Human Reproduction, Vol.28, No.10 pp. 2765–2773, 2013
Advanced Access publication on August 14, 2013 doi:10.1093/humrep/det325

human reproduction

ORIGINAL ARTICLE *Puberty, aging and HRT*

Phthalates may promote female puberty by increasing kisspeptin activity

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Submitted on February 17, 2013; resubmitted on June 29, 2013; accepted on July 18, 2013

STUDY QUESTION: Is there an association between exposure to phthalates and the timing of female puberty?

SUMMARY ANSWER: Our study suggests that the early onset of puberty is related to increased kisspeptin secretion.

WHAT IS KNOWN ALREADY: Girls are maturing earlier than in past decades and the quantity of phthalates used in consumer products has concurrently risen. The hypothesis that exposure to phthalates may increase kisspeptin secretion and thereby cause early-onset puberty is unexplored.

STUDY DESIGN, SIZE, DURATION: This case–control study ran from 2006 to 2009. We enrolled 104 girls. Girls in the central precocious puberty (CPP) (case) group were recruited from a pediatric endocrinology policlinic in Taiwan; prepubescent controls were recruited from local elementary schools and all were categorized based on a pediatrician's diagnosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The physical characteristics of puberty were assessed and levels of LH, FSH estradiol and kisspeptin-54 in blood samples were evaluated using radioimmunoassay. Reversed-phase high-performance liquid chromatography-tandem mass spectrometry was used to analyze seven urinary phthalate metabolites. Non-parametric analyses, trend tests and linear regressions were performed on the data.

MAIN RESULTS AND THE ROLE OF CHANCE: All seven urinary phthalate metabolites in the CPP group were significantly ($P < 0.05$) higher than in prepubescent controls. Serum kisspeptin-54 levels were higher ($P = 0.022$) in the CPP group than controls and were still significantly higher after adjusting for age ($P = 0.03$). There was a significant increasing trend ($P_{trend} = 0.005$) between levels of kisspeptin and the stages of puberty. The concentration of kisspeptin-54 did not change in girls treated with leuprorelin acetate. There was a significant positive correlation between kisspeptin-54 and urinary mono-n-butyl phthalate (ng/ml: $R^2 = 0.251$, $P < 0.001$; μg/g-creatinine: $R^2 = 0.109$, $P = 0.024$).

LIMITATIONS, REASONS FOR CAUTION: The study duration was short and the sample size relatively small; therefore, we were unable to collect sufficient evidence to support the temporality between exposure to phthalates and the subsequent occurrence of PP.

WIDER IMPLICATIONS OF THE FINDINGS: Kisspeptin may promote the onset of puberty in girls who are exposed to a high level of phthalates, especially di-n-butyl phthalate. These data suggest that developing a kisspeptin antagonist might be an alternative strategy for treating PP.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants NSC 96-2621-Z-006-013 and NSC 97-2621-M-006-001 from the Taiwan National Science Council. The authors have no conflicts of interest to disclose.

Key words: precocious puberty / kisspeptin / urinary phthalate metabolites / leuprorelin acetate / estrogen receptor α

Introduction

Puberty is an inevitable process: humans must pass through this developmental phase to be able to reproduce. It is a complex series of events involving the activation and maturation of the hypothalamic–pituitary–gonadal (HPG) axis. Once the hypothalamus starts its pulsatile delivery of GnRH, the secretion of LH and FSH will also begin. This turns on the switch for the onset of puberty. However, there is growing evidence that children are maturing earlier than in the past, and that the incidence of precocious puberty (PP) is rising worldwide, especially

in girls (de Muinich Keizer and Mul, 2001; Teilmann et al., 2005). Idiopathic PP in girls is defined as the appearance, for no apparent reason, of secondary sex characteristics before 8 years old (Colaco, 1997). Girls who mature sexually earlier than do their peers grow faster, which leads to reduced adult height (Partsch and Sippell, 2001). Other studies also point out that early puberty is associated with an earlier diagnosis of breast cancer in some susceptible populations (Hamilton and Mack, 2003), and that girls who mature early are more likely to engage in several risk-taking behaviors, such as consuming alcohol or drugs, smoking, resorting to risky sexual behavior and becoming a victim of adolescent pregnancy (Deardorff et al., 2005).

There has been a growing desire to investigate the effect of endocrine-disrupting chemicals (EDCs), i.e. environmental toxins that disrupt endocrine function. Phthalate esters, one kind of EDCs, are a family of industrial chemicals widely used as plasticizers in various products. Several studies explore the relationship between exposure to phthalates and early puberty. Colón et al. (2000) first raised concerns about premature thelarche (the beginning of breast development at the onset of puberty) in girls possibly being associated with exposure to phthalates. Chou et al. (2009) also reported that mean urine concentrations of mono-methyl phthalate (MMP), mono-n-butyl phthalate (MBP) and mono-2-ethylhexyl phthalate (MEHP) were higher in Taiwanese girls than in girls in the same age groups reported by the National Health and Nutrition Examination Survey (NHANES), and that urinary MMP concentrations were associated with early breast development. Wolff et al. (2010) found that high-molecular-weight phthalate metabolites in urine had a modest inverse association with pubic hair growth, but that low-molecular-weight phthalate metabolites were positively correlated with the development of breasts and pubic hair.

