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Endocrine Modulation & Metal Exposure: Long Term Effects on the Children of Agricultural Working Mothers in Thailand

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Master of Public Health

Environmental Health

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B.S.
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Abstract

Endocrine Modulation & Metal Exposure: Long Term Effects on the Children of Agricultural Working Mothers in Thailand

By Grant Walter

Changes in pubertal timing (precocious/delayed) increases risks for specific outcomes in both genders. Characterization of factors modulating timing of puberty has produced evidence suggesting a role of environmental exposure. Previous studies correlated delayed puberty and environmental toxicants, specifically heavy metals (HMs) but the mode of toxicity is unknown. Animal studies have shown possibilities with HM exposure and puberty modulation. Large focus has been framed around the Hypothalamic–pituitary-gonadal (HPG) axis. HMs have shown in multiple animal, and few human, studies to affect hormone production released from the axis, especially Luteinizing Hormone (LH). The HPG axis begins development during embryogenesis but whether HM exposure during this critical window of development and subsequent effects still needs answering. To answer this, the **S**tudy of **A**sian **W**omen **A**nd their off**S**pring's **D**evelopment and **E**nvironmental **E**xposures (SAWASDEE) birth cohort was used. Metal concentrations were measured over pregnancy and compared to neonatal LH and anthropometrics. Measurement of metals was performed through ICP-MS and LH through EIA. Comparison was made through multivariate linear and logistic regression. Adjusted average maternal HM concentration for individual HMs and LH logistic regression yielded ORs of 0.59 (0.07, 4.69), 0.04(<0.01, 1.03), 0.44(0.04, 5.30) and 2.55(0.48, 13.49) for Pb, Cd, As and Hg, respectively. Earliest individual maternal HM concentrations showed adjusted ORs of 1.37 (0.14, 13.35) for Pb, 0.54 (0.06, 4.90) for Cd, 0.94 (0.19, 4.68) for As and 0.99(0.32, 3.00) for Hg. Only Cd showed significant point estimates but lost this status after adjustment for other variables deemed important. This study is the first study looking at HM exposure and neonatal hormone release from the HPG axis. Findings suggest that HMs do modulate LH release in neonates at birth. Caution, however, should be used when interpreting results from this study. Use of the SAWASDEE birth cohort was originally intended to study organophosphate insecticide exposure and subsequent neurodevelopment. Information and samples were taken in regard to the aforementioned study and complicate the interpretive ability found from results here. Estimates and prevalence found have applicability in construction of future study sampling calculations and timing of sample collection for adequate study.

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Background & Significance:

Modulating onset of puberty and health implications: Onset of puberty is an important process that involves the development of secondary sexual characteristics, accelerated growth, behavioral changes, and eventual fruition of reproductive capabilities. Expert panels convened in 2008 to establish modulation of pubertal timing, for both sexes, should be considered an adverse event although assessment as to the gravity of the adverse event from changes in timing was not conducted[1]. After describing the gaps in data available for answering, a call to research was released[1,2]. Alteration of pubertal timing onset reportedly increased the likelihood of behavioral disorders in girls and risk for testicular cancer in boys[3]. Other health effects for precocious puberty include: associations with metabolic disorders, increased risk for breast cancer and high BMI levels in girls later in life with conflicting evidence. Currently, longitudinal studies are being conducted in order to minimize these descrepancies[3]. Delayed puberty has shown increased risk of substance abuse in males and increased risk of bone fragility in later life for girls[4,5]. As researchers continue to study the health implications associated with pubertal timing, factors associated with modulating this timeline have garnered attention. Genetic variation reportedly contributes 70-80% of the variation observed in the timing of pubertal onset but leaves a large portion of variability unexplained[6,7]. Environmental exposures to exogenous substances may play a large role in either precocious or delayed puberty[8]. The 2008 expert panel described seven research questions or gaps under three domains that future research should have as its focus (Table 1). In an attempt to add to the body of evidence linking environmental exposures to pubertal onset, this project will focus on the critical prenatal window(s) of exposures that may be associated with puberty in a prospective cohort study originally designed to evaluate prenatal pesticide exposures and neurodevelopment in children.

Known health outcomes for heavy metal exposure: Lead (Pb) is well-characterized and widely accepted as a causal agent for adverse developmental outcomes resulting from gestational exposures with the

particular outcome and severity differing between low level chronic and high level acute exposures[9]. Low-level exposures during critical windows of fetal vulnerability have been linked to altered fetal development, decreased IQ and behavioral and emotional problems. Mercury (Hg), primarily methyl mercury which comprises ~90% of total Hg, is similar to Pb in that effects differ with levels of exposure. Low-level Hg exposures have been linked to neurodevelopment alterations but no conclusive answer has been found[10]. Gestational exposure to arsenic (As) differs from other heavy metals (HM) with higher risk for developing cancer or heart disease and low birth weight [11,12]. Evidence is lacking to suggest long term neurotoxic effects caused by fetal exposure to cadmium (Cd) but modulation of anthropometric measurements has been observed[13-15]. Neurodevelopment adverse outcomes or suggestive evidence of health effects during gestational exposure are seen with all four metals but there may be more susceptible development cascades. A need exists to continue research on new modes of metal toxicity as well as interactions between metals in relation to health outcomes to further characterize the full scope of toxicity in a "real world" exposure scenario.

Developing area of toxicity: New evidence suggests a relationship between higher exposures to certain (HM)s and endocrine modulation. Two cross-sectional studies using NHANES III metal data (1988-1994; females aged 8-16 yrs) found correlations between blood lead levels (BLLs) and sexual development. S.G. Selevan, et al. examined the association between BLLs and time of onset of menarche and Tanner stage of pubertal progression (i.e., pubic hair and breast development) among non-Hispanic White, non-Hispanic black, and Mexican American girls. BLL concentrations of 3ug/dL or greater were associated with delays in reaching later Tanner stages of development breast development compared to levels of 1ug/dL for non-Hispanic-Blacks and Mexican Americans but not non-Hispanic Whites[16]. However, there was no association found to be significant between time to menarche and higher concentrations of BLLs. Wu,T et al. performed similar analysis on NHANES III and maintained the association for tanner stage pubic hair development but found time to menarche significant [17]. Combination of these and other

formulated the hypothesis that Pb does reduce the timing of menarche, acting as a proxy for puberty[18]. HMs effect on puberty gained more traction after longitudinal studies noticed correlation between higher BLLs and delayed time in sexual maturity[19,20]. This effect is found within both genders through tanner scale progression in boys and menarche onset in girls. The mode of toxicity is unknown with little information available on whether timing of exposure results in sexual delay but additional HMs, Hg, have been suggested to increase the likelihood of menarche. Evidence suggests that higher BLLs modulate pubertal onset but continued work is required to devise the mode of toxicity for exposure to HM.

Timing of exposure to heavy metals: Previous research has elucidated the effect of HMs on puberty timing but still lacks exposure duration, frequency, and timing effects. Since answering this questions in humans has been difficult, animal studies provide guidance. Dosing of Sprague-Dawley rat mothers with Pb for differing exposure time periods resulted in statistical significant increases in time for vaginal opening compared to control animals and delays in growth and sexual maturation of male Sprague-Dawley rates[21,22]. Exposure during pregnancy plus lactation and pregnancy gave significant increases in time until vaginal opening in female rats. Male rats showed decreased testicular size for those exposed during pregnancy plus lactation and pregnancy. There was no significant decrease in gonad weight or delayed vaginal opening for groups exposed during lactation only. Both sexes had significant decreases in body weight when exposed during pregnancy plus lactation and pregnancy only, no differences were seen when compared to control for rats exposed during lactation only[23]. Similar exposure to Cd in female Wistar rats resulted in delayed puberty[24]. As and Hg lack research into gestational exposure and subsequent effects on pubertal onset but other effects have been seen with unique timing of gestation for both HMs.

Window of vulnerability: Gestational development is an important and highly susceptible period in which organ/organ system formation begins. This time period is highly susceptible to perturbations from

toxicants due to undeveloped detoxification processes, creating a window of vulnerability. Developmental delays or abnormalities introduced during gestation can have long-lasting or permanent effects [10]. The placenta is the primary factor preventing HMs from entering fetal blood supply during development. Evidence shows the ability for multiple HM to enter fetal blood supply through the placenta with subsequent entry into fetal circulation [25,26]. Hg and Pb accumulate within placental tissue but correlation between maternal and umbilical cord blood has been shown, suggesting the placentas inability to completely buffer fetal circulation[27]. Cd, however, is prevented from crossing the placenta[28]. As flow transference across the placenta is contested but assumed to cross the barrier[29-31]. Knowing the agent/s of toxicity present within fetal blood supply leaves the suggestion of a mode of toxicity.

Pubertal development: Growth and sexual development is controlled by two main overlapping systems: the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes. The HPG consists of the Hypothalamus, Pituitary, and Gonads while the HPA consists of the same first two organs of the HPG but includes the adrenal cortex, replacing the gonads[8]. The two systems differ in the characteristics that are developed. The HPG dictates gonadarche which begins with the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. Release of GnRH then works to initiate the release of Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) from the anterior pituitary. The primary trophic hormone for males during puberty is LH while FSH is the primary trophic for females. Each respective trophic hormone then initiates the production of sexual hormones for each gender, androgens (primarily testosterone) for males and estrogen (primarily estradiol) for females. Each respective sex hormone results in zygote production as well as structural development specific to each sex (Figure 1). Each sexual hormone then acts in a negative feedback role on the release of GnRH and LH/FSH for males/females. The second arm of sexual development, the HPA axis, is involved in the process of adrenarche, otherwise known as adrenal activation, between the ages of six and eight[8]. This process is not distinguished between the sexes where corticotropin releasing hormone (CRH) is released

form the hypothalamus to initiate the release of adrenocorticotropic hormone (ACTH). ACTH then works to promote the production and release of Andrestenedione, cortisole, and Dehydroepiandrosterone (DHEA) from the adrenal cortex that progress the development of pubic hair, armpit hair, and acne (Figure 1). All three effector hormones from the adrenal cortex (Andrestenedione, cortisole, and DHEA) also work in a negative feedback capacity to maintain the HPA axis. The process of adrenarche leads to pubarche, the production of secondary sexual characteristics (e.g. pubic hair development).

