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A preliminary study on the relationship between environmental endocrine disruptors and precocious puberty in girls

https://doi.org/10.1515/jpem-2021-0691
Received November 21, 2021; accepted May 19, 2022;
published online June 14, 2022

Abstract

Objectives: To explore the associations of environmental endocrine disruptors on precocious puberty in girls.
Methods: This was a case-control study in which 30 girls with precocious puberty and 46 age- and race-matched prepubertal females were enrolled. The concentrations of 10 environment endocrine disruptors (bisphenol A, bisphenol B, butylparaben, propylparaben, ethylparaben, methylparaben, mono-butyl phthalate, mono-2-ethylhexyl phthalate, monoethyl phthalate, and monomethyl phthalate) in urine and 10 steroid hormones (dihydrotestosterone, corticosterone, hydrocortisone, 11-deoxycortisol, 17α-hydroxy progesterone, 4-androstene-3,17-dione, estrone, deoxycorticosterone, pregnenolone, and dehydroepiandrosterone) in serum were detected with the liquid chromatography-mass spectrometry (LC-MS).
Results: According to the Mann–Whitney U test, urinary levels of bisphenol A, monobutyl phthalate, and monomethyl phthalate were significantly higher in the precocious group than in the prepubertal group, and blood levels of hydrocortisone, 11-deoxycortisol, corticosterone, deoxycorticosterone, and pregnenolone were significantly lower in the precocious group than in the prepubertal group ($p<0.05$, VIP>1).
Conclusions: Our findings confirm the association between phthalate exposure and the incidence of precocious puberty in girls. Control and reduction of children exposure to phthalate esters should be considered as a health priority.

Keywords: environmental endocrine disruptor; precocious puberty; steroid hormone; targeted metabolomics.

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Introduction

Precocious puberty (PP) is a kind of abnormal growth and development disease. With the advance of society and economy and the change of environment, the incidence of precocious puberty is gradually increasing, and it has been one of the common endocrine diseases in children [1]. Currently, PP currently affects one in 5,000 children, and girls have a higher incidence (female-to-male ratio is approximately 10:1) and a higher risk of breast cancer than boys [2, 3]. PP is defined as the appearance of secondary sexual characteristics before the age of eight for a girl or nine for a boy, or the appearance of menarche before the age of 10 for a girl [4]. PP includes central precocious puberty and peripheral precocious puberty, and the main difference between them is whether the hypothalamus–pituitary–gonadal axis is involved or not [5]. The pathogenic factors may include heredity, central nervous system aberrations, and tumors [6]. And meanwhile the health status, nutritional status, and environmental conditions also count [7].

Environmental endocrine disruptors (EDCs) are a group of hormonally active agents to which we are directly or indirectly exposed on a daily life and that have been used in industry for almost a century, for a wide variety of uses in personal care and consumer products, including building materials, food packaging, medical devices, toys, and cosmetics [8]. They interfere with natural hormones, stimulating or inhibiting their action by binding their receptors or affecting their synthesis, transport, metabolism, and elimination [9]. As a result, they may cause different kinds of diseases. Concerning EDCs' adverse effects involving endocrine disruption on health; many studies have confirmed that fetuses, infants, and children are more vulnerable than adults, primarily because of the important role of hormonal balance in both growth and development [10, 11]. Direct or indirect exposure can increase the incidence of PP in children. In 2001, researchers conducted a study on 145 PP patients from 22 different developing countries or cultural backgrounds, and found that the cause of PP may be closely related to estrogen EDCs [12]. In 2009, a study of Chinese girls found that 26 girls with PP had significantly higher levels of monomethyl

phthalate (MMP) in their urine than prepubertal girls [13]. In 2017, researchers used liquid chromatography to measure the MMP and monoethyl phthalate (MEP) in the urine of 59 Thai girls with PP, and combined with their clinical examination information, then it was found that MEP was correlated with the PP in girls [14].

