In utero and peripubertal metals exposure in relation to reproductive hormones and sexual maturation and progression among girls in Mexico City

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ABSTRACT

There is increasing evidence that several metals are endocrine disrupting chemicals (EDCs). In utero development and adolescence are critical windows of susceptibility to EDC exposure. With the exception of a few heavy metals, few human studies have evaluated the impact of metal exposure on pubertal development. Our aim was to investigate measures of in utero and peripubertal metal exposure in relation to reproductive hormone levels and sexual maturation and progression among girls from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohorts. We measured urinary concentrations of aluminum (Al), arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), antimony (Sb), selenium (Se), and zinc (Zn) in samples collected from women during their third trimester of pregnancy and from their female children at 8–13 years (n = 132). We measured serum testosterone, estradiol, dehydroepiandrosterone sulfate (DHEA-S), inhibin B, and sex hormone-binding globulin (SHBG) at age 8–13, and assessed Tanner stages for sexual maturation (breast, pubic hair development, and menarche status), at two time points (8–13, 14–18 years). We used linear regression to independently examine in utero and peripubertal metal concentrations as predictors of peripubertal hormones. In a longitudinal analysis using generalized estimation equations, we evaluated Tanner stage and menarche progression in relation to individual in utero and peripubertal metal concentrations. We found that higher in utero Zn was associated with increased inhibin B. Several metals at 8–13 years were associated with higher DHEA-S and estradiol, while Ni was positively but Cu was negatively associated with testosterone. In utero Ni, Al, and Cd were associated with slower progression of breast development after adjustment for child age and BMI z-score. For example, an IQR increase in in utero Al exposure was associated with 0.82 times lower odds of progressing to a higher Tanner stage for breast development per year (95% CI: 0.68, 0.99). Peripubertal concentrations of Ba and Al were also associated with being at a higher pubic hair Tanner stage and menarche at 8–13, but lower odds of progressing to the next stage at 14–18 years. We used Bayesian kernel machine regression (BKMR) to model the joint effect of multiple metals while accounting for correlated exposures, as well as potential non-linear relationships between metals and outcomes of interest, which yielded results similar to individual analyses. These findings suggest that female reproductive development may be vulnerable to the effects of metal exposure, and using both Tanner stages and hormone levels may provide clues about underlying mechanisms in two sensitive periods of development.

1. Introduction

Puberty, the process by which adolescents reach sexual maturity and become capable of reproduction, is a period of rapid physical and psychological development, including increased weight and height velocity, sexual maturation, and cognitive and social maturation (Blakemore et al., 2010; Sisk and Foster, 2004). Several studies have reported a temporal trend of earlier pubertal onset among girls (Anderson et al., 2003; Herman-Giddens et al., 1997; Wyshak and Frisch, 1982). This trend is partly attributable to changes in diet (Cheng et al., 2012; Villamor and Jansen, 2016; Wyshak and Frisch, 1982; Zacharias and Wurtman, 1969), obesity (Anderson et al., 2003; Kaplowitz, 2008; Lee et al., 2007), and changes in various types of stress (Lee and Styne, 2013; Parent et al., 2003). However, a growing body of evidence suggests that environmental factors may also be contributing to earlier pubertal onset and sexual maturation, with potential adverse effects (Buck Louis et al., 2008; Lee and Styne, 2013).

Girls who begin puberty early may be at greater risk for alcohol and substance abuse (Castellanos-Ryan et al., 2013; Collado-Rodriguez et al., 2014; Patton et al., 2004), and psychological disorders (Klump, 2013; Mendle et al., 2012; Tremblay and Lariviere, 2009) during adolescence. Earlier pubertal onset is also associated with increased risk for certain types of cancer (Ali, 2014; Collaborative Group on Hormonal Factors in Breast, 2012; Jordan et al., 2005; Lacey et al., 2009; Stockl et al., 2011), metabolic syndrome and type 2-diabetes (Elks et al., 2013; Frontini et al., 2003; Janghorbani et al., 2014; Stockl et al., 2011; Widen et al., 2012), cardiovascular disease (Jacobsen et al., 2009; Lakshman et al., 2009; Prentice and Viner, 2013), and other illnesses later in life (Mueller et al., 2014; Widen et al., 2012).

Early life exposure to endocrine disrupting chemicals (EDCs), including metals, are thought to play a role in altered pubertal timing. In utero exposure can pose estrogenic or anti-androgenic effects potentially resulting in long term deviation from normal homeostatic control of the hypothalamic-pituitary-gonadal (HPG) axis or hypothalamic-pituitary-adrenal (HPA) axis (Bellingham et al., 2009; Buck Louis et al., 2008; Den Hond and Schoeters, 2006; Diamanti-Kandarakis et al., 2009; Jacobson-Dickman and Lee, 2009; Massart et al., 2006; McGivern et al., 1991; Pescovitz and Walvoord, 2007; Roy et al., 2009). Because *in utero* development is a period of organogenesis and increased hormonal activity, effects of exposure might persist long after birth and result in effects not observed with exposure at other life stages (Doherty et al., 2010; Su et al., 2010). Adolescence is also a susceptible window to the impact of endocrine disruptors, as dramatic hormonal changes during adolescence have profound effects on brain maturation, behavior, and reproductive development (Cahill, 2006; Parent et al., 2015; Sisk and Foster, 2004; Spear, 2000).

Metals enter the human body through ingestion of food, water, and dietary supplements, and the use of metal-containing products, through inhalation and skin contact (Martin and Griswold, 2009; Singh et al., 2011). In the United States, reports from the National Health and Nutrition Examination Survey (NHANES) show that children and adults, including pregnant women, have detectable concentrations of a range of metals in their bodies (Centers for Disease Control and Prevention (CDC) 2019). Because metals readily transfer cross human placenta and through breast milk, fetuses/infants are also exposed to multiple metals (Caserta et al., 2013; Chen et al., 2014; Ettinger et al., 2014; Punshon et al., 2016). Some metals are essential for human health such as cobalt (Co), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn), however, they can be toxic depending on their concentration; while other nonessential metals like cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As) can be toxic if present even at low concentrations (Singh et al., 2011). A number of metals are reproductive toxicants and suspected endocrine disruptors (Bloom et al., 2010; De Coster and van Larebeke, 2012; Diamanti-Kandarakis et al., 2009; Mendiola et al., 2011). Although exposures are highly prevalent (Centers for Disease Control and

Prevention, 2018), metals have received limited attention with respect to their endocrine disrupting potential. There has been some consistency with regard to the positive relationship between Cd exposure and testosterone levels among both males and females (Garcia-Morales et al., 1994; Jurasovic et al., 2004; Meeker et al., 2010; Menke et al., 2008; Nagata et al., 2005; Telisman et al., 2007; Zeng et al. 2002, 2004). Urinary Cd was associated with increased serum testosterone in postmenopausal Japanese women when examined cross-sectionally (Nagata et al., 2005). In another study, Cd-treated human breast cancer cells showed a significant decrease in transcription of the estrogen receptor gene (Garcia-Morales et al., 1994).

