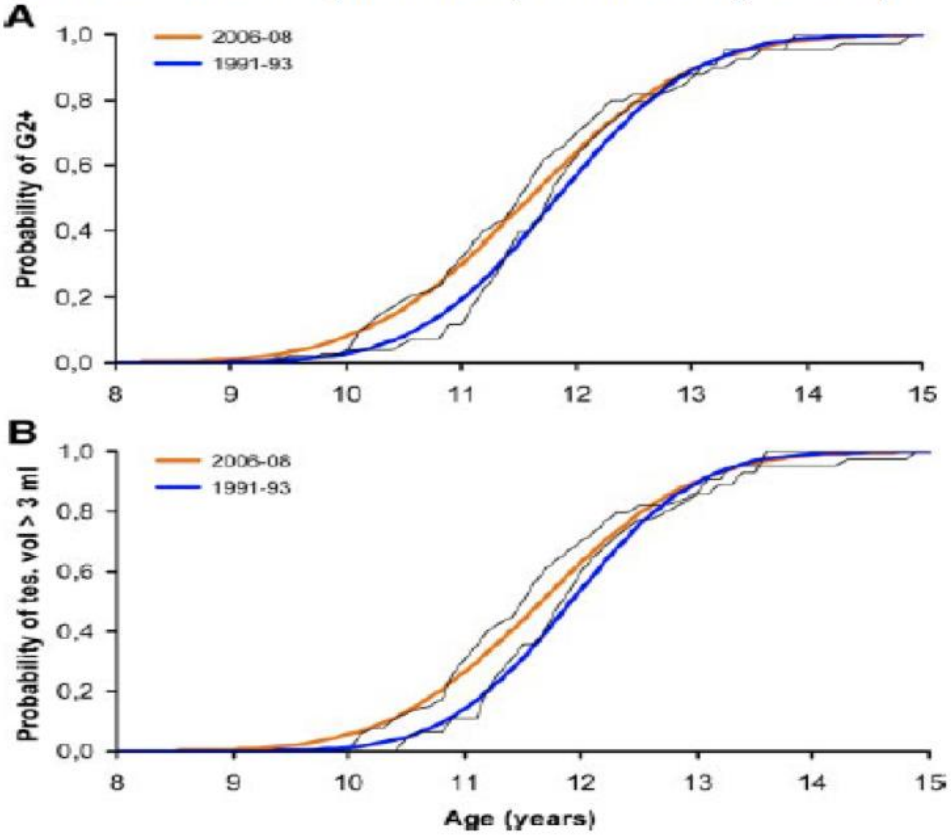


FIG. 1. Pubertal development in healthy boys from the Copenhagen Puberty Study. *Lines* represent the probabilities of observing genital stage 2 or more (G2+) (A) or a testicular volume greater than 3 ml (TV >3) (B) in relation to age in 1528 healthy school boys in two study periods; the 2006–2008 (*orange line*) and the 1991–1993 (*blue line*) study periods. The nonparametric Turnbull estimates are presented as *thin black lines*. Mean age at entry into G2+ and attainment of TV >3 were lower in the 2006-cohort compared with the 1991-cohort ($P = 0.052$ and $P = 0.025$, respectively).

Figure taken from Sørensen et al., 2010

Figure 1.3: Regulation of pubertal development

FIGURE 1

Regulation of human puberty onset and progression via the HPG and the HPA axes. The key regulatory changes that occur for the initiation and progression of puberty in boys and girls are shown. The HPG activation requires signaling from the central nervous system (CNS) to the hypothalamus. The hypothalamus releases GnRH, which in turn stimulates the production of LH and FSH from the pituitary. LH and FSH activate the gonad to produce sex hormones, which in turn initiates pubertal changes in many of the target organs. The HPA activation requires signaling from the CNS to the hypothalamus. The hypothalamus releases adrenocorticotropin releasing hormone (CRH) that in turn stimulates the production of adrenocortical tropic hormone (ACTH) from the pituitary. ACTH activates the adrenal cortex to produce androstenedione and dehydroepiandrosterone (DHEA) that in turn initiates armpit hair, pubic hair, and skin changes. Androgen action is also considered to be part of the initiation of armpit and pubic hair development. Positive (+) and negative (−) indicate stimulatory and inhibitory signals and "+/−" in the CNS indicates a combination of excitatory and inhibitory neuronal signals.

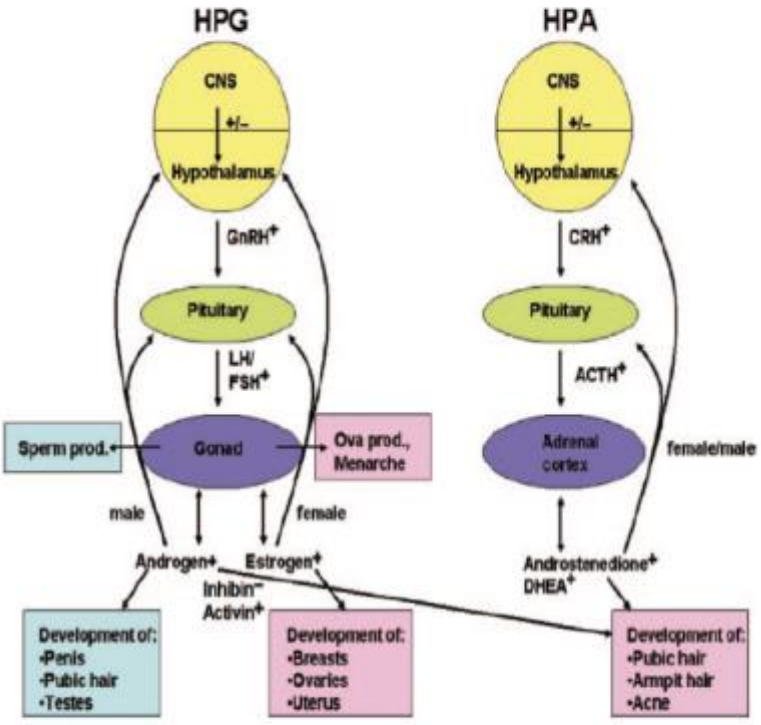


Figure taken from Buck Louis et al., 2007

CHAPTER 2

Prenatal and Peripubertal Fluoride in Relation to Sexual Maturation and Its Related Serum Hormones in Mexican Children

2.1 Abstract

Background: Fluoride exposure has been associated with alterations in developmental outcomes in children. One animal experiment and two ecologic studies in humans have suggested that fluoride may be associated with earlier pubertal onset in females; however, study designs have limited inferences about causal relationship at an individual level. Moreover, few studies have considered these relationships in boys.

Objective: Taking advantage of a birth cohort study conducted in Mexico City with validated fluoride biomarkers, we examined the effect of fluoride exposure during each stage of pregnancy and adolescence on physical markers of pubertal onset and levels of serum hormones related to puberty at ages 8-15 years among 118 boys and 132 girls.

Methods: Urine samples were collected from 250 pregnant women during each trimester and their children from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) birth cohorts. Clinical observations of Tanner staging for thelarche and pubarche along with self-reported questionnaires with regard to the status of menarche were obtained to represent sexual

maturation in girls. Physician-observed Tanner staging of genitalia and pubarche along with measured testicular volumes was obtained to represent sexual maturation in boys. Urinary fluoride levels were measured using ion-selective electrode-based diffusion assays following standardized analytic methods. Serum concentrations of several pubertal hormones were measured and used as additional indicators of puberty. Multiple imputation methods were applied to handle missing information. We used multivariable ordinal regression models and linear regression models to identify the effect of *in utero* and peripubertal fluoride exposure upon different markers of puberty. Results: Mean ± SD fluoride levels in urine during late pregnancy were 0.8±0.5 mg/L for girls and 1.1±0.8 mg/L for boys. In girls, 25.8% and 24.1% had reached Tanner stage ≥2 for pubic hair and breast development respectively, and 22.7% had attained menarche. In boys, 18.3% and 50.4% had reached Tanner stage ≥2 for pubic hair and genital development respectively, and 85.2% had testicular volume>3mL. Our analysis showed that higher urinary fluoride level during late pregnancy was negatively associated with serum testosterone (OR: -33.96, 95% CI: -48.63, -15.10) in boys, and positively associated with serum IGF-1 (insulin-like growth factor 1) (OR:10.27, 95% CI:3.93, 17.00) in girls.

Conclusions: Fluoride during pregnancy but not peripubescence at the levels observed in our study may have an impact on the concentrations of hormones related to puberty in Mexican children aged 8-15 years.

2.2. Introduction

January 25, 2015 marked the 70th anniversary of fluoridated water in U.S. with the intention of reducing dental caries while at the same time, debate over water fluoridation has been continuous. Results of earlier studies (Kaminsky et al. 1990; Murray and Rugg-Gunn 1982) have demonstrated that optimally fluoridated water prevents dental caries without posing serious risks to human health. However, a National Research Council of the National Academies of Science (NRC) review on fluoride toxicity indicated that high fluoride levels in drinking water (4 ppm) may be associated with increased risk of enamel fluorosis, bone fractures and joint pain (National Research Council (U.S.). Committee on Fluoride in Drinking Water. 2006). Therefore, the NRC committee recommended that the EPA's Maximum Contaminant Level Goal for fluoride (4 ppm) should be lowered. In addition to its effects on bone and teeth, there is growing evidence showing potential effects of high fluoride exposure on neurotoxicity and neurobehavioral deficits, alterations in reproductive hormones, fertility, and possibly timing of sexual maturity (National Research Council (U.S.). Committee on Fluoride in Drinking Water. 2006)

Among the potential adverse health problems associated with high fluoride exposure, sexual maturity requires additional attention due to its significant role in the physiological transition from childhood to adulthood. However, only 3 studies have published results describing the relationship between exposure to fluoride and physiological index of pubertal development. An animal study from birth through 28 weeks (Luke 1997) revealed that sexual maturation in 24 female Mongolian gerbils occurred earlier in high-fluoride group (3.7 mg/kg/day) (79% versus 42% having vaginal opening at 7 weeks and 70% versus 16% having differentiated ventral glands at 11.5 weeks) versus

low-fluoride group (0.7 mg/kg/day). Moreover, lower testicular weight was observed in the high-fluoride group versus low-fluoride group at 16 weeks in male Mongolian gerbils (Luke 1997). An ecological study of 405 girls aged 7-18 years who had been exposed to fluoridated water up to 10 years showed that the average age at menarche was 12 years among girls in fluoridated Newburgh, New York (0.01-0.2 mg/kg/day), versus 12 years 5 months among girls in essential fluoride free Kingston (0.001-0.02 mg/kg/day) (Schlesinger et al. 1956). A later study using an ecological design reported post-menarcheal girls present at younger ages in the higher fluoride town Kunszentmárton (with life-long exposure to fluoridated drinking water at 1.09 mg/L) than in the low-fluoride town Kiskunmajsa among 804 Hungarian girls (Farkas et al. 1983).

In addition to evaluation of physical makers, earlier studies have suggested associations of fluoride exposure with sex and growth hormones contributing to puberty (Long 2009; Ortiz-Perez et al. 2003). Several lines of evidence suggest that fluoride may lead to a reduced level of testosterone in animals (Ghosh et al. 2002; Zhou et al. 2013) and human adults (Ortiz-Perez et al. 2003; Susheela and Jethanandani 1996) by affecting the structures and enzyme functions of Leydig cells and affecting the activities of hypothalamus-hypophysis-testis axis (Long 2009). It has also been proposed that fluoride may cause a reduction in estradiol level based on animal studies using rats (Jiang et al. 2005; Zhou et al. 2013). Researchers reported that serum inhibin B was lower in males from 20-50 years to high fluoride (3–27 mg/day) compared with those exposed to low fluoride (2–13 mg/day) (Ortiz-Perez et al. 2003). Furthermore, the relationship between fluoride exposure and other hormones may modulate the timing of pubertal development including leptin and IGF-1 (insulin-like growth factor-1) (Chou and Mantzoros 2014; Divall et al. 2010). Previous analysis found that the serum IGF-1 in young adult female rabbits who were given fluoride in drinking

water at 100 mg/L for 6 months increased by 40% (Turner et al. 1997). However, to our knowledge, no studies have explored the association of fluoride with leptin levels.

The above studies have some limitations. Firstly, current epidemiological data are limited by using ecologic design, which examines the relationship with population-level rather than individual-level exposure data. Additionally, available human studies are restricted by the lack fluoride biomarkers. Furthermore, these studies did not provide information on the developmental exposure to fluoride. For example, no analysis has evaluated the association of pubertal development with early life fluoride exposure, such as fluoride exposure during pregnancy. Exposure during pregnancy may be a more sensitive period, playing a critical role in disease susceptibility later in life (Bateson et al. 2004). In addition, current reports have only focused on one pubertal outcome in girls, which is the age at menarche, providing no other physiological indicators to represent the stage of sexual maturation (e.g. development of breast, genitalia, and pubic hair). Finally, most published analyses only reported associations with fluoride in a single sex, thus failing to assess the potential differential gender effects of fluoride on sexual maturation in the same population. No studies of early life exposure to fluoride with hormones linked to puberty have been conducted in children.

To address these gaps, particularly the role of fluoride exposure during developmental periods such as pregnancy, we studied the effects of *in utero* and peripubertal fluoride exposure on various physical markers of pubertal onset along with levels of serum hormones related to puberty at ages 8-15 years among 118 boys and 132 girls by using a birth cohort study conducted in Mexico City.

2.3 Materials and Methods

Study Population

Participants in our study consisted of individuals from the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project. These individuals were recruited at three maternity hospitals (Manuel Gea Gonzalez Hospital, Mexican Social Security Institute and the National Institute of Perinatology) representing low- to middle-income populations in Mexico City, starting from year 1994 to 2006. ELEMENT has followed participants for over two decades to examine the impacts of environmental toxicants in early life on child development of their offspring. In this analysis, we include pregnant women from ELEMENT cohort who were recruited from 1997 to 2004 during their first trimester and followed for 12 months post-partum, offspring were followed up to 5 years during the initial cohort study. Urine and blood samples as well as questionnaires regarding socio-demographic information were collected from mothers during pregnancy. Exclusion criteria have been described elsewhere (Hu et al. 2006).

In 2010, 250 ELEMENT children (8-15 years old at this visit) were re-contacted to participate in a follow-up study based on availability of maternal bio-specimens during pregnancy and included in this analysis. During this follow-up visit, urine and blood samples, anthropometric measures, questionnaires as well as physician-assessed Tanner stages were collected. Research protocols were approved by the research ethics committees of all 3 participating institutions including the University of Michigan, the Mexico National Institute of Public Health, and the University of Indiana. Prior to enrollment, informed consent from mothers and informed assent from their children were obtained.

Exposure Assessment

Pregnant mothers were requested to provide a spot urine sample during each trimester visit. The offspring were also asked to provide a spot urine sample at the time of their sexual maturation examinations at age 8-15. Urine samples of about 5 mL were collected in fluoride-free vials and frozen immediately at the research site. All urine samples were then shipped and stored at -20°C at the Harvard School of Public Health (HSPH), and later shipped and stored at -80°C at the University of Michigan School of Public Health (UMSPH).

These samples were analyzed at the University of Michigan lab and validated by the fluoride laboratory at Indiana University School of Dentistry Oral Health Research Institute, as described previously (Thomas et al. 2016). Urinary fluoride was evaluated by using ion-selective electrode-based diffusion assays following standardized analytic methods described in previous studies (Martinez-Mier et al. 2011), adapted from "Preparation of Standard Solutions for Analysis for Fluoride" and "Fluoride Analyses of Biological and Bob-Biological Samples" (Diffusion Method). Diffusion of fluorides is the process by which fluorides are released by acid hydrolysis, and subsequently concentrated. It is a reliable method for measuring samples where fluoride may be in a covalent or complex form.