Kisspeptins are neuropeptides encoded by the KiSS-1 gene, and are plentiful in the brain and sex steroid organs. This gene encodes a 145-amino acid precursor peptide that is cleaved into a 54-amino acid peptide called kisspeptin-54, and can be further truncated to kisspeptin-14, -13 and -10 (Popa et al., 2008). Dhillo et al. (2005, 2007) reported that after men had been given i.v. infusions of kisspeptin-54 and women given s.c. bolus injections, plasma LH concentrations dose-dependently rose. Keen et al. (2008) monitored the release of kisspeptin-54 and luteinizing hormone-releasing hormone (LHRH)-1 in female monkeys during puberty. They also found a significant secretion of kisspeptin-54 related to pubertal development associated with LHRH-1 release, and a prepubertal nocturnal increase of kisspeptin-54 release that continued through puberty. These signs of an increase in LH are similar to the clinical characteristics in girls with central PP (CPP). This implies that an out-of-control puberty process may be associated with the early secretion of kisspeptin in girls with early-onset puberty. Tena-Sempere (2010) reviewed recent evidence and pointed out that exposure to EDCs during critical periods might be mechanistically relevant to the disruption of puberty onset because it affects the kisspeptin/G-protein coupled receptor (GPR54) system.

Although Kisspeptin-54 secretion may be critical for puberty in animal (Keen et al., 2008) and human studies (Dhillo et al., 2005, 2007), no study has reported on kisspeptin and development in children. Moreover, a correlation between GPR54 and impaired pubertal development of the HPG axis was reported by de Roux et al. (2003) and Seminara et al. (2003). It is also necessary to clarify the association between exposure to phthalates and kisspeptin secretion: both might be related to early puberty. We investigated the association between exposure to phthalates and female puberty, and assessed the effect of leuprorelin acetate treatment on kisspeptin-54 secretion in girls with CPP.

Materials and Methods

Participants

This case–control study ran from 2006 to 2009. We enrolled 104 girls. The participants in the CPP (case) group were recruited from the Pediatric Endocrinology policlinic at National Cheng Kung University (NCKU) Hospital, Tainan City, Taiwan. Prepubescent controls were recruited from local elementary schools, which precluded several biases from hospital-based controls. The inclusion criteria for the CPP group were the appearance of secondary sexual characteristics in girls under 8 years old: a girl with any one characteristic of thelarche, pubic hair or menarche (first menstrual period) was recruited for the CPP group. The examination results of pelvic ultrasound, computerized axial tomography (CT) and magnetic resonance image scans had to be normal. If the PP was caused by a tumor, hydrocephalus or congenital adrenal hyperplasia, the case was excluded. The inclusion criterion for controls was no development of secondary sexual characteristics before 8 years old. Each participant was categorized according to the diagnosis of a pediatrician using the same rule (Tanner stage for breasts and pubic hair). There were two pediatricians involved in the present study. Finally, 73 girls with CPP and 31 prepubescent controls were recruited. All participants were given physical and clinical examinations and were asked to fill out interview questionnaires. One of their parents (usually the mother) was asked to fill out the questionnaire. The questionnaire requested the following data: personal characteristics (age, height, weight, maternal age at menarche, parents' education level, family income); medical history; usage habits for personal care products; and dietary habits. We analyzed kisspeptin-54 in this study because it is more effective than the shorter fragments (Thompson et al., 2006) and it increases plasma concentrations of gonadotrophins and induces ovulation in prepubertal female rats (Matsui et al., 2004). Ethical approval for the study protocol was obtained from the NCKU Hospital Human Experiment and Ethics Committee. Informed consent was obtained from all participants and their parents. We divided the CPP-group girls into three subgroups: those diagnosed with premature thelarche and a negative LHRH test (Thel + $LHRH^-$); girls whose LH concentration was >10 on the LHRH test but who had not yet been treated with leuprorelin acetate ($LHRH^+$); and girls being treated with leuprorelin acetate or girls who had experienced menarche and for whom a pediatrician had confirmed that it was too late to treat (Leup/Men). Peak LH concentrations >10 mIU/ml on the LHRH test were considered positive and required leuprorelin acetate treatment. One girl in this study was classified as Leup/Men and treated with leuprorelin acetate at a marginal peak of LH (9.72) because she had developed enlarged breasts when she was 1-year old, and a CT scan showed that her pituitary gland was mildly enlarged.

Urine sampling and analysis

The first spot urine samples were collected in the early morning for all participants on the appointment day. Urine samples were collected in 250-ml brown glass bottles and stored at −20°C until analyzed for phthalate monoesters. Creatinine in urine was measured in the pathology laboratory of NCKU Hospital. To prevent possible contamination of the urine samples, all glassware was first washed in acetone and methanol, and then immediately blown dry with nitrogen gas. The bottles were then sealed with aluminum foil until the urine sample was collected. We modified slightly a previously described isotope dilution method (Huang et al., 2007, 2009) to determine urinary concentrations of seven phthalate metabolites: the five primary metabolites: (1) MMP, (2) mono-ethyl phthalate (MEP), (3) MBP, (4) monobenzyl phthalate (MBzP),and (5) MEHP; and two oxidized metabolites:

