

Research Article

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# Co-exposure of phthalates, bisphenols, parabens, and polycyclic aromatic hydrocarbons in follicular fluid of women undergoing assisted reproductive technologies and the associations with hormone levels

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**How to cite this article:** Dou, X.; Li, X.; Huang, S.; Long, C.; Chen, C.; Chen, X.; Yu, Y. Co-exposure of phthalates, bisphenols, parabens, and polycyclic aromatic hydrocarbons in follicular fluid of women undergoing assisted reproductive technologies and the associations with hormone levels. *J. Environ. Expo. Assess.* 2025, 4, 14. <https://dx.doi.org/10.20517/jeea.2025.07>

**Received:** 11 Feb 2025 **First Decision:** 7 Apr 2025 **Revised:** 16 Apr 2025 **Accepted:** 23 Apr 2025 **Published:** 16 May 2025

**Academic Editor:** Shan Liu **Copy Editor:** Pei-Yun Wang **Production Editor:** Pei-Yun Wang

## Abstract

Endocrine-disrupting chemicals (EDCs), particularly phthalates (PAEs), bisphenols, parabens, and polycyclic



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aromatic hydrocarbons (PAHs), constitute pervasive environmental contaminants with demonstrated potential to adversely affect female reproductive health. Although these compounds are known to exert adverse effects, critical knowledge gaps persist concerning their specific associations with reproductive outcomes. The present study analyzed 144 follicular fluid samples from women undergoing assisted reproductive technology procedures, quantifying concentrations of PAE metabolites (mPAEs), bisphenols, parabens, and hydroxylated PAHs (OH-PAHs). Analytical results demonstrated a descending concentration gradient: mPAEs exhibited the highest median concentration (6.14 ng/mL), followed by parabens (2.17 ng/mL), bisphenols (1.33 ng/mL), and OH-PAHs (0.26 ng/mL). Notably, the study identified a positive correlation between follicular fluid bisphenol concentrations and testosterone levels, along with a potential association between PAE exposure and elevated risk of secondary infertility. Application of Bayesian kernel machine regression and Quantile g-computation models revealed that EDCs predominantly influence hormone levels through mixture effects, with increasing chemical mixture concentrations corresponding to decreased estradiol levels on hCG trigger day and reduced basal progesterone. The models specifically identified bisphenol S (BPS) and bisphenol P (BPP) as the predominant mediators of these endocrine disruptions, respectively, suggesting that bisphenols may disrupt female reproductive health through endocrine interference mechanisms.

**Keywords:** Endocrine-disrupting chemicals, follicular fluid, hormone level, female fertility, women's health risks

## INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are exogenous compounds that interfere with hormonal signaling, thereby disrupting the development and function of the female reproductive system<sup>[1]</sup>. The most prevalent EDCs in daily life include phthalates (PAEs), bisphenols, parabens, and polycyclic aromatic hydrocarbons (PAHs). As essential industrial chemicals, PAEs, bisphenols, and parabens are widely incorporated into personal care products, food preservatives, and plastic consumer goods<sup>[2,3]</sup>. As products of the incomplete combustion of organic substances, human activities are the most important source of PAHs<sup>[4]</sup>. Women are routinely exposed to these EDCs via multiple pathways including dietary ingestion, respiratory inhalation, and dermal absorption<sup>[5]</sup>. These compounds have been consistently detected across various biological matrices, including urine, serum, and breast milk samples<sup>[6-8]</sup>.

Many studies have demonstrated the adverse effects of EDCs on female reproductive health<sup>[9-12]</sup>. Specifically, Peng *et al.* identified a potential association between PAE exposure and unexplained recurrent spontaneous abortion (URSA) in reproductive-aged women<sup>[13]</sup>, and significantly elevated concentrations of di(2-ethylhexyl) phthalate (DEHP) in 594 URSA cases compared to 569 healthy controls were also observed by our previous study<sup>[14]</sup>. Furthermore, epidemiological evidence links bisphenol compounds including bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) to increased risks of polycystic ovary syndrome (PCOS) and endometriosis<sup>[15,16]</sup>. Similarly, several studies have established correlations between paraben exposure and both ovarian volume abnormalities and endometriosis incidence<sup>[17,18]</sup>. In addition, PAH exposure has been significantly associated with elevated premature ovarian failure (POF) risk<sup>[19]</sup>.