A number of studies indicate that Pb exposure leads to delay in pubertal development in girls (De Craemer et al., 2017; Denham et al., 2005; Jansen et al., 2018; Liu et al., 2019; Selevan et al., 2003; Wu et al., 2003). Results from Denham et al., (2005) suggested that Hg concentrations were associated with earlier menarche, while other studies found that Hg was associated with increased estradiol levels in adults in Cambodia and among women with repeated miscarriages (Agusa et al., 2007; Gerhard et al., 1998). Human research regarding pubertal timing in relation to As exposure has been limited, but animal studies have found exposure to As resulted in delayed sexual maturation in female rats (Davila-Esqueda et al., 2012; Reilly et al., 2014).

In summary, most studies performed to assess the reproductive effect of metals focused on non-essential metals (Cd, Hg, As, and Pb) and adults, whereas information on a number of other metals that may act as endocrine disruptors is still scant and incomplete. A few recent studies found associations between certain essential or trace metals such as Cu, Mn, and Mo and adverse effects on male reproduction (Jeng et al., 2015; Meeker et al., 2008; Telisman et al., 2000). However, limited studies have measured associations in adolescents, and none have examined the impact of *in utero* exposures on sex hormone levels and timing of puberty. Therefore, our objective was to examine the association between *in utero* exposure to both essential and non-essential metals measured in maternal urine collected during the 3rd trimester of pregnancy, and sex hormone levels in girls aged 8–13 years, and measures of sexual maturation at age 8 to 13 and again at 14–18 years. Additionally, we examined cross-sectional associations between metal exposures, hormone levels, and sexual maturation assessed in adolescence.

2. Method

2.1. Study population

This study used data collected from participants in the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) project. ELEMENT is a longitudinal cohort study of women in Mexico City and their children. Mothers were recruited at maternity hospitals during their first trimester between 1997 and 2004 as previously described (Lewis et al., 2013; Liu et al., 2019; Wu et al., 2018). Spot urine samples were collected from pregnant women at third trimester visits [mean gestational age at visit: 34.4 (range: 28–43) weeks].

Between 2008 and 2011, 250 child participants (132 girls and 118 boys), then aged 8–13 years (childhood/early adolescence), were selected based on the availability of archived maternal biological specimens and invited to participate in a follow-up study (i.e. early-teen visit). Of those 250 children, 223 (89%) were enrolled again at age 14–18 years (late adolescence/adulthood) in a second follow-up study (i.e. late-teen visit), in 2013–2017. Among those 223 children, 114 were girls (86% retention rate from early to late-teen visit). Fig. 1 shows the study design and the timing of biological sample collection and physical examination. Participants provided spot urine and fasting blood samples, anthropometry, and reported socio-demographic information via an interviewer-administered questionnaire. In the current analyses, we included female children who finished the early-teen visit (majority also completed late-teen visit) and had maternal urinary
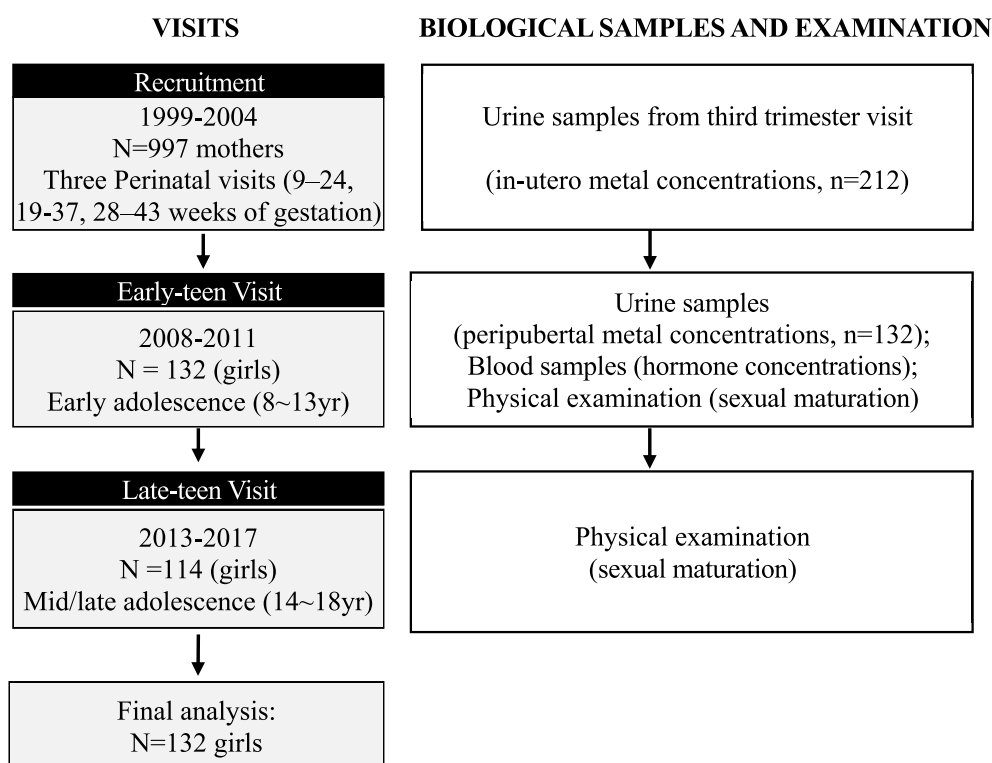
VISITS   **BIOLOGICAL SAMPLES AND EXAMINATION**

Recruitment
1999-2004
N=997 mothers
Three Perinatal visits (9–24, 19-37, 28–43 weeks of gestation)

Urine samples from third trimester visit
(in-utero metal concentrations, n=212)

Early-teen Visit
2008-2011
N = 132 (girls)
Early adolescence (8~13yr)

Urine samples
(peripubertal metal concentrations, n=132);
Blood samples (hormone concentrations);
Physical examination (sexual maturation)

Late-teen Visit
2013-2017
N =114 (girls)
Mid/late adolescence (14~18yr)

Physical examination
(sexual maturation)

Final analysis:
N=132 girls

Fig. 1. Recruitment, sample collection and examination timeline for the ELEMENT study, a prospective birth cohort in Mexico City, Mexico.

metal concentration measurements and/or their early-teen visit urinary metal measurements available (n = 132). Research protocols were approved by the ethics and research committees of the Mexico National Institute of Public Health and the University of Michigan, and all participants provided informed consent prior to enrollment.