The causes of PP in girls are intricate, so doctors generally complete the clinical diagnosis based on the results of previous medical history, clinical characteristics, bone age determination, pelvic ultrasound detection, and hormone content determination [15]. There are six diagnostic markers for the clinical diagnosis of PP in children, including luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), testosterone (TTE), progesterone (PRG), and estradiol (E2). But there is no special focus on adrenal hormone changes in children with PP.

There is some evidence that adrenarche and gonadarche are interdependent in the physiological situation. A prospective cohort study in 109 healthy children showed that adrenal androgens do influence pubertal timing and most importantly, it also indicated that adrenarche triggers the pattern of subsequent pubertal development: higher prepubertal urinary androgen excretion correlated with an earlier onset of breast development and penile growth respectively, and a shorter duration of pubertal growth spurt [16]. Furthermore, adrenal androgens are positively correlated with diaphyseal bone strength during adrenarche and late puberty [17, 18]. These data suggest that adrenal androgens may either directly or after peripheral conversion (e.g. to estrogens) modulate the GNRH pulse generator, thereby fine-tuning pubertal development. Therefore, in addition to the six traditional sex hormone tests it is crucial to compare the adrenal hormone levels in children with PP to further understand the hormonal changes in the body of children with PP. In recent years, the development of metabonomics has provided a new direction for disease diagnosis, and it is a potential technology to detect and analyze more metabolic indicators, showing great application value in the study of PP [19].

EDCs have estrogen-like effects and can interfere with the biological synthesis, secretion, transport, binding and metabolism of natural estrogens in the body, thus affect the normal growth and development of children. Recently due to human are exposed to putative endocrine disrupters with estrogen-like properties, it has become increasingly important to clarify the role of estrogen metabolism to pubertal children. To provide further insight into the possible role of phthalates in premature pubertal development, we compared urinary concentrations of 10 common household EDCs (e.g. toys, food packaging materials, etc.) and serum concentrations of 10 steroid hormones in girls with PP and in age- and race-matched prepubertal females.

Materials and methods

Instruments and reagents

The targeted metabonomics analysis platform in the study was AB Sciex 5,500 Ultra-performance Liquid Chromatography Tandem Mass Spectrometer (Agilent-AB Sciex, Boston, USA). HPLC-grade Acetonitrile, HPLC-grade methanol and AR-grade ethylacetate were purchased from Merk (Merck KGaA, Darmstadt, Germany). HPLC-grade ammoniumacetat, AR-grade formic acid and β-glucuronidase were purchased from CNW (CNW Technologies GmbH, Duesseldorf, Germany). AR-grade hydroxylamine hydrochloride was purchased from Alfa Aesar (Alfa Aesar, Lancashire, UK).

Ten EDCs including monomethyl phthalate (MMP), bisphenol A (BPA), butylparaben (BP), mono-butyl phthalate (MBP), mono-2-ethylhexyl phthalate (MEHP), monoethyl phthalate (MEP), methylparaben (MP), ethylparaben (EP), propylparaben (PP), extrasynthese, and bisphenols B (BPB) were purchased from Dr. Ehrenstorfer (Dr. Ehrenstorfer GmbH, Augsburg, Germany), AccuStandard (AccuStandard, Inc., New Haven, USA), (Extrasynthèse S.A., Genay, France) or CNW (CNW Technologies GmbH, Duesseldorf, Germany).

Ten steroid hormones including hydrocortisone, corticosterone, 4-Androstene-3, 17-Dione, deoxycorticosterone, dihydrotestosterone, estrone, 17α-hydroxyprogesterone, dehydroepiandrosterone, 11-deoxycortisol, progesterone, testosterone, and pregnenolone were purchased from Dr. Ehrenstorfer (Dr. Ehrenstorfer GmbH, Augsburg, Germany), TRC (Toronto Research Chemicals, Toronto, Canada) or ANPEL (ANPEL Laboratory Technologies (Shanghai) Inc., Shanghai, China).

Study population

All study subjects were recruited from Zhuji Hospital of Traditional Chinese Medicine pediatric endocrinology clinics between 2019 and 2021. The study population comprised 30 girls with PP and 46 age- and race-matched prepubertal females. The relevant basic information collected including physical signs physical tests, routine biochemical tests and common imaging test results.