Estrogen and progesterone, as critical reproductive hormones, play indispensable roles in female reproductive health<sup>[15,20,21]</sup>, with well-established associations to PCOS development, endometriosis progression, and clinical pregnancy outcomes<sup>[21-23]</sup>. Numerous studies demonstrate significant correlations between circulating hormone levels and urinary EDC concentrations. Sathyanarayana *et al.* identified positive associations between serum estradiol (E2) and urinary concentrations of monoisobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), and di(2-ethylhexyl) phthalate (DEHP) metabolites<sup>[24]</sup>. Similarly, Yuan *et al.* reported comparable findings for urinary 1-OH-PHE and E2 levels<sup>[25]</sup>. However, an inverse relationship between urinary BPA concentrations and peak serum E2 levels was reported by MokLin

*et al.*<sup>[26]</sup>. Moreover, elevated urinary methylparaben (MeP) concentrations have been consistently associated with lower serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels<sup>[27,28]</sup>. These results indicate that EDC exposure may disrupt reproductive endocrine homeostasis, potentially contributing to the pathogenesis of various reproductive disorders.

The ovary represents the female-specific reproductive organ whose functional status serves as a direct indicator of the reproductive health of women. Within ovarian physiology, follicles constitute the fundamental endocrine and reproductive functional units<sup>[29]</sup>. Follicular fluid contaminant concentrations can reflect the actual exposure doses reaching the female reproductive system, thereby offering critical insights into potential reproductive health impacts. A study in South American women undergoing assisted reproduction found that specific urinary and follicular fluid phthalate metabolites were associated with altered ovarian response and embryo development parameters, revealing both detrimental and unexpected beneficial correlations, warranting further investigation into the complex role in assisted reproductive technology (ART) outcomes by phthalate exposure<sup>[10]</sup>. Despite this significance, current research on EDCs in follicular fluid remains very limited<sup>[10,30–35]</sup>. Most existing studies have predominantly investigated contaminant effects on female reproductive health using other biological matrices such as urine and serum<sup>[6,8,36]</sup>.

Given the unclear effects of EDC exposure on female infertility and the crucial role of reproductive hormones in female reproduction, we hypothesized that EDCs may promote infertility development through hormonal disruption. Therefore, the present study aimed to: (1) quantify EDC concentrations in follicular fluid; (2) investigate their associations with reproductive hormones and infertility subtypes; and (3) elucidate their specific effects on estradiol and progesterone levels. By analyzing the concentrations of EDCs or their metabolites in follicular fluid, the present findings provide novel insights into mixture effects on hormonal secretion and infertility pathogenesis, establishing a valuable foundation for female reproductive health risk assessment.