Lead, Mercury, Arsenic, and Cadmium involvement in the Axis: First, Do HMs cause substantial changes to the axis of interest, the HPG? Animal studies of Pb have shown statistically significant delays in puberty with both perinatal and postnatal exposure. Fisher 344 female rats were dosed with Pb at differing windows of exposure showed marked decreases in vaginal opening, first diestrus event and decreased serum IGF-1, LH, and E_2[21]. This finding was supported by Ronis et al who additionally observed significant increases in pituitary LH concentrations in addition to Pb disruption in the secretory pathway[32]. Hg showed modulation of endocrine function in animal and human studies. Multiple mouse species exposed to increasing doses of Hg within fathead minnows lead to decreasing levels of plasma T and E_2 levels in males and females respectively[33]. Female Sprague-Dawley rats exposed to aerosolized Hg resulted in decreased E_2 concentration in a dose-response fashion[34].Exposure to As has also shown affect to this axis, where Wistar rats exposed to As correlated with decreased serum levels of FSH, LH, and T[35]. Cd also showed modulate of hormones released from the pituitary (e.g. GH, FSH, and LH)[23]. Exposure to Cd during gestation has led to reduced birth weight as well as increases in spontaneous abortions and preterm birth[36]. Further work has been seen with combination of Cd and Pb and modulations of the HPG axis in peripubertal girls[37]. A cross sectional study looking at Inhibin B (released from the pituitary and gonads) found increased BLLs significantly decreased blood concentration of the hormone with similar trends seen with LH but lacked significance. Cd worked in a similar fashion in decreasing LH and Inhibin B but was not found to be significant.

Initiation of puberty and in-utero development: The main mechanism of what initiates puberty is still unknown, leaving a gap in what questions can be answered of the relationship between environmental effects of puberty timing[8]. Natural progression of puberty requires both axes be studied but due to simplicity and limitations, only the HPG axis will be studied. Development of the HPG axis begins with GnRH neurons migration the begin formation with the hypothalamus around 15 weeks gestation, suggesting the initiation point of the HPG within fetal development but this relationship has yet to be elucidated[38,39]. Fetal activation of the HPG axis begins near the second trimester with hormone secretion levels similar to post-menopausal women[38,40]. Increased oestrogenesis during the third trimester by the placenta results in decreases of LH through negative feedback mechanism[41]. At birth, release of LH is low within umbilical cord blood and increases two weeks after birth. The maximum release of LH within males is reached between four to ten weeks and steadily declines to lower levels after 6 months[39]. Females also see increases in LH but along a differing timeframe with gradual increase over the first two to three years[39]. Due to the initiation of the HPG axis during fetal development and susceptibility of this time period, the effect of HM exposure must be characterized.

Hypothesis: Fetuses are highly susceptible to toxicants because of reduced detoxifying capacity as compared to adults, thus are more likely to have developmental alterations resulting from those exposures. As previously stated, Pb, As and Hg have the ability to cross the placental barrier while Cd has shown effects on neonatal growth. Introduction of these agents have been shown to cause adverse health effects but adding to their potential toxicity is the perturbation of the HPG axis and subsequent disruption of sexual development and growth. Our hypothesis focuses on the mode of toxicity within the fetus. ***We hypothesis that higher maternal blood concentrations of Pb, As, Hg, and Cd will be associated with lower hormone concentrations (i.e., urinary LH) as primary outcomes of interest.*** Anthropometric measurements in neonates are also hypothesized to be lower with higher

concentrations of HMs. Specifically, anthropometric measurements will be head circumference, body weight, and body length.

Methods

Participants and recruitment: All study protocols were reviewed and approved by the Institutional Review Boars of Emory University and the Ethics Boards of Chiang Mai University and the Thai Ministry of Health. The **S**tudy of **A**sian **W**omen **A**nd their off**S**pring's **D**evelopment and **E**nvironmental **E**xposures (SAWASDEE) birth cohort is a longitudinal pilot birth cohort of farmworker women and neonates residing in the Chiang Mai Province of northern Thailand. Between March 2011 and February 2012, 59 pregnant women were recruited into the cohort during their first prenatal to the antenatal clinic at Fang Hospital also located in northern Thailand. Inclusion criteria were: ages between 18 to 40 years, Thai identification card permitting hospital and antenatal clinic access, Thai is the primary language at home, residence in their regional district for ≥6 months and planned to remain in residence for 1 month after delivery, good general health (i.e. no major medical conditions such as hypertension, diabetes, etc) and consumption of two alcoholic beverages or less per day and no illegal drugs. Thai identification cards allowed each pregnant women a minimum of one monthly prenatal visit to an obstetrician/ gynecologist. Mothers were followed longitudinally at each prenatal and postnatal visit until three days after delivery. Participation was high (59/59 or 100%) but three women were lost to follow up and one was excluded due to spontaneous abortion (95% retention).

Participants were administered a comprehensive questionnaire at the time of enrollment, at 28 weeks and 36 weeks to evaluate their knowledge, attitudes, and practices (KAPs). Demographic data collected included: maternal age and education, household income and occupations. Exposure information was also collected through KAP surveys on pesticide and metal exposure behaviors at home and work. Information included in the KAP questionnaire included: pesticide related hazards, pesticide safe-use practices, and maternal health and lifestyle factors. Additional information was taken from medical

records that included infant sex, body weight (BW), body length (BL), gestational age, and head circumference (HC).

Exposure assessment: Measurement of HM exposure for mothers was performed through blood metal concentrations. Mothers provided blood samples at three time points: recruitment, 28 and 36 weeks gestation coinciding with administration of KAP questionnaire. Blood samples collected in metal free-Vacutainer tubes by a licensed nurse or aid and stored at -20°C. Metal concentrations for neonates were assessed from umbilical cord blood samples collected at parturition by a licensed nurse or aid. Samples were stored at -20°C in Vacutainer tubes. Finally, neonatal samples were collected through application of a collection bag during the first week of life then stored at -20°C. All samples were held at this storage temperature until analysis at Emory University Rollins School of Public Health.

Analysis of HMs within collected blood samples was performed with Agilent 7700 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) connected to an ASX-500 Series autosampler (Agilent technologies). The ICP-MS system was outfitted and controlled with ICP-MS Mass Hunter Software version B.01.01 (Agilent Technologies). Quality of analytical data was assessed with the use of appropriate internal standards for each metal analyzed with an LOD for each respective metal (Pb as 0.5ng/µl, and Cd, As, and Hg were 0.05ng/µl) and the upper bounds for each metal (Pb at 200 ng/µL and As, Hg, and Cd at 20 ng/µL). Sample preparation for analysis was as follows. A total of seven samples were used for one extraction procedure with one blank included and treated identically to samples. Polypropylene tubes, 16mL, were rinsed with 1% nitric acid (Optima®, Fisher Scientific, Canada) and 1mL of whole blood sample was added. 1ml of internal standard, containing 250ug/mL of Lu and Sn. Samples and blank were mixed with 2mL of Nitric Acid and heated in stepwise temperature program on a heating block (from 25°C to 95°C). Samples were then cooled to 35°C and 2ml of H_2O_2 (Suprapure®, Merck KGaA, Germany) were added. Once added, samples were heated again to 75°C to which 1mL of H_2O_2 was added. Heating continued to 95°C and held for 40min. Samples and blanks were then cooled to room temperature and

added to 50mL propylene tubes and diluted to 25mL with 1mL of 25ug/mL $AuCl_3$ solution (made with 2% HCO_3) and 2% HCO_3. The samples and blanks were then stored at room temperature until analysis.

Spot urine samples were collected at each prenatal visit at the antenatal clinic using 50mL polypropylene cups. Each sample was aliquoted to smaller vials and stored at -20°C until analysis was conducted at Chiag Mai University in Thailand. Diakly phosphate (DAP) metabolites were measured using gas chromatography (GC) coupled with flame photometric detection (FPD) with isotope dilution methods. More detailed description of the analytical methods and quality control can be found elswhere[42] with a relative recovery range of 94.4-119% and relative standard deviation of less than 20%.The limit of detection(LOD) was reported as 0.1ng/mL to 2.5ng/mL in urine for all six common DAP metabolites

Maternal urine samples were collect at multiple time points throughout pregnancy, with an average of 8 samples for each woman, as well as one postnatally. Given the short half-life of Organophosphate pesticides, metabolite levels measured in postnatal urine samples reflect postpartum rather than in utero exposure.

Diethyl and dimethyl phosphate metabolites were converted to molar equivalents by dividing by their respective molecular weights and summed on a molar basis using equation 1.

$$\boldsymbol{\Sigma DEP} = \frac{[DEP]}{149\frac{ng}{nmol}} + \frac{[DETP]}{165\frac{ng}{nmol}} + \frac{[DEDTP]}{181\frac{ng}{nmol}} = \frac{nmol}{mL} x \frac{1000mL}{1L} = nM$$

$$\boldsymbol{\Sigma DMP} = \frac{[DMP]}{125\ ng/nmol} + \frac{[DMTP]}{141\ ng/nmol} + \frac{[DMDTP]}{157\ ng/nmol} = \frac{nmol}{mL} x \frac{1000mL}{1L} = nM$$

$$\boldsymbol{\Sigma DAP} = \boldsymbol{\Sigma DEP} + \boldsymbol{\Sigma DMP}$$

Equation 1 produced summary measurements for total diethyl phosphates (∑DEP) and total dialkyl phosphates (∑DAP) yielding three summary measures for each urine sample. Each participant's samples were averaged across trimesters to create a total average for the entirety of pregnancy.