Inclusion criteria for PP: patients with a confirmed diagnosis of central PP (2015 version) [20]: (i) Early appearance of secondary sexual characteristics: Girls before the age of 8 years with the first appearance of breast nodules appearance. (ii) Accelerated linear growth: higher annual growth rate than normal children. (iii) Premature bone age: bone age is 1 year or more above the actual age. (iv) Enlarged gonads: pelvic ultrasound shows increased volume of the girl's uterus and ovaries, and multiple follicles>4 mm in diameter are seen in the ovaries. (v) HPGA function initiation, serum gonadotropins, and sex hormones at pubertal levels: peak LH≥5.0 U/L and peak LH/FSH>0.6 by stimulation test of GnRHa, detected by immunochemical luminescence assay (ICMA).

Exclusion criteria: (i) Serious illness such as combined cardiovascular disease, liver disease, kidney disease and mental illness etc.; (ii) Long medication experience or taking medication within the last

three days; (iii) Incomplete relevant underlying information; (iv) Failure to sign informed consent.

To avoid possible systematic bias due to sample selection during small-sample histological analysis, two groups of samples should have no significant differences in age, physical examination of signs, and clinical and biochemical indices. All parents and guardians were informed and consent, same as all subjects. All subjects' urine, blood and related clinical information were collected. In clinical tests, six sex hormones were detected by e601 chemiluminescence analyzer (Roche, Germany) with matching original reagents. Bone age diagnosis was according to the bone age standard TW3-C RUS in "Standard of carpal bone development in Chinese human hands-China 05" [21]. Subjects' height and weight were measured with light colored clothes and no shoes. Body mass index (BMI), height and weight percentiles, and *z*-scores were calculated by using Epi Info, version 3.5.1. Serum and urine samples were stored in refrigerator at −80 °C for the study.

Measurement of EDCs in urine

1 mL of urine sample added with 500 μL of buffer and 20 μL of β-glucuronidase, mixed well and shaken in a 37 °C water bath for 90 min. After cooling to room temperature, added ethyl acetate for extraction and centrifuged at 3,000 rpm for 2 min. Then placed the supernatant in a 45 °C water bath, dried with nitrogen, and redissolved with 1 mL of 50% methanol for LC-MS analysis.

Measurement of steroid hormones in serum

A total of 600 μL of precipitant containing the internal standard was added to 150 μL of serum sample and centrifuged at 13,000 rpm for 15 min. Lyophilized the supernatant in a vacuum freeze dryer, redissolved the lyophilized sample by adding 50 μL hydroxylamine hydrochloride solution and lyophilized in a water bath at 60 °C for 20 min in an electric thermostat. After lyophilization, added 65 μL of 30% methanol solution to re-dissolve the sample at 13,000 rpm for 6 min and took the supernatant for LC-MS analysis.

Statistical analysis

The statistical software SPSS (version 22, IBM, USA) was used for the statistical analyses. 10 EDCs and 10 steroidal hormones mass concentrations were non-normally distributed and expressed as M (P_{25}~P_{75}), and the Mann–Whitney U test was used for comparison between multiple groups. Clinical data of each group were normally distributed and analyzed by independent sample t-test; $p<0.05$ indicates statistically significant differences.

The resulting data sets were imported separately into the SIMCA 14.1 software package (Umetrics, Umeå, Sweden). After mean centering and unit variance scaling, we used orthogonal partial least-squares-discriminant analysis (OPLS-DA) to identify differences between the PP group and prepubertal group. The variable importance in projection (VIP) generated in OPLS-DA processing represents the contribution to the discrimination of each metabolite ion between groups. Variables with a VIP>1 and $p<0.05$ were considered to be different variables. The S-plot is used to visualize the covariance and the correlation structure between the X-variables and the predictive score t [1]. Thus, variables situated in the upper right and lower left of the S combine high model influence with high reliability and are potential markers [22, 23].