## MATERIALS AND METHODS

### Reagents and materials

Four kinds of standards containing parabens including MeP, propylparaben (PRP), butylparaben (BUP), ethylparaben (ETP), heptylparaben (HEP), and benzylparaben (BzP), bisphenols including BPA, BPS, BPF, bisphenol B (BPB), bisphenol AF (BPAF), bisphenol Z (BPZ), bisphenol P (BPP), and bisphenol AP (BPAP), hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) including 1-hydroxynthalene (1-OH-NAP), 2-hydroxynthalene (2-OH-NAP), 2-hydroxyfluorene (2-OH-FLU), 3-hydroxyfluorene (3-OH-FLU), 1-OH-PHE, 3-hydroxyphenanthrene (3-OH-PHE), 4-hydroxyphenanthrene (4-OH-PHE), and 9-hydroxyphenanthrene (9-OH-PHE), and phthalate metabolites (mPAEs) including MiBP, MBzP, mono-methyl phthalate (MMP), mono(2-ethylhexyl) phthalate (MEHP), mono-n-butyl phthalate (MBP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-n-octyl phthalate (MOP), and monoethyl phthalate (MeP), were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The isotopically labeled internal standards of d<sub>4</sub>-MeP, d<sub>4</sub>-PRP, d<sub>4</sub>-ETP, d<sub>7</sub>-BzP, d<sub>4</sub>-HEP, and d<sub>4</sub>-BUP were bought from CDN Isotopes, Inc. (Quebec, Canada), while the internal standards of d<sub>7</sub>-2-OH-NAP, d<sub>9</sub>-2-OH-FLU, <sup>13</sup>C<sub>6</sub>-3-OH-PHE, <sup>13</sup>C<sub>12</sub>-BPA, <sup>13</sup>C<sub>4</sub>-MMP, d<sub>4</sub>-MiBP, <sup>13</sup>C<sub>4</sub>-MeP, <sup>13</sup>C<sub>4</sub>-MBP, <sup>13</sup>C<sub>4</sub>-MEHHP, <sup>13</sup>C<sub>4</sub>-MEHP, <sup>13</sup>C<sub>4</sub>-MOP, and <sup>13</sup>C<sub>4</sub>-MBzP were purchased from Cambridge Isotope Laboratories, Inc (Andover, USA).

β-glucuronidase was obtained from Sigma Aldrich Corp (St. Louis, MO, USA). Methanol, acetonitrile, and MAX solid-phase extraction (SPE) cartridges (10 mg/1 mL) were purchased from CNW Technologies (Shanghai, China).

### Sample collection and preparation

This study enrolled 144 women aged  $\leq 40$  years with anti-Müllerian hormone (AMH) levels  $> 1.1$  ng/mL who were undergoing ART treatment at Shunde Hospital of Southern Medical University (2021-2022). From each participant, follicular fluid ( $> 2$  mL) and corresponding serum samples for hormonal analysis were collected. Participants completed brief lifestyle questionnaires. All samples were stored at  $-80$  °C prior to laboratory analysis. All the volunteers signed the informed consent form and to participate in the present study. The experiments were approved by the Ethics Committee of Shunde Hospital of Southern Medical University (20210301).

### Sample pretreatment and instrumental analysis

In the present study, mPAEs, bisphenols, parabens, and OH-PAHs in the follicular fluid were detected, and the pretreatment was similar to a previous study<sup>[31]</sup>. In brief, the internal standards of mPAEs, bisphenols, parabens, and OH-PAHs were added to the follicular fluid samples (200  $\mu$ L), which were hydrolyzed by  $\beta$ -glucuronidase at  $37$  °C for 180 min. Then, the mixture was subsequently loaded onto MAX SPE cartridges, with target compounds eluted using 1 mL of 2% formic acid in methanol. All target compounds were separated by a Poroshell 120 EC-C18 column (100 mm  $\times$  4.6 mm, 2.7  $\mu$ m particle diameter, Agilent) and determined by ultra-performance liquid chromatography-tandem mass spectrometry. Parabens and mPAEs were analyzed by applying a gradient of Milli-Q water and acetonitrile, while bisphenols and OH-PAHs were analyzed by applying a gradient of 2 mM ammonium acetate and methanol. Complete methodological details are provided in the [Supplementary Text 1 and 2](#), with instrumental parameters summarized in [Supplementary Table 1](#).

### Quality assurance and quality control

To ensure analytical accuracy, each sample batch included one procedural blank, one reagent blank, and one matrix-spiked sample for recovery assessment and background contamination evaluation. The recoveries were 77%-109% for mPAEs, 74%-97% for parabens, 63%-108% for bisphenols, and 64%-105% for OH-PAHs. The background contamination concentrations were 4.14-8.91 ng/mL (mPAEs), 12.19-20.04 ng/mL (parabens), 1.88-6.78 ng/mL (bisphenols), and 0.87-3.68 ng/mL (OH-PAHs), with specific values detailed in [Supplementary Table 2](#). The calibration curves were 0.01-100  $\mu$ g/L, with correlation coefficients being greater than 0.994.