Outcome assessment: Trophic sex hormones released form the Pituitary were the primary outcomes of interest. Measurement of LH was used as a proximal measurement of the health of the HPG axis. Due to the short half-life of LH within the infant blood stream, urine samples were also used for measurement[43]. LH was measured through Enzyme Immuno-Assay (Cayman Chemical, Ann Arbor, MI) with an LOD of 0.5mIU/mL and a range of 0-200mIU/mL. 1mL of Umbilical cord blood and urine were centrifuged at 1,000-2,000 xg for 15min and serum was transferred to separate tubes. Samples were ran in duplicate, with standard curves and a blank during each run. 20µL of sample, blank, and standard were added to each well. 100µL of LH-conjugate was added to each well except the blank and incubated at room temperature while rotating for 1hr. Wells were emptied and washed with specified buffer twice. Hydrogen peroxide substrate was then added to each well and incubated for 15min in the dark. A stop solution was added after incubation and read at 450nm with Synergy HT multi-mode reader (BioTek® Instruments).

Statistical analysis: Metals analyzed were reviewed for skew and log-transformed to fit normality assumptions. Survey data provided during all three visits was condensed into one observation for each mother. The mode value of dichotomous data of mothers whom provided information for all three time points was used. If any value was missing during follow-up, mode value was used unless values were discordant, then the mother was treated as missing data for the variable. Continuous data from the KAP survey was converted to dichotomous data for further analysis. Outcome levels were assessed for normality and dichotomized. LH was dichotomized based on previous studies searched for important thresholds[17]. HC, BW, and BL were dichotomized based on the WHO international growth charts. Due to

the small sample size and small changes in anthropometrics based on previous literature the 15th percentile was used as a threshold with any measurement below was considered low[44,45].

Due to the variability in half-life of each metal (Pb[46,47],Cd [48], Hg[49,50], MeHg[51-53], As[54]) an average concentration was estimated for average fetal exposure through gestation but required validation. This was accomplished through MANOVA analysis of metal concentration for mothers at each time point. Differences between average and earliest time point HM concentration of mothers to neonatal HM concentration was calculated through t-test. The average concentration was used for comparison to fetal HM concentrations. Correlation between maternal exposure and fetal exposure was conducted through Pearsons correlation coefficient of each metal. Correlation was calculated for earliest maternal and average HM concentrations.

Dichotomized LH was assessed with individual HMs for earliest and average mother concentration through univariate logistic regression. Multivariate logistic regression was used for variables available from the KAP survey, hospital chart review, and DAP analysis. These included: gestational age, total DAP congeners (DEAP and DMP) and iodine consumption of the mother. Odds ratios (ORs) for each individual metal were generated for individual metals in univariate and multivariate logistic regression with and without controlling for aforementioned variables. Continuous and dichotomous anthropomorphic variables were assessed with univariate and multivariate linear and logistic regression, respectively. Individual HMs were included in each regression for univariate analysis of anthropometrics then combined for estimation of ORs or β coefficients. Control of factors modulating anthropometrics were included in multivariate linear and logistic regression. Factors included: mothers height, weight and age, total maternal DAP congeners, parity, iodine consumption, and fathers smoking status. Confidence intervals were calculated with asymptotic methods due to limitation of sample size.

Results

Basic demographics: Basic descriptive statistics for mothers and fathers is provided in Table 2. The average (±S.D.) age at birth was 26.32(±4.73) years where height and weight of the mothers was 153(±5)cm and 48.85(±6.76)kg, respectively. The majority of mothers lived as though they were married (91. 1%) with the greatest ethnicity being Thai Yai (60.7%),the largest portion of the Shan people that constitute a majority of Northern Thailand. The second largest ethnicity were those identifying as Thai (19.6%), the dominant peoples across Thailand. The majority of mothers spoke phasa tai yai (58.9%), a largely used language within the Shan people with Northern Thai (26.8%), similar to the national language of Thailand, second. The majority of mothers had no education (64.3%) or primary school education only (17.9%). The metric of poverty was those receiving <6000 Bhats per month (~200 USD), the local currency of Thailand, where over 95% of the cohort earned below this in one year. Over 70% of mothers said that the father spent all of their time with the mother while the remaining 19% spent most of their time with them. There was an even split between fathers ethnicity, 50% Thai and 50% Thai Yai. Lastly, fathers smoking status was 62%. The estimated gestational age when follow up began was 14.79(±3.09) weeks with an average gestational age at birth of 38.66(±1.51) weeks with even divide between males and females children within the cohort.

Occupational categories: Mothers worked more than one job over the duration of their pregnancy and each occupation had differing exposures (Table 3). The majority of women worked in occupations with fertilizer and agricultural pesticides, 63.0% and 61.1% respectively. Less women, however, had agricultural foreman jobs (34.5%). A small proportion of the cohort worked as food packers (9.1%) but there were many that worked within facilities where food packing occurred (30.4%). Father's occupations followed mother's occupation with the majority of fathers worked within agricultural work (79.6%). A little under 70% (69.8%) also worked with pesticides in their jobs.

Accuracy and precision of analysis: Analysis of metals through ICP-MS was reviewed through accuracy and precision of methodology (Table 4). The metric for accuracy was recovery percentage for each spiked internal standards through extraction protocols, Pb and Cd were validated with an Indium(In) internal standard and As and Hg were validated with Lucinium (Lu). Accuracy for In showed all analyzed samples were between the range of 80 to 120% (mean=100.60%, range=83.14 – 113.05%). Lu was also deemed accurate with all sampled between 80 to 120% recovery (mean=95.41%, range=85.40 – 117.12%). Precision of metal analysis was measured with the relative standard deviation (RSD) for each sample in triplicate. All RSD values calculated were below an *a priori* threshold of 20%: Pb mean of 1.32%, Cd mean of 6.32%, As mean of 6.53% and Hg mean of 4.13%. Precision was also measured for EIA hormone analysis. Precision was based on the coefficient of variance (CV) estimated through duplicates of each sample. All CV values were below the *a priori* 15% CV limit. The mean CV for analysis was 4.92% with the range of 0% to 11.34%.

Exposure, confounder and outcome summary: Analysis of the differences between measurement time points of metal concentrations showed no significant difference. The average concentration over the three measurement points and earliest HM concentration was used in analysis (Table 5). The average Pb concentration within mothers was 2.78 µg/dL (±1.24 µg/dL), Cd 0.06 µg/dL (±0.04 µg/dL), As 0.21 µg/dL (±0.16 µg/dL) and Hg 0.125 µg/dL (0.06 µg/dL). Earliest time point HM concentration was: Pb 2.91(±1.34)µg/dL, Cd 0.08(±0.04) µg/dL, As 0.21(±0.24)µg/dL and Hg 0.13(±0.08)µg/dL. Concentrations of each metal was also found within umbilical cord samples representing each child. The mean concentration within neonates was 2.05 µg/dL (±1.28 µg/dL) for Pb, 0.01 µg/dL (±0.01 µg/dL) for Cd, 0.17 µg/dL (±0.15 µg/dL) for As, and 0.21 µg/dL (±0.15 µg/dL) for Hg. Umbilical cord concentrations in neonates was significantly lower in Pb (<0.05%), Cd and Hg (each P<0.001%) for average HM concentration over pregnancy. Comparison of earliest time point measurement and Umbilical cord blood concentration yielded significant differences to umbilical cord HM concentration. Pb and Hg both

had P<0.005 and Cd had a P<0.0001.Correlation between mother's average concentration and umbilical cord concentration was plotted (Graph 1-4) with R^2 values for Pb, As, and Hg were significant (P<0.001%, P<0.001% and P<0.05% respectively). Earliest time point concentration compared to umbilical cord HMs was significant for Pb (P<0.001) only. Average total DAP (∑DAP) concentration was 374.00nM (±394.764nM). Two congeners of DAP were analyzed for total DEAP (∑DEAP) and total DMAP (∑DMP). ∑DEAP mean concentration was 394.76nm (±384.272nM) and ∑DMP mean concentration of 41.03nM (±67.093nM).

Confounders of interest collected through the study are summarized in Table 6. The mean mother's age at birth was 26.32 (±4.73) and height of 153cm (±5cm). The mean mother's weight before pregnancy was 48.85kg (±6.76kg). Gestational age at beginning of follow up was 14.79 weeks (±3.09 weeks) with gestational age at birth being 38.66 weeks (±1.51 weeks). 50% of the neonates fathers had smoked during the follow-up period and consumption of iodine was high in over 50% of the families interviewed (51.9%). The arithmetic mode of mother's parity was one child with a range of the current child in the study being first to three previous pregnancies.

The primary and secondary outcomes of interest can be seen in Table 7. LH had a mean value of 2.04 mIU/mL (±1.71 mIU/mL). Dichotomization showed the prevalence of low LH just under 20% (19.6%). Mean children birth weight and length were 2862.5g (±420.25g) and 51.54cm (±2.71cm) and HC of 32.84mm (±1.68mm). Prevalence of each dichotomized anthropometric outcome was: low BW = 51.8%, low BL = 5.4% and low HC = 66.1%.

Primary outcome: Logistic regression of the relationship between average maternal HMs concentrations and low LH levels in neonatal urine began with unadjusted point estimates (Table 8). Cd was the only metal with a significant unadjusted point estimated (OR=0.05, 95%CI=<0.01, 0.66) with all other metals with non-significant estimates. Pb and As had unadjusted values with decreased odds of low LH (OR =

0.51 and 0.42 respectively) while Hg showed protection (OR of 0.46). Unadjusted values for the earliest time point HM concentration of mothers compared to neonatal LH resulted in a higher OR for Pb (1.83) exposure while all other metals showed protection (Table 8). Pb, Cd, and As average maternal concentration estimates indicating lower odds of low LH (OR = 0.59, 0.04 and 0.44 respectively) while Hg exposure increased odds (OR equal to 2.55) after adjustment. Cd lost the significant protective OR after adjustment (OR=0.04 95%CI=<0.01, 1.03). Adjustment of the earliest time point maintained no significant relationship but higher odds of low LH from Pb exposure while all others where protective. Combining all metals in logistic regression, Cd was the only metal to significantly change the odds of low LH before adjustment for average maternal concentration (OR = 0.02 95%CI = <0.01, 0.71) (Table 9). Pb showed decreased odds of low LH while As and Hg showed higher odds of low LH (OR=0.405, 1.06 and 2.22 respectively). Unadjusted earliest time point maternal HM concentration with combined metals resulted in increased odds of LH for Pb (OR=1.62). When adjusted for all variables in combination for the effect of each metal for average HM concentration of mothers, Cd lost significance (OR=0.01, 95%CI=<0.01, 1.04) and Pb (OR=0.49) showed protection. As (OR=1.24) and Hg (OR=2.38) showed higher odds of low LH. Earliest time point HM concentrations in others showed higher odds of low LH for Pb (OR=1.62), As (OR=1.06) and Hg (OR=1.07) while Cd indicated protection (OR=0.48) with no significance.