All urine samples were analyzed in duplicate, and relative standard deviation (RSD) values were calculated to assess the precision and repeatability. Samples containing fluoride levels ≥0.2mg/L and RSD values >10%, or fluoride levels <0.2mg/L and RSD values >20%, were deemed to have low precision and were removed from analysis. To correct for variations in urine dilution at the time of measurement, specific gravity adjusted urinary fluoride concentrations were obtained for

each urinary sample in children. Specific gravity was evaluated using a handheld digital refractometer (Atago Co., Ltd., Tokyo, Japan). Specific gravity adjusted fluoride (UF_{sg}) in children was obtained by using following formula: $UF_{sg} = UF_{child} * (1.02-1) / (specific\ gravity-1)$. 1.02 is the suggested median of specific gravity (Carrieri et al. 2001), which was consistent with the level in our population. Since specific gravity was not measured at the time of the fluoride analysis, creatinine was used instead for correcting urinary fluoride concentrations in pregnant women. Creatinine in urine was analyzed by using MicroLab AT Plus (Hamilton Co., Reno, NV, USA) and Microplate Spectrophotometer (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA). To obtain creatinine adjusted maternal fluoride (UF_{cr}), urinary fluoride concentration (UF_{mom}) was divided by corresponding urinary creatinine concentration for each trimester (UC_{mom}), and then was multiplied by the mean concentration of urinary creatinine (UC_{mean}) available at each trimester using the formula: $UF_{cr} = (UF_{mom}/UC_{mom}) \times UC_{mean}$ (Thomas et al. 2016). The concentration (mg/L) for UC_{mean} was 103.74 for 1st trimester, 80.68 for 2nd trimester and 75.28 for 3rd trimester.

Outcome Assessment

Tanner staging was used to assess pubertal onset of all female and male participants. Tanner staging of breast and pubic hair growth in girls (Marshall and Tanner 1969) as well as Tanner staging of genitalia, and pubic hair in boys (Marshall and Tanner 1970) were examined by a trained physician with a range of stages from 1 indicating prepuberty to stage 5 indicating fully matured. For instance, pubic hair stage 1 represents no pubic hair. Pubic hair stage>1 represents pubic hair growth from sparse (stage 2) to full development (stage 5) (Marshall and Tanner 1969, 1970). Among girls, breast stage 1 represents only having elevation of papilla. Breast stage>1 represents breast development from bud (stage 2) to full growth (stage 5) (Marshall and Tanner 1969).

Menarche was measured via a self-reported questionnaire, where girls at ages 8-15 years were asked if and when they had initiated menses. Among boys, genital stage 1 represents prepuberty in terms of development status of penis, testes, and scrotum. Genital stage >1 represents the enlargement and change in texture and color of gonads indicating puberty (Marshall and Tanner 1970). Testicular volume was used as an additional indicator of puberty. Right and left testicular volume was determined by comparing with an orchidometer (range 1–25 mL). In the present analysis, the larger volume of the right or left testicles was used. A cutoff of 3 mL was applied to represent no puberty (≤3 mL) vs. puberty (>3 mL) (Mouritsen et al. 2013).

Fasting serum samples for both female and male participants at 8-15 years of age were collected for hormone analysis. All serum samples of sex hormones were measured by the Clinical Ligand Assay Service Satellite (CLASS) Laboratory at the University of Michigan. Total T, estradiol, DHEAS, and SHBG were analyzed by using an automated chemiluminescent immunoassay (Bayer Diagnostics ACS:180). Active inhibin B was measured using Gen II ELISA (Beckman Coulter, Webster, TX). IGF-1 and leptin were measured at Michigan Diabetes Research and Training Center Chemistry Lab. IGF-1 was analyzed by using automated chemiluminescence immunoassay (Immulite 1000), and leptin was measured using RIA (Millipore). Undetectable levels (values below the limit of detection (LOD)) were replaced with the LOD/$\sqrt{2}$ (Ferguson et al. 2014).

Covariates

Height of child was measured by trained nurses using standardized protocols (Habicht 1974; Lohman et al. 1988). Then height was converted to Z-score based on the World Health

Organization (WHO) standard curves for children aged from 5 to 19 years old with adjustment of age and gender (WHO 2007).

Statistical analysis

Descriptive statistics were carried out and distributions of fluoride and hormone levels were examined. All serum hormones were log normally distributed, and were ln-transformed before conducting linear regression analysis. SAS macro program was used to calculate the z scores for height, based on the WHO growth charts adjusted for age and sex (WHO 2006). Covariates were considered in this analysis provided that they are predictors of child's puberty or potential confounders, including child age, BMI and height Z-score, maternal age and education, maternal smoke status, parity, delivery weight and height as well as social economic status. Only variables that were significantly associated with pubertal outcomes were included in the final regression models.

To replace missing values of exposure variables, multiple imputation was applied. Missing values were filled in using regression-based prediction to produce a complete dataset. Conventionally 5-10 imputed datasets are generated, but a larger number is needed when the percent of missing is large (Lee and Simpson 2014), as was the case here. Therefore, the process was repeated 40 times (40 complete datasets) and each time a different value was imputed to reflect uncertainty in the true exposure values. We used PROC MI in SAS to impute the missing values. Each of the 40 complete data sets was then analyzed using linear regression models for hormone analysis, and logistic regression models for pubertal onset. The parameter estimates from each data set were then combined using established formulae to obtain final results (UCLA 2007). We then performed

multivariable logistic regression models to examine the association of creatinine adjusted urinary fluoride at each stage of pregnancy and specific gravity-adjusted urinary fluoride during peripubescence with pubertal onset. The onset of puberty was indicated by Tanner staging for pubic hair ≥2, breast growth≥2, or imitating menses in girls. The pubertal onset of boys was defined by Tanner stage ≥2 for genital growth, by Tanner stage ≥2 for pubic hair development, or having testicular volume >3mL. Results for logistic regression models were reported as the odds of having pubertal onset with 0.5 mg/L increase in urinary fluoride concentration.

Multivariable linear regression models were applied to assess the effect of prenatal and peripubertal urinary fluoride with serum concentration of pubertal hormones. Trimester-specific fluoride and childhood fluoride in urine was used in separate models as primary independent variable, and hormones including estradiol, total testosterone, inhibin B, SHBG, DHEAS, leptin and IGF-1 were considered as dependent variable in each model. Results for linear regression models were reported as the percent difference in serum hormone level in relation to 0.5 mg/L increase in urinary fluoride concentration. We defined statistical significance as $p < 0.05$. We performed SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) to analyze data.

2.4 Results

A total of 132 female subjects and 118 male subjects with various percentages of missing information on selected variables of interest were included in the final analysis (**Table 2.1 & Table 2.2**). Among these participants, 219 had at least one urinary fluoride measured during pregnancy. Due to inadequate sample volume, maternal urinary fluoride levels were missing for 31%-36% of the samples in first trimester (early pregnancy), 38%-45% in second trimester (mid pregnancy)

and 61%-67% in the third trimester (late pregnancy). In addition, a few subjects (0.8%-0.9%) had missing information on serum levels of hormones (**Table 2.2**). The mean age of children in the analysis was 10.3 years. The urinary fluoride levels did not differ significantly by sex (**Table 2.1**).

In our study population, we had an overall low rate of pubertal onset. Among 132 female participants, 98 girls (74.2%) were Tanner stage=1 for pubic hair growth (pre-pubertal), 87 girls (65.9%) were at Tanner stage=1 for breast growth (pre-pubertal), and 102 girls (77.3%) had not started menses (**Table 2.3**). Among 118 male participants, 94 boys (81.7%) were at Tanner stage= 1 for pubic hair growth (pre-pubertal), 57 boys (49.6%) were at Tanner stage=1 for genitalia (pre-pubertal), and 17 boys (14.8%) had testicular volume ≤ 3mL(pre-pubertal) (**Table 2.3**).

We did not find significant associations of urinary fluoride during any stage of pregnancy and during adolescence with the odds of having pubarche, thelarche or menarche in girls (**Table 2.4a**). Similar to our findings in girls, prenatal and peripubertal urinary fluoride were not significantly associated with the odds of pubarche, genitalia and testicular volume (>3mL) in boys (**Table 2.4b**). Despite our lack of significant results, overall, there was a suggestive positive association of female pubertal onset and a suggestive negative association of male pubertal onset with prenatal and peripubertal fluoride levels in urine.

Regarding the fluoride-hormone association, our findings showed that a 0.5 mg/L increase in urinary fluoride concentration during late pregnancy was associated with 10.27% higher serum IGF-1 (95% CI: 3.93%, 17.00%) in girls independent of child's age at visit and height z-scores (Table 2.5a). In boys, we observed a significant negative association of prenatal fluoride in urine

with serum testosterone concentration during peripubescence (**Table 2.5b**). With 0.5 mg/L increase in urinary fluoride during late pregnancy was associated with 33.96% lower serum testosterone concentrations (95% CI: -48.63%, -15.10%) adjusted for child's age at visit and height z-scores. Maternal fluoride concentration in urine during early or mid-pregnancy was not significantly associated with any concurrent serum hormones. No significant results were found using peripubertal measures of urinary fluoride in relation to sex hormones in the adjusted analysis (**Table 2.5b**).

2.5 Discussion

Our results suggest that maternal fluoride exposure during pregnancy, rather than concurrent fluoride exposure during adolescence, may be a predictor of sex and growth hormones linked to pubertal development in children aged from 8-15 years residing in Mexico City. Moreover, the effect of fluoride exposure on pubertal hormones may vary by child sex. No evidence was found in the present analysis supporting the hypothesis that fluoride exposure during sensitive periods significantly impacts the onset of puberty in children, although a suggestive positive association was observed in girls and a negative association was observed in boys.

Overall, our findings are consistent with previous animal and human studies suggesting fluoride can affect the levels of hormones such as testosterone (Ghosh et al. 2002; Ortiz-Perez et al. 2003; Susheela and Jethanandani 1996; Zhou et al. 2013) and IGF-1 (Turner et al. 1997). Notably, our study is the first study showing *in utero* fluoride exposure in the 3rd trimester, was associated with levels of puberty-related hormones in children.

The association between fluoride and physical markers of pubertal onset observed in this study did not reach statistical significance at p=0.05; therefore, we were not able to establish a positive association of fluoride with initiation of puberty as suggested in previous animal and human studies (Farkas et al. 1983; Luke 1997; Schlesinger et al. 1956). The proposed mechanism is that higher fluoride exposures increase the release of gonadotropins and subsequently stimulate sex hormones by reducing the production of melatonin in pineal gland, eventually accelerate the development of puberty in girls (Attanasio et al. 1985; Garcia-Patterson et al. 1996; Waldhauser et al. 1988). The small sample size, the low rate of pubertal onset and the use of spot urine sample may contribute to the inconsistencies observed between our findings and those previous analyses.

This work demonstrated that prenatal fluoride may positively predict levels of male testosterone and negatively predict female IGF-1 during adolescence. Since melatonin is an anti-gonadotropic hormone (Silman et al. 1979), it is possible that fluoride potentially interferes with melatonin production indirectly, but elevates serum gonadotropins secreted by gonadotrope cells of the anterior pituitary (Long 2009; Ortiz-Perez et al. 2003; Tokar and Savchenko 1977). Normally, elevated levels of gonadotropic hormones such as follicle-stimulating hormone (FSH), and luteinizing hormone (LH) cause an increase in the testosterone levels. However, when the ability to produce testosterone is disrupted by fluoride exposure, increased levels of gonadotropins may inhibit elevated testosterone levels (Long 2009). Animal studies also have revealed that melatonin can reduce release of LH and FSH by disrupting hypothalamus and pituitary activity (Martin et al. 1977; Tamarkin et al. 1977). Human evidence showed that melatonin can disturb ovarian and testicular function by working directly on gonads (MacPhee et al. 1975). On the other hand, we found that fluoride during late pregnancy may positively affect serum concentrations of IGF-1 in

girls, which has been found in several animal studies to regulate GnRH (gonadotropin-releasing hormone) neuron functions to advance the timing of pubertal development (Danilovich et al. 1999; Hiney et al. 1996; Keene et al. 2002)

This study has several advantages over the existing human studies. Our analysis featured a longitudinal birth cohort, validated individual biomarkers of fluoride exposure during multiple developmental periods, included physician-examined measures of puberty, and measured testicular volumes and a panel of sex and growth hormones. In addition, our study is one of very few studies that examines the effect of fluoride on pubertal onset in both sexes. Our findings should encourage more epidemiological research to understand the effect of early life exposure to fluoride on the sexual maturation in later life. The study has some limitations. The sample size of our analysis was relatively small, which may restrict the power of this study to detect differences. The age range of the study participants was limited to reflect sensitive windows of fluoride exposure during early childhood. The use of spot urine samples (vs. 24 hour collections) also provides an additional limitation, since fluoride concentration values have been reported to fluctuate at different collection points (Rugg-Gunn et al. 2011). Finally, the high percentage of missing values is also a limitation to this study.

2.6 Conclusions

Our findings are in line with previous evidence suggesting fluoride exposure is a determinant of sex and growth hormones. In this study, we found that prenatal fluoride exposure affects serum levels of testosterone and IGF-1 in a sex-specific fashion. However, no significant associations between fluoride and physical measures of pubertal onset were found. Given that the associations

we observed were confined to the assessment of fluoride in a spot urine sample, small sample size, and a low rate of pubertal onset, these findings need to be confirmed in other analyses.

Table 2.1 Characteristics of the study population[a] from ELEMENT

	Girls				Boys			
Characteristics	N	Percent missing	Mean	SD	N	Percent missing	Mean	SD
Child age (years)	132	0	10.3	1.7	118	0	10.3	1.6
Child height Z-score[b]	132	0	-0.2	0.9	118	0	-0.1	0.8
UF (mg/L) [c]								
Early pregnancy	91	31%	0.8	0.3	76	36%	1.0	0.6
Mid pregnancy	82	38%	0.9	0.4	65	45%	0.9	0.3
Late pregnancy	51	61%	0.8	0.5	37	67%	1.1	0.8
Peripuberty	117	11%	0.7	0.4	110	7%	0.7	0.3

[a] Included 250 children at ages 8-15
[b] Constructed using the 2007 SAS macro WHO growth reference for 5-19 year olds
[c]Adjusting for creatinine for maternal urine; adjusting for specific gravity for peripubertal urine
UF represents urinary fluoride; SD represents standard deviation

Table 2.2 Distribution of serum hormone levels in Mexican children by sex

	Girls										
Hormone	N	Percent missing	Geometric mean	Minimum	5th	25th	50th	75th	90th	95th	Maximum
Estradiol (pg/mL)	131	0.8	26.9	4.2	9.8	16.5	23.0	43.1	70.7	95.7	482.7
Testosterone (ng/dL)	131	0.8	13.6	0.1	0.1	13.4	19.8	29.6	41.0	47.7	73.6
Inhibin B (pg/mL)	131	0.8	25.8	10.0	10.0	11.8	22.4	47.9	86.2	105.5	283.1
SHBG (nmol/L)	131	0.8	61.5	12.6	22.7	39.2	64.0	92.7	119.1	151.6	172.4
DHEAS (μg/dL)	131	0.8	42.7	15.0	15.0	23.2	42.6	74.0	98.9	150.0	214.0
Leptin (ng/ml)	131	0.8	11.0	2.4	3.6	6.3	10.7	18.5	26.8	34.3	62.2
IGF-1(ng/ml)	131	0.8	259.9	102.0	141.0	198.0	250.0	359.0	436.0	468.0	606.0

SHBG, sex hormone binding globulin; DHEAS, dehydroepiandrosterone sulfate; IGF-1, insulin-like growth factor 1

	Boys										
Hormone	N	Percent missing	Geometric mean	Minimum	5th	25th	50th	75th	90th	95th	Maximum
Estradiol (pg/mL)	117	0.9	16.8	3.2	9.1	13.3	16.7	20.1	29.1	32.8	83.5
Testosterone (ng/dL)	117	0.9	20.3	0.1	0.1	11.1	21.3	59.8	273.6	442.3	719.7
Inhibin B (pg/mL)	117	0.9	104.6	20.9	40.4	64.9	103.2	176.5	231.4	251.4	353.4
SHBG (nmol/L)	117	0.9	72.2	18.0	24.9	48.3	76.3	105.2	150.5	162.6	224.1
DHEAS (μg/dL)	117	0.9	52.5	15.0	15.0	32.6	51.0	90.6	130.0	183.0	326.0
Leptin (ng/ml)	117	0.9	6.4	1.4	2.2	3.6	6.5	10.7	16.8	21.8	34.2
IGF-1(ng/ml)	117	0.9	215.4	92.9	124.0	166.0	206.0	268.0	385.0	453.0	568.0