Results

Clinical characteristics

Clinical characteristics of the two study groups are summarized in Table 1. The bone age of girls in the PP group was one year older than that in the prepubertal group ($p<0.01$). The serum levels of luteinizing hormone and estradiol in the PP group were higher than those in the prepubertal group ($p<0.05$).

The OPLS-DA analysis

10 EDCs and 10 steroidal hormones were analyzed by a multivariate statistical method based on the OPLS-DA model. Score diagram and S-plot diagram were obtained from the modeling results (Figures 1 and 2). Score diagram shows that the samples of PP girls and prepubertal girls were clustered and distributed on both sides of the figure, which suggesting they were effectively discriminated in the model. S-plot diagram shows that BPA, MBT, and MMT levels in the PP group were higher than those in the prepubertal group, 11-deoxycortisol, corticosterone, deoxycorticosterone, and pregnenolone levels in the PP group were lower than those in the prepubertal group ($p<0.05$ and VIP>1). The levels of eight significantly different diagnostic

Table 1: Clinical information of girls (6–8 years old) in the PP group and the prepubertal group.

Clinical characteristics	Prepubertal group (n=46)		PP group (n=30)		p-Value
	Mean	SD	Mean	SD	
Age, year	7.263	0.679	7.119	0.742	0.388
Height, cm	125.583	5.570	126.283	5.640	0.621
Weight, kg	25.487	5.119	24.083	4.683	0.231
BMI, kg/m^2	16.083	2.387	15.000	2.003	0.043
Bone age, year	7.167	1.062	8.206	1.119	<0.001
Luteinizing hormone, IU/L	0.102	0.015	0.443	0.257	<0.001
Follicle-stimulating hormone, IU/L	2.485	0.515	2.487	1.200	0.994
Prolactin, ng/L	13.020	1.745	11.193	5.448	0.037
Testosterone, nmol/L	0.128	0.074	0.184	0.217	0.112
Progesterone, nmol/L	0.426	0.132	0.607	0.277	<0.001
Estradiol, pmol/L	23.937	10.691	73.980	21.547	<0.001

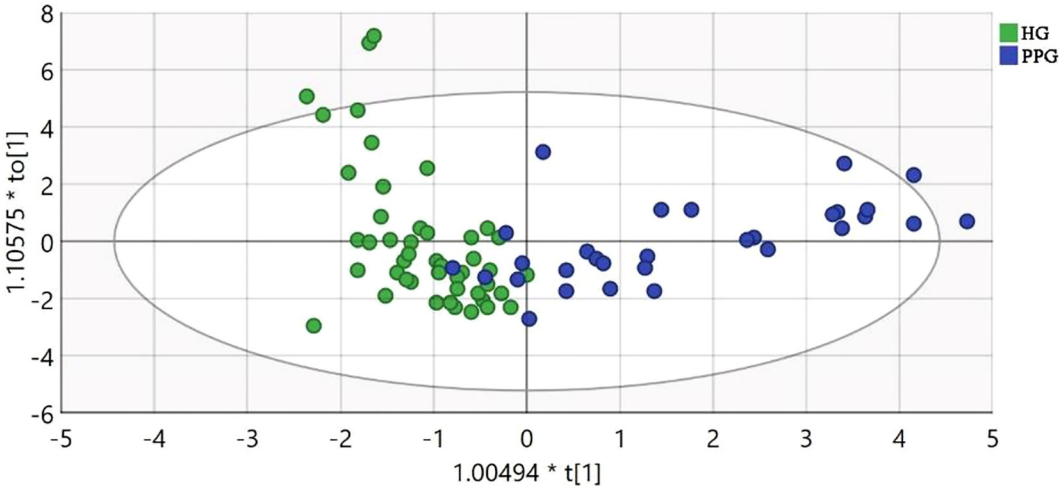

Figure 1: The score diagram from the OPLS-DA model. The axis represents the principal component.
The green scatters represent samples in the prepubertal group and the blue scatters represent samples in the PP group (R^2X=0.337, R^2Y=0.640, Q^2=0.563).