Secondary outcomes: Infant Anthropometrics were compared to each individual (Table 10, 11 and 12) and combined HMs (Table 13, 14 and 15). For HC, unadjusted values of average maternal HM concentration of individual metals did not show significance. Pb, Cd and Hg showed negative coefficients (β = -0.06, -0.49, and -0.07 respectively) but only Cd, As and Hg had higher odds of low HC when dichotomized (OR = 1.64, 1.62 and 2.36 respectively). Earliest time point maternal HM concentration coefficients were negative for Cd (-0.71), As (-0.28) and Hg (-0.16) but not Pb (0.91). This persisted when HC was dichotomized (OR=0.33, 2.17, 2.33, and 1.95 for Pb, Cd, As and Hg respectively). Average maternal HM concentration, for individual HMs, coefficients after adjustment resulted in Pb, As, and Hg

with positive values (0.87, 0.56 and 0.01 respectively) and a negative Cd value (-0.19), all non-significant. When dichotomized, Pb showed protection (OR=0.38) while Cd, As and Hg had higher odds of low HC (OR=1.61, 1.84, and 2.58 respectively). Adjustment of earliest time point maternal HM concentration yielded negative coefficients for Cd (-0.45) and Hg (-0.356) only. Dichotomized outcome variable for earliest time point maternal HM concentration, after adjustment, added As to Cd and Hg for higher odds of low HC (Cd=1.88, As=1.77, Hg=2.38), all non-significant.

Unadjusted linear regression of BW with average maternal HM concentration produced negative coefficients for Cd, As and Hg (-140.2, -186.66 and -76.72) and a positive Pb coefficient (65.32). Dichotomized BW confirmed continuous outcomes with Pb as protective (OR=0.85) and Cd, As and Hg as aversive (OR=2.13, 2.91 and 1.43). Use of the earliest maternal HM concentration before adjustment resulted in negative coefficients for all HMs (Pb=-1.03, -140.74, -251.99 and -86.80 for Pb, Cd, As and Hg respectively). Dichotomization of the outcome for earliest maternal HM concentration flipped Pb (0.49) and Hg (0.92) to protective and maintained Cd (1.98) and As (4.52) as aversive. Adjustment of individual metal linear regression of average maternal concentration showed all metals with negative coefficients (Pb=-30.64, Cd=-142.04, As=-209.93 and Hg=-51.75). Dichotomization of BW resulted in increased odds for Pb (OR=1.45), Cd (OR=1.93), and As (OR=2.86) with protection with Hg (OR=0.64). Earliest time point maternal concentration after adjustment showed all metals had negative β's but when BW was dichotomized, only Cd and As maintained negative effects (Cd OR=1.53, As OR=2.86) while Pb and Hg showed protection (Pb OR=0.64, Hg OR=0.82). Unadjusted linear regression with all metals for average maternal concentrations produced negative coefficients for Cd (-110.43), As (-147.49) and Hg (-55.84) while Pb's coefficient was positive, β=78.71. Dichotomization of BW with average maternal concentrations yielded similar results (Pb OR=0.83, Cd OR=1.65, As OR=2.18 and Hg OR=1.28). Earliest time point maternal HM concentration with all metals combined yielded negative coefficients for Cd, As, and Hg (-87.28, -237.89, and -70.57 respectively) and a positive Pb coefficient (18.86). Dichotomization

of BW with earliest maternal HM concentration yielded higher odds for Cd (OR=1.75) and As (OR=5.00) exposure but protection with Pb (OR=0.43) and Hg (OR=0.79) exposure. As resulted in a statistically significant decrease in body weight when the earliest time point was compared to the continuous outcome. This significance disappeared once bodyweight was dichotomized. Adjusted average maternal concentration for combined metals resulted in negative coefficients for Cd As, and Hg (-96.82, -207.92 and -9.65 respectively) while Pb was positive (4.19). Dichotomization yielded increased odds of low BW for Pb (OR=1.60), Cd (OR=2.18) and As (OR=4.75) while Hg (OR=0.80) showed lower odds. Use of the earliest time point maternal concentration after adjustment produced negative β's for all metals (Pb=-67.00, Cd=-101.69, As=-230.85 and Hg=-60.53) after all metals where added to the regression. Dichotomization of BW for comparison to earliest time point resulted in higher odds ratios for Cd (OR=1.56) and As (OR=5.28) while Pb and Hg showed lower odds ratios (OR=0.43 and 0.59 respectively) when all metals were included after adjustment.

BL linear regression for unadjusted individual average maternal metal concentration resulted in negative β's for Pb (-0.36) Cd (-0.94) and As (-0.25) with a positive Hg coefficient (0.09). Dichotomized BL yielded higher odds for Pb (OR=3.71) only. Cd, As and Hg were protective compared to dichotomized body length (OR=0.48, 0.98 and 0.40 respectively). Unadjusted earliest time point for maternal HM concentration yielded negative β's for all metals (Pb=-0.06, Cd=-0.77, As=-0.84 and Hg=-0.41). Dichotomization of BL for earliest maternal metal concentration yielded higher odds for Pb (OR=3.31), As(OR=1.71) and Hg (OR=1.32). Cd yielded lower odds of low BL (OR=0.34). Adjusted BL estimates for average maternal HM concentration resulted in negative values for Cd and As (-1.15 and -0.18 respectively). Pb and Hg showed positive coefficients (0.02 and 0.59 respectively). Dichotomization of BL in adjusted regression yielded higher odds for Pb only (OR=1.69). Cd, As, and Hg showed lower odds (OR=0.01, 0.34 and <0.001 respectively). Adjusted regression with the earliest maternal HM concentration resulted in negative β's for all metals (Pb=-0.06, Cd=-1.22, As=-0.80 and Hg=-0.36).

Dichotomization of BL with earliest time point maternal HM concentration, with adjustment, resulted in only Pb having higher odds of low BL (OR=5.47). Cd, As and Hg showed protective ORs (0.05, 0.90 and 0.38 respectively). Regression with average maternal HM concentration with all metals combined without adjustment showed negative coefficients for Pb and Cd (-0.34 and -0.76) while As and Hg were positive (0.03 and 0.13 respectively). Dichotomization of BL with average maternal HM concentration altered results with Pb and As showing higher odds of low BL (2.03 and 1.52 respectively). Cd and Hg showed protection from low BL (0.48 and 0.36 respectively). Earliest maternal HM concentration with all HMs combined without adjustment yielded negative β's for all metals (Pb=-0.05, Cd=-0.59, As=-0.74 and Hg=-0.30). Dichotomization of BL with the same parameters altered Cd effect on protection (OR=0.31) while all other metals maintained higher odds of low BL (Pb=2.79, As=1.96 and Hg=1.38). Adjusted average maternal HM concentration with all metals included showed positive coefficients for Pb (0.23) and Hg (0.81) with Cd and As were negative (-1.14 and -0.36 respectively). OR for dichotomized BL resulted in incalculable values after adjustment for extraneous variables. Adjusted coefficients for earliest time point maternal HM concentration with all metals included yielded negative values for Cd, As and Hg (-1.15, -0.74, and -0.05 respectively) while lead showed a positive coefficient (0.08). Dichotomization of the outcome with earliest concentration and all metals included yielded incalculable results for odds.

Discussion

Maternal exposure: To date, this is the second study to characterize the relationship of HMs relationship with the HPG axis in respect to modulation of puberty, and is the only study looking at in-utero exposure and immediate changes of the HPG axis at birth[37]. A large strength of the SAWADSEE birth cohort is the multiple samples collected throughout follow-up. Increased sampling gives greater characterization of maternal exposure during pregnancy. Grouping of time points when mothers blood samples were collected and analysis through MANOVA indicated that exposure and subsequent HM blood

concentrations were independent/consistent throughout follow-up. Validity of this assumption is suspect given blood Pb levels decrease then increase during the second and third trimesters[55]. Due to the variability in sample collection throughout pregnancy, the possibility of missing this dip in concentration is possible. Even with a biological half-life greater than repetitive measurements within this study, the true maternal blood Pb concentration requires further validation. As has a biological half-live below the difference in time of sample collection meaning the assumption of independent exposure of the mother may not be correct. Hg's initial effluence and differences between organic and inorganic half-lives also added complexity to the assumption. Rapid metabolism, allocation to the kidneys and excretion of inorganic mercury falls short of the assumption but methyl mercury could adhere to the assumption. Cd does follow the assumption with an extensive half-life within the body but body burden of Cd sequesters to the Kidneys and Liver. Due to difficulty in assumption for the independent exposure assumption, the earliest measurement in mothers may prove more pertinent to the question at hand. This is due to the unique window of vulnerability specifically seen within the first trimester when GnRH begin migration to establish the HPG axis. The formation of the HPG axis may result in decreased in function not observed if exposure occurred later in pregnancy.