2.2. Metal concentrations

Urinary metal concentrations [aluminum (Al), As, barium (Ba), Cd, Co, Cu, Fe, Mn, Mo, Ni, Pb, antimony (Sb), Se, Zn] were measured in maternal third trimester urine and peripubertal urine samples collected during the early-teen visit at age 8–13 years. Prenatal and peripubertal urine samples were collected in sterile cups, aliquoted within 1 h after collection, frozen and stored at −80 °C, and shipped on dry ice to McGill University (Montreal, Canada) for analysis. Urinary metals were measured using inductively coupled plasma mass spectrometry (ICPMS; Varian 820-MS, Inc., Palo Alto, California) as described previously (Basu et al., 2010; Srigboh et al., 2016). Accuracy and precision were measured using certified reference standards (Institut National de Santé Publique du Québec, or INSPQ) with coefficients of variation (CVs) ranging from 3 to 14%, and each batch run contained procedural blanks and replicate runs (Lewis et al., 2018; Srigboh et al., 2016). More details regarding quality control (QC) were previously described (Lewis et al., 2018).

Values below the limit of detection (LOD) were replaced with the LOD/√2. Urinary specific gravity (SG) was measured using a handheld digital refractometer. Metal concentration values were corrected for urinary SG using the following equation: $P_c = P [(SG_p - 1)/(SG_i - 1)]$ where P_c is the SG corrected metal concentration (μg/L), P is the measured metal concentration, SG_p is the median urinary specific gravity, and SG_i is the individual's urinary specific gravity. Pb exposure was also measured in maternal patella/blood and early childhood blood, and results of these biomarkers in relation to pubertal development within this population have already been published (Jansen et al., 2018; Liu et al., 2019). As patella and blood are better biomarkers of Pb

exposure, we excluded urinary Pb from the current analyses.

2.3. Hormones

Children provided fasting blood samples during the early-teen visit at age 8–13 years. Serum aliquots were separated and frozen at −80 °C, and then sent to the Clinical Ligand Assay Service Satellite (CLASS) Laboratory at the University of Michigan (Ann Arbor, MI) for hormone analysis. Estradiol, testosterone, inhibin B, and sex hormone-binding globulin (SHBG) were measured in serum samples as biomarkers of puberty, and dehydroepiandrosterone sulfate (DHEA-S) was measured as a biomarker of adrenarche. Estradiol, total testosterone, SHBG, and DHEA-S were measured using an automated chemiluminescent immunoassay (Bayer Diagnostics ACS:180). Active inhibin B was assayed using Gen II ELISA (Beckman Coulter, Webster, TX). Values below the LOD were replaced with the LOD/√2.

2.4. Sexual maturation

Each participant underwent a physical examination during both follow-up visits at age 8–13 and 14–18 years. Exams were completed by two pediatricians at the early-teen visit, and by one of the same pediatricians at the late-teen visit. To ensure consistency, pediatricians were trained prior to the start of each follow-up as previously described (Chavarro et al., 2017) to evaluate Tanner staging in female participants using standardized protocols. Breast developmental stage and pubic hair stage were assessed as indicators of puberty and adrenarche, respectively. Tanner Stage 1 corresponds to the no development with progression to Tanner Stage 5, indicating full development (Marshall and Tanner, 1969). Girls were also asked if they had had their first period (menarche) at both visits as an additional marker of sexual maturation.

2.5. Covariates

Age-specific BMI z-scores were calculated based on the World Health Organization child reference curves for age and sex (WHO, 2007) for each study visit. Socioeconomic status (SES) was estimated using a validated scale consisting of thirteen questions on housing quality, services, material goods and head of household education (Asociación Mexicana de Agencias de Investigación de Mercados y Opinión Pública, AMAI version 13×6), which classifies households into six SES categories (AMAI, 2000; López, 2008).

2.6. Statistical methods

We calculated geometric mean concentrations of each metal in prenatal and peripubertal urine samples. The percent of urine samples with concentrations below the LOD were reported for each metal, and metals that were detected in less than 50% of samples were excluded from further analysis.

Serum hormones were natural log-transformed prior to analysis to achieve normal distribution. Associations of prenatal and peripubertal urinary metal concentrations with peripubertal serum hormone concentrations were assessed using separate linear regression models for each metal, adjusting for potential confounders including child age and BMI z-score. SG was included as a covariate in all models as a measure of urinary dilution. The percent difference in hormones (95% confidence interval) per interquartile range (IQR) increase in urinary metal concentrations were calculated from model estimates. As a sensitivity analysis, we restricted analyses to girls that had not yet undergone menarche at the early-teen visit.

Longitudinal analyses were conducted to explore the association between metal exposure and repeated measures of sexual maturation using repeated generalized estimating equation models (GEE). With separate models for each metal, the GEE approach was used to fit ordinal regression models for pubertal stages at each visit:

$$g\left(E\left[Y_{ij}\right]\right) = \beta_0 + \beta_1 M_{ij} + \beta_2 Age_i + \beta_3 Time_{ij} + \beta_4 M_{ij}*Time_{ij} + \beta_5 Age_i *Time_{ij}$$

where Y represents the repeated measures of Tanner stage and menarche at two visits, g () is a link function (cumulative logit), i denotes subject number (1, …,n), and j denotes visit number (1, 2). M represents the ln-transformed, sg-corrected metal concentration, Age is the age at the early-teen visit, and $Time$ is the change in time between the early-teen and late-teen visit. We included age at early-teen visit and change in time between two visits to account for 1) the effect of baseline age on attained Tanner stage or menarche status, 2) the natural pubertal progression across time, and 3) the effect of baseline age on the natural pubertal progression. Hence, the coefficients of interest are the cross-sectional effects of prenatal or peripubertal metal concentrations on Tanner stage or menarche status at baseline (β_1) and the effect of metal concentrations on the progression of Tanner stage or menarche status (β_4). These were estimated using GEE with working independence correlation to ensure the validity of parameter estimates (Sullivan Pepe and Anderson, 1994). We included SG-corrected metal concentrations rather than entering SG as a separate covariate to minimize the number of covariates in the models. In our final model we adjusted for BMI, specifically adding terms $\beta_6 BMIbase + \beta_7 BMIvar$ to the model described above, where $BMIbase$ is BMI z-score at the early-teen visit and $BMIvar$ is the change in the BMI z-score measure that occurred between the two visits. Results are presented as odds ratios (OR) and 95% confidence intervals (95% CI) per IQR increase in exposure.

To evaluate the joint effect of multiple metals, interactions between metals, and potential non-linear relationships between metals and outcomes of interest, we conducted mixture analyses using Bayesian kernel machine regression (BKMR) (Bobb et al., 2015). BKMR is designed for cross-sectional studies and can analyze continuous and binary outcomes in relation to exposure mixtures (Bobb et al., 2018). Therefore, for this analysis, only reproductive hormone levels and measures of sexual maturation from the early-teen visit were included and Tanner stages were converted to dichotomous variables (stage = 1, stage > 1). Because metal exposures in our study are correlated, we implemented BKMR with hierarchical variable selection (10,000 iterations by a Markov Chain Monte Carlo (MCMC) algorithm). This approach requires grouping of exposures based on similar potential mechanisms of action (e.g. toxic metals vs essential metals) and correlations between exposures. Therefore, we grouped Ni, Cu, Ba, Se, Al, and Zn into group 1, Mo, Mn, and Co into group 2, and As and Pb into group 3. The BKMR model for a continuous outcome (hormones) can be expressed as:

$$Y_i = h[group_1 = (Ni, Cu, Ba, Se, Al, Zn), \ group_2 = (Mo, Mn, Co),$$
$$group_3 = (As, Cd)] + x_i'\beta + e_i$$

where h denotes the unknown exposure-response function of the predictor variables, β represents the effect of the covariates (we controlled for the same covariates as in the main analysis). The same model can be extended to binary outcomes (sexual maturation measurements) via generalized linear modeling using the probit regression function. Posterior inclusion probabilities (PIP) were extracted from each BKMR model, which provides a measure of variable importance for each exposure group (groupPIP) and how each exposure in that group is driving that group-outcome association (condPIP). To determine the importance of each group/exposure for each study outcome a threshold of PIP > 0.5 was used (Coker et al., 2018; Zhang et al., 2019).