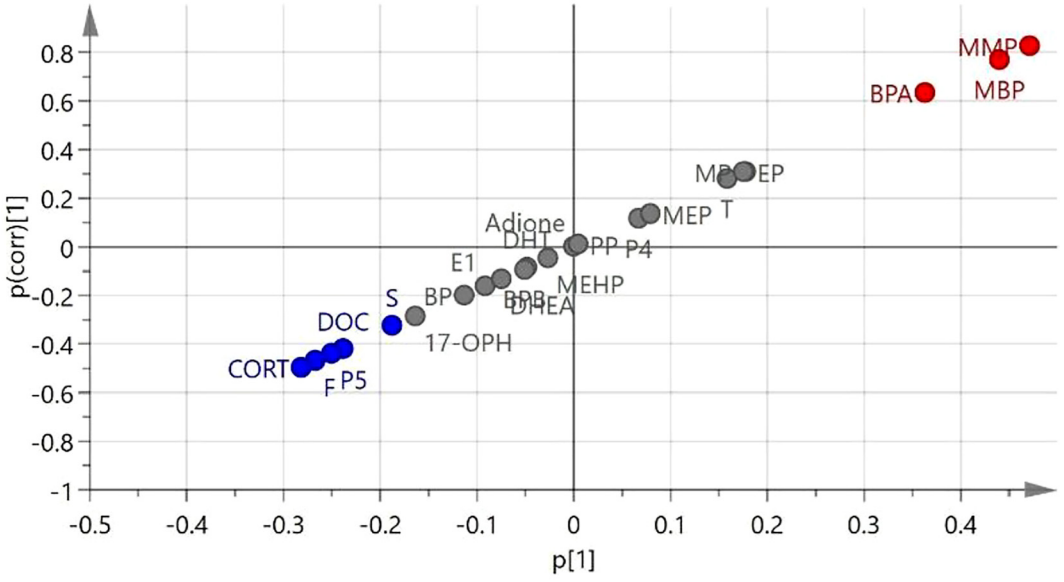

Figure 2: The S-plot diagram from the OPLS-DA model.
The red scatters represent the diagnostic markers level in the PP group were higher than those in the prepubertal group and the blue scatters represent the diagnostic markers level in the PP group were lower than those in the prepubertal group ($p<0.05$, VIP>1).

markers in the prepubertal and PP groups are shown in Figures 3 and 4, Table 2, and the distribution of all 10 EDCs as well as 10 steroid hormones is shown in Supplementary Tables 1, 2. The results show that the detection rate of EDCs were higher in PP girls compared with prepubertal girls, and all levels in the PP group were higher than those in the prepubertal group, and all levels of steroid hormones in the PP group were lower than those in the prepubertal group. In the OPLS-DA model, BPA, MBT, MMT, hydrocortisone, 11-deoxycortisol, corticosterone, deoxycorticosterone, and pregnenolone had high biomarker potential.

The correlation between urinary EDCs levels, serum sex hormones and steroid hormones was analyzed by using Spearman's correlation test. The results showed that there is no statistically significant correlation between urinary EDCs levels, serum sex hormones and steroid hormones.

Discussion

In school-aged girls, accumulated data indicated the incidence of PP is increasing throughout the world in a secular