Fetal exposure: Pearson correlation coefficients indicated stronger positive relationships for Pb and Hg with weaker correlation values for As for earliest collected HM concentrations and average HM concentrations. Lack of significance for both of Hg's and As' correlation decreased confidence in their to determine neonatal concentration. Interpretation of Pb exposure for neonates from mothers has the stronger evidence to show possible effect from exposure with correlation coefficients and biological plausibility of exposure. As is limited by the short biological half-life in mothers where exposure may not be accurately represented by a correlation based on one to three samples and lack biological plausibility due to a shorter half-life. The scientific literature supports neonatal exposure from mothers but is not seen within this analysis. This called for reservation of conclusions made about direct exposure to and

health outcomes of Hg and As. Cd differs from other metals where evidence showed an inability to cross the placenta. Correlation between mother and neonatal blood within the cohort did enforce this relationship.

Outcomes interpretation: Prevalence of low LH and low BL were low within the cohort when categorized. Low HC and Low BW, however, were extremely high. Validity of this prevalence is suspect with regards to the threshold chosen. HC's threshold was based on international standards that were not necessarily specific to populations found within children living in northern Thailand. The same issue could explain similar high prevalence within BW. Consideration of thresholds for LH is under scrutiny as well due to lack of evidence suggesting what values are considered important. Threshold chosen for low LH was designated based on previous research on LH concentration deemed important for pubertal onset. Translation of this value may be inappropriate due to differing timelines of hormone release during fetal life necessary for later development (i.e. population of peripubertal individuals versus neonates).

The relationship between mothers HM concentration and LH in neonatal urine showed significant estimates only for Cd. Univariate analysis multivariate, when other HMs where added, analysis showed Cd maintained a protective effect indicating that the odds of low LH at birth were lower when the mothers exposure to cadmium was higher. This relationship lost significance after adjustment for other factors affecting LH production were controlled for. Confidence intervals for the adjusted Cd estimate was, however, close to significance for all estimated in relation to low LH. Caution should be taken when considering the relationship of Cd and LH in neonates at birth. Multiple comparison adjustments were not performed during analysis and may dissuade any relationship seen from initial results. Biological feasibility of Cd's effect on hormone modulation during fetal development and birth is still possible. One study has noted Cd's interference of oestradiol production in the placenta[56]. Cd's ability to interfere with oestradiol production within the placenta can result in decreases of E_2 and protection from the negative

feedback mechanism within HPG axis. Cd could modulate the natural progression of neonatal hormone milieu that leads to the natural decrease of trophic hormone production but the significant health effects of this progression are not known.

The hypothesized relationship of HMs decrease of LH during fetal life is contradictory to the statistically significant relationship observed. Categorization of either protective or harmful fluctuated based on exposure classification, i.e. average or earliest time point, with large changes in magnitude for point estimates. Pb point estimates using the earliest maternal HM concentrations did follow the hypothesized relationship, although lacking significance, but did not when considering the average maternal HM concentration. Consideration of the time of exposure, especially during gestation, is important when determining a toxicants effect. Pb exposure during later in gestation may not have effects on the fetal HPG axis. Later exposure in fetal development may be important when considering Cd. Cd did show statistically significant protection for average maternal concentration of HMs. This later exposure time point needs to be considered due dynamic hormone levels from the placenta and subsequent modulation of this progression.

Association between maternal HM exposure and neonatal anthropometrics showed mixed results. Prenatal Pb exposure and low HC is shown form many studies but results observed disagreed with use of average and earliest time point. Dichotomization yielded concordance with previous literature but issues with this methods have been stated within this paper. BW did show statistically significant estimates supported by previous study in neonates. Observation of As effect on BW was only with the use of the earliest time point of maternal HM concentration further supporting the argument for consideration of the time of exposure. Concordance with previous studies helps validate results even without lack of significance and strengthens interpretations of the effect of HMs on neonatal anthropometrics. Inversely, lack of robustness with estimates deters conclusion to support HMs effect

on anthropometrics at birth and further study is required to answer this question within the Thai population.

Limitations: The SAWADSEE birth cohort was originally designed to assess the relationship between organophosphates prenatal exposure and neurodevelopmental changes[57]. Consideration of the focus of the pilot cohort is a large limitation where hypothesis generation *post hoc* and lacked appropriate study designs to capture the true relationship between tropic hormone releases in neonates and heavy metal exposure in mothers. Per nature of pilot studies, smaller samples were used for hypothesis generation and preliminary study, not conclusions.

Analysis of neonatal hormones was performed through EIA but the kit used was not validated for the use of urine. The EIA used in analysis was validated for measurement of LH within serum only and may have led to incorrect interpretation. Validation of the tools used for measurement may lead to overestimation or incorrect characterization of the relationship of HMs effect on LH. Additionally, natural secretion of LH within neonates is modulated with fluctuations of effector sex hormones from the mothers HPG axis as well as the placenta, requiring time after parturition for the neonatal HPG axis to acclimate to the new environment. Subsequent effects on trophic hormones from the mother's hormones may distort HMs effect on LH measured within this study. Effects of extraneous maternal/placental hormones may result in a strong signal that will increase LH above noticeable levels that HMs could modulate. Inversely, collection of fetal blood samples during the second trimester would act as a viable method to determine the effect of HMs on the HPG axis. Future study focused on LH must require sufficient spacing from birth or before the placenta decreases trophic hormone release to collect samples reflecting neonatal function of the HPG axis without external hormonal influence.

Lack of information gathered from the mothers HPG axis, placental hormone release and differing trophic hormones used by the neonatal HPG axis produced large gaps in understanding the

hypothesized relationship. Focus on one biomarker of effect within the neonate is largely flawed due to possible susceptibility to other environmental exposures. Exposure was measured for two main toxicants: HMs and Organopesticides. HMs have been shown to modulate LH but the effects of organophosphates on this biomarker, LH, are lacking. Possible susceptibility of LH to other exogenous chemicals could have contributed to the relationships observed. Reliance on one biomarker leaves large susceptibility to chance and possible overestimation of HMs role in endocrine modulation due to complexity of the system with each hormone of the HPG axis overlapping cascades. Inclusion of separate hormones as biomarkers of effect would overcome this shortfall but must be relevant to the pathway, i.e. chosen from the Hypothalamus or Pituitary.

Conclusion

HM exposure effects the normal progression of puberty within boys and girls measured by tanner stage progression and menarche respectively. Answering whether HMs exposure during fetal life causes changes in the system propagating puberty is still contested. Our hypothesis that increased HM exposure would decrease the level of LH was supported and refuted based on the timing of exposure considered. Maternal Cd exposure increased LH levels at birth in neonates. This was only seen with the average maternal exposure and disappeared when the earliest time point was used. Inversely, increased As maternal concentration at the earliest time point showed significant decreases in BW but disappeared when the average maternal concentration was used. Consideration of the time of exposure during fetal life is extremely important due to fleeting windows of susceptibility during gestational development. Our hypothesis of lowering neonatal LH concentration with higher Pb levels did occur but only when measured closest to the first trimester. Pb's neurotoxicity is accepted and explains the increased odds of low LH with affecting neurodevelopment of the GnRH neuron migration, the preceding step to release of LH from the pituitary. When the average value is used, this disappears and becomes protective. Pb's effect on the axis may be miniscule compared the effect of placental

hormones during the third trimester which decrease with increased Cd concentration. With the major limitations stated, Observations within this study allow hypothesis formation and research planning for future study, not conclusion.

Recommendations for future study of HM's and the HPG axis include shifting the timeline of sample collection, diversity in biomarkers of effect and increased media type analyzed. Sample collection time is crucial to capture the dynamic process of hormone effects during fetal life. Capturing neonatal HPG activity would require sample collection either pre-conception to the second trimester or from the second trimester to 1 month after partition. Recruitment of mothers pre-conception is a difficult task so the second timeframe is suggested. LH secretion in neonatal male's peaks around 1 month post parturition where maternal HM concentration during birth would be compared to time until levels begin to decline. Neonatal females gradually increase LH levels in the first years of life. Analysis would consist of high and low maternal HM levels compared to high and low LH concentrations. Biomarkers of effect should include multiple hormones from the HPG axis associated with pubertal development. FSH and GnRH are two suggestions due to their direct involvement in the HPG axis and production during fetal life. Inclusion of these hormones allows analysis of differing target organs of HMs and greater characterization of the mode of toxicity that HMs play in puberty modulation. Lastly, multiple media should be used for exposure and outcome analysis. The short biological half-life of LH indicates that urine is important to capture the total amount produced over the period of collection. Even with a short-half-life, knowing the concentration within blood is important for understanding concentrations sufficient to progress normal development. Equally as important is determination of exposure. Cd was measured through maternal blood samples but sequesters to the kidneys and liver, removing its ability to modulate placental and neonatal hormone levels. Collection of urine samples would further describe the amount of HM within fetal circulation after elimination from the body.