SHBG, sex hormone binding globulin; DHEAS, dehydroepiandrosterone sulfate; IGF-1, insulin-like growth factor 1

Table 2.3 Distribution of secondary sex characteristics by sex

Girls (N=132)			Boys (N=115)		
Secondary sex characteristic	Stage	N (%)	Secondary sex characteristic	Stage	N (%)
Pubic hair	1	98 (74.2)	Pubic hair	1	94 (81.7)
	2 or above	34 (25.8)		2 or above	21 (18.3)
Breast	1	87 (65.9)	Genitalia	1	57 (49.6)
	2 or above	45 (34.1)		2 or above	58 (50.4)
Menarche	Had their first period	30 (22.7)	Testicular volume	>3ml	98 (85.2)

Table 2.4a Odds ratio (95% confidence interval) of pubertal onset with 0.5 mg/L increase in creatinine adjusted prenatal and specific gravity adjusted peripubertal urinary fluoride concentrations in girls with the imputed data set (N=132)

Secondary sex characteristic	UF (mg/l)	Model 1[a] Odds ratio (95% CI)	Model 2[b] Odds ratio (95% CI)
Pubic hair ≥2	Early pregnancy	1.42 (0.66, 3.06)	0.89 (0.28, 2.80)
	Mid pregnancy	2.04 (0.97, 4.82)	1.21 (0.41, 3.54)
	Late pregnancy	1.55 (0.99, 2.45)	1.66 (0.73, 3.79)
	Adolescent	0.97 (0.53, 1.75)	1.67 (0.62, 4.48)
Breast ≥2	Early pregnancy	1.42 (0.76, 2.66)	0.90 (0.34, 2.35)
	Mid pregnancy	1.70 (0.96, 2.99)	0.78 (0.35, 1.71)
	Late pregnancy	1.45 (0.98, 2.14)	1.08 (0.66, 1.77)
	Adolescent	1.06 (0.62, 1.80)	1.28 (0.62, 2.65)
Having started menarche	Early pregnancy	1.36 (0.64, 2.92)	0.95 (0.38, 2.36)
	Mid pregnancy	1.68 (0.91, 3.10)	0.87 (0.41, 1.83)
	Late pregnancy	1.34 (0.91, 1.97)	1.02 (0.62, 1.68)
	Adolescent	0.83 (0.44, 1.60)	1.14 (0.47, 2.75)

[a] Unadjusted odds ratio estimates
[b] Adjusted for adolescent's age at visit and height z-score

Table 2.4b Odds ratio (95% confidence interval) of pubertal onset with 0.5 mg/L increase in creatinine adjusted prenatal and specific gravity adjusted peripubertal urinary fluoride concentrations in boys with the imputed data set (N=118)

		Model 1[a]	Model 2[b]
Secondary sex characteristic	UF (mg/l)	Odds ratio (95% CI)	Odds ratio (95% CI)
Pubic hair ≥2	Early pregnancy	0.86 (0.48, 1.56)	0.70 (0.27, 1.78)
	Mid pregnancy	1.04 (0.49, 2.24)	1.10 (0.31, 3.85)
	Late pregnancy	0.80 (0.54, 1.18)	0.65 (0.26, 1.59)
	Adolescent	0.18 (0.04, 0.76)	0.21 (0.03, 1.60)
Genitalia ≥2	Early pregnancy	1.06 (0.70, 1.60)	1.00 (0.66, 1.50)
	Mid pregnancy	0.89 (0.55, 1.43)	0.86 (0.46, 1.62)
	Late pregnancy	0.86 (0.65, 1.13)	0.79 (0.56, 1.10)
	Adolescent	0.47 (0.23, 0.96)	0.61 (0.27, 1.38)
Testicular volume>3ml	Early pregnancy	1.06 (0.62, 1.79)	1.01 (0.58, 1.74)
	Mid pregnancy	0.90 (0.45, 1.82)	0.88 (0.38, 2.04)
	Late pregnancy	0.89 (0.68, 1.16)	0.85 (0.62, 1.16)
	Adolescent	0.93 (0.39, 2.24)	1.17 (0.45, 3.03)

[a] Unadjusted odds ratio estimates

[b] Adjusted for adolescent's age at visit and height z-score

Table 2.5a Percent difference (95% confidence interval) in serum hormone levels with 0.5 mg/L increase in creatinine adjusted prenatal and specific gravity adjusted peripubertal urinary fluoride concentrations in girls with the imputed data set (N=132)

		Model 1[a]	Model 2[b]
Hormone	UF (mg/l)	%Difference (95%CI)	%Difference (95%CI)
Estradiol	Early pregnancy	17.42 (-10.26, 53.65)	6.40 (-9.62, 25.44)
	Mid pregnancy	20.33 (-2.35, 48.28)	1.59 (-12.52, 17.98)
	Late pregnancy	9.34 (-2.60, 22.73)	-1.18 (-9.02, 7.34)
	Adolescent	-9.70 (-24.91, 8.59)	-6.74 (-17.88, 5.91)
Testosterone	Early pregnancy	13.81 (-23.71, 69.77)	-1.26 (-32.65, 44.76)
	Mid pregnancy	-3.62 (-38.14, 50.18)	-22.15 (-53.83, 31.25)
	Late pregnancy	4.02 (-24.13, 42.61)	-7.08 (-32.55, 28.02)
	Adolescent	-15.60 (-43.31, 25.67)	-7.37 (-36.31, 34.71)
Inhibin B	Early pregnancy	-1.19 (-23.63, 27.85)	-9.33 (-28.97, 15.75)
	Mid pregnancy	20.45 (-2.14, 48.25)	3.94 (-11.97, 22.72)
	Late pregnancy	19.05 (4.07, 36.18)	10.40 (-0.15, 22.06)
	Adolescent	8.10 (-11.27, 31.71)	10.08 (-5.89, 28.76)
SHBG	Early pregnancy	-2.24 (-17.05, 15.21)	3.32 (-10.59, 19.39)
	Mid pregnancy	-3.42 (-16.98, 12.35)	1.26 (-15.50, 21.33)
	Late pregnancy	-9.66 (-17.55, -1.02)	-7.17 (-15.64, 2.16)
	Adolescent	5.29 (-8.24, 20.81)	0.63 (-11.34, 14.21)
DHEAS	Early pregnancy	-0.99 (-18.93, 20.91)	-11.83 (-23.01, 0.98)
	Mid pregnancy	11.53 (-9.84, 37.97)	-4.80 (-20.27, 13.67)

	Late pregnancy	7.83 (-4.37, 21.59)	-1.92 (-11.19, 8.32)
	Adolescent	-12.92 (-27.13, 4.07)	-7.90 (-18.90, 4.59)
Leptin	Early pregnancy	20.29 (-1.60, 47.04)	13.83 (-5.38, 36.94)
	Mid pregnancy	9.37 (-9.54, 32.23)	4.08 (-17.17, 30.78)
	Late pregnancy	0.15 (-11.07, 12.79)	-4.28 (-15.11, 7.94)
	Adolescent	-9.12 (-23.38, 7.80)	-4.61 (-18.74, 11.97)
IGF-1	Early pregnancy	2.36 (-8.70, 14.77)	-3.37 (-11.72, 5.78)
	Mid pregnancy	4.79 (-4.92, 15.49)	-3.87 (-11.04, 3.88)
	Late pregnancy	**14.57 (6.48, 23.27)**	**10.27 (3.93, 17.00)**
	Adolescent	3.46 (-6.26, 14.20)	6.63 (-1.01, 14.85)

[a] Unadjusted percent difference
[b] Adjusted for adolescent's age at visit and height z-score

Table 2.5b Percent difference (95% confidence interval) in serum hormone levels with 0.5 mg/L increase in creatinine adjusted prenatal and specific gravity adjusted peripubertal urinary fluoride concentrations in boys with the imputed data set (N=118)

Hormone	UF (mg/l)	Model 1[a] %Difference (95%CI)	Model 2[b] %Difference (95%CI)
Estradiol	Early pregnancy	-5.30 (-12.96, 3.03)	-6.03 (-12.82, 1.28)
	Mid pregnancy	-3.72 (-17.80, 12.77)	-3.47 (-15.84, 10.73)
	Late pregnancy	-1.23 (-5.87, 3.63)	-1.01 (-5.82, 4.04)
	Adolescent	-3.50 (-16.06, 10.95)	2.78 (-9.93, 17.28)
Testosterone	Early pregnancy	-2.08 (-36.67, 51.41)	-6.72 (-28.92, 22.40)
	Mid pregnancy	-18.63 (-49.29, 30.55)	-14.79 (-45.34, 32.84)
	Late pregnancy	**-34.58 (-50.54, -13.47)**	**-33.96 (-48.63, -15.10)**
	Adolescent	-54.19 (-74.57, -17.47)	-29.69 (-56.03, 12.44)
Inhibin B	Early pregnancy	3.25 (-9.22, 17.43)	2.14 (-6.50, 11.57)
	Mid pregnancy	-5.61 (-24.32, 17.72)	-6.23 (-23.29, 14.62)
	Late pregnancy	-3.05 (-10.16, 4.62)	-2.94 (-10.25, 4.97)
	Adolescent	-7.67 (-23.38, 11.25)	5.19 (-8.46, 20.89)
SHBG	Early pregnancy	-1.08 (-10.53, 9.36)	0.03 (-9.21, 10.21)
	Mid pregnancy	0.54 (-15.85, 20.13)	-1.54 (-17.70, 17.80)
	Late pregnancy	-0.39 (-7.89, 7.71)	-1.15 (-7.33, 5.44)
	Adolescent	21.54 (2.91, 43.64)	10.64 (-4.72, 28.48)
DHEAS	Early pregnancy	2.71 (-11.01, 18.55)	1.14 (-8.37, 11.64)
	Mid pregnancy	-9.40 (-24.43, 8.62)	-8.76 (-22.28, 7.10)
	Late pregnancy	-3.28 (-13.39, 8.02)	-2.77 (-10.30, 5.39)

Leptin	Adolescent	-4.57 (-23.38, 18.85)	12.26 (-5.57, 33.46)
	Early pregnancy	1.18 (-11.30, 15.42)	0.82 (-11.83, 15.30)
	Mid pregnancy	-15.59 (-31.56, 4.10)	-14.83 (-31.32, 5.62)
	Late pregnancy	2.12 (-9.44, 15.17)	2.68 (-8.96, 15.80)
IGF-1	Adolescent	-4.71 (-23.69, 18.99)	-3.01 (-22.48, 21.35)
	Early pregnancy	-0.12 (-8.29, 8.78)	-1.14 (-6.53, 4.57)
	Mid pregnancy	7.81 (-5.78, 23.36)	9.65 (-1.21, 21.71)
	Late pregnancy	-1.72 (-6.26 3.04)	-1.25 (-4.87, 2.51)
	Adolescent	-15.07 (-24.39, -4.60)	-6.92 (-14.69, 1.56)

[a] Unadjusted percent difference
[b] Adjusted for adolescent's age at visit and height z-score

CHAPTER 3

Maternal Intake of Micronutrients during Pregnancy in Relation to Sexual Maturity in Mexican Children

3.1 Abstract

Background: Alterations in the timing of puberty has been associated with long-term health implications. Researchers have suggested that maternal nutrition may play an important role in the timing of sexual maturation in offspring, potentially by programming the development of physical growth and the reproductive axis during fetal life. A few studies have examined the association of micronutrients with onset of puberty, while studies of prenatal micronutrients and their influence on pubertal development more broadly are limited.

Objective: Utilizing a longitudinal cohort study in Mexico City, we explored the association between maternal dietary intake of various micronutrients during each trimester and pubertal development in 400 children at ages 9-17.

Methods: Daily intake of micronutrients was estimated using a validated semi-quantitative FFQ collected from women during pregnancy from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) birth cohort study. Pubertal development of offspring aged 9-17 was assessed by a trained physician using Tanner staging for breast and pubic hair development and by self-reported questionnaire with regard to menarcheal status in girls. Pubertal development was

assessed by a trained physician using Tanner staging for development of genitalia and pubic hair growth and by measuring volume of testicle in boys. Trimester-specific multiple logistic regression models and ordinal regression models were used to capture the association of prenatal micronutrient intake on sexual maturity in boys and girls, controlling for maternal energy intake, child age and height Z-score.

Results: Maternal dietary intake of selenium (OR=1.57, 95% CI:1.08-2.28) and zinc (OR=1.52, 95% CI:1.06-2.18) during the 2nd trimester was associated with increased odds of a higher stage of pubic hair growth in male participants corresponding to a 11.1 μg increase in selenium and 1.3 mg increase in zinc, respectively. Prenatal intake of phosphorus (OR=1.72, 95% CI:1.17-2.52) and riboflavin (OR=1.60, 95% CI:1.09-2.34) during the 2nd trimester was associated with increased odds of a higher stage of genital development in boys per 172.2 mg increase in zinc and per 0.4 mg increase in riboflavin, respectively. No significant associations were found in girls.

Conclusions: In this population, prenatal consumption of micronutrient may have long-term effects on the tempo of sexual maturation in boys. Mid-pregnancy was identified as a more vulnerable period to the effects on puberty in relation to micronutrient consumption compared with other stages during pregnancy. Future work is needed to confirm our findings and improve current knowledge of the role of micronutrients in child development.

3.2 Introduction

Puberty, the transitional period between childhood and adult sexual maturity, is considered a complex process involving a series of biological events. Changes in the timing of pubertal onset have been observed over the past few decades. Studies from American and European countries have reported a trend towards earlier menarche and breast development in girls, as well as earlier genital development in boys (Sorensen et al. 2012). Altered pubertal timing has received special attention due to its implications for development of hormone-related cancers and chronic diseases in later life (Golub et al. 2008).

Identifying elements associated with perturbations in pubertal timing may improve the understanding of these diseases, and it is of public health importance to understand modifiable factors particularly early in life such as maternal nutrition affecting the onset of sexual maturation. Nutrition during pregnancy, which has been shown to influence intrauterine growth (Robinson 1999) and prenatal development of the hypothalamic–pituitary–gonadal axis (Brooks et al. 1992; Caldani et al. 1995; McNatty et al. 1995), may have long-term effects on sexual maturation later in life (Da Silva et al. 2002; Rhind et al. 2001; Wu et al. 2004).

The majority of existing studies have focused on the association of dietary macronutrient intake with the timing of puberty while only a few have examined the role of micronutrient intake in the timing of sexual development. Most of these investigations were aimed at the influence of vitamins and trace minerals during childhood, primarily on the age at menarche. A cohort study of 213 girls from middle-income towns in Massachusetts, who were followed up from 1984-1988, reported that intakes of vitamin A, B12, C, and thiamin were related to earlier age at menarche (Maclure et

al. 1991). In a Korean population, both daily intakes of thiamine and riboflavin were found to be higher in 433 menarcheal girls than 187 pre-menarcheal girls aged from 10 to 19 years (Cho et al. 2010). Previous analysis found that dietary intake of zinc was associated with earlier age at menarche in 3298 British girls who participated in the Avon Longitudinal Study of Parents and Children (ALSPAC) (Rogers et al. 2010). Positive associations with intake of vitamin C were reported using data from 2299 girls in the Quebec City area aged from 10-13 years (Moisan et al. 1990a).

Contrary to these findings, negative associations of menarcheal age with intake of vitamin A were reported in a nested case-control study performed among 666 Canadian girls (Moisan et al. 1990b). One U.S. study conducted in 230 girls residing in southern California showed the intake of thiamine (2.1-6.1 mg/d) and iron (16-40 mg/d) in the upper quartile was associated with a 7-8 months later menarcheal age than in the lowest quartile of intake of thiamine (0-1.25 mg/d) and iron (0-10 mg/d) aged 9–15 years (Kissinger 1987).

Other than menarcheal age, a few studies have centered on the effects of micronutrients using different pubertal indicators. Several earlier studies of the role of selenium in child sexual maturity reported that zinc supplementation led to the acceleration of genital development in 22 Egyptian village boys (Sandstead et al. 1967) and in a larger population of 279 village boys in Egypt with dwarfism and hypogonadism (Carter et al. 1969), and the improvement of breast development based on 2 case studies of Iranian girls (Ronaghy and Halsted 1975). In addition, recent animal data related maternal dietary selenium supplementation to several ovarian characteristics and reproductive function in 44 ewe lambs (Grazul-Bilska et al. 2014).