(6) mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and (7) mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). Because the analytical methods for MEHHP and MEOHP were set up late, urinary data for only 39 CPP-group girls and 9 controls were obtained. Native-standard and ^{13}C-labeled internal-standard were purchased from Cambridge Isotope Laboratories, Andover, MA, USA. Briefly, 1 ml of urine sample was thawed, buffered with ammonium acetate (250 μl, 1 M) and then spiked with a mixture of isotope phthalate monoester standards (30 ng/ml) and β-glucuronidase enzyme (5 μl, 200 U/ml). After the sample had been incubated at 37°C for 90 min, it was loaded into a solid-phase extraction cartridge (Nexus; Varian, Palo Alto, CA, USA). One milliliter each of formic acid and H_2O was added to remove hydrophilic compounds, and then 2 ml each of acetonitrile and ethyl acetate was added to elute phthalate metabolites. The extract was dried with nitrogen gas and reconstituted with 1 ml of 85% methanol, and then directly analyzed using a high-performance liquid chromatograph (HPLC 1200; Agilent Technologies, Waldbronn, Germany) coupled with tandem mass spectrometry (6410B tandem quadrupole mass spectrometer; Agilent) with electro-spray ionization. A C18 (1.8 μm, 2.1 × 100 mm) analytical column (Extend-C18; Agilent) was used for chromatographic fractionation. The fractionation was performed using a linear gradient program with an organic solvent (acetonitrile and 0.05% acetic acid) and an aqueous solvent (H_2O and 0.05% acetic acid) at a flow rate of 0.3 ml/min. The calibration range of the seven phthalate metabolites was 1–1000 ppb. One blank and one quality control (QC) sample were included in each batch. The recoveries for a spike QC sample and a ^{13}C-labeled internal-standard of each phthalate monoester in samples were >80 and >40%, respectively. The limit of detection for each phthalate metabolite was MMP: 0.08 ng/ml; MEP: 0.1 ng/ml; MBP: 0.18 ng/ml; MBzP: 0.14 ng/ml; MEHP: 0.04 ng/ml; MEOHP: 0.09 ng/ml; and MEHHP: 0.05 ng/ml. All metabolite concentrations were presented as both unadjusted and creatinine-standardized calculations.

Estrogen receptor α binding effect

People are exposed to mixtures of phthalates every day. Each phthalate may have a different estrogen-like activity that may make a different contribution toward promoting puberty. Popa *et al.* (2008) detailed the role of kisspeptin in neuroendocrine regulation. We hypothesized that phthalates bind with estrogen receptor (ER)α and stimulate the secretion of kisspeptin, which then induces PP in humans. ERα has been expressed on the KiSS-1 neuron in the arcuate (Arc) nucleus and anteroventral periventricular (AVPV) nucleus in animal models (Smith *et al.*, 2005). It is required for mediating the stimulation of estradiol (E_2) and controlling the positive and negative secretion of GnRH (Smith *et al.*, 2005). To explore the total estrogen-like activity for all the phthalates we detected, we developed an equation (Equation (1)) to estimate the total ERα binding effect. We calculated the phthalate daily intake (DI) dose [e.g. DI_{DMP}: DI dose of dimethyl phthalate (DMP)] from urinary phthalate primary and second metabolites in each participant using the method developed by David (2000) and Wittassek *et al.* (2007). Takeuchi *et al.* (2005) investigated the estrogenic activity of 22 phthalates on human ERα. We applied the relative estrogenic activity (%) of each phthalate [DMP: 0%; diethyl phthalate: 1%; di-n-butyl phthalate (DBP): 35%; benzyl butyl phthalate (BBP): 99%; di(2-ethylhexyl) phthalate (DEHP): 30%] from Takeuchi *et al.* (2005) and multiplied it by its DI. All calculated results finally aggregated to a dose that represented the ERα binding effect for each participant.

$$\begin{aligned} ER\alpha_{\text{binding effect}}(\mu g/kg/day) = {} & DI_{DMP} \times 0\% + DI_{DEP} \times 1\% \\ & + DI_{DBP} \times 35\% + DI_{BBP} \times 99\% \\ & + DI_{DEHP} \times 30\%. \end{aligned} \quad (1)$$

Blood sampling and determinations

Blood samples were drawn by NCKU Hospital phlebotomists via venipuncture into red (7 ml) and lavender (10 ml) tubes (Vacutainer; BD Biosciences, San Jose, CA, USA) and analyzed for sex hormones and kisspeptin-54. Some of the girls who were being regularly treated with leuprorelin acetate had their blood drawn on a clinic visit the day before the regular monthly injection. To overcome the possibility that blood collections at different times might affect the concentration of kisspeptin-54, all participants were asked to donate blood samples between 1:00 p.m. and 5:00 p.m. on the appointment day. The blood samples were centrifuged at 1200*g* to derive serum. Serum LH, FSH and E_2 were analyzed by the NCKU Hospital Department of Nuclear Medicine, using a radioimmunoassay (RIA) kit (Coat-A-Count; Siemens Healthcare Diagnostics, Tarrytown, NY, USA). All serum samples were stored at −80°C until analyzed.

Only 40 CPP-group girls and 11 controls agreed to provide blood samples for kisspeptin analysis. Ten-milliliter blood samples were collected in lavender tubes containing ethylene diamine tetraacetic acid, immediately transferred to centrifuge tubes containing aprotinin (0.6 trypsin-inhibiting units (TIU)/ml of blood), and then gently rocked to inhibit proteinase activity. After they had been centrifuged at 1600*g* for 15 min at 4°C, the plasma samples were collected for analysis. The plasma was extracted using a column containing 200 mg of C18 (Strata; Phenomenex, Torrance, CA, USA). The concentration of kisspeptin-54 in the eluent was measured using an RIA kit (RK-048–59; Phoenix Pharmaceuticals, Burlingame, CA, USA). An automatic gamma counter (1470 Wizard; Perkin Elmer, Turku, Finland) recorded the RIA counts per minute of the pellets. The calibration curve ranged from 10 to 1280 pg/ml. The antibody cross-reacted 100% with human Kiss-1 protein (68–121), 25% with human metastin (the former name of kisspeptin) (26–54), 4% with human neuropeptide AF and 0% with human neuropeptide FF, human RFRP-3 and FMRF-NH2. One positive control was analyzed per batch.