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Appendix

Table 1. Research Focus from Expert Panel		
Priority Research Questions	*Recommended Studies*	*Recommended Species*
Etiologic Research		
1. Are environmental levels of EDCs affecting onset and progression of puberty and aberrant pubertal development such as delayed or precocious puberty in children?	*Prospective epidemiologic research inclusive of existing cohort currently being followed (eg, Seveso, Yucheng, Yusho, PBB, DES grandchildren) or newly designed epidemiologic research capturing environmentally relevant exposures (single chemical and mixtures) Cross-sectional studies for monitoring population changes particularly across geographic areas and inclusive of high-risk subgroups of the population*	*Humans*
2. Do body shape and adiposity affect onset and progression of puberty and aberrant pubertal development such as delayed or precocious puberty in children?	*Prospective epidemiologic research inclusive of existing cohort currently being followed (eg, Seveso, Yucheng, Yusho, PBB, DES grandchildren) or newly designed epidemiologic research capturing environmentally relevant exposures (single chemical and mixtures) Cross-sectional studies for monitoring population changes particularly across geographic areas and inclusive of high-risk subgroups of the population*	*Humans*
Critical windows of exposure		
3. What are the critical windows, ranging from periconception through adolescence, for human pubertal development in relation to environmental exposures?	*Prospective research comprising couples attempting pregnancy to capture parentally mediated exposures from periconception window to prospective pregnancy cohorts Research focusing on the early origins of human health and disease*	*Humans and nonhuman primates*
Mechanistic		
4. What is the primary signal(s) for the onset of GnRH-dependent (central) puberty?	*Genomics and proteomic studies using nonhuman primates and humans with alterations in the onset of puberty*	*Nonhuman primates and humans*
5. What is the primary signal(s) for the onset of GnRH-independent (peripheral) puberty?	*Genomic and proteomic studies using transgenic rodent model.*	*Rodents*
6. What is the molecular basis for sexual dimorphism in puberty onset and progression?	*Identifying the similarities and differences in molecules involved from puberty initiation to final outcome (described in 4 and 5) in males and females*	*Rodents, nonhuman primates*
7. How is the tempo of puberty progression including the temporal relationship among the different markers regulated?	*Environmental exposure and puberty-timing studies that measure >1 puberty-timing end point/outcome.*	*Rodents, nonhuman primates, humans*

Table 1. Description of the future research focuses and needs to answer the question of environmental factors and onset of puberty. Provided by Buck Louis, G. M. et al.[8]

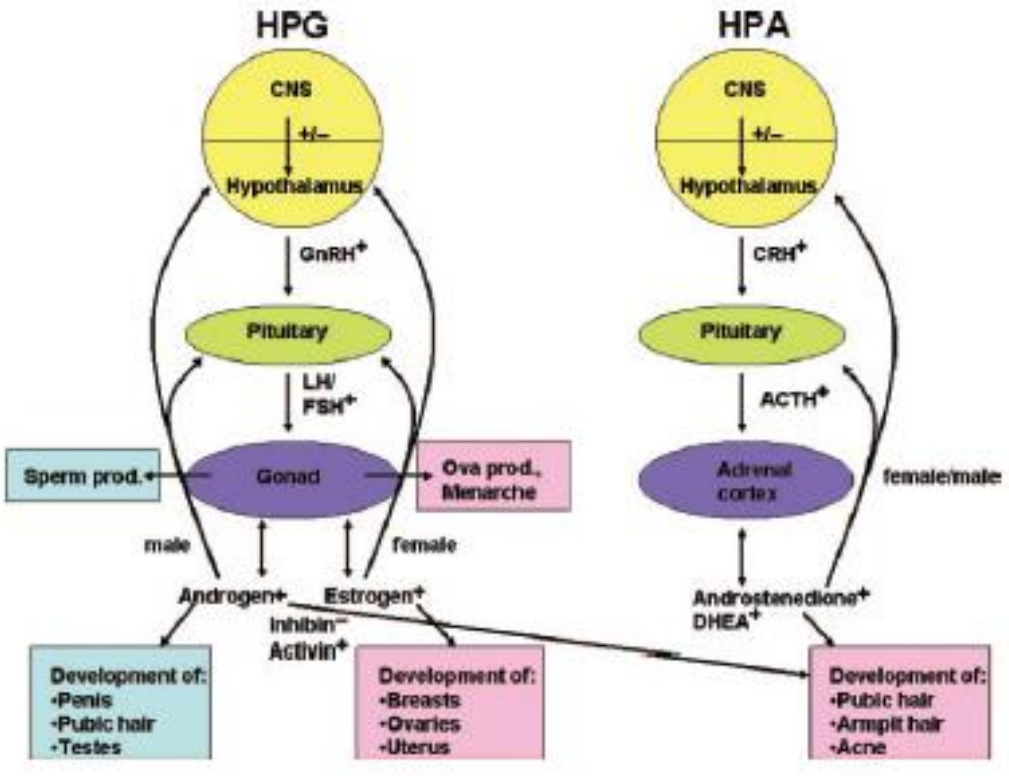

Figure 1. (Left) the Hypothalamic-Pituitary-Gonadal axis controls the development of sexually reproductive organs. (Right) The Hypothalamic-Pituitary-Adrenal axis controls the development of secondary sexual characteristics for both sexes. Provided by Buck Louis, G. M. et al.[8]

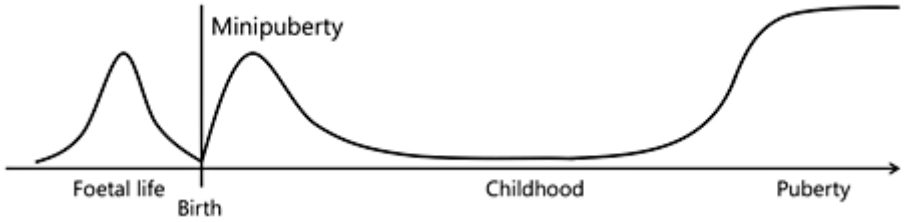

Figure 2. Progression of Puberty through fetal, infant and adolescent life. Provided by Kuiri-Hannien, T. et al[58].

Table 2 Mother and Father Basic Demographics

Marital Status	N	%
Living as Married	51	91.1
Married	5	8.9
Mother's Ethnicity		
Thai	11	19.6
Tai Yai	34	60.7
Chinese	2	3.6
Palong	3	5.4
Muser	4	7.1
Larhu	2	3.6
Language		
Northern Thai	15	26.8
Thai Yai	33	58.9
Other	8	14.3
Education		
None	36	64.3
Primary School	10	17.9
Middle School	2	3.6
High School	7	12.5
Some College	1	1.8
Income		
<6000 Bahts/month	50	96.2
>6000 Bahts/month	1	1.9
NR/DK	1	1.9
Father Smoke Status		
Yes	26	61.9
Father Ethnicity		
Thai	28	50.0
Thai Yai	28	50.0
Time father spends with mother		
All of the time	39	70.9
Most of the time	16	29.1
Sex of Child		
Male	28	50

Table 2. Basic demographic descriptors for the birth cohort in Chiang Mai, Thai.

Table 3 Occupational Variables

	N	Yes	%
Mother			
Work with fertilizer	54	34	63.0
Job with ag pesticides	54	33	61.1
Ag foreman Job	55	19	34.5
Food package job	55	5	9.1
Work in food packing or cannery	56	17	30.4
Father			
Agricultural work	54	43	79.6
work with pesticides	53	37	69.8
food processing	52	9	17.3

Table 3. Occupational categories for mothers and fathers in birth cohort

Table 4 Accuracy and Precision of Metal Analysis

		Accuracy(Recovery)		Precision(RSD)
Metal		Mean (%)	Range	Mean (%)
Lead	In	100.60	83.14 - 113.05	1.32
Cadmium	In	100.58	83.14 - 113.06	6.32
Arsenic	Lu	95.41	85.40 - 117.11	6.53
Mercury	Lu	95.35	85.40 - 117.12	4.13

Table 4. Accuracy and precision of laboratory analysis of HMs by recovery percentage and RSD

Table 5. Metal and DAP distribution in Mothers and Neonates

	Neonates		Mothers					
			Earliest			Average		
Metals	N	Mean(±SD)µg/dL	N	Mean(±SD) µg/dL	P value	N	Mean(±SD) µg/dL	P value
Lead	45	2.05(±1.28)	56	2.91(±1.34)	P<0.005	55	2.78(±1.24)	P <0.005
Cadmium	45	0.01(±0.01)	56	0.08(±0.04)	P<0.0001	55	0.075(±0.038)	P<0.001
Arsenic	45	0.17(±0.15)	56	0.21(±0.24)	P=0.307	55	0.21(±0.16)	P=0.206
Mercury	45	0.21(±0.15)	56	0.13(±0.08)	P<0.005	55	0.125(±0.064)	P<0.001
Total						N	Mean(±SD)nM	
DAP		n/a		n/a		56	374(±394.76)	
DEAP		n/a		n/a		56	331.64(±384.27)	
DMAP		n/a		n/a		55	41.03(±67.09)	

Table 5. Metal and DAP congener distribution and t-test analysis for earliest and average metal concentration in mothers

Table 6. Confounders/Covariates Evaluated

	N	Mean (±SD)	
Mother's Age at Birth	56	26.32(±4.73)	
Mother's height	56	153(±5)	
Mother weight before pregnant	55	48.85(±6.76)	
Gestational age at start	56	14.79(±3.09)	
Gestational age at birth	56	38.66(±1.51)	
	N	**%**	
Father Smokes	56	50	
High Iodine Consumption	54	51.9	
	N	**Mode**	**Range**
Parity	56	01	0-3

Table 6. Confounders collected through medical record review and KAP survey for birth cohort

Table 7. Outcome variables Distribution and Prevalence

Continuous	***N***	***Mean(±SD)***
Child Birth weight*(g)*	*56*	*2862.5(±420.25)*
Child Birth Length(cm)	*56*	*51.54(±2.71)*
Child Head Circumference*(cm)*	*56*	*32.84(±1.68)*
LH (mIU/mL)	*46*	*2.04(±1.71)*
Dichotomous	***N***	***(%)***
Low LH	*46*	*19.6*
Low BW	*56*	*51.8*
Low BL	*56*	*5.4*
Low HC	*56*	*66.1*