All analyses were performed using R version 3.5.2 and SAS 9.4. R package bkmr was used to implement BKMR.

3. Results

3.1. Exposure and outcome distributions

Distributions of metal concentrations (geometric means, standard deviations, and selected percentiles) from prenatal and early-teen visits, as well as the percent of samples with concentrations below the limit of detection are shown in Table 1. Spearman correlations between the prenatal and early-teen visit metal concentrations adjusted for SG are also presented in the table. With the exception of Sb and Fe, the majority of metals were detected in > 50% of the urine samples, therefore, Sb and Fe were excluded from further analysis. Distributions and Spearman correlations of metals among all children (including male children) and their mothers in this cohort were previously reported elsewhere (Lewis et al., 2018); the analysis found weak correlations between maternal and childhood metal levels, weak to moderate correlations between different urinary metal concentrations within maternal and child samples were also reported. The associations between metal concentration and the covariates child age and child BMI-z-score are presented in SI Table S1.

The distribution of measures of Tanner stages of sexual maturation at the two visits among this population is reported (SI Table S2). Among 132 female participants at the early-teen visit, 98 girls (74.2%) were at stage 1 for pubic hair development, 87 girls (65.9%) were at stage 1 for breast development, and only one girl had reached stage 5 (full maturity) for pubic hair development. At the late-teen visit, only 9 girls (8%) were still at stage 1 for pubic hair development and only 5 (4.4%) were at stage 1 for breast development, while 14 (12.4%) and 18 girls (15.9%) had reached full maturity (stage 5) for pubic hair and breast development, respectively. There were 22 girls (19.3%) at the early-teen visit and 90 girls (78.9%) at the late-teen visit who had attained menarche.

Table 1
Distribution of uncorrected metal concentration during pregnancy (µg/L) among ELEMENT mothers who gave birth to girls and in their female children.

	In utero								Peripubertal							P value [a]
	LOD	% < LOD	GM	GSD	25%	50%	75%	MAX	% < LOD	GM	GSD	25%	50%	75%	MAX	
SG																< 0.01
Ni	3.0	0.9	9.3	2.2	5.9	8.1	12.2	950.5	0.8	7.9	1.7	5.7	7.6	10.5	48.0	0.56
Cu	48.2	43.6	113.3	5.4	34.1	52.9	111.5	2610.4	47.0	46.9	1.4	34.1	49.3	62.1	119.8	0.71
Mo	2.9	23.1	14.5	3.4	7.1	20.9	37.8	85.9	0.0	41.6	1.9	28.4	44.6	64.8	186.4	0.93
Ba	1.1	6.8	3.7	2.2	2.1	4.3	6.4	23.3	7.6	2.8	2.0	1.7	2.8	4.2	24.4	0.55
As	0.3	0.0	12.4	2.3	8.7	12.4	18.2	546.1	0.0	12.9	1.9	8.4	12.9	19.3	126.8	0.29
Se	10.5	6.0	30.4	1.8	20.9	35.8	45.5	95.2	2.3	44.6	1.8	32.2	45.4	67.6	130.0	0.23
Al	8.6	12.1	23.7	2.3	12.8	28.3	39.5	486.1	13.6	16.1	1.9	10.4	15.1	23.1	114.4	0.19
Mn	0.4	6.8	0.8	1.9	0.6	0.7	1.0	9.1	5.3	1.0	1.8	0.7	1.0	1.6	5.9	0.09
Co	0.4	0.0	1.1	2.2	0.7	1.1	1.8	26.5	0.0	0.7	1.8	0.5	0.7	1.0	4.3	0.71
Zn	0.1	3.7	268.3	2.1	182.7	305.9	449.4	1545.4	0.8	336.2	1.9	218.9	357.4	543.4	1120.6	0.51
Cd	54.0	1.7	0.2	2.3	0.1	0.2	0.3	7.9	6.1	0.1	1.9	0.1	0.1	0.2	2.0	0.43

[a] P value from Spearman correlation test between *in utero* and peripubertal specific urinary specific gravity and metal concentration measurements.
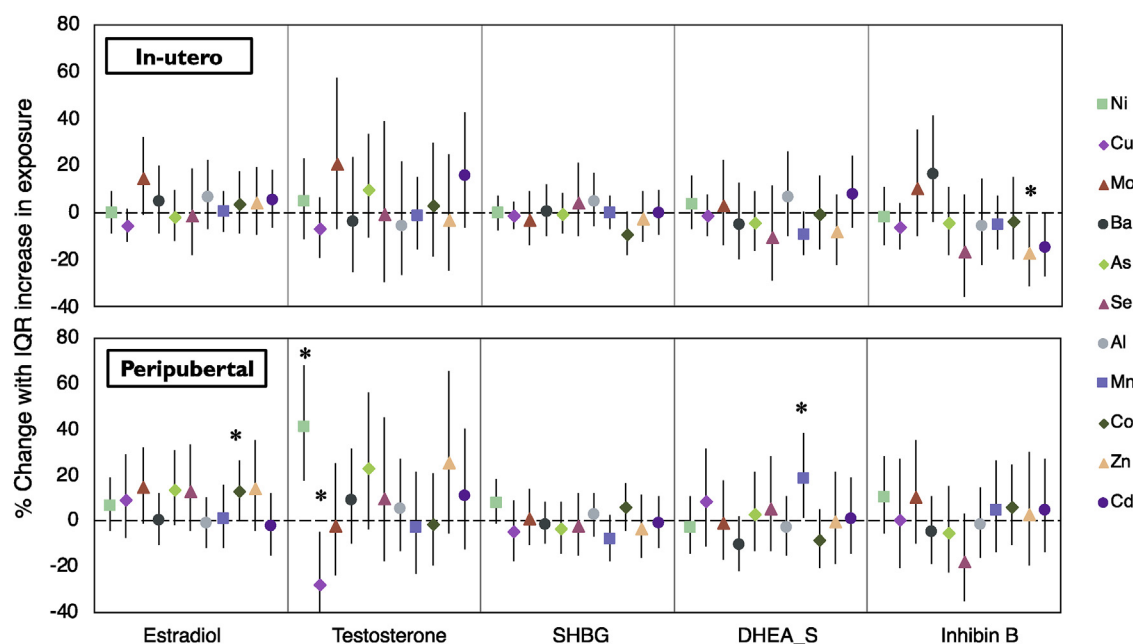
3.2. In utero and peripubertal metal exposure and peripubertal hormone levels