### Statistical analysis

Statistical analysis was carried out using an SPSS software version 27.0 (IBM, USA) with concentrations below the limit of detection (LOD) assigned zero values. Values between LOD and limit of quantitation (LOQ) were calculated as one-fourth of the LOQ when detection frequencies were below 50%, or as one-half of the LOQ when detection frequencies exceeded 50%<sup>[37]</sup>. The LOQs and LODs are presented in [Supplementary Table 2](#). Nonparametric analyses included the Kruskal-Wallis H test and Mann-Whitney U test for variable comparisons, while Spearman's correlation analysis evaluated both inter-compound relationships and compound-hormone associations, with all tests being two-tailed at a significance level of  $P < 0.05$ . In addition, 1/9-OH-PHE was used to represent the combined signal of 9-OH-PHE and 1-OH-PHE because of unresolved chromatographic coelution of the two chemicals.

To comprehensively investigate the relationships between target compounds and hormone levels, Python 13.0 (Guido van Rossum, Netherlands) for advanced statistical modeling was used. Initial multiple nonlinear regression analysis revealed complex nonlinear associations and dependencies among variables. Subsequent multivariate analyses incorporated logistic regression, support vector classification (SVC), classification trees, and neural networks to rigorously examine and verify the correlations from different perspectives and with different modeling approaches. This multi-methodological approach enabled robust

characterization of subtle compound-hormone interactions while ensuring the reliability and validity of findings.

Bayesian kernel machine regression (BKMR), a robust method for assessing mixture health effects<sup>[38]</sup>, was also used to explore the influence of chemical mixtures on hormone regulation. The BKMR model generated posterior inclusion probabilities (PIPs) to quantify individual chemical contributions, with a PIP threshold of 0.50 considered statistically significant<sup>[39]</sup>. Model adjustments incorporated age, body mass index (BMI), and therapeutic regimen as covariates. Complementary analysis using the Quantile g-computation (Qgcomp) model determined chemical-specific weight contributions. All analyses were conducted in R 4.1 (R Foundation for Statistical Computing, Vienna, Austria).

### Sensitivity analysis

Sensitivity analyses confirmed the robustness of the results to methodological choices. Correlation-based screening retained only chemicals significantly associated with hormones, excluding weakly correlated ones, to reduce potential confounding. In the BKMR model, varying the kernel bandwidth and iteration parameters yielded stable exposure importance rankings, despite minor fluctuations in PIPs. The exposure-response curves also exhibited consistent trends, supporting the robustness of variable selection. For the Qgcomp model, sensitivity to quantile grouping parameters was evaluated by testing different discretization levels. Although slight variations in magnitude were observed, the weight trends and directional consistency of mixture effects remained stable across scenarios.

## RESULTS AND DISCUSSION

### Demographic characteristics of the study population

The demographic characteristics and serum hormone levels of the subjects are shown in Table 1. The cohort comprised women aged 22-40 years (mean age = 32 years) with an average infertility duration of approximately 3 years. All participants reported being non-smokers and abstaining from alcohol consumption.

Moreover, the present study analyzed hormonal parameters during days 2-3 of the menstrual cycle, measuring basal testosterone (Basal\_T), basal progesterone (Basal\_P), and basal antral follicle count (Basal\_AFC). On human chorionic gonadotropin (HCG) trigger day, estradiol levels (HCG\_E2) and follicles  $\geq 14$  mm in diameter (HCG\_number) were also evaluated. Furthermore, variations were observed in the forms of infertility among the subjects, specifically including primary infertility and secondary infertility. It was also found that most of them received *in vitro* fertilization (IVF) as a therapeutic regimen.