Table 7.Outcome distributions and prevalence's for birth cohort neonates

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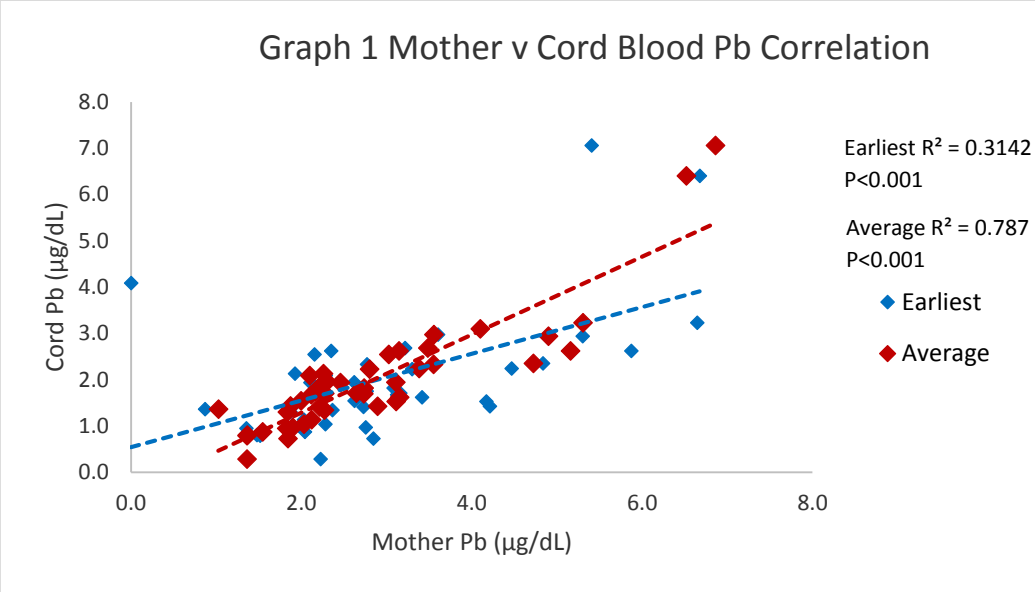
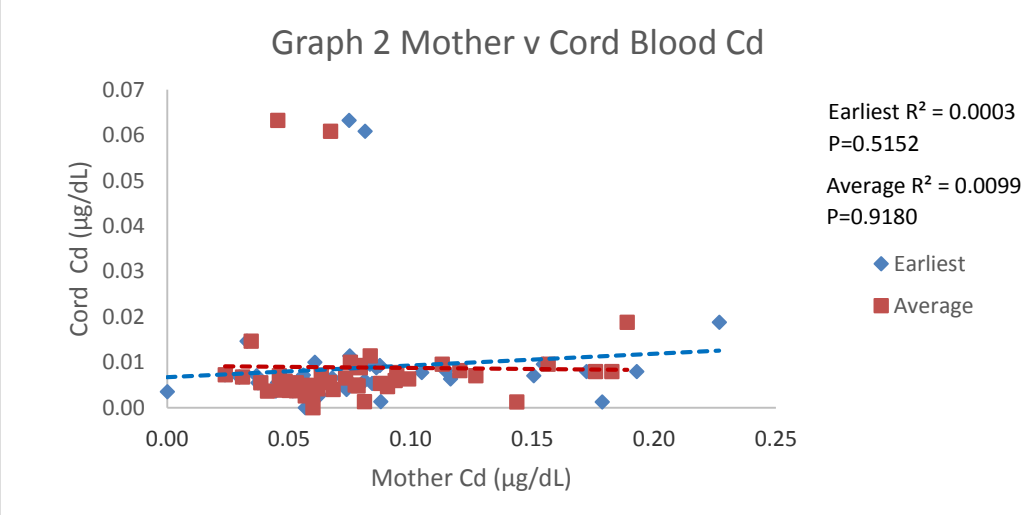
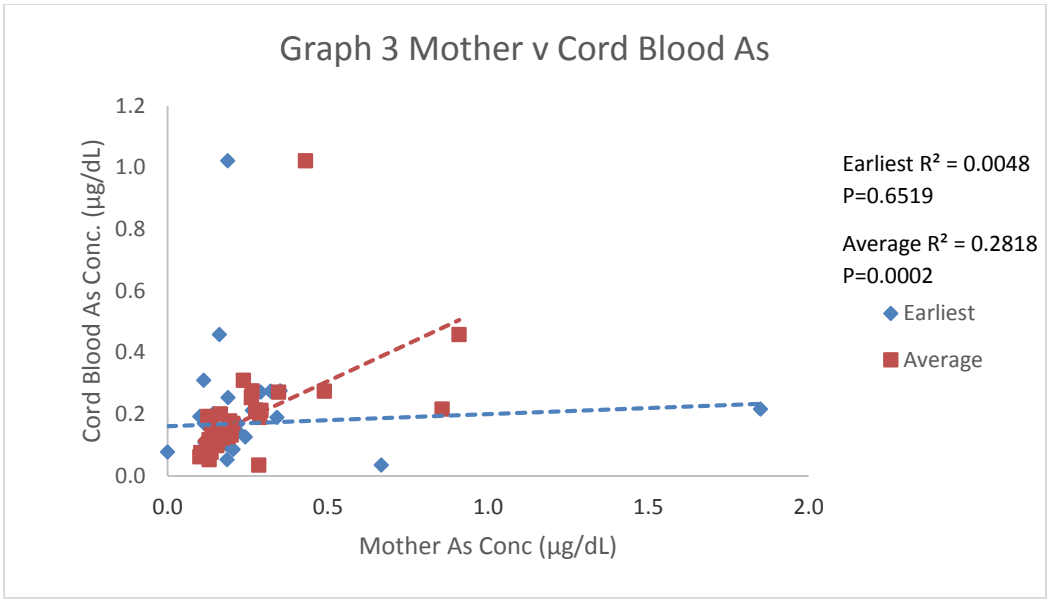
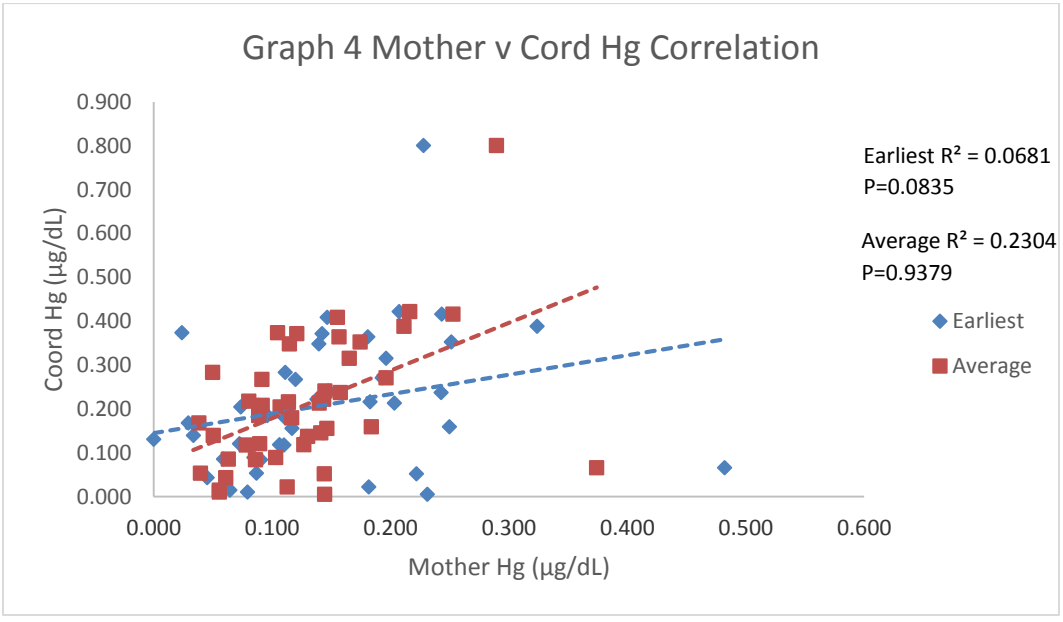




Graph 1(top). Pearson correlation of Maternal Pb concentration of earliest time point (blue) or average (red) concentration to umbilical cord blood.
Graph 2(bottom). Pearson correlation of Maternal Cd concentration of earliest time point (blue) or average (red) concentration to umbilical cord blood





Graph 3(top). Pearson correlation of Maternal As concentration of earliest time point (blue) or average (red) concentration to umbilical cord blood.
Graph 4(bottom). Pearson correlation of Maternal Hg concentration of earliest time point (blue) or average (red) concentration to umbilical cord blood

Table 8. Unadjusted and Adjusted Odds Ratios for Individual HMs and LH

	Average	Earliest
Unadjusted	OR(95%CI)	OR(95%CI)
Pb	0.51(0.06, 3.36)	1.61(0.25,10.23)
Cd	0.05(<0.01, 0.66)	0.53(0.08, 3.64)
As	0.42(0.03, 3.78)	0.77(0.13, 4.53)
Hg	2.15(0.45, 12.62)	0.85 (0.29, 2.49)
Adjusted[1]	OR(95%CI)	OR(95%CI)
Pb	0.59(0.07, 4.69)	1.37(0.14 13.35)
Cd	0.04(<0.01, 1.03)	0.54(0.06, 4.90)
As	0.44(0.04, 5.30)	0.94(0.19, 4.68)
Hg	2.55(0.48, 13.49)	0.99(0.32, 3.00)

Table 8. Unadjusted and adjusted OR for individual HM concentrations for earliest time point and average over pregnancy with the primary outcome of interests, LH.
1. Adjusted for DEAP, DMAP, iodine consumption, and gestational age at birth

Table 9. Unadjusted and Adjusted Odds Ratios for Combined HMs and LH

	Average	Earliest
Unadjusted	OR(95%CI)	OR(95%CI)
Pb	0.41(0.03, 5.19)	1.83(0.27, 12.31)
Cd	0.02(<0.01, 0.71)	0.51(0.07, 3.87)
As	1.06(0.03, 33.49)	0.845(0.14, 5.21)
Hg	2.25(0.32, 15.22)	0.95(0.29, 3.08)
Adjusted[1]	OR(95%CI)	OR(95%CI)
Pb	0.49(0.04, 6.45)	1.62(0.15, 17.08)
Cd	0.01(<0.01, 1.04)	0.48(0.05, 4.80)
As	1.24(0.03, 52.29)	1.06(0.20, 5.63)
Hg	2.38(0.30, 19.22)	1.07(0.33, 3.45)

Table 9. Unadjusted and adjusted OR for combined HM concentrations or earliest time point and average over pregnancy with the primary outcome, LH.
1. Adjusted for DEAP, DMAP, iodine consumption and gestational age at birth

Table 10. Unadjusted and Adjusted β coefficients and ORs for Individual HMs and Head Circumference

	Average		Earliest	
Unadjusted	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	-0.06(-0.05, 2.23)	0.36(0.08, 1.53)	-0.06(-0.05, 2.23)	0.36(0.08, 1.53)
Cd	-0.49(-1.53, 0.54)	1.64(0.47, 5.75)	-0.49(-1.53, 0.54)	1.64(0.47, 5.75)
As	0.32(-0.94, 1.58)	1.62(0.34, 7.63)	0.32(-0.94, 1.58)	1.62(0.34, 7.63)
Hg	-0.07(-1.02, 0.88)	2.36(0.73, 7.68)	-0.07(-1.02, 0.88)	2.36(0.73, 7.68)
Adjusted[2]	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	0.87(-0.45, 2.19)	0.38(0.05, 2.86)	0.87(-0.45, 2.19)	0.38(0.05, 2.86)
Cd	-0.19(-1.38, 1.01)	1.61(0.31, 8.33)	-0.19(-1.38, 1.01)	1.61(0.31, 8.33)
As	0.56(-0.77, 1.88)	1.84(0.22, 15.48)	0.14(-0.91, 1.19)	1.77(0.28, 11.36)
Hg	0.01(-1.04, 1.06)	2.58(0.57, 11.72)	-0.356(-1.11, 0.40)	2.38(0.76, 7.43)

Table 10. Unadjusted and adjusted β estimates for individual HW with secondary outcome, Head Circumference.