Associations of *in utero* and peripubertal metal concentrations with sex hormone levels are presented in Fig. 2 and SI Table S4. *In utero* concentrations of metals were not associated with peripubertal serum hormone levels in girls, with the exception of Zn, for which an IQR increase was associated with 17.4% (95%CI: 31.5, −0.6) lower serum inhibin B after adjustment for child age, BMI z-score, and urinary SG. Although only marginally significant, girls exposed to higher Mo concentrations *in utero* had higher estradiol levels (%△/IQR: 14.7%, 95%CI: 0.6, −32.2). In models where sex hormones were regressed on concurrent peripubertal urinary metal concentrations, an IQR increase in Mn was associated with 18.4% higher (95%CI: 1.2, 38.5) DHEA_S and an IQR increase in Co was associated with 12.6% higher estradiol

(95%CI: 0.04, 26.8). Peripubertal Ni concentrations were associated with higher testosterone levels (%△/IQR: 40.8, 95%CI: 18.0, 68.0). In contrast, peripubertal exposure to Cu was associated with lower testosterone levels (%△/IQR: 28.0, 95%CI: 45.5, −4.8). No significant associations were detected between peripubertal metal levels and inhibin B or SHBG.
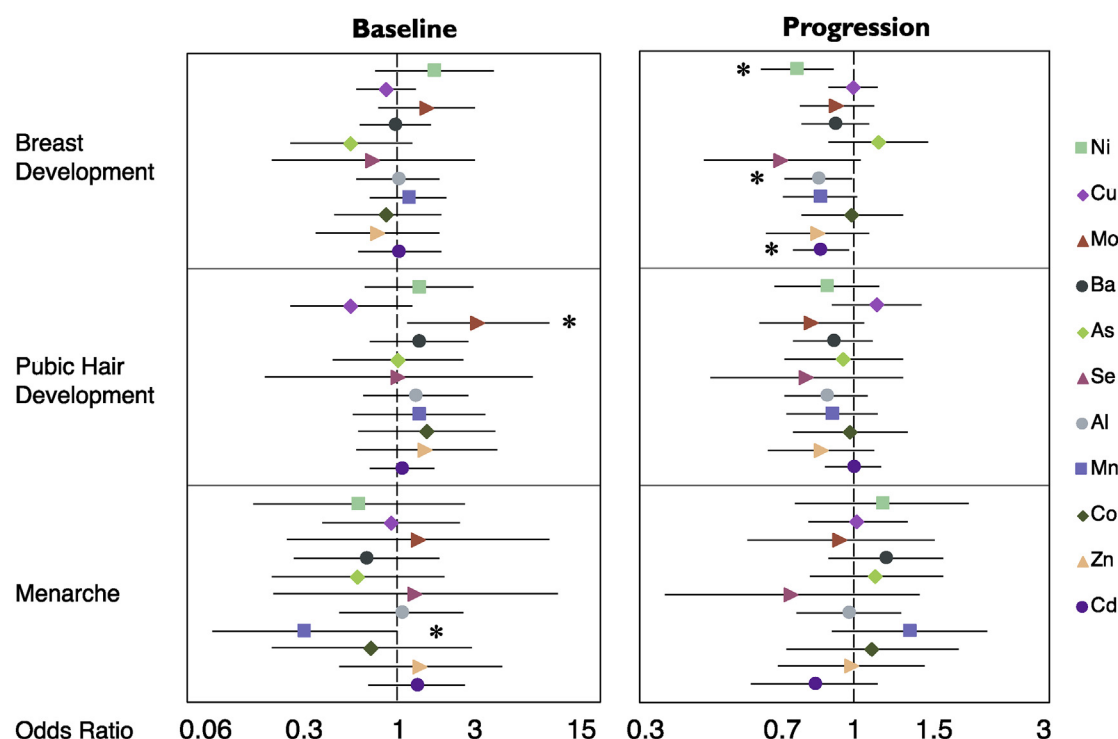
3.3. In utero and peripubertal metal exposure and sexual maturation

Results from multiple ordinal regression models of *in utero* metal concentrations and Tanner stage and menarche are shown in Fig. 3 and SI Table S5. An IQR increase in *in utero* Mo concentrations was associated with 2.9 times greater odds (95% CI: 1.14, 7.55) of being at a higher stage of pubic hair maturation versus any lower stages. *In utero* concentrations of other metals were not significantly associated with

a Linear regression models were adjusted for child age, BMI z-score and specific gravity
* Significant associations detected (p value<0.05)

Fig. 2. Percent difference in peripubertal hormone levels associated with an interquartile range (IQR) increase in *in utero* and peripubertal metal concentration among ELEMENT girls[a]. [a] Linear regression models were adjusted for child age, BMI z-score and specific gravity. * Significant associations detected (p value < 0.05).

Fig. 3. Odds Ratios and 95% Confidence Intervals for the Ordinal Generalized Linear Regression of *in utero* Metal Exposure and Tanner Stage[a]. [a] GEE models were adjusted for child age and BMI z-score (baseline and change). * Significant associations detected (p value < 0.05).

Tanner stages. The odds of having undergone menarche at the early-teen visit was reduced with higher *in utero* Mn concentrations (OR/IQR: 0.29, 95% CI: 0.08, 0.98).

As shown in Fig. 4 and SI Table S5, peripubertal concentrations of some urinary biomarkers of exposure to metals were associated with higher odds of being at higher Tanner stage at age 8–13 years, adjusting for child age and BMI z-score, most notably with Al exposure. For an IQR increase in peripubertal Al, the odds of being at higher versus any lower stages for pubic hair development at the early-teen visit was 1.9 times greater (95% CI: 1.11, 3.36); similar associations were observed between odds of being at higher pubic hair stage and peripubertal exposure to Ba (OR/IQR: 1.69, 95% CI: 1.04, 2.75). Menarche was also cross-sectionally associated with peripubertal Co concentrations, for which an IQR increase was associated 3.8 times higher odds of having had menarche at the early-teen visit (95%CI: 1.14, 12.58).