Statistical analysis

Mann–Whitney *U*-tests and Fisher's Exact tests were used to assess the difference between CPP groups and controls for the questionnaires, concentrations of urinary phthalate metabolites and clinical demographic data. A Kruskal–Wallis test was used to compare the age, sex hormones, bone age and concentrations of kisspeptin-54 of the controls and the three subgroups of girls with CCP. A trend-test analysis was done to explore the association between the concentration of kisspeptin-54 and the different stages of puberty in all participants. Simple linear regression analyses were done to evaluate the association between concentrations of kisspeptin-54 and urinary phthalate metabolites. Because all urinary metabolite concentrations were not normally distributed, the data were transformed to natural logarithms (ln) before the simple linear regression analysis. Multiple linear regression was used to test whether age was a confounding factor for kisspeptin-54 secretion in the four groups. Significance was set at $P < 0.05$. The Statistical Product and Service Solutions 17.0 and SAS 9.2 for Windows were used for all statistical analyses.

Results

Demographic data of participants

Although we had 104 participants in the study, two girls in each group refused to fill out the questionnaire or provide a urine sample. Therefore, only 71 questionnaires and urine samples from CPP girls and 29 from controls were examined. The CPP group were significantly older

($P < 0.001$), taller ($P < 0.001$) and heavier ($P < 0.001$) than the controls (Table I). Previous studies pointed out that psychological stress and family socioeconomic status seem to be suspected of triggering early onset sexual maturation. Therefore, we integrated several items on maternal age at menarche, parental education and family income and did not differ between the two groups.

Urinary phthalate monoesters

The absolute concentrations of seven urinary phthalate metabolites in the CPP group were all significantly ($P < 0.05$) higher than those in the control group (Table II). After normalizing to creatinine, only the median concentrations of MEP ($P = 0.001$), MBzP ($P = 0.005$), MEHHP ($P = 0.002$) and MEOHP ($P = 0.001$) were significantly higher in the CPP group. The median ERα binding effect dose for the CPP group was 3.25 μg/kg/day, significantly higher than the 2.71 μg/kg/day for the control group ($P = 0.022$). The calculated results of the ERα binding effect revealed a significantly higher binding potency in girls of the CPP group.

Kisspeptin

To investigate the profile of kisspeptin-54 secretion at different stages in CPP-group girls, and to assess the effect of leuprorelin acetate treatment, we divided the CPP group into three subgroups: Thel + $LHRH^-$, $LHRH^+$ and Leup/Men. Control-group girls were not given bone age or LHRH tests, because they are invasive and unrelated to their health. Kisspeptin-54 concentrations were significantly different between the four groups ($P = 0.022$). The Leup/Men subgroup had higher concentrations of kisspeptin-54 than the $LHRH^+$ subgroup and the Thel+$LHRH^-$ (1.97 pmol/l) subgroups, as well as the control group (1.95 pmol/l) (Table III). A multiple linear regression was carried out to determine whether age was a confounding factor for kisspeptin-54 secretion, and results showed that kisspeptin-54 concentrations were still significantly correlated with the four different development groups after adjusting for age (R^2 for the whole model = 0.21; P for the whole model = 0.028; P for the four clinical features = 0.03; P for age = 0.79). This implied that kisspeptin-54 secretion was significantly different between the four groups. Trend-test analysis showed a significantly increasing trend of kisspeptin-54 concentrations from the control group to the Leup/Men subgroup ($\beta = 0.12$, $P_{trend} = 0.005$ after adjusting for age). Five girls had experienced menarche in the Leup/Men subgroup; therefore we excluded them and significant differences in bone age ($P < 0.001$), basal LH, basal FSH ($P = 0.001$) and basal E_2 ($P = 0.006$) between the four groups remained while differences in kisspeptin were not significant ($P = 0.054$). When PP was more advanced, the average concentration (dotted line) of kisspeptin-54 was higher (Fig. 1). There were significant ($P < 0.001$) differences in bone age between the CPP subgroups (Table III). The bone growth rate in the $LHRH^+$ and Leup/Men subgroups was significantly faster than in the Thel + $LHRH^-$ subgroup (Table III). The concentrations of basal LH, FSH and E_2 were quite low in all participants except for several girls who had experienced menarche (Table III). The average peak LH in the Leup/Men subgroup was significantly higher than that in the $LHRH^+$ and Thel + $LHRH^-$ subgroups ($P < 0.001$). Peak LH in the Thel + $LHRH^-$ subgroup was significantly lower than in the $LHRH^+$ ($P < 0.001$) and Leup/Men ($P < 0.001$) subgroups.

Table I Demographic data of girls with CPP and prepubescent controls.

Item	Control group (n = 31)[a]	CPP group (n = 73)[a]	P-value[b]
Age (years)[c]	6.8 (2.2–8.3)	8.1 (2.5–11.5)	<0.001
Age at diagnosis (years)[c]	–	7.0 (1.3–8.5)	–
Maternal age at menarche (years)[c]	13 (11–18)	13 (9–15)	0.207
Height (cm)[c]	120.8 (90.0–136.7)	129.6 (83.0–152.1)	<0.001
Weight (kg)[c]	22.6 (12.0–35.1)	29.0 (9.9–55.3)	<0.001
BMI (kg/m^2)[c]	16.0 (12.9–21.1)	16.8 (11.9–24.8)	0.05
Father's education level (n)			
High school	6	27	0.16
College	19	32	
Graduate school	4	12	
Mother's education level (n)			
High school	8	32	0.25
College	19	36	
Graduate school	2	3	
Family's monthly income (n)			
Less than 70 000 NT$	16	42	0.71
More than 70 000 NT$	13	29	

CPP, central precocious puberty; NT$, New Taiwan Dollar.
[a]Although we had 104 participants in the study, 2 girls in each group refused to fill out the questionnaire or provide a urine sample.
[b]Continuous variables were tested using Mann–Whitney U-tests, and categorical variables using Fisher's Exact tests.
[c]Values are medians (range).