### Detection frequencies of the target chemicals in the follicular fluid

The detection frequencies of mPAEs, bisphenols, parabens, and OH-PAHs in follicular fluid are shown in Table 2. The majority of target compounds were reliably detected, demonstrating that EDCs including PAEs, bisphenols, parabens, and PAHs can enter the female reproductive organs with potentially adverse effects on female reproductive health.

Among the four classes of target compounds, mPAEs exhibited the highest detection frequencies, demonstrating widespread PAE exposure among participants. With the exception of non-detectable MBzP, all the other mPAEs showed detection frequencies exceeding 50%. The detection frequencies followed a descending order of MeP (92%), MBP (84%), MiBP (83%), MMP (82%), MOP (71%), MEHHP (68%), and MEHHP (59%). Li *et al.* also reported detection frequencies above 60% for mPAEs in follicular fluid from women undergoing *in-vitro* fertilization/intracytoplasmic sperm injection and embryo transfer

**Table 1. Characteristics of the study population**

Items	Subject (n = 144)	Items	Subject (n = 144)
<b>Age</b>		<b>Infertile years</b>	
Mean (SD)	31.9 (4.04)	Mean (SD)	3.13 (2.54)
<b>BMI</b>		<b>Endometrial thickness (mm)</b>	
Mean (SD)	21.37 (3.26)	Mean (SD)	10.04 (2.57)
<b>AMH (ng/mL)</b>		<b>Basal AFC</b>	
Mean (SD)	3.71 (3.62)	Mean (SD)	16.31 (8.88)
<b>Basal_T (nmol/L)</b>		<b>Basal_P (nmol/L)</b>	
Mean (SD)	3.04 (17.36)	Mean (SD)	0.47 (14.8)
<b>HCG_E2 (pg/mL)</b>		<b>HCG_number</b>	
Mean (SD)	3,163.9 (2,683.1)	Mean (SD)	9.94 (5.67)
<b>Alcohol, n (%)</b>	0	<b>Smoking, n (%)</b>	0
<b>Infertility form</b>	<b>n (%)</b>	<b>Therapeutic regimen</b>	<b>n (%)</b>
Primary infertility	77 (53.5%)	ICSI	1 (0.7%)
Secondary infertility	67 (46.5%)	IVF	143 (99.3%)

BMI: Body mass index; AMH: anti-Müllerian hormone; Basal AFC: basal antral follicle count; Basal\_T: basal testosterone; Basal\_P: basal progesterone; HCG\_E2: estradiol on HCG trigger day; HCG\_number: follicles were observed with a diameter of  $\geq 14$  mm on HCG trigger day.

procedures<sup>[40]</sup>. The consistent detection of these compounds provides compelling evidence for PAE accumulation in female reproductive tissues.

Bisphenols, parabens, and OH-PAHs showed significantly lower detection frequencies compared to mPAEs. Among bisphenols, BPA exhibited the highest detection frequency (56%), while other analogs were detected in < 50% of samples: BPP (36%), BPS (33%), and BPB (non-detectable). For parabens, PRP demonstrated exceptionally high detection (99%), whereas others showed lower frequencies in descending order of MeP (24%), ETP (19%), BUP (8%), BzP (2%), and HEP (non-detectable). Bellavia *et al.* reported that both bisphenols and parabens showed certain non-detection rates, with bisphenols were significantly higher, exceeding 80% (such as 95.5% for BPA and 80.8% for BPS) in the follicular fluid of Sweden and Estonia women undergoing ART treatment, while those of most parabens were less than 50% (such as 33.6% for PRP and 45.9% for ETP)<sup>[31]</sup>. The variable detection patterns suggest regional differences in exposure profiles, potentially reflecting lifestyle variations affecting chemical accumulation in reproductive tissues.