Although the possible mechanisms of action involved in these relationships remain unclear, it is possible that micronutrients can influence pubertal development by affecting the levels of puberty-related hormones and/or levels of body fat, which have been related to puberty (Copeland et al. 1982; German et al. 2015; Hiney et al. 1991; Hiney et al. 1996; Kaplowitz 2008; Luna et al. 1983; Siervogel et al. 2003; Wolfe et al. 2014).

Taken together, existing findings of the influence of micronutrients on puberty are equivocal and studies of dietary intake of these nutrients during early life are quite sparse. Therefore, research is needed to better understand the potential long-term effects of maternal consumption of micronutrients on sexual development in offspring.

Therefore, the objective of the present study was to determine the association of pubertal development with maternal dietary intake of targeted vitamins and trace minerals during each stage of pregnancy in a Mexican population of both boys and girls aged 9-17. Micronutrients included in this analysis were based on their associations with predictors of sexual maturity such as hormones related to puberty including IGF-1 (insulin-like growth factor 1) and leptin, and body composition. Trace minerals such as selenium, zinc, phosphorus and iron, and vitamins such as vitamins A, C, D and B vitamins were targeted for this analysis based on their associations with IGF-1, leptin and/or body fat mass.

3.3 Materials and Methods

Study population

Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) is an ongoing longitudinal cohort that recruited pregnant women at three maternity hospitals in Mexico City (Manuel Gea Gonzalez Hospital, Mexican Social Security Institute and the National Institute of Perinatology) representing population of low to medium socioeconomic status starting from the year 1994 to 2006 (Ettinger et al. 2009; Hu et al. 2006; Tellez-Rojo et al. 2004). We have followed up these pregnant participants for over two decades in order to understand the influence of environmental factors on the development of their offspring in later life. In our study, we included participants who were enrolled from 1997 to 2004 during their early pregnancy and followed until 12 months post-partum. Questionnaires regarding dietary intake of nutrients and socio-demographic information were collected from pregnant participants. A follow-up investigation among 400 Mexican mothers were enrolled in previous ELEMENT studies was conducted in 2015. During this visit, anthropometric measures, self-reported questionnaires of menarcheal age as well as data on physician-assessed Tanner stages were obtained. Research protocols were approved by the research ethics committees of both participating institutions including the University of Michigan and the Mexico National Institute of Public Health. Prior to enrollment, both mothers and their offspring provided consent for participation. Detailed information about exclusion criteria in this investigation has been described elsewhere (Gonzalez-Cossio et al., 1997).

Dietary intake

Daily dietary energy and micronutrient intakes of pregnant women were estimated using a semi-quantitative food frequency questionnaire (FFQ) administered by trained personnel at each visit.

This questionnaire, for evaluating the usual dietary intake over the previous month including pregnancy specifically for Mexican women, has been validated in this population previously (Hernandez-Avila et al. 1998) using methods adopted in the Harvard Nurses' Health Study and Health Professionals Study (Willett et al. 1985; Willett et al. 1987).

One hundred and sixteen foods based on the most common urban Mexican diet were used (Cantoral et al. 2015). Values used to assess the frequency of usual intake range from "0" to "6 or more times per day," which was then converted to a daily intake. The micronutrient component of each item was obtained using food tables from the US Department of Agriculture (USDA) and the Mexican National Institute of Nutrition and compiled by National Institute of Public Health of Mexico (INSP) Center for Research in Nutrition and Health (CINyS) (Cantoral et al. 2015). The daily intake of total energy (kcal) and of these micronutrients in the diet was calculated using a program developed by INSP staff by multiplying nutrient content in each food item with the frequency of reported usual intake of that item (servings per day) and then summing over the all food items containing that specific nutrient (Arora et al. 2008). All the micronutrients derived from the FFQ and food tables were adjusted for total energy intake using nutrient residual model described previously (Willett et al. 1997) to control for confounding effects and to remove extraneous variation. In this process, we regressed daily intakes of micronutrients of all the mothers on their total energy intakes. The residuals generated from the linear regression models demonstrate the differences between each mother's actual daily intake and the predicted intake of these nutrients by their total energy intakes (Willett et al. 1997)

Pubertal measurement

Sexual maturity of girls was evaluated by both Tanner staging of breast (thelarche) and pubic hair growth (pubarche) (Marshall and Tanner 1969) as well as self-reported menarche status. In boys, pubertal development was assessed using Tanner staging of genitalia, and pubarche (Marshall and Tanner 1970) as well as testicular volume. Tanner staging of sexual development of all the participants was examined by a trained clinician with scales ranging from 1 (no sexual development) to stage 5 (full sexual development). Specifically, Stage of pubarche=1 indicates no pubarche. Stage of pubarche>1 indicates pubic hair growth from sparse (stage 2) to full development (stage 5) (Marshall and Tanner 1969, 1970). Stage of thelarche=1 indicates girls merely having elevation of papilla. Stage of thelarche>1 indicates breast enlargement from bud (stage 2) to fully matured (stage 5) (Marshall and Tanner 1969). Stage of genitalia=1 indicates no development in terms of growth of penis, testes, and scrotum. Stage of genitalia>1, during which the appearance of enlargement and changes in texture and color of gonads indicating full development (Marshall and Tanner 1970). Menarche was measured via self-reported questionnaire, where girls were asked if they have started menses at the time of visit. Right and left testicular volume, as another indicator of male sexual maturation, was measured using an orchidometer (range 1–25 mL). In this study, the larger volume of the right and left testicles was used. A cutoff of 20 mL was applied to indicate onset (>3 mL and <20mL) vs. matured (≥ 20 mL) (Mouritsen et al. 2013).

Covariates

Child height was measured by trained staff using standardized protocols at the time of visit (Lohman et al. 1988). Child age was also collected at the same time as the measurement of pubertal

development. Height for age Z-score of all the children then was achieved by using the World Health Organization (WHO) standard curves specifically for children aged 5-19 years old adjusting for their age and sex (WHO 2007).

Statistical analysis

Descriptive statistics (mean and SD) were calculated for all variables. A list of variables suspected to be potential confounders based on their biological plausibility include maternal education and age, gestational age, delivery weight, breastfeeding duration, smoking status and maternal bone lead during pregnancy based on previous analysis done by our group members. Only factors that were significantly associated with any indicator of sexual maturation in bivariate analyses were included in the final analysis. Taken together, we only included child's age and height for age Z-score in our adjusted regression models.

Multivariable logistic and ordinal regression models were applied to examine the associations of maternal dietary intake of trace minerals, including selenium, zinc, iron and phosphorus as well as several vitamins including vitamin A, thiamine, riboflavin, vitamin B6, B12, vitamin C, D and E during pregnancy with indices of pubertal development in both sexes; we adjusted for maternal energy intake alone and fully adjusted for maternal energy intake, child age, and height Z-score. The maternal usual daily intakes of micronutrients during each trimester of pregnancy were used as the primary predictors. For girls, we used menarcheal status and ordinal Tanner staging (stage 1, stage 2 or 3, stage 4 or 5) for breast and pubic hair growth as the outcome variables. For boys, the amount of testicular volume (onset versus matured) and ordinal Tanner staging (stage 1, stage 2 or 3, stage 4 or 5) for genital and pubic hair growth was considered as the outcome variables.

We defined statistical significance as $p < 0.05$. We performed SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) to analyze all the data.

3.4 Results

A total of 400 participants were included in this analysis, 205 girls and 195 boys. The mean values for child age were 13.7 and 14 years for girls and boys, respectively. The maternal consumption of micronutrient during pregnancy did not differ significantly by sex or by the stage of pregnancy (**Table 3.1**).

Table 3.2 shows the distributions of clinician-assessed Tanner stages and testicular volume in boys and distributions of physician-observed Tanner stages and self-reported menarcheal status in girls. Among 199 girls with available data on puberty, 9.1% had not started pubarche, 6.0% had not started thelarche and 22.7% had not initiated menses. Among 185 boys with available data on puberty, 25.4% had not initiated pubarche, 6.5% had not started development of genitalia, and 33.0% had testicular volume <20 mL.

Our data showed no significant association between maternal consumption of micronutrients during pregnancy and sexual maturation in girls (**Table 3.3**). However, we observed a positive association in boys. We found that prenatal dietary intake of selenium and zinc during 2nd trimester was positively associated with the development of pubic hair growth (selenium: OR (95% CI): 1.57 (1.08-2.28); zinc: 1.52 (1.06-2.18)) (**Table 3.4**). These associations correspond to a one standard deviation (SD) increase in selenium and zinc intake, respectively. Positive associations were also found for phosphorus (OR=1.72, 95% CI:1.17-2.52) and riboflavin (OR=1.60, 95%

CI:1.09-2.34) consumption during 2nd trimester and development of genitalia in boys at age 9-17 independent of maternal energy intake, child age and height Z-scores **(Table 3.4)**. Contrary to positive associations observed for 3 minerals and 1 vitamin, we found that intake of prenatal vitamin B6 during 3rd trimester negatively predicted the development of genitalia in male participants **(Table 3.4).**

3.5 Discussion

This analysis showed that higher prenatal intakes of micronutrients were associated with accelerated sexual maturation in Mexican boys aged 9-17 years. These associations were not found in Mexican girls, suggesting differential effects on pubertal development by sex. In this analysis, we did not observe any relationships between prenatal intakes of micronutrients and menarche.

Overall, our findings provided support for the association of maternal micronutrient intake during pregnancy with pubertal development in boys. To the best of our knowledge, this is the first cohort study suggesting sexual development of offspring may be altered by maternal prenatal consumption of micronutrients, and *in utero* is a possible critical window of development. In addition, 2nd trimester was identified as the most sensitive period compared with other trimesters. It is possible because 2nd trimester is the period when major fetal organs are formed. These findings are biologically plausible since studies from a wide range of scientific disciplines provide strong evidence that prenatal and early postnatal factors, particularly nutrition, play a vital role in the programming of reproductive function (Gunn RG 1995; Rhind et al. 2001). Previous animal data have revealed that maternal nutrition alters the development of the fetal reproductive axis, and then subsequently change the timing of pubertal onset in lambs (Da Silva et al. 2001).

Specifically, in terms of the biological pathways, it is well known that the growth of pubic hair is highly androgen-dependent, primarily regulated by dehydroepiandrosterone (DHEA)/ dehydroepiandrosterone sulfate (DHEA-S) (Auchus and Rainey 2004). Previous investigations suggested that DHEA may reduce body fat mass and increase muscle mass in normal men (Nestler et al. 1988). Other studies found that the effects of DHEA-S on body fat distribution were sex-related by showing a negative association of DHEA-S levels with visceral fat in men and with abdominal body fat in women (Hernandez-Morante et al. 2008). There is evidence that concurrent selenium, and zinc supplementation has an effect on body composition by decreasing body mass index (BMI), waist circumference (WC) and percentage of body fat (%BF) in adults (Spina et al. 2013; Wang et al. 2016) and increasing FFM in children, respectively (Gunanti et al. 2016). Taken together, it is reasonable for us to hypothesize that these micronutrients may increase DHEA/DHEAS levels by reducing body fat mass, eventually leading to an accelerated pubic hair growth in children. Finally, it has been recognized that the body composition of pre-pubertal children varies by sex. Before the onset of puberty, compared to boys with the same age, weight and height, girls have lower FFM and higher %BF (Mast et al. 1998). This may explain the differential effect of maternal micronutrient intake on pubic hair growth by sex in this study.

In addition, other researchers have illuminated the possible pathway involved with genital development. For example, a cross-sectional study showed that intake of phosphorus is associated with increased IGF-1 (insulin-like growth factor-1) levels in a population of middle-old aged male health professionals (Giovannucci et al. 2003). Another cross-sectional analysis of 2109 European women reported a positive relationship between serum IGF-1 and intake of riboflavin (Norat et al. 2007), while no studies have examined such relationship in men. Furthermore, IGF-1 has been

suggested to promote the development of puberty with increased testosterone levels in serum, pituitary LH (luteinizing hormone) content (Pazos et al. 1999), and testicular growth (Keene et al. 2002) in male mice. Therefore, it is possible that intake of phosphorus and possibly riboflavin positively predict the development of genitalia through upregulating IGF-1 levels in serum. However, the precise underlying mechanism remains largely unknown.

However, given that we were aiming at examining the potential developmental effects of trace mineral and vitamins during pregnancy on pubertal development, our study is not quite comparable to previous studies regarding the relationship of contemporaneous micronutrients and puberty. As far as we know, only one animal study (Grazul-Bilska et al. 2014) looking at maternal dietary intake of selenium supplements suggested possible influence on ovarian characteristics and reproductive functions in offspring. In the present study, we did observe associations of prenatal selenium intake with pubic hair development in boys, showing potential effects of selenium during early life on the sexual maturity for the first time. We also found some evidence that dietary zinc, phosphorus, and riboflavin are predictors of male pubertal development. However, we did not observe any associations between vitamin A, thiamine or vitamin C with puberty, which have been suggested by previous cross-sectional analyses (Cho et al. 2010; Kissinger 1987; Maclure et al. 1991; Moisan et al. 1990a, b). The use of different study design, physical makers, exposure periods and quantitative research method used in nutritional assessment may contribute to the inconsistency between our findings and those of other researchers.

In addition, our study has several advantages compared to previous studies. For the first time, we examined dietary intake of various micronutrients estimated using a validated FFQ in the Mexican

population among women of childbearing age. Our analysis provides insights into how micronutrient intake during pregnancy may influence sexual maturity in both boys and girls. Unlike the majority of previous reports focusing on menarche only, we were able to demonstrate the impact of these micronutrients on various indexes of pubertal development such as the development of pubic hair, breast, genitalia and testicular volume. One limitation of this study is the relatively small sample size, which may affect the power of this study to detect meaningful differences. Another potential limitation of our analysis is we do not have biomarkers of these nutrients (e.g. plasma) to establish the link between dietary intake and nutrient status in this population.

3.6 Conclusions

In summary, we found that maternal consumption of micronutrients during 2nd trimester was predictive of accelerated pubertal development in male participants. Additional studies with a larger sample size and biomarkers of micronutrient status are warranted to confirm this finding and to better understand the early programming of nutritional factors and their effects on puberty. If future studies confirm this finding, effective strategies should be identified to closely monitor micronutrient levels during pregnancy.