Table II Unadjusted and adjusted (per g creatinine) concentrations of urinary phthalate monoesters and aggregated dose of ER α binding effect in girls with CPP and prepubescent controls.

	Control group[a] (n = 29)	CPP group[a] (n = 71)	P-value[b]	NHANES 1999–2008[c]	GerES IV 2003–2006[d]
Creatinine unadjusted (ng/ml)					
MMP	4.53 (0.70–14.1)	6.95 (0.70–48.3)	0.033	<LOD–1.90	–
MEP	7.87 (0.50–332)	19.1 (0.50–558)	<0.001	45.2–53.9	–
MBP	40.2 (9.93–163)	60.4 (6.14–1324)	0.049	28.7–40.0	93.4
MBzP	2.45 (0.70–18.4)	6.22 (0.70–167)	0.002	17.6–29.0	18.1
MEHP	5.10 (0.45–125)	8.23 (0.45–85.1)	0.002	2.20–4.90	6.7
MEHHP[e]	27.6 (13.8–106)	59.6 (15.4–432)	0.004	27.0–36.5	36.3
MEOHP[e]	25.0 (13.6–92.7)	56.9 (11.2–392)	0.004	16.6–25.8	46.0
Creatinine-adjusted (μg/g creatinine)					
MMP	6.34 (0.94–31.3)	8.10 (0.83–128)	0.141	<LOD–2.32	–
MEP	11.3 (1.06–337)	25.4 (1.21–379)	0.001	50.1–57.4	–
MBP	67.2 (20.5–275)	94.6 (22.3–910)	0.195	33.9–39.1	–
MBzP	3.74 (0.95–50.4)	9.00 (1.14–172)	0.005	20.7–27.8	–
MEHP	9.04 (0.95–185)	10.8 (4.03–69.3)	0.059	2.80–5.38	–
MEHHP[e]	27.7 (12.3–202)	57.4 (24.5–291)	0.002	31.4–37.0	–
MEOHP[e]	25.8 (13.8–177)	52.0 (21.9–244)	0.001	18.5–25.3	–
Aggregated dose of ERα binding effect with phthalates	2.71 (0.70–26.6)	3.25 (1.03–12.6)	0.022	–	–

CPP, central precocious puberty; MMP, mono-methyl phthalate; MEP, mono-ethyl phthalate; MBP, mono-n-butyl phthalate; MBzP, mono-benzyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; NHANES, National Health and Nutrition Examination Survey; GerES, German Environmental Survey for Children; LOD, limits of detection; ERα, estrogen receptor α.
[a]Median (range).
[b]Mann–Whitney *U*-tests were used to test the difference between the CPP and control groups.
[c]The minimum-to-maximum median in 6- to 11-year-old US children from the NHANES 1999–2008 (Centers for Disease Control and Prevention, 2012).
[d]Medians in 3- to 14-year-old German children from GerES IV 2003–2006 (Becker *et al*., 2009).
[e]Urinary data from only 39 girls of the CPP group and 9 of the control group were analyzed in this study.

Table III Average (range) plasma kisspeptin level and clinical examination results of girls with CPP and prepubescent controls.

	Control group (n = 11)	CPP group (n = 40)			
		Thel + LHRH$^-$ subgroup (n = 13)	LHRH$^+$ subgroup (n = 12)	Leup/Men subgroup (n = 15)	P-value[a]
Age (years)	7.4 (6.1–8.3)	7.0 (2.5–9.1)	8.9 (7.7–10.6)	9.0 (4.6–11.5)	<0.001
Kisspeptin (pmol/l)	1.95 (1.69–2.18)	1.97 (1.39–2.51)	2.16 (1.71–2.61)	2.29 (1.92–2.86)	0.022
Bone age (years)	–	6.6 (1.75–9.0)	10.3 (8.8–12)	10.7 (5.0–12.5)	<0.001[b]
Basal LH (mIU/ml)	<0.15	0.25 (<0.15–1.84)	0.99 (<0.15–2.54)	1.03 (<0.15–8.65)	0.003
Basal FSH (mIU/ml)	1.65 (1.11–2.31)	2.96 (1.37–8.14)	4.81 (1.42–8.31)	2.66 (0.92–8.29)	0.006
Basal E_2 (mIU/ml)	<8	4.88 (<8–15.5)	10.5 (<8–19.0)	16.6 (<8–102)	0.032
Peak LH (mIU/ml)	–	5.43 (3.11–7.89)	21.2 (10.9–51.8)	27.6 (9.72–90.2)	<0.001[b]

CPP, central precocious puberty; Thel + LHRH$^-$, girls with premature thelarche and a negative LHRH test result; LHRH$^+$, girls with a positive LHRH test result; Leup/Men, girls being treated with leuprorelin acetate or who had experienced menarche; E_2, estradiol; LHRH, luteinizing hormone releasing hormone.
[a]Kruskal–Wallis test: *P*-value for the difference between the four groups.
[b]Kruskal–Wallis test: *P*-value for the difference between the CPP subgroups.