2. Adjusted for DEAP, DMAP, mother's age, mother's weight, mother's height, parity, iodine consumption and fathers smoking status

Table 11. Unadjusted and Adjusted β coefficients and ORs for Individual HMs and Bodyweight

	Average		Earliest	
Unadjusted	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	65.32(-231.30, 361.94)	0.85(0.22, 3.22)	-1.03(-282.78, 280.72)	0.49(0.13, 1.88)
Cd	-140.2(-398.12, 117.72)	2.13(0.62, 7.25)	-140.74(-378.91, 97.43)	1.98(0.62, 6.35)
As	-186.66(-499.22, 125.90)	2.91(0.61, 13.87)	-251.99(-478.26, -25.73)	4.52(0.89, 22.97)
Hg	-76.72(-319.96, 166.52)	1.43(0.49, 4.19)	-86.80(-259.83, 86.24)	0.92(0.41, 2.07)
Adjusted[2]	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	-30.64(-395.66, 334.37)	1.45(0.20, 10.70)	-119.75(-454.57, 215.08)	0.64(0.11, 3.64)
Cd	-142.04(-462.70, 178.63)	1.93(0.34, 10.95)	-146.61(-444.04, 150.82)	1.53(0.31, 7.50)
As	-209.93(-565.86, 146.01)	2.86(0.30, 27.06)	-252.45(-521.48, 16.58)	2.86(0.37, 22.22)
Hg	-51.75(-334.51, 231.01)	0.64(0.23, 1.78)	-104.93(-307.07, 97.23)	0.82(0.30, 2.25)

Table 11. Unadjusted and adjusted estimates for combined HMs with secondary outcomes, Bodyweight

2. Adjusted for DEAP, DMAP, mother's age, mother's weight, mother's height, parity, iodine consumption and fathers smoking status

Table 12. Unadjusted and Adjusted β coefficients and ORs for Individual HMs and Body Length

	Average		Earliest	
Unadjusted	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	-0.36(-2.30, 1.57)	1.71(0.11, 26.43)	-0.12(-1.93, 1.70)	3.31(0.19, 57.57)
Cd	-0.94(-2.62, 0.74)	0.48(0.04, 6.60)	-0.77(-2.32, 0.77)	0.34(0.02, 5.14)
As	-0.25(-2.32, 1.82)	0.98(0.05, 19.80)	-0.84(-2.35, 0.67)	1.71(0.28, 10.57)
Hg	0.09(-1.47, 1.64)	0.40(0.05, 3.05)	-0.41(-1.53, 0.72)	1.32(0.20, 8.56)
Adjusted[2]	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	0.02(-2.40, 2.43)	1.69(0.03, 88.85)	-0.06(36.82, 88.73)	5.47(0.13, 227.93)
Cd	-1.15(-3.26, 0.95)	0.01(<0.001, 46.38)	-1.22(-3.14, 0.69)	0.05(<0.001, 9.62)
As	-0.18(-2.57, 2.22)	0.34(0.01, 21.05)	-0.80(-2.61, 1.00)	0.90(0.06, 14.28)
Hg	0.59(-1.21, 2.40)	<0.001(<0.001, >999.99)	-0.36(-1.69, 0.96)	0.38(0.01, 12.18)

Table 12. Unadjusted and adjusted estimates for combined HMs with secondary outcomes, Bodyweight
2. Adjusted for DEAP, DMAP, mother's age, mother's weight, mother's height, parity, iodine consumption and fathers smoking status

Table 13. Unadjusted and Adjusted β coefficients and ORs for Combined HMs and Head Circumference

	Average		Earliest	
Unadjusted	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	1.00(-0.18, 2.20)	0.36(0.08, 1.74)	0.92(-0.21,2.04)	0.25(0.05, 1.22)
Cd	-0.65(-1.72, 0.42)	1.76(0.43, 7.23)	-0.62(-1.61, 0.37)	1.70(0.44, 6.48)
As	0.60(-0.71, 1.91)	0.89(0.17, 4.68)	-0.22(-1.17, 0.73)	2.09(0.49, 8.97)
Hg	-0.14(-1.08, 0.81)	2.44(0.72, 8.32)	-0.13(-0.84, 0.58)	2.05(0.79, 5.31)
Adjusted[2]	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	0.82(-0.64, 2.28)	0.36(0.04, 3.32)	0.71(-0.58, 2.00)	0.12(0.01, 1.23)
Cd	-0.40(-1.72, 0.91)	1.72(0.25, 12.03)	-0.27(-1.49, 0.94)	1.60(0.24, 10.77)
As	0.59(-0.87, 2.06)	1.09(0.12, 9.89)	0.11(-0.96, 1.18)	1.53(0.22, 10.49)
Hg	0.003(-1.09, 1.10)	2.38(0.48, 11.77)	-0.35(-1.18, 0.48)	2.67(0.70, 10.12)

Table 13. Unadjusted and adjusted estimates for combined HMs with secondary outcome, Head Circumference
2. Adjusted for DEAP, DMAP, mothers age, mothers weight, mothers height, parity, iodine consumption and fathers smoking status

Table 14. Unadjusted and Adjusted β coefficients and ORs for Combined HM and Bodyweight

	Average		Earliest	
Unadjusted	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	78.71(-234.47, 391.89)	0.83(0.21, 3.28)	18.86(-256.80, 294.53)	0.43(0.10, 1.76)
Cd	-110.43(-393.09, 172.23)	1.65(0.47, 5.84)	-87.28(-328.91, 154.34)	1.75(0.50, 6.05)
As	-147.49(-493.84, 198.87)	2.18(0.44, 10.89)	-237.89(-470.31, -5.46)	5.00(0.87, 28.67)
Hg	-55.84(-305.92, 194.25)	1.28(0.42, 3.89)	-70.57(-244.55, 103.40)	0.79(0.32, 1.93)
Adjusted[2]	β(95%CI)	OR(95%CI)	β(95%CI)	OR(95%CI)
Pb	4.19(-392.95, 401.33)	1.60(0.17, 14.75)	-67.00(-403.73, 269.73)	0.43(0.06, 2.93)
Cd	-96.82(-454.79, 261.15)	1.19(0.15, 9.54)	-101.69(-416.77, 213.40)	1.56(0.26, 9.46)
As	-207.92(-606.40, 190.56)	4.75(0.22, 101.01)	-230.85(-510.07, 48.38)	5.28(0.41, 68.34)
Hg	-9.65(-307.91, 288.61)	0.80(0.18, 3.65)	-60.53(-276.71, 155.66)	0.59(0.18, 1.93)

Table 14. Unadjusted and adjusted estimates for combined HMs with secondary outcome, Bodyweight
2. Adjusted for DEAP, DMAP, mothers age, mothers weight, mothers height, parity, iodine consumption and fathers smoking status

Table 15. Unadjusted and Adjusted β coefficients and ORs for Combined HMs and Body Length

	Average		*Earliest*	
Unadjusted	*β(95%CI)*	*OR(95%CI)*	*β(95%CI)*	*OR(95%CI)*
Pb	*-0.34(-2.36, 1.68)*	*2.03(0.12, 34.31)*	**-0.05(-1.90, 1.80)*	*2.79(0.14, 55.26)*
Cd	*-0.76(-2.56, 1.07)*	*0.48(0.03, 7.55)*	**-0.59(-2.21, 1.03)*	*0.31(0.02, 4.63)*
As	*0.03(-2.21, 2.27)*	*1.52(0.08, 30.65)*	**-0.74(-2.30, 0.82)*	*1.96(0.26, 14.77)*
Hg	*0.13(-1.49, 1.75)*	*0.36(0.04, 3.35)*	**-0.30(-1.47, 0.86)*	*1.38(0.19, 10.20)*
Adjusted[2]	*β(95%CI)*	*OR(95%CI)*	*β(95%CI)*	*OR(95%CI)*
Pb	*0.23(-2.32, 2.78)*	*>999.99(<0.001, >999.99)*	*0.08(-2.18, 2.33)*	*>999.99(<0.001, >999.99)*
Cd	*-1.14(-3.44, 1.16)*	*>999.99(<0.001, >999.99)*	**-1.15(-3.26, 0.96)*	*<0.001(<0.001, >999.99)*
As	*-0.36(-2.92, 2.20)*	*<0.001(<0.001, >999.99)*	**-0.74(-2.61, 1.13)*	*<0.001(<0.001, 117.04)*
Hg	*0.81(-1.10, 2.73)*	*<0.001(<0.001, >999.99)*	**-0.05(-1.50, 1.40)*	*0.001(<0.001, >999.99)*

Table 15. Unadjusted and adjusted estimates for combined HMs with secondary outcome, Body Length
2. Adjusted for DEAP, DMAP, mothers age, mothers weight, mothers height, parity, iodine consumption and fathers smoking status