Table 3.1 Characteristics by sex at ages 9-17 among 400 Mexican children

	Girls			Boys		
Variable	N	Mean	SD	N	Mean	SD
Child characteristics						
Age (years)	205	13.7	2.0	195	14.0	2.0
Height z-score[1]	205	-0.3	0.9	195	-0.1	0.9
Maternal prenatal intake of energy adjusted micronutrients						
Selenium (μg)						
T1	175	44.7	12.3	173	44.5	12.9
T2	175	45.6	11.7	174	44.5	11.1
T3	171	44.9	11.6	167	44.6	11.7
Zinc (mg)						
T1	175	9.4	1.7	173	9.3	1.3
T2	175	9.5	1.6	174	9.5	1.3
T3	171	9.5	1.6	167	9.4	1.4
Iron (mg)						
T1	175	12.8	3.6	173	12.7	2.8
T2	175	12.8	3.1	174	13.3	3.1
T3	171	12.9	3.4	167	12.7	3.1
Phosphorus (mg)						
T1	175	1377.9	199.7	173	1382.4	184.2
T2	175	1438.5	208.0	174	1438.1	172.2
T3	171	1446.0	211.9	167	1467.0	199.2
Vitamin A (IU)						
T1	175	7921.2	3772.3	173	8039.2	3675.4
T2	175	7514.8	3643.2	174	8037.0	3508.4
T3	171	7736.1	3801.9	167	7543.1	3882.8
Vitamin B1 (Thiamine) (mg)						

T1	175	1.4	0.4	173	1.4	0.3
T2	175	1.4	0.3	174	1.4	0.3
T3	171	1.5	0.4	167	1.4	0.3
Vitamin B2 (Riboflavin) (mg)						
T1	175	1.9	0.5	173	1.9	0.4
T2	175	2.0	0.4	174	2.1	0.4
T3	171	2.1	0.5	167	2.1	0.4
Vitamin B6 (mg)						
T1	175	1.9	0.5	173	1.9	0.3
T2	175	1.9	0.4	174	2.0	0.4
T3	171	2.0	0.4	167	1.9	0.4
Vitamin B12 (μg)						
T1	175	5.6	1.6	173	5.4	1.5
T2	175	5.7	1.5	174	5.7	1.4
T3	171	5.8	1.6	167	5.8	1.5
Vitamin C (mg)						
T1	175	178.8	79.7	173	178.6	78.2
T2	175	170.3	91.0	174	168.1	62.6
T3	171	170.6	84.8	167	160.8	68.5
Vitamin D (IU)						
T1	175	32.2	27.9	172	27.9	27.4
T2	175	31.7	28.4	173	26.7	24.2
T3	171	27.6	25.8	167	27.5	26.8
Vitamin E (mg)						
T1	175	8.0	3.0	173	8.0	2.7
T2	175	8.1	2.9	174	8.6	2.8
T3	171	7.9	2.6	167	7.9	2.6

[1]Constructed using the 2007 SAS macro WHO growth reference for 5-19 year olds
SD = standard deviation; T1= 1st trimester; T2= 2nd trimester; T3= 3rd trimester

Table 3.2 Distribution of secondary sex characteristics by sex

	Girls (n=199)			Boys (n=185)		
Secondary sex characteristic	Stage	N	Percentage %	Stage	N	Percentage %
Pubic hair	1	18	9.1	1	47	25.4
	2 or 3	109	54.8	2 or 3	73	39.5
	4 or 5	72	36.2	4 or 5	65	35.1
Genitalia				1	12	6.5
				2 or 3	65	35.1
				4 or 5	108	58.4
Testicular volume (TV)				TV<20mL	61	33.0
				TV≥20mL	124	67.0
Breast	1	12	6.0			
	2 or 3	88	44.2			
	4 or 5	99	49.8			
Menarche	Having started menarche	161	79.3			

Table 3.3 Odds ratio (95% confidence interval) of female pubertal development per standard deviation increase in prenatal intake of trace minerals and vitamins during pregnancy

	Pubic hair		Breast		Menarche	
Prenatal nutrient intake	Model 1[1]	Model 2[2]	Model 1[1]	Model 2[2]	Model 1[1]	Model 2[2]
Selenium (μg)						
T1	0.87 (0.64, 1.18)	0.84 (0.57, 1.21)	0.88 (0.66, 1.18)	0.79 (0.54, 1.15)	0.83 (0.58, 1.19)	0.76 (0.47, 1.21)
T2	0.87 (0.64, 1.19)	0.87 (0.60, 1.27)	0.97 (0.72, 1.31)	1.07 (0.73, 1.58)	0.72 (0.50, 1.04)	0.76 (0.48, 1.20)
T3	0.82 (0.60, 1.11)	0.80 (0.55, 1.16)	0.83 (0.61, 1.13)	0.81 (0.54, 1.20)	0.87 (0.61, 1.25)	1.02 (0.63, 1.66)
Zinc (mg)						
T1	0.71 (0.52, 0.97)	0.89 (0.60, 1.32)	0.79 (0.58, 1.08)	1.04 (0.72, 1.52)	0.74 (0.52, 1.06)	0.87 (0.56, 1.35)
T2	0.74 (0.54, 1.01)	1.01 (0.68, 1.51)	0.81 (0.59, 1.10)	1.21 (0.82, 1.81)	0.72 (0.50, 1.04)	0.88 (0.56, 1.40)
T3	0.66 (0.48, 0.92)	0.86 (0.58, 1.29)	0.77 (0.56, 1.06)	1.13 (0.76, 1.68)	0.70 (0.47, 1.04)	0.92 (0.58, 1.46)
Iron (mg)						
T1	1.00 (0.99, 1.00)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (0.99, 1.00)	1.00 (0.99, 1.01)
T2	1.00 (0.99, 1.00)	1.00 (0.99, 1.01)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (0.99, 1.00)	1.00 (0.99, 1.01)
T3	1.00 (0.99, 1.00)	0.99 (0.99, 1.00)	1.00 (1.00, 1.01)	1.00 (0.99, 1.01)	1.00 (0.99, 1.00)	1.00 (0.99, 1.00)
Phosphorus (mg)						
T1	0.93 (0.69, 1.27)	1.10 (0.76, 1.58)	0.99 (0.74, 1.33)	1.15 (0.80, 1.65)	0.85 (0.59, 1.22)	0.92 (0.60, 1.41)
T2	0.93 (0.69, 1.27)	0.96 (0.67, 1.39)	1.13 (0.83, 1.54)	1.24 (0.86, 1.80)	0.90 (0.62, 1.30)	0.97 (0.60, 1.56)
T3	0.84 (0.62, 1.13)	0.69 (0.47, 1.01)	1.05 (0.78, 1.41)	1.00 (0.68, 1.46)	0.86 (0.59, 1.23)	0.83 (0.54, 1.28)
Vitamin A (IU)						
T1	1.20 (0.89, 1.63)	1.19 (0.83, 1.69)	1.07 (0.80, 1.44)	0.99 (0.70, 1.41)	1.19 (0.80, 1.77)	1.22 (0.73, 2.05)
T2	1.02 (0.76, 1.39)	0.81 (0.57, 1.16)	0.94 (0.70, 1.27)	0.75 (0.52, 1.09)	0.99 (0.69, 1.41)	0.92 (0.58, 1.47)
T3	1.15 (0.85, 1.55)	0.96 (0.65, 1.39)	1.12 (0.83, 1.51)	0.96 (0.63, 1.44)	0.97 (0.68, 1.38)	0.81 (0.51, 1.30)
Vitamin B1 (Thiamine) (mg)						
T1	0.90 (0.66, 1.25)	1.04 (0.70, 1.54)	1.00 (0.73, 1.37)	1.21 (0.83, 1.78)	1.19 (0.80, 1.79)	1.56 (0.89, 2.71)
T2	0.92 (0.71, 1.19)	1.07 (0.78, 1.47)	0.87 (0.67, 1.13)	1.05 (0.75, 1.47)	0.92 (0.67, 1.27)	1.06 (0.71, 1.59)

T3	0.72 (0.51, 1.00)	0.93 (0.62, 1.40)	0.85 (0.61, 1.17)	1.28 (0.82, 1.98)	0.89 (0.60, 1.30)	1.03 (0.65, 1.61)
Vitamin B2 (Riboflavin) (mg)						
T1	0.83 (0.60, 1.14)	0.97 (0.65, 1.44)	0.93 (0.68, 1.27)	1.14 (0.78, 1.66)	0.92 (0.63, 1.34)	1.09 (0.69, 1.73)
T2	0.91 (0.69, 1.19)	0.99 (0.72, 1.37)	1.01 (0.77, 1.32)	1.19 (0.85, 1.66)	0.91 (0.65, 1.26)	1.06 (0.68, 1.63)
T3	0.73 (0.53, 1.00)	0.76 (0.51, 1.12)	0.87 (0.63, 1.19)	1.00 (0.66, 1.51)	0.86 (0.59, 1.25)	0.94 (0.61, 1.45)
Vitamin B6 (mg)						
T1	0.86 (0.64, 1.16)	0.98 (0.68, 1.42)	0.90 (0.67, 1.20)	1.03 (0.72, 1.47)	0.88 (0.63, 1.24)	0.98 (0.63, 1.53)
T2	0.92 (0.70, 1.22)	1.08 (0.77, 1.52)	0.85 (0.65, 1.12)	1.04 (0.74, 1.46)	0.92 (0.66, 1.28)	1.07 (0.71, 1.60)
T3	0.78 (0.58, 1.05)	0.85 (0.58, 1.23)	0.86 (0.65, 1.15)	1.06 (0.74, 1.51)	0.93 (0.66, 1.30)	1.10 (0.71, 1.69)
Vitamin B12 (μg)						
T1	0.86 (0.64, 1.17)	0.95 (0.66, 1.38)	0.97 (0.72, 1.31)	1.14 (0.79, 1.64)	0.72 (0.50, 1.02)	0.73 (0.47, 1.14)
T2	0.93 (0.68, 1.26)	1.02 (0.70, 1.50)	1.07 (0.79, 1.45)	1.35 (0.91, 1.99)	0.70 (0.48, 1.02)	0.78 (0.50, 1.22)
T3	0.73 (0.53, 1.01)	0.74 (0.50, 1.10)	0.82 (0.60, 1.12)	0.93 (0.63, 1.37)	0.78 (0.54, 1.13)	1.04 (0.65, 1.65)
Vitamin C (mg)						
T1	1.37 (1.01, 1.85)	1.16 (0.80, 1.68)	1.19 (0.88, 1.60)	0.95 (0.64, 1.40)	1.38 (0.93, 2.05)	1.41 (0.80, 2.49)
T2	1.02 (0.76, 1.37)	0.92 (0.65, 1.32)	0.93 (0.70, 1.25)	0.86 (0.59, 1.26)	1.04 (0.72, 1.50)	1.03 (0.68, 1.56)
T3	0.98 (0.73, 1.32)	0.85 (0.58, 1.24)	1.09 (0.81, 1.46)	1.07 (0.72, 1.59)	1.23 (0.83, 1.80)	1.20 (0.75, 1.90)
Vitamin D (IU)						
T1	1.29 (0.95, 1.74)	1.43 (0.97, 2.10)	1.00 (0.74, 1.34)	0.88 (0.61, 1.28)	1.21 (0.82, 1.80)	1.16 (0.72, 1.87)
T2	0.98 (0.73, 1.31)	0.94 (0.66, 1.35)	1.05 (0.78, 1.40)	1.17 (0.81, 1.69)	0.79 (0.56, 1.10)	0.91 (0.60, 1.38)
T3	0.84 (0.62, 1.15)	0.88 (0.62, 1.26)	0.72 (0.53, 0.97)	0.72 (0.50, 1.05)	0.81 (0.58, 1.13)	1.06 (0.71, 1.60)
Vitamin E (mg)						
T1	0.97 (0.72, 1.31)	0.92 (0.63, 1.35)	1.03 (0.77, 1.39)	1.10 (0.76, 1.60)	0.81 (0.57, 1.15)	0.85 (0.56, 1.29)
T2	1.17 (0.87, 1.58)	1.28 (0.88, 1.86)	1.04 (0.78, 1.40)	1.18 (0.81, 1.72)	1.08 (0.75, 1.56)	1.11 (0.71, 1.73)
T3	1.04 (0.76, 1.41)	1.12 (0.75, 1.65)	1.06 (0.79, 1.44)	1.23 (0.83, 1.83)	1.25 (0.86, 1.81)	1.51 (0.93, 2.45)

[1]Adjusted for energy intake

[2]Adjusted for energy intake, child age and height z-score

T1= 1st trimester; T2= 2nd trimester; T3= 3rd trimester

Table 3.4 Odds ratio (95% confidence interval) of male pubertal development per standard deviation increase in prenatal intake of trace minerals and vitamins during pregnancy

	Pubic hair		Genitalia		Testicular volume	
Prenatal nutrient intake	Model 1[1]	Model 2[2]	Model 1[1]	Model 2[2]	Model 1[1]	Model 2[2]
Selenium (μg)						
T1	1.08 (0.81, 1.43)	1.29 (0.88, 1.88)	1.05 (0.77, 1.41)	1.07 (0.73, 1.56)	0.93 (0.68, 1.27)	0.90 (0.59, 1.37)
T2	1.29 (0.97, 1.72)	**1.57 (1.08, 2.28)**	1.10 (0.81, 1.49)	1.07 (0.73, 1.57)	1.04 (0.75, 1.43)	0.99 (0.65, 1.49)
T3	1.17 (0.88, 1.56)	1.14 (0.79, 1.64)	0.96 (0.71, 1.30)	0.74 (0.50, 1.10)	1.11 (0.81, 1.53)	0.99 (0.66, 1.49)
Zinc (mg)						
T1	0.76 (0.57, 1.00)	1.06 (0.73, 1.54)	0.72 (0.54, 0.97)	0.88 (0.61, 1.27)	0.89 (0.66, 1.21)	1.19 (0.79, 1.80)
T2	1.00 (0.76, 1.33)	**1.52 (1.06, 2.18)**	0.91 (0.68, 1.23)	1.07 (0.75, 1.53)	0.98 (0.72, 1.33)	1.15 (0.77, 1.71)
T3	0.69 (0.52, 0.92)	1.18 (0.81, 1.72)	0.58 (0.42, 0.79)	0.74 (0.51, 1.08)	0.90 (0.66, 1.23)	1.43 (0.93, 2.19)
Iron (mg)						
T1	1.00 (1.00, 1.00)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)	1.00 (1.00, 1.01)
T2	1.01 (1.00, 1.01)	1.00 (1.00, 1.01)	1.01 (1.00, 1.01)	1.01 (1.00, 1.02)	1.00 (1.00, 1.01)	1.00 (0.99, 1.01)
T3	1.00 (0.99, 1.00)	1.00 (1.00, 1.01)	1.00 (1.00, 1.00)	1.00 (1.00, 1.01)	1.00 (0.99, 1.00)	1.00 (1.00, 1.01)
Phosphorus (mg)						
T1	1.00 (0.76, 1.32)	1.01 (0.71, 1.46)	1.14 (0.85, 1.54)	1.21 (0.83, 1.76)	1.15 (0.84, 1.58)	1.24 (0.83, 1.85)
T2	1.29 (0.97, 1.71)	1.22 (0.84, 1.76)	**1.57 (1.15, 2.15)**	**1.72 (1.17, 2.52)**	1.10 (0.80, 1.50)	0.92 (0.61, 1.38)
T3	0.86 (0.65, 1.14)	1.10 (0.76, 1.58)	0.95 (0.70, 1.28)	1.12 (0.76, 1.64)	0.92 (0.67, 1.27)	1.11 (0.74, 1.65)
Vitamin A (IU)						
T1	1.04 (0.79, 1.37)	0.95 (0.66, 1.36)	0.97 (0.72, 1.30)	0.81 (0.57, 1.17)	1.02 (0.75, 1.40)	0.99 (0.67, 1.46)
T2	1.09 (0.82, 1.44)	1.14 (0.80, 1.62)	0.99 (0.74, 1.33)	0.86 (0.61, 1.23)	0.99 (0.73, 1.36)	1.00 (0.67, 1.49)
T3	1.07 (0.80, 1.43)	0.81 (0.54, 1.22)	1.13 (0.82, 1.56)	0.89 (0.59, 1.34)	1.02 (0.74, 1.40)	0.84 (0.55, 1.30)
Vitamin B1 (Thiamine) (mg)						
T1	0.96 (0.73, 1.27)	1.41 (0.96, 2.08)	0.84 (0.63, 1.13)	1.06 (0.73, 1.54)	0.89 (0.65, 1.22)	1.08 (0.72, 1.62)
T2	0.82 (0.63, 1.08)	1.20 (0.83, 1.73)	0.92 (0.69, 1.23)	1.46 (0.99, 2.14)	0.83 (0.61, 1.13)	1.10 (0.73, 1.64)