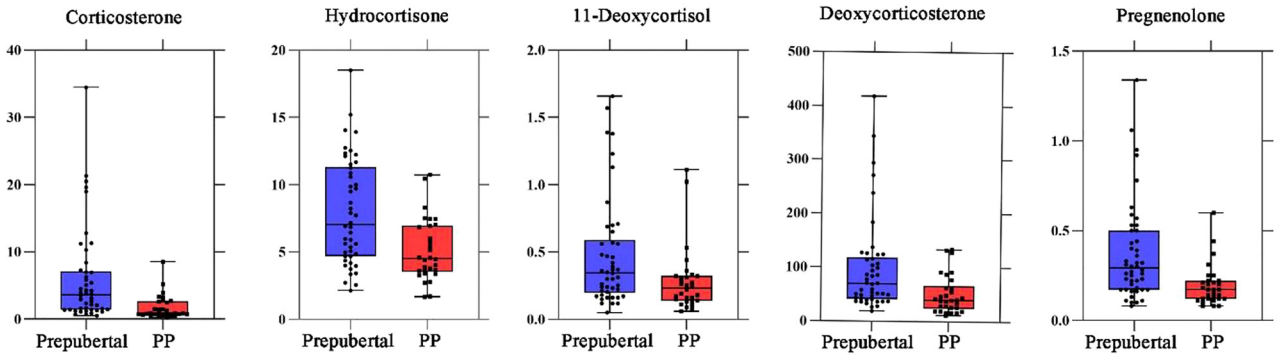

Figure 3: Scatter plot of five significantly different steroid hormones.
The blue column represents the prepubertal group and the red column represents the PP group. The black scatters represent samples within their respective groups.

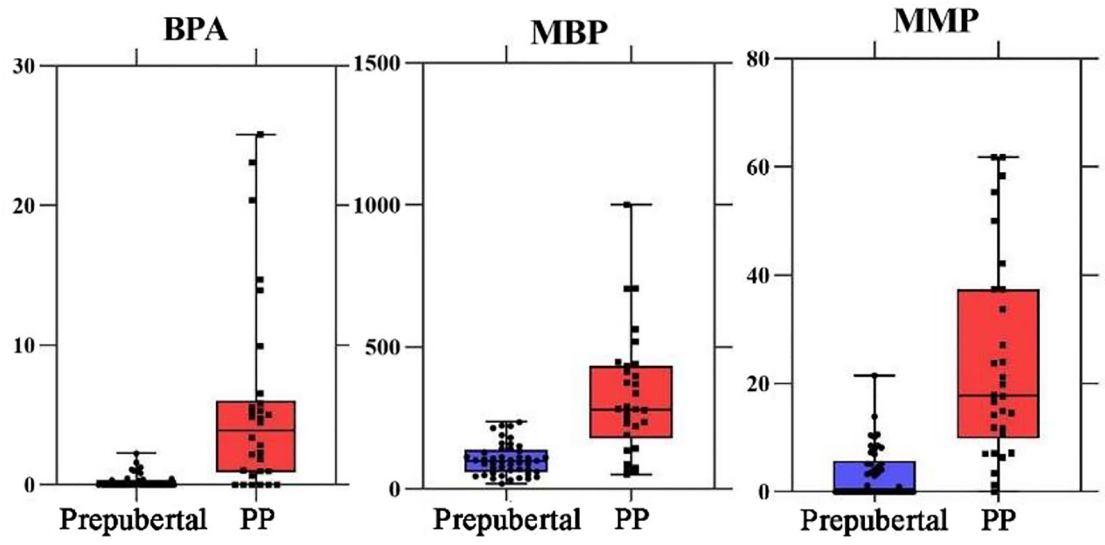

Figure 4: Scatter plot of three significantly different EDCs.
The blue column represents the prepubertal group and the red column represents the PP group. The black scatters represent samples within their respective groups.

trend [7]. Although the prevalence of PP in different countries and the factors causing these conditions have become the top concern among pediatricians and endocrinologists, the available data are still very scarce. The previous observation of elevated serum phthalate concentrations in girls with premature thelarche raised concerns about the estrogenic effects of phthalate exposure in children [24]. Phthalates, as plasticizers, can affect reproductive system in women [25]. It might also promote adolescent development in girls by increasing kisspeptin activity [26]. In addition to phthalates, Parabens are also common endocrine disruptors in the environment. As a broad spectrum preservative in cosmetics and food, parabens are inevitably used in daily life [27]. It will be absorbed by the body surface, hydrolyzed, conjugated and discharged in urine, causing metabolic disorders of human body and leading to changes in sexual development in the end [28].