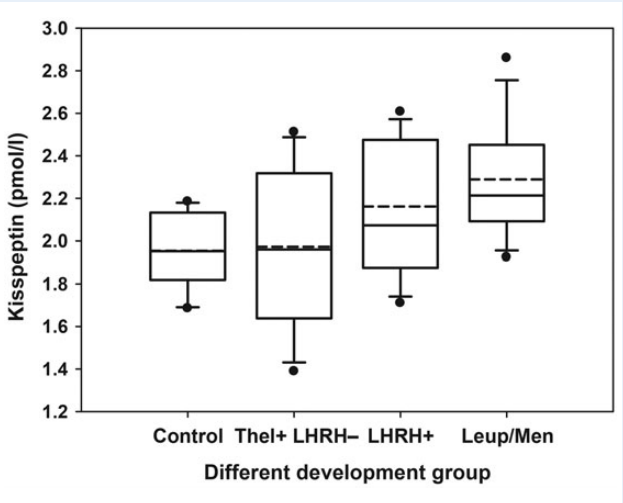

Figure 1 Box plot of plasma kisspeptin levels in prepubescent controls and three subgroups of girls of the CPP subgroup. (1) Controls ($n = 11$); (2) Thel + LHRH$^-$: premature thelarche and a negative LHRH test result ($n = 13$); (3) LHRH$^+$: positive LHRH test result ($n = 12$); (4) Leup/Men: being treated with leuprorelin acetate or had experienced menarche ($n = 15$). Dotted line: the mean level of kisspeptin-54 in each group. The boundary of the box indicates the 25th and 75th percentiles. The line within the box is the median. The error bars above and below the box indicate the 90th and 10th percentiles. The points outside the error bars are outlying points. (P_{trend} among the four groups after adjusting for age = 0.005). CPP, central precocious puberty.

Association between urinary phthalate monoesters and serum kisspeptin

We also investigated the association between urinary phthalate and serum kisspeptin-54 concentrations. Linear regression analysis showed an increasing trend for kisspeptin-54 with the absolute concentration of ln-transformed MMP ($R^2 = 0.083$; $\beta = 0.139$; $P = 0.05$) (Fig. 2A) and ln-transformed MBzP ($R^2 = 0.076$; $\beta = 0.089$; $P = 0.06$) (Fig. 2B). There was a significantly increasing trend between kisspeptin-54 and the absolute concentration of ln-transformed MBP ($R^2 = 0.251$; $\beta = 0.174$; $P < 0.001$) (Fig. 2C). After adjusting for creatinine, the ln-transformed MBP (μg/g-creatinine) was still significantly correlated with kisspeptin-54 secretion ($R^2 = 0.109$; $\beta = 0.148$; $P = 0.024$) (Fig. 2D), i.e. a 1% increase in MBP (μg/g-creatinine) was associated with a 0.001 pmol/l increase in kisspeptin-54 secretion.

Discussion

We found that the body burden of phthalate metabolites in the group of girls with CPP was significantly higher than in the prepubescent controls. Even when we analyzed these groups using ERα binding-effect indices, the results were similar. We first developed for the ERα binding effect a new index that reflects the estrogenic effect of the internal phthalate dose. In addition, we showed that kisspeptin-54 secretion was correlated with the onset of puberty and that it increased as CPP progressed. Moreover, we showed that the increasing body burden of phthalates in girls is positively related to the rising concentration of kisspeptin, suggesting that this may accelerate female sexual maturation. The present study is the first investigation of whether exposure to phthalates is correlated with neuropeptide secretion, which is associated with the onset of puberty in girls. This implied that the increase in the amount of plasticizer used in contemporary products might be associated with the rising incidence of PP around the world in recent decades.

The profile of urinary phthalate metabolites in this study was compared with the results from NHANES 1999–2008 (6- to 11-year-old US children) (Centers for Disease Control and Prevention, 2012) and GerES IV 2003–2006 (3- to 14-year-old German children) (Becker *et al*., 2009) (Table II). Generally, Taiwanese girls and German children had higher concentrations of MBP, MEOHP and MEHHP, but US children had higher concentrations of MEP. The median urinary MBP concentrations in the CPP group in the present study were twice as high as in US children, but lower than in German children. The median urinary MEHHP and MEOHP concentrations of the girls of the CPP group in the present study were one to three times higher than in US and German children. This suggests that high concentrations of DBP and DEHP were accumulated in the girls of the CPP group in the present study. MBP, MEHHP and MEOHP showed the highest levels in both groups, which indicated that the girls in these groups were exposed primarily to DBP and DEHP in their daily life.

Several studies have investigated the relationship between exposure to phthalates and hormone disturbance and other health effects. Svechnikova *et al*. (2007) found that orally gavaged DEHP had two effects on immature female rats: it stimulated the pituitary to secrete LH and reduced plasma concentrations of progesterone and E_2 by inhibiting steroidogenesis in granulosa cells. Ma *et al*. (2006) reported that inhaled DEHP may accelerate the onset of puberty and disturb postpubertal reproductive function in female rats. It also showed that inhaling high-concentration DEHP accelerated the age at which the vagina opened and of the first estrous cycle. Moreover, they found that serum cholesterol, LH and E_2 were elevated in immature female rats exposed to high-dose DEHP but that serum cholesterol concentrations decreased in adulthood. These findings are consistent with our observation that exposure to phthalates may advance the onset of puberty in female mammals.