Among OH-PAHs, 1-OH-NAP showed the highest follicular fluid detection frequency (56%), followed by 2-OH-NAP (44%), 2-OH-FLU (27%), and 3-OH-FLU (3%), while monohydroxy PHE metabolites (1/3/4/9-OH-PHE) were non-detectable. This contrasts with our previous urinary biomonitoring data, which reported substantially higher detection rates: 2-OH-NAP (91.3%), 1-OH-NAP (82.5%), 3-OH-PHE (53.3%), 4-OH-PHE (36.3%), 2-OH-FLU (16.7%), and 3-OH-FLU (7.1%)<sup>[41]</sup>. The marked discrepancy between follicular fluid and urinary profiles supports the established metabolic pathway wherein PAHs are preferentially metabolized to monohydroxy compounds and excreted renally<sup>[42]</sup>, resulting in lower accumulation in reproductive tissues.

#### Levels of mPAEs, bisphenols, parabens, and OH-PAHs in the follicular fluid

As shown in [Supplementary Figure 1](#) and [Table 2](#),  $\Sigma$ mPAEs exhibited the highest median concentration (6.14 ng/mL, range: 0.37–87.53 ng/mL) among the measured contaminants. For individual mPAE compounds, MMP demonstrated the highest median concentration (1.52 ng/mL), followed by MBP (1.43 ng/mL) and MeP (1.29 ng/mL), indicating these mPAE congeners readily accumulate in the female



**Table 2. Distributions and levels of quantification of chemicals evaluated in primary analyses (ng/mL)**

Compounds	Mean (SD)	Median [min, max]	Quantified, n (%)
<b>mPAEs</b>			
MMP	2.86 (4.16)	1.52 [0.07, 31.1]	118 (82%)
MeP	2.67 (4.00)	1.29 [0.07, 25.0]	133 (92%)
MBP	2.91 (4.39)	1.43 [0.09, 29.9]	121 (84%)
MiBP	1.29 (1.32)	1.03 [0.13, 11.0]	120 (83%)
MEHP	0.99 (3.67)	0.25 [0.01, 33.0]	85 (59%)
MEHHP	0.71 (1.99)	0.39 [0.06, 19.5]	98 (68%)
MBzP	0	0	0
MOP	0.93 (2.88)	0.25 [0.25, 28.8]	102 (71%)
<b>ΣmPAEs</b>	10.1 (11.7)	6.14 [0.37, 87.5]	
<b>Bisphenols</b>			
BPAF	0.08 (0.01)	0.08 [0.06, 0.10]	13 (9%)
BPA	4.43 (5.86)	2.37 [0.46, 36.0]	81 (56%)
BPS	0.69 (1.04)	0.22 [0.04, 4.72]	48 (33%)
BPB	0	0	0
BPF	0.97 (0.33)	0.85 [0.68, 1.55]	9 (6%)
BPAP	0.04 (0.01)	0.04 [0.03, 0.08]	26 (18%)
BPZ	0.09 (0.06)	0.08 [0.04, 0.37]	37 (26%)
BPP	0.07(0.05)	0.05 [0.01, 0.24]	52 (36%)
<b>ΣBisphenols</b>	2.84 (4.93)	1.33 [0, 36.14]	
<b>Parabens</b>			
MeP	1.76 (1.31)	1.49 [0.16, 4.40]	34 (24%)
ETP	0.67 (1.57)	0.21 [0.07, 8.00]	28 (19%)
PRP	2.40 (2.03)	1.90 [0.27, 10.2]	143 (99%)
BUP	0.04 (0.04)	0.03 [0.01, 0.13]	11 (8%)
BzP	0.13 (0.08)	0.10 [0.07, 0.22]	3 (2%)
HEP	0	0	0
<b>ΣParabens</b>	2.94 (2.72)	2.17 [0, 16.1]	
<b>OH-PAHs</b>			
1-OH-NAP	0.76 (0.69)	0.52 [0.03, 3.55]	80 (56%)
2-OH-NAP	0.77 (0.72)	0.54 [0, 2.84]	63 (44%)
2-OH-FLU	0.10 (0.11)	0.08 [0, 0.50]	39 (27%)
3-OH-FLU	0.02 (0.06)	0.00 [0, 0.24]	4 (3%)
1/9-OH-PHE	0	0	0
3-OH-PHE	0	0	0
4-OH-PHE	0	0	0
<b>ΣOH-PAHs</b>	0.84 (1.24)	0.26 [0, 5.75]	