In this study, we have detected 10 EDCs in girls' urine. The results showed that BPA, MBP, and MMP levels in the PP group were higher than those in the prepubertal group (Supplementary Table 1). BPA is considered as a substance to promote PP, and studies on the estrogenic activity of BPA have reported that it contributes to immature glucuronidation activity [29]. In this study, the detection rate of BPA in the group of girls with PP (80%) was significantly higher than that in the prepubertal group (28.26%), and the level of BPA in the urine of the group of girls with PP was 24 times higher than that of the prepubertal group. This is the same as some research results in Asian populations. Durmaz et al. [30] tested the difference in urinary BPA between 28 non-obese Turkish girls affected by idiopathic central PP

Table 2: Significantly different diagnostic markers levels between the PP group and the prepubertal group.

Abbreviation	Prepubertal group			PP group		
	Detection rate, %	Scope	M (P_{25}~P_{75})	Detection rate, %	Scope	M (P_{25}~P_{75})
Corticosterone, ng/mL	100.000	0.500–34.480	5.980 (1.430–6.980)	100.000	0.250–8.510	1.740 (0.720–2.590)
Hydrocortisone, μg/dL	100.000	2.120–18.530	7.950 (4.730–11.150)	100.000	1.660–10.370	5.140 (3.590–6.920)
11-Deoxycortisol, ng/mL	100.000	0.050–1.660	0.480 (0.200–0.570)	100.000	0.060–1.110	0.280 (0.140–0.320)
Deoxycorticosterone, pg/mL	100.000	18.990–418.160	97.500 (41.710–115.510)	100.000	11.470–133.820	50.700 (26.290–61.730)
Pregnenolone, ng/mL	100.000	0.080–1.340	0.370 (0.170–0.490)	100.000	0.080–0.600	0.190 (0.120–0.220)
BPA, μg/g.Cr	28.260	0.000–2.270	0.240 (0.000–0.270)	80.000	0.000–25.090	5.870 (0.000–25.090)
MBP, μg/g.Cr	100.000	19.580–236.200	103.990 (62.970–133.220)	100.000	52.180–447.430	202.620 (99.710–276.790)
MMP, μg/g.Cr	50.000	0.000–21.510	3.430 (0.000–5.240)	96.670	0.000–37.430	12.260 (6.700–16.210)

and 25 healthy age- and ethnicity-matched control girls, the results showed that the level of urinary BPA in the affected group of 26 girls was significantly (p=0.001) higher than that in the control group. Another more recent study by Supornsilchai et al. [31] considered the values of urinary BPA and pubertal stage in 41 patients affected by PP and 47 healthy age-matched controls. Those with signs of early puberty had higher levels of BPA in the urine compared to the control group. Similar results can be extrapolated from previous works conducted by Qiao et al. in 2010 [32] and Lee et al. in 2009 [33].

However, some studies have shown that there is no significant correlation between BPA and puberty. In 2015, Wolff M et al. evaluated 1,239 girls taken from BCERC, all of whom were 6–8 years old at enrolment, in order to detect first puberty appearance age in a 7-year follow-up program and correlated this moment with urinary values of 10 different phenols, including BPA [34]. This study estimated a relative risk of early or late puberty which linked to phenol exposure. In approximately 85% of girls, signs of pubertal activation (growth of breasts and pubic hair) appeared in the extended follow-up time, but no statistical correlation was found between levels of BPA and early pubertal development. There are also some studies that show, urinary BPA levels were not significantly associated with age at menarche [35–38].

The MBP levels (202.62 μg/g. Cr) in the serum of girls were 1.95 times higher than those of the prepubertal group (103.99 μg/g. Cr) (p<0.001). In consistent with previous reports (33), the results of this study suggest that the onset of PP may be affected by exposure to phthalates. But this result is different from a study in the United States, which reported no correlation between phthalates levels in urine and central PP of pre-pubescent girls [39]. Meanwhile, the MMP levels (12.26 μg/g. Cr) in the serum of PP girls were 3.57 times higher than those of the prepubertal group (3.43 μg/g. Cr) (p<0.001), which was similar to the findings of Mahin Hashemipour et al. [40]. The conflicting results of the BPA and MBP studies may be due to the lack of standardization of the studies or the influence of the different geographical areas in which the epidemiological surveys were conducted.