We also explored associations between *in utero* and peripubertal metal concentrations and pubertal development over time in girls. Results are also shown in Fig. 3 and Fig. 4 (SI Table S5). After controlling for age, BMI, and Tanner stage at the early-teen visit, *in utero* concentrations of Ni, Al, and Cd were associated with decreased odds of breast development progression during follow-up. The change in odds of being at higher Tanner stage for breast development per year after the early-teen visit was decreased by 27% (OR/IQR: 0.73 95% CI: 0.59, 0.89), 18% (OR/IOR: 0.82, 95% CI: 0.68, 0.99), and 17% (OR/IQR: 0.83, 95% CI: 0.71, 0.97) for an IQR increase in *in utero* concentrations of Ni, Al, and Cd respectively. No significant associations were found between *in utero* urinary metal concentrations with the progression of pubic hair development or menarche, or between peripubertal urinary metal concentrations and the progression of puberty estimated by Tanner stage of breast development. However, higher peripubertal urinary Ni and Ba were associated with decreased odds of pubic hair development progression, for which IQR increases were associated with

25% (OR/IQR: 0.75, 95% CI: 0.59, 0.95) and 18% (OR/IQR: 0.82, 95%CI: 0.67, 0.1) lower odds of being at a higher pubic hair Tanner stage, respectively. Similarly, we found associations between peripubertal Cd concentrations with decreased odds of menarche status progression (OR/IQR: 0.69, 95% CI: 0.48,1).

3.4. Sensitivity analyses

When we restricted the hormone analysis to girls that had not yet undergone menarche at the early-teen visit (102 out of 132), the direction and magnitude of observed associations from the main analysis did not change (SI Table S6), and we observed one additional association: An IQR increase in *in utero* Mo was associated with 25% higher estradiol level (95% CI: 1.36, 53.98). Because BMI may be on the causal pathway between exposure and puberty, we ran the GEE models both with and without BMI z-score. The magnitude of estimates from both models were similar when BMI z-score was not included (SI Table S7), with slightly attenuated associations between *in utero* Mo concentration and pubic hair development, and peripubertal Cd and menarche progression, as well as a slightly stronger association between peripubertal As concentrations and pubic hair development. In another sensitivity analysis, SES was included as a covariate in models of hormones and maturation stages because it could be a potential confounder, associated with metal exposure and puberty. Findings were generally consistent with metal and hormone associations observed in our main analyses; in SES-adjusted models, association between *in utero* Cd concentrations and lower inhibin B (%△/IQR = −15.27, 95%CI = −26.72,-2.04), was stronger and statistically significant, while the association between peripubertal Cu and testosterone was slightly attenuated and no longer significant. In GEE models for Tanner stage or menarche status including SES, cross-sectional associations between pubic hair Tanner stage and Al (OR/IQR: 2.5, 95% CI:1.04,
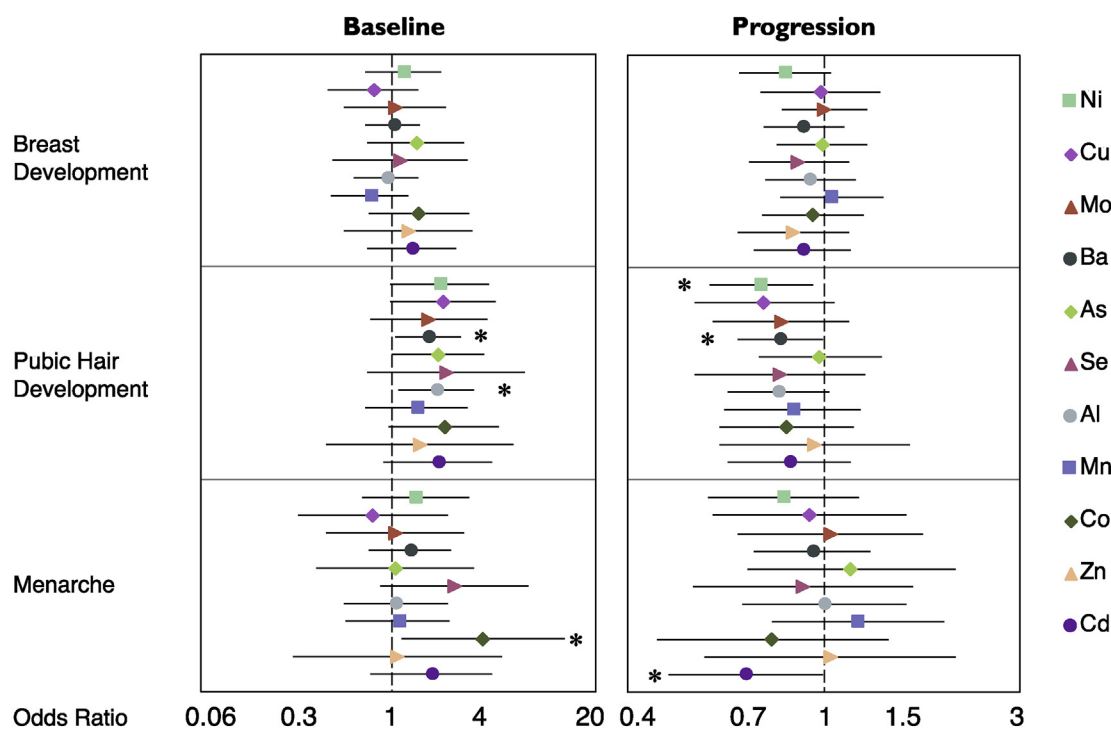
Fig. 4. Odds Ratios and 95% Confidence Intervals for the Ordinal Generalized Linear Regression of peripubertal Metal Exposure and Tanner Stage[a]. [a] GEE models are adjusted for child age and BMI z-score (baseline and change). * Significant associations detected (p value < 0.05).

5.99) and Zn (OR/IQR: 2.8, 95% CI: 1.01, 7.77) were significant while other findings remain consistent with the main models. As Cu was below the detection limit in 44% of samples, we categorized urinary Cu concentration into three groups in a secondary analysis. The low group consisted of values below the LOD, while the medium and high groups were made up of equalized bins among the detected values. We estimated model parameters using this categorical variable, and the parameter results were similar to those from the main analysis.

3.5. Mixture analysis

Finally, the estimates of PIPs from sexual maturation and reproductive hormone BKMR models are presented in Table 2 and Table S8, respectively. Mixture analysis results were comparable to the findings from individual analyses; 1) Among both models where breast development regressed on in utero and prepubertal metal exposure mixtures, all three group PIPs were < 0.5. (i.e. no group was identified as "important") 2) In the model for menarche and in utero metal mixtures, PIP for group 2 (Mo, Mn, and Co) was > 0.5 and condPIP of Mn was the highest (0.71) within this group; 3) Similarly, peripubertal Co was identified as the most important exposure for menarche in relation to peripubertal metal mixture; 4) In reproductive hormone BKMR models, few metals with both group PIPs and CondPIPs > 0.5 were in agreement with the significant or borderline significant associations in the individual analyses. However, for pubic hair development models regressed on exposure mixtures, all three groupPIPs were < 0.5, whereas individual analysis found associations between in utero Mo, peripubertal Ba and Al and pubic hair stages at the early-teen visit. BKMR also generated figure outputs corresponding to exposure-response relationship for each metal when fixing a second metal at

various quantiles (10th, 50th, 90th percentiles). SI Fig. S1 shows an example of the exposure-response relationship where menarche status was regressed on the peripubertal metal mixture which indicated that there is no interaction between different metals. Similarly, no interaction between any of the metals with any other outcome is detected.