mPAEs: Phthalate metabolites; MMP: mono-methyl phthalate; MeP: methylparaben; MBP: mono-n-butylphthalate; MiBP: monoisobutyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MBzP: monobenzyl phthalate; MOP: mono-octyl phthalate; BPAF: bisphenol AF; BPA: bisphenol A; BPS: bisphenol S; BPB: bisphenol B; BPF: bisphenol F; BPAP: bisphenol AP; BPZ: bisphenol Z; BPP: bisphenol P; ETP: ethylparaben; PRP: propylparaben; BUP: butylparaben; BzP: Benzylparaben; HEP: Heptylparaben; OH-PAHs: hydroxyl polycyclic aromatic hydrocarbon; OH-NAP: hydroxynaphthalene; OH-FLU: hydroxyfluorene; OH-PHE: hydroxyphenanthrene.

reproductive system. Similar results were found in a previous study<sup>[33]</sup>, which reported comparable median concentrations of MBP (1.72 ng/mL) and MeP (1.62 ng/mL) in follicular fluid from women undergoing IVF treatment. Although PAEs undergo rapid metabolism and excretion, their classification as non-persistent chemicals belies the reality of continuous exposure through ubiquitous sources including polyvinyl chloride

(PVC) products, cosmetics, and personal care items, resulting in detectable concentrations reaching reproductive tissues<sup>[1,43,44]</sup>.

For bisphenols, the analysis revealed the concentrations of  $\Sigma$ bisphenols ranging from 0 to 36.1 ng/mL (median: 1.33 ng/mL) in follicular fluid [Supplementary Figure 1 and Table 2]. BPA demonstrated the highest median concentration (2.37 ng/mL), followed by its alternatives BPF (0.85 ng/mL) and BPS (0.22 ng/mL). Similarly, a previous study reported a predominance of BPA (0.21  $\mu$ g/L) over BPS (0.04  $\mu$ g/L) and BPF (non-detectable) in follicular fluid samples<sup>[33]</sup>. The consistent detection pattern identifies BPA, BPF, and BPS as the primary bisphenol contaminants in reproductive fluids. As these compounds are extensively incorporated into food packaging and personal care products<sup>[45]</sup>, the studied population likely experiences exposure primarily through oral ingestion and dermal contact pathways.