Exposure to endocrine disruptors can affect the content of steroid hormones in the body, thus affecting the development of target organs. The research by Nakano et al. showed, when H295R cells were exposed to 10 μM forskolin, increases were observed in cortical hormone. Conversely, when cells were exposed to 1 μM prochloraz, decreases were observed in cortical hormone. This indicated that cortical hormone pathway may be influenced by EDCs [41]. But the relationship between EDCs and levels of

endogenous steroids is still not clear. To elucidate the metabolic changes of endogenous steroid hormones in body, the levels of serum steroids were compared between groups and its connection with EDCs exposure. We have detected several main hormones in the serum, including androgenic hormones (dihydrotestosterone, 4-androstene-3,17-dione, dehydroepiandrosterone), estrogenic hormones (estrone), progestational hormones (17α-hydroxy progesterone, pregnenolone), and cortical hormones (corticosterone, hydrocortisone, 11-deoxycortisol, deoxycorticosterone). Finally, hydrocortisone, 11-deoxycortisol, corticosterone, deoxycorticosterone, and pregnenolone levels were lower in serum from PP girls (Table 2). According to the result, we found that cortical hormone pathway in PP girls might be influenced by EDCs. Pregnenolone was catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) and 21-hydroxylase (CYP21) to form deoxycorticosterone and 11-deoxycortisol, deoxycorticosterone and 11-deoxycortisol was catalyzed by 11β-hydroxylase (CYP11B1) to form corticosterone and hydrocortisone. According to the measured results, we speculated that EDCs might affect pregnenolone related protease, leading to changes in its downstream metabolic network. Meanwhile, some studies have shown that deoxycorticosterone is converted to active androgens in the gonads and peripheral target tissues of androgen action including the skin, resulting in the development of pubic and axillary hair [42]. Physiologically, increasing androgen production during adrenarche manifests with distinct changes in body odor and oily skin and hair, followed by the first appearance of pubic and axillary hair. In addition, the rise in adrenal androgens can result in transient growth acceleration and contributes to bone maturation [17, 43, 44]. However, there was no significant difference in the content of deoxycorticosterone in the results of this study.

The research by Lee et al. showed, altered steroid metabolism was associated with urinary BPA levels, and levels of testosterone, 17β-estradiol, and pregnenolone were significantly increased among individuals with high BPA levels. In girls, BPA exposure causes metabolic changes in steroidogenesis [45]. However, in this study, the correlation analysis of urinary EDCs with serum sex hormones and steroid hormones showed that urinary EDCs levels did not correlate with either serum sex hormone or steroid hormone levels. The limitations of this study are mainly represented that only 76 girls aged 6–8 years were selected for urine and blood testing, and the sample size was too small and not representative enough to be limited in the generalization of the findings. In addition, the use of a single random urine sample for exposure assessment is not fully representative of long-term EDCs exposure levels in children. Given that EDCs exposure may be potentially harmful to children, there is a strong need for a large sample size survey and long-term follow-up assessment of EDCs exposure levels in children. To more accurately assess the relationship between children's urine EDCs levels and children's daily exposure, this study also requires a questionnaire survey of children's general conditions, lifestyle habits, and parents' education and economic income.

In conclusion, we found that EDCs exposure may trigger PP in girls, and at the same time affect changes in the metabolism of steroid hormones in the body. Although the mechanism of the effect of EDCs exposure on the changes of steroid hormone metabolism is still unclear and needs further study, it is certain that the different levels of EDCs in girls' urine are evidence of different exposures in girls' lives. Therefore, it is important to further understand and explore the level of EDCs exposure in children and its influencing factors to protect children's physical and mental health. As far as the results we have obtained, we think control and reduction of children exposure to phthalate esters should be considered as a health priority.

Acknowledgments: This work was supported by the Shaoxing Health and Family Planning Science and Technology Plan Project (No. 2020002032).
Research funding: This work was funded by Shaoxing Scientific Research Fund Project, Shaoxing city health committee (No. 2017CX027).
Author contribution: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The local Institutional Review Board deemed the study exempt from review.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/jpem-2021-0691).