Several studies investigated the secretion profile of kisspeptin-54 in humans. Horikoshi *et al*. (2003) found that plasma concentrations of kisspeptin-54 were ~1.3 pmol/l in men and non-pregnant women. They also found high plasma concentrations of kisspeptin-54 in pregnant women in different trimesters (1230 pmol/l in the first trimester, 4590 pmol/l in the second, 9590 pmol/l in the third), which decreased to 7.63 pmol/l 5 days post-partum. Pita *et al*. (2011) analyzed kisspeptin-54 in cord blood (127.01 pmol/l) and in plasma from women versus men (3.71 versus 1.77 pmol/l) and found that the kisspeptin-54 significantly correlated with LH levels. Seminara and Crowley (2008) concluded that kisspeptin is powerful for connecting peripheral sex steroids and GnRH secretion. Serum concentrations of kisspeptin-10 in girls with CPP were significantly higher than in hospital-based controls (de Vries *et al*., 2009). This suggests that kisspeptin is an important gatekeeper for reproduction or a key regulator of sexual hormones, especially for females. The concentrations of kisspeptin-54 for the girls in this study seem to be very close to those in adults in other reports, and the difference in kisspeptin-54 concentrations between girls of the CPP group and those of the prepubescent control group was not very large. The reason that a minor increase can turn on the puberty process might be explained by the high susceptibility to the

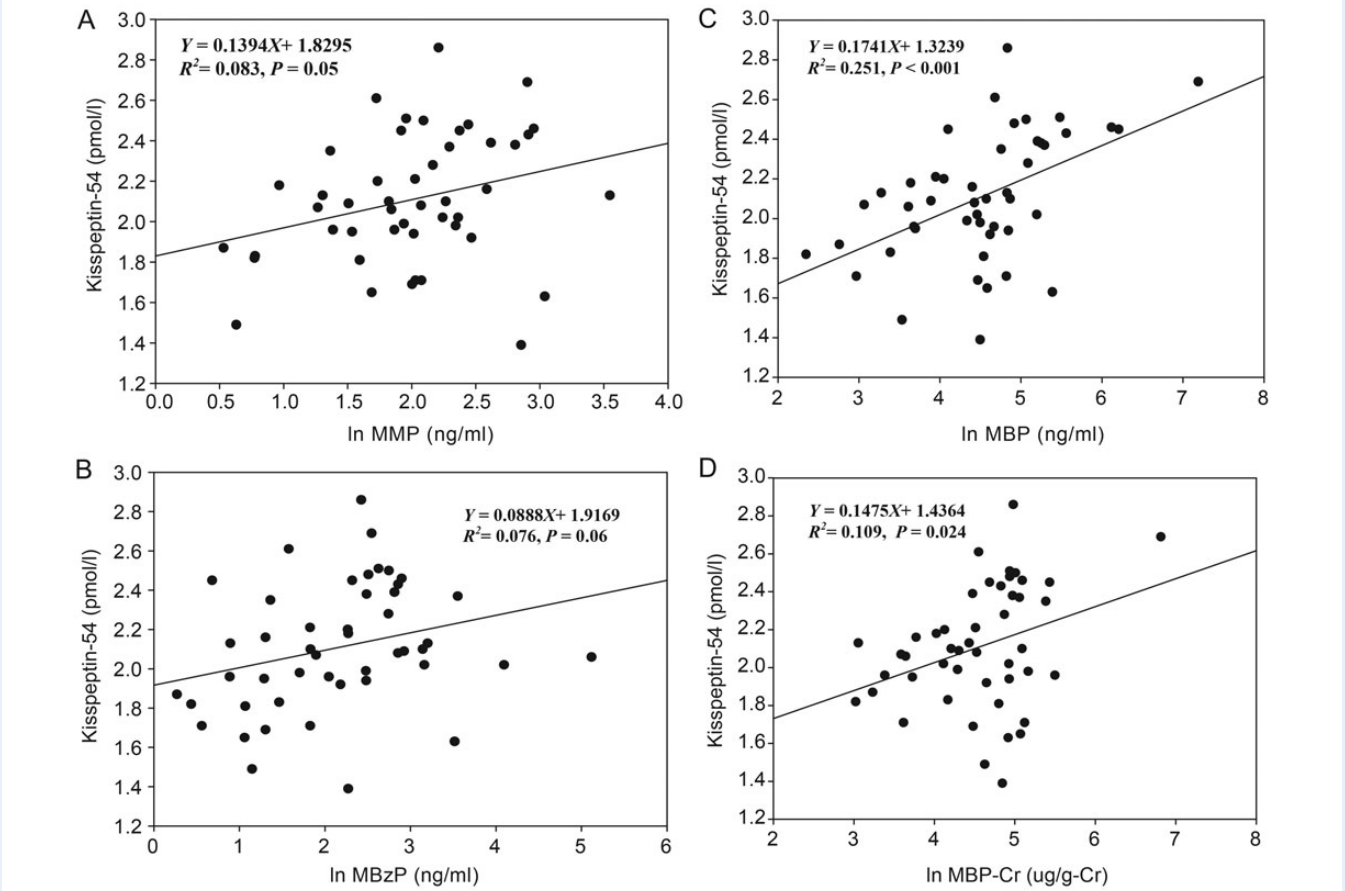

Figure 2 The association between kisspeptin-54 and urinary (**A**) ln-transformed MMP, (**B**) ln-transformed MBzP, (**C**) ln-transformed MBP and (**D**) ln-transformed MBP-Cr (μg/g creatinine) for all participants in this study (ln = natural logarithm).