T3	0.82 (0.64, 1.06)	1.04 (0.76, 1.43)	0.93 (0.71, 1.21)	1.23 (0.89, 1.71)	0.85 (0.64, 1.12)	1.00 (0.72, 1.40)
Vitamin B2 (Riboflavin) (mg)						
T1	0.82 (0.63, 1.07)	1.04 (0.74, 1.46)	0.85 (0.64, 1.12)	1.05 (0.74, 1.50)	0.94 (0.70, 1.27)	1.16 (0.80, 1.68)
T2	0.96 (0.73, 1.27)	1.09 (0.76, 1.56)	1.18 (0.87, 1.59)	**1.60 (1.09, 2.34)**	0.95 (0.70, 1.30)	1.04 (0.70, 1.52)
T3	0.81 (0.61, 1.08)	1.00 (0.77, 1.41)	1.01 (0.75, 1.35)	1.31 (0.92, 1.87)	0.88 (0.64, 1.20)	1.04 (0.72, 1.49)
Vitamin B6 (mg)						
T1	0.87 (0.68, 1.12)	0.99 (0.71, 1.38)	0.77 (0.59, 1.00)	0.73 (0.52, 1.02)	0.94 (0.71, 1.23)	1.01 (0.70, 1.46)
T2	0.89 (0.66, 1.20)	1.38 (0.93, 2.06)	0.71 (0.52, 0.98)	0.81 (0.54, 1.19)	0.89 (0.63, 1.24)	1.17 (0.76, 1.81)
T3	0.86 (0.62, 1.18)	0.87 (0.57, 1.33)	0.73 (0.52, 1.03)	**0.61 (0.39, 0.95)**	1.05 (0.73, 1.50)	1.20 (0.75, 1.91)
Vitamin B12 (μg)						
T1	0.88 (0.68, 1.16)	1.02 (0.71, 1.47)	0.84 (0.63, 1.11)	0.91 (0.63, 1.31)	1.06 (0.78, 1.44)	1.31 (0.87, 1.96)
T2	1.07 (0.80, 1.43)	1.21 (0.83, 1.75)	1.06 (0.78, 1.45)	1.22 (0.83, 1.78)	1.09 (0.78, 1.51)	1.19 (0.79, 1.81)
T3	0.82 (0.61, 1.09)	1.10 (0.76, 1.58)	0.71 (0.52, 0.96)	0.75 (0.51, 1.10)	0.93 (0.67, 1.27)	1.13 (0.76, 1.68)
Vitamin C (mg)						
T1	0.96 (0.73, 1.27)	0.80 (0.54, 1.19)	1.03 (0.77, 1.38)	0.94 (0.65, 1.37)	1.02 (0.74, 1.39)	0.97 (0.63, 1.48)
T2	1.08 (0.81, 1.43)	1.10 (0.76, 1.56)	0.87 (0.64, 1.17)	0.69 (0.47, 1.01)	1.04 (0.75, 1.43)	1.04 (0.68, 1.58)
T3	1.08 (0.81, 1.44)	0.96 (0.65, 1.44)	1.03 (0.76, 1.40)	0.87 (0.56, 1.37)	0.99 (0.72, 1.37)	0.82 (0.52, 1.31)
Vitamin D (IU)						
T1	1.12 (0.84, 1.48)	1.15 (0.81, 1.63)	1.08 (0.79, 1.46)	1.01 (0.70, 1.45)	1.16 (0.82, 1.64)	1.17 (0.74, 1.85)
T2	0.74 (0.55, 0.99)	0.95 (0.65, 1.38)	0.70 (0.52, 0.95)	0.83 (0.58, 1.19)	0.85 (0.62, 1.16)	1.03 (0.68, 1.56)
T3	0.89 (0.67, 1.18)	1.03 (0.73, 1.45)	0.74 (0.55, 1.00)	0.71 (0.50, 1.01)	0.85 (0.63, 1.16)	0.85 (0.58, 1.25)
Vitamin E (mg)						
T1	0.81 (0.61, 1.08)	0.94 (0.65, 1.36)	0.74 (0.55, 1.00)	0.81 (0.55, 1.18)	0.93 (0.68, 1.27)	1.10 (0.73, 1.66)
T2	1.02 (0.76, 1.37)	1.05 (0.72, 1.54)	0.88 (0.64, 1.19)	0.80 (0.55, 1.16)	1.22 (0.87, 1.71)	1.35 (0.89, 2.04)
T3	1.08 (0.81, 1.44)	0.98 (0.67, 1.43)	0.91 (0.67, 1.24)	0.71 (0.48, 1.06)	1.26 (0.90, 1.76)	1.19 (0.79, 1.79)

[1]Adjusted for energy intake

[2]Adjusted for energy intake, child age and height z-score

T1= 1st trimester; T2= 2nd trimester; T3= 3rd trimester

CHAPTER 4

Life Stage Lead Exposures and Development and Tempo of Puberty

4.1 Abstract

Background: Lead exposure has been associated with changes in the timing of puberty according to animal and human studies. However, few studies have considered exposures at multiple developmental stages influencing the development and tempo of pubertal process.

Objective: We examined *in utero,* early childhood and peripubertal lead exposure in relation to attained pubertal development and tempo at ages 9-18 in a Mexican birth cohort.

Methods: Maternal bone lead was measured with K-XRF at 1 month postpartum (proxy for cumulative prenatal exposure). Lead concentration in blood was measured to represent cumulative exposure from 1-4 yr of age and peripubertal exposure. Pubertal development was characterized using Tanner stage of breast, pubic hair and attainment of menarche in girls, and Tanner stage of genitalia, pubic hair and attainment of matured testicular volume in boys measured at visit in 2015. Repeated measures on sexual maturation at visit in 2011 and 2015 were used to examine the association of lead with pubertal tempo. Logistic and ordinal regression analyses were carried out to assess the effect of lead on attained pubertal development and on pubertal tempo.

Results: After accounting for child age at visit, a one IQR increase in maternal patella bone lead was associated with decreased odds of reaching a higher stage of breast growth (OR: 0.71, 95% CI: 0.51, 0.99), and decreased odds of having menarche (OR: 0.42, 95% CI: 0.21, 0.84). One IQR increase in cumulative 1-4 yr blood lead concentration was associated with decreased odds of reaching a higher stage of breast growth (OR: 0.87, 95% CI: 0.75, 0.99), decreased odds of reaching a higher stage of pubic hair growth (OR: 0.84, 95% CI: 0.73, 0.96) and decreased odds of having menarche (OR: 0.76, 95% CI: 0.59, 0.99).

Conclusions: *In utero* and early childhood lead exposure was associated with delayed pubertal development in girls, while peripubertal lead exposure (8-15 years) was associated with increased tempo of puberty in girls. No such associations were observed in boys. Our findings need to be confirmed by other studies to improve current knowledge of lead exposure during multiple developmental stages and its role in sexual maturation later in life.

4.2 Introduction

Lead, a ubiquitous environmental toxicant, is associated with a variety of adverse health effects (Bellinger 2011). Infants, children, and pregnant women are the most vulnerable to lead toxicity (National Research Council (U.S.). Committee on Measuring Lead in Critical Populations. et al. 1993). A growing body of studies have shown that blood lead levels as low as 3 to 10 μg/dL *in utero* and during childhood are associated with impaired nervous, reproductive and cardiovascular systems (Silbergeld 1990; WHO 2010). Although blood lead levels have decreased over time mainly due to the phase-out of leaded gasoline (Caravanos et al. 2014; Falk 2003), other routes of exposure to lead still exist and pose a continued public health burden, especially in developing countries (Falk 2003; Schnur and John 2014) and many US inner cities and underserved areas (Bellinger 2016).

Previous studies have elucidated that lead is readily transferred from mother to offspring by crossing the placental-fetal barrier during gestation and via breast milk during lactation (Koyashiki et al. 2010; WHO 2010). Many existing studies have investigated the association between early life lead exposure and neurodevelopment in children (Bellinger et al. 1987; Sanders et al. 2009; Schnaas et al. 2006). Fewer analyses have characterized the detrimental effects of lead on pubertal development across sensitive periods of exposure.

Earlier animal experiments have demonstrated a delay in pubertal onset and development as a results of prenatal and postnatal lead exposure (Dearth et al. 2002; Dearth et al. 2004; Ronis et al. 1996), while only a few epidemiological studies have looked at the impact of lead exposure on pubertal process. In girls, a cross-sectional analysis using the Third National Health and Nutrition

Examination Survey (NHANES III) found that blood lead was negatively associated with development of breast, pubic hair and menarche in adolescent girls (Selevan et al. 2003; Wu et al. 2003). Another cross-sectional study using the Birth to Twenty cohort (Bt20) also reported that higher blood lead levels (≥ 5 μg/dL) were associated with delayed breast and pubic hair growth as well as attainment of menarche (Naicker et al. 2010). In 8-9 years-old boys from Chapaevsk, Russia, blood lead levels ≥ 5 μg/dL were associated with 43% reduced odds of reaching pubertal onset of genitalia compared with those who had lower lead (Hauser et al. 2008). A later longitudinal analysis of these boys followed annually for 3 years reported that blood lead levels ≥ 5 μg/dL were associated with 24%–31% reduction in "risk" of pubertal onset in testicular volume >3mL, genitalia and pubarche compared with those with lower lead. The timing of pubertal onset was delayed by 6–8 months on average in boys with high blood levels compared with those with low blood levels (Williams et al. 2010).

Regarding the biological pathways, previous studies have found that lead exposure could potentially disrupt the pubertal development indirectly through its effects on body fat mass. It is well established that onset, progression, and tempo of puberty are influenced by early changes in body fat (German et al. 2015; Kaplowitz 2008; Siervogel et al. 2003), possibly following a sex-specific pattern (Lee et al. 2010; Wang 2002); moreover, changes in body fat can be determined by levels of lead exposure. For instance, prenatal and childhood exposure to lead is associated with a lower body mass index (BMI) (Scinicariello et al. 2013). To our knowledge, no previous work has examined whether adiposity mediates the effect of lead on puberty.

To address these research gaps, we used longitudinal data from the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) study to: (1) examine the associations between lead exposure at three time points (*in utero*, early childhood and peripubescence) and attained sexual maturation, and (2) the associations between lead exposure during these life stages and maturational tempo. Finally, we examined the mediation effects of body adiposity measures on the association between lead exposure and measures of attained puberty and pubertal tempo.

4.3 Materials and Methods

Study population

The ELEMENT study is a longitudinal study comprising three sequentially enrolled birth cohorts of mothers and their offspring residing in Mexico City, aiming at examining the impacts of early life exposure to environmental toxicants on child growth and development. Pregnant women with low- to moderate-income were recruited at three maternity hospitals (Manuel Gea Gonzalez Hospital, Mexican Social Security Institute and the National Institute of Perinatology), followed for 12 months post-partum and their offspring were followed up to 4 years of age. Mothers provided a blood sample and completed interview-based questionnaires. A blood sample and anthropometric measurements were also obtained from children annually from 1 to 4 years of age. In this study, archived data and biospecimens were collected from participants recruited from two cohorts in 1997-2000 and 2001-2005. Participants were excluded if they had pre-existing conditions that interact with child growth and development and the same exclusion criteria applied to all birth cohorts have been described previously (Afeiche et al. 2011; Hu et al. 2006).

In 2011, a subset of children (n=250) at 8-15 years of age were invited to participate in a follow-up study based on the availability of maternal biological samples. In 2015, an expanded subset of child participants (n = 550), who were now 9-18 years of age, were reconnected and invited to participate in this new follow-up study depending on the availability of archived pregnancy biological samples. During each follow-up visit, a blood sample and an interview-based questionnaire were collected from each child. Anthropometry including weight, height, waist circumference (WC), skinfolds (subscapular, suprailiac and triceps) were collected by trained nurses. The weight of each child was measured with a digital scale (BAME Mod 420; Catálogo Médico) and read to the nearest 0.1 kg. The height of each child was evaluated using a calibrated stadiometer (BAME Mod 420; Catálogo Médico) and read to the nearest 0.1 cm. BMI=kg/m^2 was calculated from the weight and height. A non-stretchable measuring tape (QM2000; QuickMedical) was used to obtain WC, which was read to the nearest 0.1 cm. The thicknesses of subscapular, suprailiac and triceps skinfold was assessed with calibrated skin calipers (Lange; Beta Technology) (Lohman et al. 1988). In this study, the sum of skinfolds (subscapular, suprailiac and triceps) were calculated and included in the final analysis. Children were examined by a trained pediatrician for Tanner stages of breast and pubic hair growth in girls, and genitalia and pubic hair growth in boys. Additionally, each girl was asked if she had initiated menses, using an interview-based questionnaire and each boy's testicular volume was measured using an orchidometer by a trained pediatrician (Ferguson et al. 2014; Watkins et al. 2014).

Research protocols were reviewed and approved by the ethics committees of participating institutions including the University of Michigan and the Mexico National Institute of Public

Health. Informed consent from mothers and informed assent from their offspring were obtained before participation in this study.

Exposure assessment

The levels of lead in mid-tibial shaft and patella were measured at 1 month postpartum to estimate the cumulative lead exposure throughout pregnancy. Bone lead levels at these two sites were assessed using a 109Cd K X-ray fluorescence (KXRF) measurement system constructed at Harvard University and assembled at American British Cowdray Medical Center in Mexico City. This is a non-invasive and low-radiation method of measuring bond lead concentration and an appropriate instrument to use on pregnant women. Given that mobilization of maternal skeletal lead stores is a main route of fetal lead exposure, maternal bone lead has the potential to serve as a better biomarker of cumulative fetal lead exposure over the course of pregnancy than either maternal venous blood or umbilical cord blood lead (Gomaa et al. 2002). Detailed information regarding the protocol, application, validation and quality control of using this system has been described elsewhere (Gonzalez-Cossio et al. 1997; Hu et al. 1989, 1991; Hu et al. 1995).

Blood samples were obtained from children and stored in trace-metal–free tubes by trained research assistants using standardized protocols. Blood lead levels were measured using graphite-furnace atomic-absorption spectroscopy (model 3000; Perkin-Elmer, Chelmsford, MA, USA) at the research facility of the American British Cowdray Hospital in Mexico City and validated by the Maternal and Child Health Bureau (MCHB) and the Wisconsin State Laboratory of Hygiene (WSLH) Cooperative Blood Lead Proficiency Testing Program (LEADPTP). The quality control tests have been described previously (Afeiche et al. 2011; Gonzalez-Cossio et al. 1997). All blood

lead levels were above the limit of detection (<1 μg/dL) and the precision of this instrument is within 1 μg/dL. Cumulative early childhood lead exposure was obtained by calculating the area under curve of repeat measures from 1-4 years.

Outcome assessment

In girls, the stage of sexual maturation was defined using Tanner staging scales for the development of breast and pubic hair (Marshall and Tanner 1969). For breast growth, Stage 1 represents having elevation of papilla only. Stage 2 represents the onset of puberty having elevation of papilla and breast on a small mount (bud) and increase in areola. The breast tissues further enlarge at Stage 3 and 4 and reach full growth at adult level at Stage 5 (Marshall and Tanner 1969). For pubic hair growth, Stage 1 represents pre-pubertal with no pubic hair. Stage 2 represents the onset of puberty having sparse and long slightly pigmented hair. The pubic hair grows darker and coarser at Stage 3 and 4 and reach adult level at Stage 5 (Marshall and Tanner 1969, 1970). The attainment of menarche, an additional measure of sexual maturation, was measured via a self-reported questionnaire, where girls were asked if they have started menstruating. In boys, the stage of sexual maturation was defined using Tanner staging scales for the development of genitalia and pubic hair. For genital development, stage 1 represents pre-puberty with no enlargement of the penis, testes, and scrotum. Stage 2 represents the onset of puberty with enlargement of scrotum and testes with the scrotum becoming reddened and coarser. The penis continues to enlarge and further grow at stage 3 and 4 and reach adult genitalia at stage 5 (Marshall and Tanner 1970). The volume of the testis was used as another indicator of puberty for boys. Right and left testicular volume was determined using an orchidometer ranging from 1 to 25 mL. In our analysis, the larger

volume of the right and left testicles was used. A cutoff of 20 mL was applied to represent adult level (≥20 mL) (Burns et al. 2016).

Statistical analysis

Descriptive statistics and distributions of lead levels during pregnancy, cumulative 1-4 years, and peripubescence were examined. Cumulative blood lead during age 1-4 years and peripubertal blood lead appeared log normally distributed, and were ln-transformed before fitting regression models. Only variables were considered that are known as potential confounders of the association between lead and pubertal development based on biologic plausibility and covariates considered to be predictors of outcomes of pubertal measures; these included child age, maternal age and education, maternal smoking status, parity and social economic status. Only those that reached statistical significance ($p < 0.05$) in bivariate analyses were included in the final analysis models.

Multiple ordinal regression models were used to examine the association of life stage lead exposure with each of the Tanner stages separately. The dependent variables in this analysis were Tanner staging for pubic hair and breast growth (stage 1-stage 5) in girls. In boys, dependent variables were Tanner staging for pubic hair and genitalia (stage 1-stage 5). Multiple logistic regression analysis was used to model the association of life stage lead exposure with the attainment of menarche ("0" denotes prepuberty, "1" denotes puberty) in girls and the attainment of matured testicular volume ("0" denotes <20mL, "1" denotes ≥20mL) in boys. Results for ordinal regression models were reported as the odds of a higher Tanner stage versus the combined low and mid stage associated with per IQR increase in a given lead exposure. Results for logistic

regression models were reported as the odds of having attained menarche or matured testicular volume with per IQR increase in a given lead exposure.