4. Discussion

In this study, we investigated the effects of in utero and peripubertal metal exposure on subsequent peripubertal steroid hormone levels and progression of puberty in Mexican girls. Average urinary concentrations of metals measured in the present study were generally lower than levels reported in studies of pregnant women and their children living in regions of Mexico where metals-related industries are prevalent (Garcia-Vargas et al., 2014; Moreno et al., 2010; Roy et al., 2011). However, most of the metal concentrations among women and their children in this study were largely higher than the US women and children within 12–19 years old age group, participants of NHANES, including various cycles from 1999 to 2014 (Centers for Disease Control and Prevention, 2018) (SI Table S3). We found associations between in utero and peripubertal exposure to a number of metals, reproductive hormones, and progression of pubertal development.

4.1. In utero exposure

Higher in utero Zn exposure was negatively associated with inhibin B levels among female children, and marginally associated with decreased odds of pubic hair development progression. This finding is consistent with one animal study where zinc-supplemented testicular tissue from adult male rats showed reduced levels of inhibin-B and

Table 2
Posterior inclusion probabilities (PIPs) for group inclusion and conditional inclusion into sexual maturation measurements models, using Bayesian kernel machine regression (BKMR) model (N = 132)[a].

| Metals | Group | *In utero* exposure | | | | | | *Peripubertal* exposure | | | | | |
| | | Breast Development | | Pubic Hair Development | | Menarche | | Breast Development | | Pubic Hair Development | | Menarche | |
		groupPIP	condPIP	groupPIP	condPIP	groupPIP	condPIP	groupPIP	condPIP	groupPIP	condPIP	groupPIP	condPIP
Ni	1	0.38	0.48	0.34	0.03	0.27	0.11	0.33	0.13	0.34	0.11	0.28	0.27
Cu	1	0.38	0.05	0.34	0.17	0.27	0.06	0.33	0.23	0.34	0.23	0.28	0.19
Mo	2	0.22	0.28	0.32	0.52	0.72	0.19	0.40	0.28	0.42	0.29	0.59	0.10
Ba	1	0.38	0.09	0.34	0.09	0.27	0.26	0.33	0.11	0.34	0.11	0.28	0.04
As	3	0.20	0.76	0.20	0.58	0.29	0.30	0.37	0.66	0.36	0.65	0.23	0.43
Se	1	0.38	0.14	0.34	0.29	0.27	0.34	0.33	0.34	0.34	0.31	0.28	0.16
Al	1	0.38	0.16	0.34	0.24	0.27	0.03	0.33	0.03	0.34	0.10	0.28	0.05
Mn	2	0.22	0.26	0.32	0.17	**0.72**	**0.71**	0.40	0.31	0.42	0.32	0.59	0.08
Co	2	0.22	0.46	0.32	0.32	0.72	0.10	0.40	0.41	0.42	0.39	**0.59**	**0.82**
Zn	1	0.38	0.08	0.34	0.19	0.27	0.19	0.33	0.17	0.34	0.14	0.28	0.28
Cd	3	0.20	0.24	0.20	0.42	0.29	0.70	0.37	0.34	0.36	0.35	0.23	0.57

[a] Models were adjusted for age and BMI z-score at early teen visit.

spermatogenetic activity (Semercioz et al., 2017). The "inhibin B pubertal surge" signals gonadal maturation among girls as it is a prominent marker of follicular development through the peripubertal stages (Burger et al., 2000; Lahlou and Roger, 2004; Welt et al., 1999); therefore, the observed inverse association between *in utero* Zn exposure, inhibin B, and progression of pubic hair development adds further evidence that *in utero* Zn exposure may be associated with the timing of pubertal progression.

We found that one IQR increase *in utero* Mo concentrations was associated with 2.9 times greater odds of being at a higher stage of pubic hair development at early-teen visit among girls in our study; and among those who had not undergone menarche, Mo was also associated with 25% higher estradiol levels. While no prior studies have investigated these associations in girls, inverse associations between urinary concentrations of Mo and testosterone and sperm concentration were reported among men of reproductive age in the general US population (Lewis and Meeker, 2015; Meeker et al. 2008, 2010). Although no association with higher Mo levels were evaluated in animal studies, Mo deficiency was reported to be associated with delayed puberty among female cattle; with our findings on high Mo concentrations associated with advanced sexual maturation, these may reflect the lower and higher end of the dose-response relationship between Mo and puberty (Deb et al., 2014).

We observed 17–27% decreased odds of progression to a higher stage of breast development over the study period associated with an IQR increase in Ni, Al, and Cd concentration. These findings are consistent with results of a recent cross-sectional study that showed blood Cd was associated with delayed breast and pubic hair development among Flemish adolescent females (De Craemer et al., 2017). Previously, another study suggested that higher urinary Cd was associated with decreased inhibin B levels and might be linked to pubertal delays in 705 girls 6–11 years of age who participated in NHANES III (1988–1994) (Gollenberg et al., 2010). However, both studies were cross-sectional and therefore unable to clearly establish temporal relationships between metal exposures and pubertal development. Animal studies have also found that rats exposed to Cd during gestation had delayed sexual maturation together with increased oxidative stress and impaired steroid hormone levels (Samuel et al., 2011). There have been no human studies of *in utero* Al and Ni exposure and sex hormone levels during the peripubertal period, although our findings are consistent with the previous animal and *in vitro* studies that have reported associations between Ni exposure and disruption of mammalian reproductive functions (Iscan et al., 2002).

There are no previous studies reporting relationships between Mn exposure and sexual maturation in humans, although experimental studies assessing the ability of Mn to stimulate critical hypothalamic

actions among female rats reported that Mn is capable of enhancing puberty-related hormone secretions, and thus, may facilitate the normal onset and progression of puberty (Dees et al., 2017; Pine et al., 2005). This is in contrast to our finding that *in utero* Mn concentrations were negatively associated with having experienced menarche prior to the early-teen visit.

4.2. Peripubertal exposure

Higher peripubertal Co concentrations were positively associated with both estradiol levels and odds of having had menarche at the early-teen visit. This is in contrast to two experimental studies that explored the reproductive effects of Co exposure in mice, of which one showed that Co exposure was associated with significantly reduced reproductive organ development (Pedigo et al., 1988), while the other observed a null association (Madzharova et al., 2010). However, it has been shown that circulating estradiol drives the activation of kisspeptin, which in turn activates gonadotropin-releasing hormone (GnRH) neurons, which is essential for the onset of puberty (Clarkson et al., 2009; Karapanou and Papadimitriou, 2010). Therefore, the observed positive association of peripubertal Co exposure with estradiol and odds of menarche adds further evidence that Co exposure may be associated with the timing of puberty.