action of kisspeptin-54 in girls with CPP. After a small increase in the concentration of kisspeptin, GPR54 might be more sensitive than in prepubescent girls. Girls of different ages may have different sensitivities because of changes in kisspeptin concentrations. Additional studies are needed to clarify this notion, however. Our findings indicate that the level of kisspeptin secretion is possibly related to the body burden of phthalate metabolites in girls. Smith *et al*. (2005) demonstrated that all KiSS-1-expressing neurons in the Arc and AVPV co-express ERα, which suggests that the effects of E_2 are mediated directly through KiSS-1 neurons. It was believed that the estrogenic activity of phthalates, and especially of DBP, mimicked the function of E_2 and that phthalates might bind with ERα and thereby disturb kisspeptin secretion. Once the switch turns on, GnRH will be secreted, which will activate the puberty process. Although BBP show the highest estrogenic activity in animal models, the amount of BBP consumed in Taiwan is quite low: this is possibly the reason for the low burden of MBzP in our participants. On the contrary, DBP and DEHP are widely used in consumer products in Taiwan, which might be why the body burden of MBP, MEHHP and MEOHP were the highest in our participants.

The concentrations of kisspeptin-54 rose as CPP progressed, implying that kisspeptin-54 may be involved in the human puberty process; kisspeptin-54 was reported to be involved in the animal puberty process (Keen *et al*., 2008). We also found that leuprorelin acetate treatment did not suppress kisspeptin-54 secretion, possibly because leuprorelin acetate desensitizes only the pituitary gonadotrope and suppresses only the release of GnRH (Badaru *et al*., 2006); it does not suppress Kiss-1, which is the upstream regulatory neuron. When the medication was stopped, kisspeptin still stimulated the hypothalamus to secrete GnRH, which may explain why children with CPP need a monthly injection of leuprorelin acetate. The first therapeutic use of leuprorelin acetate was reported in 1989 (Mazzei *et al*., 1989). It acts as an agonist at pituitary GnRH receptors, and by desensitizing GnRH receptors it suppresses the secretion of LH and FSH, thereby dramatically reducing E_2 and testosterone levels, which slows the growth of the bones and ovaries. It is necessary to inject leuprorelin acetate monthly to continually suppress gonadotrophin secretion. Some studies (Badaru *et al*., 2006; Lee *et al*., 2012) reported an increased injected dose of leuprorelin acetate to minimize the frequency of injections. In the present study, we found that in girls receiving leuprorelin acetate injections the levels of LH, FSH and E_2, but not kisspeptin were suppressed. In addition, developing a kisspeptin antagonist might be another strategy for treating PP. Oakley *et al*. (2009) also pointed out that the development of novel ligands for kisspeptin receptors will help when treating people with hypogonadotropic hypogonadism and reproductive disorders, and for hormonal birth control.

This is the first investigation to report that exposure to phthalates is correlated with neuropeptide secretion, which is associated with the

onset of puberty in girls. This is also the first study in which girls with CPP were categorized in three subgroups to explore the characteristic of kisspeptin secretion among these subgroups. As this is a case–control study, we could not address the cause–effect relationship between exposure to phthalates and PP. We found that a specific group of females might be more affected than others by phthalates, but additional studies are needed before our findings can be generalized to all females. Although one study (Svechnikova *et al*., 2007) reported that DEHP-treated rats produced and secreted significantly greater than normal concentrations of LH in response to GnRH, and we also found that the serum concentration of phthalates was correlated with kisspeptin secretion, additional research is needed to identify the detailed molecular mechanism(s) related to phthalate exposure and kisspeptin secretion. Another direction that might be considered is whether decreasing the internal burden of phthalates (by dietary or lifestyle interventions) in females leads to a decline in the concentration of kisspeptin.

In conclusion, we found that both urinary phthalate metabolites and serum kisspeptin-54 in girls with CPP were higher than in prepubescent controls, which implied that exposure to phthalates may promote the onset of puberty by stimulating kisspeptin secretion. We also found that serum levels of kisspeptin-54 may have a significant positive association with the progression of PP and that this was not suppressed by leuprorelin acetate treatment in girls. We suggested that developing a kisspeptin antagonist might be a new strategy for treating PP. Exposure to DBP may increase kisspeptin secretion and promote the early onset of puberty: this finding may help to explain why the sexual maturation of female children occurs earlier now than it did in the past, but the tentative hypothesis that phthalates may bind with ERα to affect kisspeptin secretion needs additional investigation.

Acknowledgements

We are indebted to the girls who participated in this study, and to their parents. We thank the Taiwan National Science Council for financial support.

Authors' roles

C.-Y.C. contributed toward the conceptualization and design, drafted the manuscript, statistically analyzed data and coordinated participant recruitment, sample collection and sample analysis. Y.-Y.C. conducted physical exams, checked medical histories, screened participants for eligibility and recruited patients in the clinic. Y.-M.W. participated in the kisspeptin analyses and recruited controls. C.-C.L. contributed toward the urinary phthalate metabolite analyses and recruited controls. S.-J.L. conducted physical exams, recruited patients in the clinic, interpreted clinical data, and reviewed and revised the manuscript. C.-C.L. coordinated and supervised sample collection at all sites, interpreted analytical data, and reviewed and revised the manuscript.

Funding

This work was supported by grants NSC 96-2621-Z-006-013 and NSC 97-2621-M-006-001 from the Taiwan National Science Council. The Council played no role in the design, conduct or interpretation of data of this study.

Conflict of interest

The authors have no conflicts of interest to disclose.

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