Repeated measures multinomial logistic regression analysis was performed to examine whether lead exposure was associated with maturational tempo using measures of pubertal outcomes at visits in 2011 and 2015. The models were estimated using a generalized estimating equations. Models were built specifically for examining such association as following:

$$\mathbf{g(E(Y)) = \beta_0 + \beta_1 \mathit{X} + \beta_2\, \mathit{age}_0 + \beta_3\, \mathit{time} + \beta_4 X{*}time + \beta_5 age_0 {*} time}$$

where the **Y** represents the repeated measures of pubertal stage at two visits; **g()** is the cumulative logistic function; **X** represents a given lead exposure; $\mathbf{\mathit{age}_0}$ is the baseline age of children at the first visit in 2011 (baseline); and ***time*** is the change in an individual child's age between the two visits. As such, $\boldsymbol{\beta_1}$ estimates the association between lead and Tanner stage at the baseline visit; $\boldsymbol{\beta_2}$ estimates the effect of baseline age on attained Tanner stage; and $\boldsymbol{\beta_3}$ estimates the effect of aging on the tempo of puberty. The term **X*time** is the interaction term between lead and the change in age, and thus $\boldsymbol{\beta_4}$ estimates the effect of lead on the tempo of pubertal changes. The term $\mathbf{age_0}$ ***time** is the interaction between baseline age and change of child age, such that $\boldsymbol{\beta_5}$ estimates the effect of baseline child age on the tempo of puberty (i.e., this accounts for the fact that children who were younger at baseline may have higher tempos). Hence, the coefficients of primary interest in this analysis are $\boldsymbol{\beta_1}$ and $\boldsymbol{\beta_4}$.

We then examined the potential mediation effects of adiposity on the association between lead and pubertal development by extending the models above to include adding adiposity variables, specifically adding terms β_6 **adiposity**$_0$ + β_7 **adiposity**$_1$, where **adiposity**$_0$ is a baseline adiposity measure (BMI, WC or sum of skinfolds (subscapular, suprailiac and triceps)) and **adiposity**$_1$ is the change in the adiposity measure that occurred between the two visits. Thus, β_6 estimates the effect of adiposity on the attained Tanner stage and β_7 estimates the effect of changes of adiposity on changes in pubertal outcomes (i.e. pubertal tempo). A significant reduction in the estimates of β_1 by adding adiposity terms compared with those without adiposity terms indicates a potential mediating effect of adiposity on lead and attained puberty. A significant reduction in the estimates of β_4 by adding adiposity terms compared with those without adiposity terms indicates a potential mediating effect of adiposity on lead and tempo of puberty. We defined statistical significance as $p < 0.05$. We performed SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) to analyze data.

4.4 Results

Our final analysis included 549 mother–offspring pairs with a total of 285 girls and 264 boys (Table 1). Child age at visit, and bone and blood lead levels did not differ significantly by sex. At the second visit in 2015, the mean age was 14.5 years, ranging from 9.8 to 18.0 years. Geometric mean±SD cumulative 1-4 yr lead was 14.88±1.46 μg/dL and 14.26±1.49 for boys and girls respectively. Geometric mean±SD peripubertal lead was 2.87±1.77 μg/dL and 2.72±1.78 for boys and girls respectively. Bone lead concentration in the patella was appreciably higher than in the tibia in girls, but not in boys (female: mean±SD patella 9.46 ± 11.01 μg/g; tibia 7.77 ± 9.91 μg/g) **(Table 4.1)**.

Among 264 male participants, 48 boys (19.1%) were pre-pubertal (stage 1) and 53 boys (21.1%) had matured at adult level (stage 5) for pubic hair growth; 14 boys (5.6%) were pre-pubertal and 57 boys (22.7%) had matured at adult level (stage 5) for genitalia. There were 176 boys (70.1%) who had matured testicular volume ≥ 20mL (Table 2). Among 285 female participants, 21 girls (7.6%) were pre-pubertal (stage 1) and 54 girls (19.5%) had matured at adult level (stage 5) for pubic hair growth; 13 girls (4.7%) were pre-pubertal and 64 girls (23.1%) had matured at adult level (stage 5) for breast growth. There were 237 girls (84.0%) who had attained menarche **(Table 4.2)**.

Multiple ordinal regression models of Tanner stage showed that maternal bone lead and early childhood blood lead were negatively associated with Tanner stage controlling for child age in girls **(Table 4.3)**. The odds of reaching a high stage of breast maturation versus combined low and middle stages decreased by 29% (OR: 0.71, 95% CI: 0.51, 0.99) per IQR increase in patella bone lead, and decreased by 13% (OR: 0.87, 95% CI: 0.75, 0.99) per IQR increase in cumulative 1-4 years lead concentration in blood. Cumulative 1-4 yr blood lead concentration was negatively associated with the development of pubic hair in girls with a OR=0.84 (95% CI: 0.73, 0.96). The odds of having menarche decreased by 58% (OR: 0.42, 95% CI: 0.21, 0.84) per IQR increase in patella bone lead, and decreased by 24% (OR: 0.76, 95% CI: 0.59, 0.99) per IQR increase in cumulative 1-4 years lead concentration in blood. Maternal tibia level was not significantly associated with pubertal development in girls. In boys, no significant associations between lead and Tanner stage or testicular volume were observed **(shown in Table 4.3)**.

We report the associations between 1-IQR increase in life stage lead concentrations and the tempo of pubertal development in boys **(Table 4.4a)** and girls **(Table 4.4b)** using separate models. After controlling for child age at visit, no significant associations were found in boys between any lead biomarkers and the tempo of puberty estimated by Tanner stage of pubic hair and genitalia (Table 4a). However, we did observe a negative relationship between cumulative 1-4 yr blood lead level and tempo of puberty estimated by the changes in testicular volume and a positive relationship between cumulative 1-4 yr blood lead level and attained testicular volume **(Table 4.4a)**. In girls, early life exposure to lead (pregnancy and early childhood) was not a predictor of puberty progression, but increased peripubertal lead exposure was consistently associated with increased tempo of pubertal development across different outcome indicators and with decreased attained Tanner stage and menarche **(Table 4.4b)**.

Since peripubertal body fat did not significantly reduce the estimate of association between lead and pubertal outcomes, we found no evidence of a mediating effect of peripubertal adiposity on the negative association between lead exposure at any time and pubertal development in females accounted for child age at visit (Table 5). Nevertheless, we did observe positive associations between peripubertal measures of body fat (BMI, WC and sum of 3 skinfolds) and pubertal development and tempo in girls (Table 5), but not in boys (data not shown). The significant positive association between adiposity and pubertal development was found consistently across all the indicators of sexual maturation, while the significant positive association between adiposity and pubertal tempo was only observed for breast development **(Table 4.5)**.

4.5 Discussion

This study is the first epidemiological study that investigated the effects of cumulative prenatal lead exposure (as measured by maternal bone lead), cumulative early childhood lead exposure (as measured by cumulative blood lead 1-4 years), and peripubertal blood lead exposure (as measured by blood lead aged from 8-15) on the pubertal development and tempo in Mexican boys and girls. To better understand the potential mechanism that could explain these associations, we examined the potential mediating effects of indicators of adiposity during peripubescence. We found a sustained delay in the pubertal development in relation to lead concentration in early life exposure to lead (patella and cumulative blood 1-4 years), and an increase in the pubertal tempo in relation to peripubertal lead exposure both in girls only.

Our findings are consistent with previous animal studies on female rats measuring the effects of lead exposure during early life on pubertal development (Dearth et al. 2002; Dearth et al. 2004; Ronis et al. 1996). However, unlike a few cross-sectional analyses performed in girls, we found no evidence of an association between lead exposure during adolesecence and pubertal development (Naicker et al. 2010; Selevan et al. 2003; Wu et al. 2003). Several factors may affect our analysis: Firstly, in contrast with previous studies, we considered the prospective association of peripubertal lead levels with Tanner stages approximately 4 years after exposure, rather than concurrent lead exposure. Secondly, these cross-sectional studies examined the effect of lead on pubertal onset among younger participants (mostly from 8-16 years) with lower prevalence of pubertal onset while we were investigating the effect of lead on pubertal development (stage 1-stage 5) among slightly older participants (9-18 years), which means a higher rate of pubertal onset.

Finally, the small sample size of our study (only 108 boys and 113 girls had peripubertal blood lead measured) limited the power to detect significant differences compared to these large cross-sectional investigations. Another possibility is that prenatal and early childhood period is more sensitive than late childhood to lead impacting the development of sexual maturation. Furthermore, only patella lead was significantly associated with delayed pubertal development while tibia was not associated with puberty. It is possible that this finding is due to the smaller sample size of tibia versus patella, or is the difference may be caused by the structure of the two bone sites with the tibia being more vascularized, reflecting more recent lead exposure; thus accumulation of lead is slower in tibia than in patella (Afeiche et al. 2011; Hu et al. 1989; Hu et al. 1998). No association between lead at any time point and any pubertal outcome was found in boys, which is inconsistent with significant findings reported by (Hauser et al. 2008; Williams et al. 2010).

To explain the impact of lead on pubertal development, we hypothesized that lead could affect pubertal outcomes indirectly through its negative effect on body fat. Therefore, we carried out tests examining the mediating effects of various indicators of body fat. Contrary to our expectation, we found that body fat during peripubescence does not mediate the negative effect of lead on the development and tempo of pubertal process. Earlier studies have shown a negative relationship between developmental exposure to lead and blood level of insulin-like growth factor 1 (IGF-1), luteinizing hormone, estradiol in female rats (Dearth et al. 2002; Dearth et al. 2004) and inhibin B in girls (Gollenberg et al. 2010), which are makers for child growth and pubertal development. Further studies are warranted to examine these hormones as potential mediators, rather than body fat.

Nevertheless, to the best of our knowledge, this is the first study demonstrating a relationship between lead and pubertal tempo in humans. A positive association between peripubertal lead exposure and the tempo of pubertal changes in the growth of breast and pubic hair was found in girls. The positive association with menarche was borderline significant. To explain the increased tempo of puberty observed in our female participants, we propose a concept similar to "catch-up growth," i.e. "catch-up puberty". Since these girls had experienced a sustained delayed pubertal development through exposure to lead as early as prenatally and continued into early childhood, their body systems may respond to fix this delay in puberty by accelerating the change from lower stages of puberty to higher stages, surpassing the restraining effect exerted by lead. Future research is needed to confirm these findings.

This study has some limitations. The sample size was small, especially for modeling ordinal outcomes, which limited the power to detect significant differences. Another limitation was the lack of concurrent lead exposure at the time of pubertal outcomes were measured. In addition, levels of serum hormones related to pubertal development were not measured to help identify the potential pathway leading to delayed pubertal development. The strengths of this study include the ability to examine the impact of lead at multiple life stages on pubertal development and tempo, the use of physician-assessed pubertal stages, the measurement of testicular volume as an additional pubertal marker, which has barely been used, and the use of maternal bone lead representing cumulative prenatal exposure, which is a better surrogate than blood lead.

4.6 Conclusions

In conclusion, we found that prenatal and early childhood lead exposure was associated with a delay in attained development of puberty. Moreover, peripubertal lead exposure was associated with an increase in tempo of puberty. Peripubertal level of body fat was not a mediator of the association between lead and attained sexual maturation or maturational tempo. The delaying effect of lead on pubertal process was observed only in female participants, suggesting lead may influence sexual maturation in a sex-dependent manner, potentially through the differential effects of lead on levels of sex and growth hormones. More epidemiological studies are needed to confirm these results to improve our understanding of lead's impact on the neuroendocrine system. More studies confirming the potential alterations in pubertal timing and tempo in relation to early life lead exposure could lead to public health interventions in pregnant and pre-pregnant women.

Table 4.1 Characteristics of the study population[a] from ELEMENT

	Boys			**Girls**		
Characteristics	N	Mean	SD	N	Mean	SD
Child age (years)	264	14.54	2.06	285	14.45	2.18
Maternal bone lead (μg/g bone) [b]						
Patella	230	8.31	10.11	229	9.46	11.01
Tibia	157	7.99	10.11	177	7.77	9.91
Child's blood lead (μg/dL) [c]						
Cumulative 1-4 yr	255	14.88	1.46	277	14.26	1.49
Peri-pubertal	108	2.87	1.77	113	2.72	1.78

[a] Included 549 children at ages 9-18
[b]K-XRF at 1 month postpartum
[c]Geometric mean and SD; SD represents standard deviation

Table 4.2 Distribution of secondary sex characteristics among children at ages 9-18 by sex

Measure	Stage	N (%)
Boys (n=251)		
Pubic hair	1	48 (19.1)
	2	32 (12.8)
	3	63 (25.1)
	4	55 (21.9)
	5	53 (21.1)
Genitalia	1	14 (5.6)
	2	33 (13.2)
	3	44 (17.5)
	4	103 (41.0)
	5	57 (22.7)
Testicular volume	Yes (>=20ml)	176 (70.1)
	No	75 (29.9)
Girls (n=277)		
Pubic hair	1	21 (7.6)
	2	66 (23.8)
	3	63 (22.7)
	4	73 (26.4)
	5	54 (19.5)
Breast	1	13 (4.7)
	2	28 (10.1)
	3	69 (24.9)
	4	103 (37.2)
	5	64 (23.1)
Menarche	Yes	237 (84.0)
	No	45 (16.0)

Table 4.3 Adjusted[a] odds ratio (95% confidence interval) of pubertal development at ages 9-18 per IQR increase in maternal bone and childhood blood lead concentrations

	Boys			**Girls**		
	Genitalia OR(95%CI)	Pubic hair OR(95%CI)	TV OR(95%CI)	Breast OR(95%CI)	Pubic hair OR(95%CI)	Menarche OR(95%CI)
Maternal bone lead (μg/g bone) [b]						
Patella	0.95 (0.67, 1.34)	1.00 (0.71, 1.42)	1.18 (0.79, 1.75)	**0.71 (0.51, 0.99)**	0.96 (0.71, 1.33)	**0.42 (0.21, 0.84)**
Tibia	1.04 (0.67, 1.62)	1.00 (0.64, 1.54)	1.01 (0.62, 1.62)	0.97 (0.66, 1.43)	1.13 (0.77, 1.30)	0.62 (0.25, 1.54)
Child's blood lead (μg/dL) [c]						
Cumulative 1-4 years	0.94 (0.81, 1.10)	0.96 (0.82, 1.12)	1.05 (0.88, 1.26)	**0.87 (0.75, 0.99)**	**0.84 (0.73, 0.96)**	**0.76 (0.59, 0.99)**
Peripubertal	1.06 (0.89, 1.26)	1.03 (0.87, 1.21)	0.90 (0.73, 1.10)	1.00 (0.84, 1.18)	1.07 (0.90, 1.26)	0.85 (0.67, 1.08)

[a]Adjusted for child age at visit
[b]K-XRF at 1 month postpartum
[c] ln-transformed

Table 4.4a Adjusted[a] odds ratio (95% confidence interval) of pubertal tempo per IQR increase in maternal bone lead concentrations and childhood blood lead concentrations in boys (baseline visit at ages 8-15, 2nd visit at ages 9-18)

Lead exposure	Terms	**Boys**		
		Genitalia OR(95%CI)	Pubic hair OR(95%CI)	TV OR(95%CI)
Maternal bone lead (μg/g bone) [b]				
Patella	β1	0.96 (0.61, 1.50)	0.92 (0.55, 1.55)	1.25 (0.65, 2.40)
	β4	1.05 (0.86, 1.28)	1.07 (0.84, 1.36)	1.09 (0.82, 1.45)
Tibia	β1	1.00 (0.50, 1.97)	1.29 (0.57, 2.94)	1.85 (0.73, 4.72)
	β4	1.20 (0.87, 1.65)	1.01 (0.71, 1.42)	1.13 (0.70, 1.83)
Child's blood lead (μg/dL) [c]				
Cumulative 1-4 years	β1	1.01 (0.83, 1.24)	0.96 (0.67, 1.36)	1.50 (1.07, 2.11)
	β4	0.96 (0.88, 1.05)	0.97 (0.86, 1.09)	0.85 (0.75, 0.95)
Peripuberty	β1	0.92 (0.79, 1.08)	0.98 (0.77, 1.24)	1.03 (0.80, 1.33)
	β4	1.04 (0.98, 1.11)	1.01 (0.92, 1.11)	0.95 (0.86, 1.06)