We found a negative association between peripubertal Cu concentration and testosterone levels among female children, which was consistent with findings from the Flemish study (De Craemer et al., 2017). We also observed that being at higher pubic hair development stage at the early-teen visit was suggestively associated with peripubertal Cu concentration, while the Flemish study found that blood Cu was associated with a delay in maturation among 14–15 year-old adolescents. However, caution has to be taken when comparing the two studies because we measured urinary Cu while the Flemish study assessed Cu in blood. Cu is a trace element which is subject to a complex system to maintain homeostasis in the body (Blazewicz et al., 2013). Non-essential metals such as Cd can affect the metabolism and urinary excretion of essential metals like Cu (Ashby et al., 1980; Chmielnicka et al., 1989). As such, differences in Cu concentrations in urine might not only be caused by a higher exposure, but also by the individual differences in non-essential metal concentrations and their impacts on homeostasis.

We found that an IQR increase in Ba concentrations was associated with a 1.7 fold increase in odds of being at higher pubic hair stage at the early-teen visit and 18% decreased odds of pubic hair development progression. No previous studies were located reporting effects of Ba on maturation. Decreased progression of maturation for those female children at higher Tanner stages at the early-teen visit (i.e. increased

progression for those at a lower stage) may be explained by a concept similar to "catch-up growth" (Wu et al., 2018). Catch up growth is defined as an increased growth following a period of growth inhibition (Ashworth and Millward, 1986; Wit and Boersma, 2002). Since some girls had experienced an advanced pubertal development in relation to exposure to metals at early-teen, their body systems may have responded by slowing down the tempo of pubertal progression, attenuating the accelerating effect exerted by metal; while others who had experienced a delayed puberty may respond by accelerating the change from lower stages of puberty to higher stages. How catch-up growth or "catch-up puberty" may be achieved is not understood and future research is needed to confirm these findings.

We also found a negative association between peripubertal Cd concentrations and timing of menarche, which is consistent with previous cross-sectional analyses of menarche onset (De Craemer et al., 2017; Gollenberg et al., 2010). However, caution has to be taken while comparing these studies as we considered a longitudinal association between metal exposure and pubertal development by introducing an interaction term with time and exposure in the model.

Results from mixture analyses using BKMR identified the same critical metals as reported in the individual analysis, except for pubic hair development models. This may be due to different distribution assumptions for the outcome in BKMR and GEE models, as the probit regression in BKMR required dichotomous Tanner stage variables, whereas ordinal generalized regression models utilized each category of the outcome.

4.3. Potential mechanisms

The onset and progression of puberty depend on a complex interplay between the central neuroendocrine system, the gonads, and the adrenal cortex modulated by a sex steroid-mediated negative feedback mechanism (Bordini and Rosenfield, 2011), and metals exposure may affect this process through a few different mechanisms. Puberty changes occur as a consequence of the activation of the HPG axis: the secretion of neuroendocrine factors stimulates the pulsatile secretion of GnRH, which eventually result in the secretion of the sex hormones estradiol and progesterone from the ovaries. Estrogen stimulates breast development and genital growth (Bordini and Rosenfield, 2011; Stattin and Magnusson, 1990). Pubertal changes are also a result of HPA axis activation: the adrenal zona reticularis increases adrenal androgen production. Pubic and axillary hair growth can be stimulated by the adrenal androgens dehydroepiandrosterone and DHEA-S (Bordini and Rosenfield, 2011).

In vitro and animal studies suggest that non-essential metals can disrupt the onset and progression of puberty via interrupting the critical neuroendocrine and hormonal pathways (Dees et al., 2017; Dyer, 2007; Garcia-Morales et al., 1994; Iavicoli et al., 2009; Nagata et al., 2005; Pine et al., 2005; Shen et al., 2016). Moreover, essential metals necessary for pubertal development can also be harmful when at deficient or excessive concentrations as they can accelerate or delay timing and progression of normal puberty (Dees et al., 2017; Kozielec et al., 1996).

Considering the positive associations between certain reproductive hormone levels and sexual maturation in normal pubertal development, the observed associations of Co and Zn with sexual maturation (increased odds of menarche; decreased odds of pubic hair development) are in line with their associations with hormone levels (increased estradiol; decreased inhibin B). Several animal studies on Pb and Cd have suggested that metals may impact sexual maturation through changes in reproductive hormones; by suppressing the secretion of sex steroid hormones involved in the initiation of puberty, sexual maturation is delayed (Iavicoli et al., 2009; Lafuente et al., 2003; Sokol and Berman, 1991). It is possible that Co and Zn also exert their influence on the endocrine system through the same mechanism of action, but different mechanisms are also possible.

Human studies also suggest that change in body fat (primarily measured by BMI) is another potential pathway for metals to affect puberty. When we did not include BMI z-score in models evaluating the relationship between metals and sexual maturation measurements, effect estimates for a few associations were attenuated whereas others became stronger compared to models with BMI z-score included (Supplemental SI Table S7). These findings suggest that *in utero* and peripubertal metal exposure may be related to pubertal development both independent of BMI as well as via this pathway. However, the relationship between *in utero* or peripubertal metal exposure and peripubertal BMI is not well understood. Some studies have shown a relationship with early growth, but these findings have also been inconsistent between studies (Fan et al., 2017; Padilla et al., 2010; Park et al., 2017), therefore, additional research is needed.

This longitudinal analysis allowed us to capture associations of both *in utero* and peripubertal metal exposure on hormone levels and measures of sexual maturation, as well as Tanner stage progression during the peripubertal period. However, this analysis also had a number of limitations, including a somewhat small sample size and few observations for certain Tanner stages, resulting in imprecise effect estimates. In addition, because we measured several metals, a large number of comparisons were made which likely increased the probability of chance findings, although the considered outcome measures are not independent of one another. As some urinary metal concentrations have been shown to vary over relatively short periods of time, a single sample collected during third trimester visit and at the early-teen visit may not fully characterize exposure during each specific window of development or reflect trends in exposure. We were not able to assess the combined effect of metals and other EDCs (e.g. phthalates) due to sample size constraints, however, metal concentrations are not likely to be correlated with other chemicals or share sources of exposure. Nevertheless, a larger cohort mixture analysis considering the multitude of environmental toxicants humans are exposed to daily is needed. Lastly, the diurnal variation of hormones may result in non-differential misclassification of the concentrations as they were measured at only one time point.

5. Conclusion

Our study found a variety of associations among several *in utero* and peripubertal metal concentrations with reproductive hormone levels and sexual maturation among girls, several of which are comparable to previous reports from human and animal research and supported by the current understanding of female pubertal development. These findings add to a growing body of literature on the reproductive effects of environmental metal exposure. Our work also suggests that aspects of reproductive development may be more vulnerable to the effects of metal exposure during critical periods of *in utero* and/or peripubertal development. The potential impact of metals exposure on pubertal timing is an important public health concern as an advance or delay in normal pubertal development is associated with adverse health outcomes during adolescence and adult reproductive life. Therefore, more epidemiological studies are needed to confirm these findings and to better understand the underlying mechanism for the effect of metals on sexual maturation and progression.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108630.

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