[a]Adjusted for child age at visit
[b]K-XRF at 1 month postpartum
[c]ln-transformed

Table 4.4b Adjusted[a] odds ratio (95% confidence interval) of pubertal tempo per IQR increase in maternal bone lead concentrations or childhood blood lead concentrations in girls (baseline visit at ages 8-15, 2nd visit at ages 9-18)

Lead exposure	Terms	**Girls**		
		Breast OR(95%CI)	Pubic hair OR(95%CI)	Menarche OR(95%CI)
Maternal bone lead (μg/g bone) [b]				
Patella	β_1	0.90 (0.48, 1.68)	0.65 (0.35, 1.19)	0.96 (0.45, 2.03)
	β_4	0.90 (0.73, 1.11)	1.22 (0.95, 1.56)	0.80 (0.56, 1.13)
Tibia	β_1	1.41 (0.64, 3.09)	0.95 (0.40, 2.28)	0.99 (0.30, 3.28)
	β_4	0.87 (0.69, 1.10)	1.10 (0.85, 1.44)	0.79 (0.49, 1.30)
Child's blood lead (μg/dL) [c]				
Cumulative 1-4 years	β_1	1.04 (0.81, 1.34)	1.05 (0.76, 1.45)	1.06 (0.71, 1.59)
	β_4	0.99 (0.91, 1.08)	0.99 (0.88, 1.12)	0.92 (0.78, 1.09)
Peripuberty	β_1	**0.80 (0.63, 1.00)**	**0.62 (0.45, 0.85)**	**0.60 (0.38, 0.94)**
	β_4	**1.07 (1.00, 1.16)**	**1.19 (1.08, 1.31)**	1.12 (0.95, 1.33)

[a]Adjusted for child age at visit
[b]K-XRF at 1 month postpartum
[c]ln-transformed

Table 4.5 Adjusted odds ratio (95% confidence interval) of pubertal tempo per IQR increase in peripubertal blood lead concentrations and adiposity levels (baseline visit at ages 8-15, 2nd visit at ages 9-18)

	Girls Breast OR (95%CI)	Pubic hair OR (95%CI)	Menarche OR (95%CI)
BMI			
β1	0.81 (0.64, 1.04)	0.62 (0.46, 0.84)	0.58 (0.37, 0.93)
β4	1.08 (1.00, 1.17)	1.21 (1.10, 1.33)	1.15 (0.95, 1.38)
BMI0	**2.31 (1.51, 3.52)**	**2.23 (1.41, 3.53)**	**2.49 (1.03, 6.02)**
BMI1	**3.43 (1.34, 8.79)**	1.74 (0.65, 4.63)	2.53 (0.49, 13.14)
WC			
β1	0.81 (0.63, 1.03)	0.62 (0.46, 0.84)	0.58 (0.35, 0.94)
β4	1.08 (1.00, 1.18)	1.21 (1.10, 1.33)	1.15 (0.95, 1.39)
WC0	**3.01 (1.96, 4.62)**	**2.44 (1.48, 4.03)**	**3.20 (1.41, 7.24)**
WC1	**3.23 (1.26, 8.32)**	1.29 (0.50, 3.34)	0.83 (0.18, 3.77)
Sum of skinfolds			
β1	0.81 (0.64, 1.02)	0.62 (0.46, 0.83)	0.57 (0.36, 0.91)
β4	1.09 (1.01, 1.17)	1.21 (1.11, 1.33)	1.15 (0.96, 1.39)
SF0	**2.91 (1.82, 4.66)**	**2.93 (1.70, 5.05)**	**3.68 (1.57, 8.59)**
SF1	**2.10 (1.04, 4.25)**	1.10 (0.50, 2.46)	1.64 (0.58, 4.66)

CHAPTER 5

Conclusions

5.1 Summary of findings

This dissertation improves our understanding of early life predictors of pubertal onset, development and tempo including important environmental toxicants such as fluoride and lead, and consumption of micronutrients including selenium, zinc, phosphorus and riboflavin. These micronutrients were selected according to their associations with factors (e.g. IGF-1, leptin and body fat mass) related to pubertal development.

Chapter 2 focused on the associations of pubertal onset reflected by physical makers of sexual maturation and serum levels of sex and growth hormones related to sexual maturation with creatinine adjusted urinary fluoride during each stage of pregnancy and specific gravity adjusted urinary fluoride during adolescence. Contrary to our hypothesis, we did not find a significant association between fluoride levels during any stage of pregnancy or during adolescence and the odds of pubarche, thelarche and menarche in girls. Similarly, we found no significant association between prenatal or peripubertal fluoride exposure and the odds of pubarche, genitalia and testicular volume (>3mL) in boys.

Although *in utero* and peripubertal fluoride exposure were not significant determinants of pubertal onset in this population, we did observe a significant association of prenatal fluoride with serum growth and sex hormones confined to sex and the stage of pregnancy. We found that late pregnancy is more sensitive than other developmental stages to fluoride's impact on growth hormone (IGF-1) in girls and sex hormone (testosterone) in boys, which provided more evidence suggesting fluoride may differentially affect sexual maturation by sex.

Similar results have been observed elsewhere. As early as in 1956, researchers found that water fluoridation at 1.2 mg/L was associated with earlier age at menarche in 8-17 American girls using an ecological design (Schlesinger et al. 1956). In 1997, for the first time, Dr. Luke proposed a positive association between early life fluoride exposure and sexual maturation, and that melatonin is a potential mediator (Luke 1997). Up to now, limited studies have examined the effect of fluoride on physiological indicators of sexual maturation. Therefore, our study is important in filling the research gap and enhancing our current knowledge of fluoride and its influence on secondary sex characteristics. Furthermore, several animal experiments (Divall et al. 2010; Ghosh et al. 2002; Jiang et al. 2005; Zhou et al. 2013) and a few human studies (Ortiz-Perez et al. 2003; Susheela and Jethanandani 1996) have reported a negative relationship between fluoride and puberty-related hormones, mostly in adults. Our studies confirmed that fluoride during late pregnancy negatively predicts the levels of IGF-1 in girls and testosterone in boys during adolescence.

The objective of chapter 3 was to examine the relationships between prenatal consumption of trace minerals and vitamins by each trimester and the odds of reaching higher stage of puberty by sex.

We found evidence of a significant positive association of prenatal intake of selenium and zinc during mid-pregnancy with development of pubic hair in adolescent boys. Also, our findings suggested a significant positive association of prenatal intake of vitamin B2 and phosphorous with development of genitalia in these boys. The positive association we observed is consistent with previous findings reported by (Carter et al. 1969; Sandstead et al. 1967) among participants with growth retardation.

In contrast, prenatal consumption of micronutrient was not a significant predictor of pubertal development of breast, pubic hair and the attainment of menarche in girls during adolescence, which is inconsistent with earlier studies showing positive association with menarche (Cho et al. 2010; Maclure et al. 1991; Moisan et al. 1990a; Rogers et al. 2010) or earlier studies showing negative association with menarche (Kissinger 1987; Moisan et al. 1990a).

The majority of currently available studies has focused on the influence of micronutrients of peripubertal consumption on the age at menarche, few studies have described the influence of micronutrient consumption on breast or pubic hair development in girls or on the pubertal development of genitalia or pubic hair in boys. No data has been reported to characterize the long-term effects of fetal exposure to fluoride on the sexual maturation in children. This work, for the first time, suggested that prenatal consumption of micronutrient assessed using the FFQ positively predict the pubertal development in boys aged 9-17 years but not in girls. We identified mid-pregnancy as a more vulnerable period to the effects on sexual maturation in relation to micronutrient consumption. Hopefully, this work can encourage more studies to confirm our

findings in order to improve current understanding of the interaction between dietary intake of nutrients and sexual maturation.

In chapter 4, we examined a reproductive toxicant that has been related to delayed pubertal onset in animal and human studies, lead. First, we assessed whether lead exposure at different developmental stages impacted pubertal development in children. Secondly, we extended our analysis from attained pubertal development to the tempo of pubertal changes in relation to various periods of lead exposure using repeated measures. To improve our knowledge of potential pathways that explain these associations, we evaluated the mediating effect of body fat during adolescence in the same population. Consistent with previous analyses, we found lead exposure during fetal life (as measured by maternal patella bone lead at 1 month postpartum) and during early childhood (as measured by cumulative blood lead from 1-4 years) were associated with reduced odds of higher stage of pubertal development for breast, pubic hair and the attainment of menarche in girls. Neither maternal tibia bone lead at 1 month postpartum nor peripubertal blood lead concentration from age 8-15 years was a significant determinant of female secondary sex characteristics in this population. In boys, we found that life stage lead exposures were not a significant predictor of pubertal development.

To the best of our knowledge, few studies have considered exposures at multiple developmental stages influencing the development of pubertal process. Until now, only one study used female rats to examine the impact of early life exposure to lead during gestation and lactation, showing a delay in pubertal onset from a normal of 33–35 days to about 40–43 days (Dearth et al. 2004). Remaining published studies have focused on the association between mid-late childhood

exposure to lead and Tanner stage 2 for pubic hair, breast development and the attainment of menarche (Naicker et al. 2010; Wu et al. 2003) or menarcheal age (Selevan et al. 2003) in girls. Only one study has characterized the effect of lead on testicle volume>3ml, pubarche and the onset of genital development in boys using a longitudinal design (Williams et al. 2010).

In addition, our work is the first analysis to assess whether lead exposures at different life stages affect the tempo of puberty. Our results showed that increased peripubertal blood lead concentration was associated with increased tempo of pubertal changes in girls. Similar to "catch-up growth," we hypothesize a "catch-up puberty" that may explain the positive association of lead with pubertal tempo. Future studies are needed to confirm our findings of the differential effects of lead on pubertal development and tempo.

To provide insight into the underlying mechanism for the delaying effect of lead on pubertal development and the accelerating effect on pubertal tempo, we examined whether adiposity is a potential mediator of lead-puberty associations: Previous studies have found that 1) Early changes in body fat mass are related to pubertal onset, progression and tempo (German et al. 2015; Kaplowitz 2008; Siervogel et al. 2003); and 2) Prenatal and childhood exposure to lead is associated with a lower body mass index (BMI) (Scinicariello et al. 2013). However, we found no evidence that the effects of lead on pubertal process were mediated by body fat mass in girls. Factors other than adiposity, such as IGF-1 and sex hormones, may play an important role in mediating the association between lead and sexual maturation. More studies examining the association between developmental stages of lead exposure and pubertal onset, development and tempo are needed.

5.2 Future considerations and implications

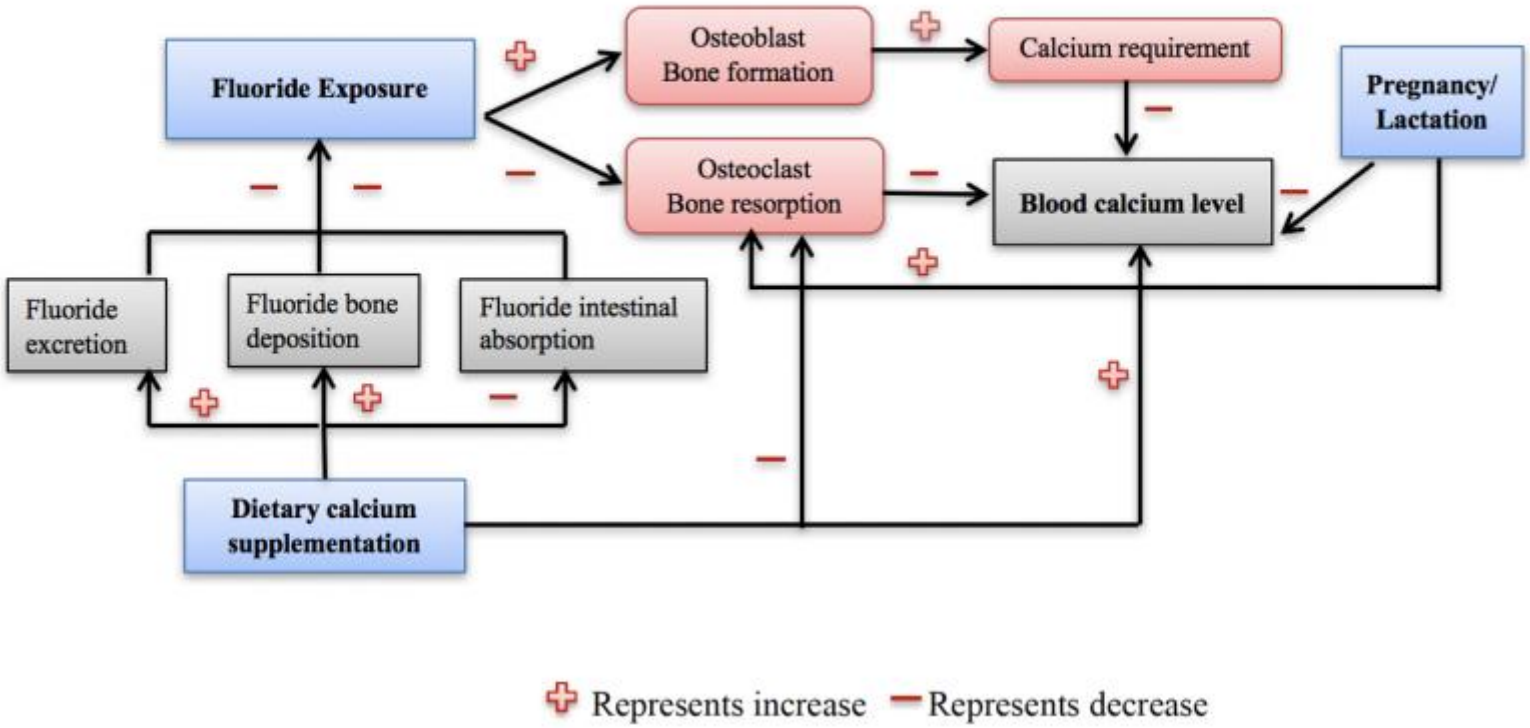
This work has demonstrated that environmental factors including toxicants and nutrients are predictors of pubertal onset, development and tempo. Overall, future research should focus on whether these alterations in pubertal process exerted by environmental factors during early life can be tracked into adult reproductive life. In addition, one next step would be examining the interplay among toxicants and nutrients and how these interactions affect pubertal development. Moreover, considering that this population is exposed to multiple toxicants such as BPA, phthalate, and cadmium, all of which have been related to child maturation, it would be intriguing to investigate the effects of exposure to the mixture of these metabolites on pubertal development.

Specifically, for chapter 2, future studies with large sample size and multiple biomarkers, e.g. 24-hours urine, blood and/or bone, are needed to elucidate the association of fluoride on sexual maturation. Additionally, it would be interesting to assess whether melatonin is a potential mediator of the fluoride-puberty associations, as found in an earlier animal study by (Luke 1997). It is well known that both fluoride and calcium are important for bone metabolism. Long-term fluoride exposure can stimulate osteoblast activity and decrease osteoclast function (National Research Council (U.S.). Committee on Fluoride in Drinking Water. 2006). Several animal studies have shown that dietary calcium intake may reduce fluoride toxicity by reducing fluoride in the non-osseous tissues via promoting the formation and deposition of calcium fluoride in bones (Shankar et al. 2013). The effect of fluoride on calcium homeostasis and bone metabolism is illustrated in Figure 5.1. Therefore, it is important to examine whether calcium modifies the effect of fluoride on childhood growth and development.

For chapter 3, future analyses with larger sample size and reliable biomarkers of micronutrients (e.g. plasma) are warranted to establish the link between nutrient status and pubertal development. Sensitive periods such as early childhood and peripubertal intake of micronutrients should be analyzed. For chapter 4, future studies should focus on the potential mediating effect of growth and sex hormones that are relevant to sexual maturation to provide better understanding of lead's influence on puberty. Studies providing the age when participants enter each stage are needed to examine the association between lead and the timing of pubertal progression.

Alterations in the timing of pubertal development have been observed for decades. Early onset of puberty is a public health issue due to its associated adverse health outcomes. Expanding our current understanding of the potential predictors of sexual maturation during early life are beneficial to public health interventions aimed at promoting a smooth transition from childhood to adulthood and avoid costly long-term health issues.

Figure 5.1: Fluoride affects calcium homeostasis and bone metabolism by regulating both bone formation and resorption



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