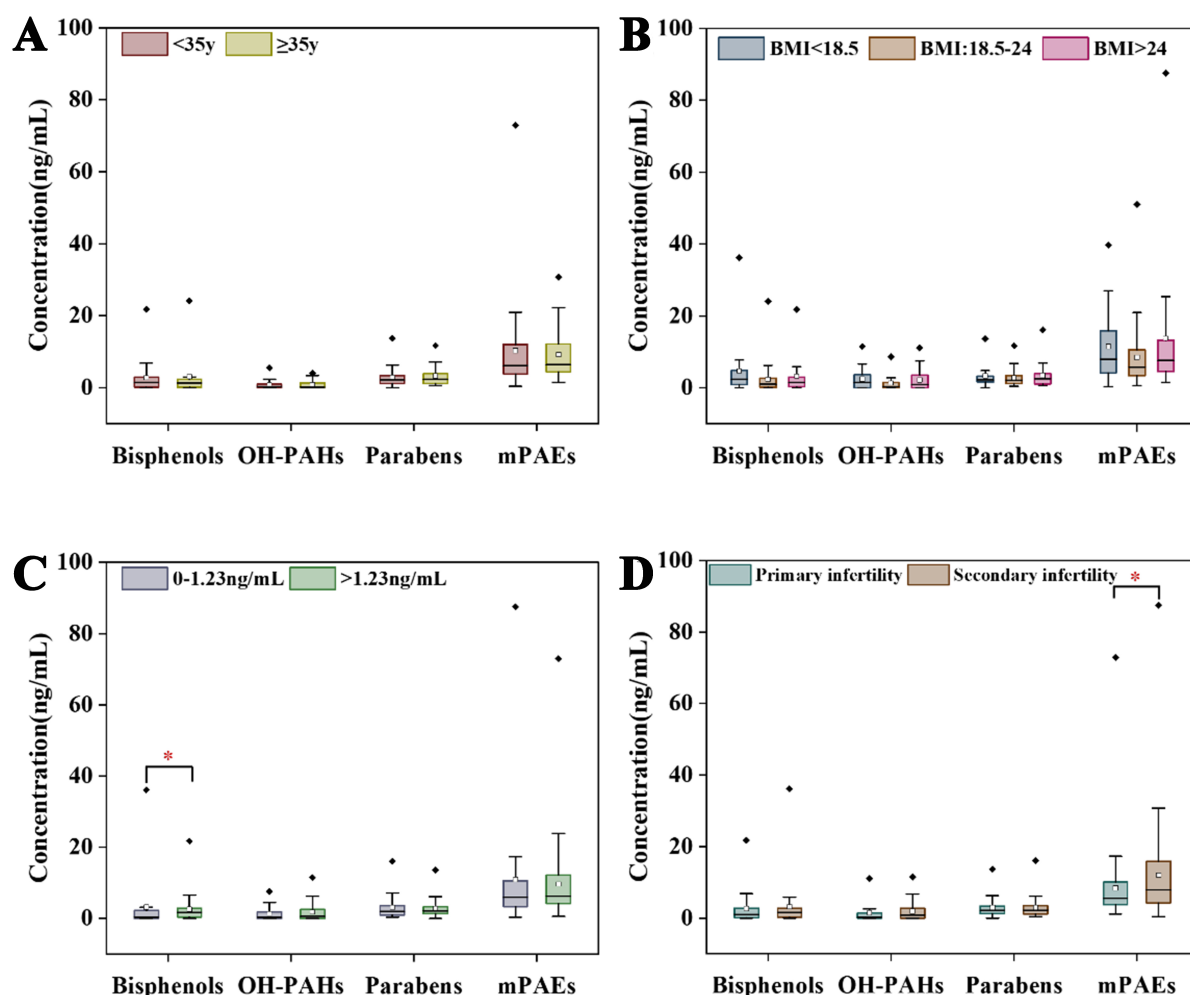
Paraben analysis revealed  $\Sigma$ parabens ranging from 0 to 16.1 ng/mL (median: 2.17 ng/mL) in follicular fluid [Supplementary Figure 1 and Table 2]. MeP and PRP emerged as the predominant compounds, with median concentrations of 1.49 and 1.90 ng/mL, respectively. Similar results were found by Bellavia *et al.*, who reported comparable paraben levels in follicular fluid from women undergoing ART treatment (MeP: 0.71 ng/mL; PRP: 6.90 ng/mL)<sup>[31]</sup>. The frequent co-occurrence of MeP and PRP reflects their widespread dual application in cosmetics and personal care formulations. Routine female exposure to these preservatives occurs through multiple pathways including cosmetic use, consumption of processed foods and beverages, and pharmaceutical applications, leading to chronic cumulative exposure<sup>[27,46]</sup>.

Compared with the above EDCs, OH-PAHs exhibited significantly lower exposure levels in follicular fluid with  $\Sigma$ OH-PAHs ranging from 0 to 5.75 ng/mL (median: 0.26 ng/mL) [Supplementary Figure 1 and Table 2]. Among these compounds, monohydroxy Nap metabolites showed the highest median concentrations (1-OH-NAP: 0.52 ng/mL; 2-OH-NAP: 0.54 ng/mL), while Flu metabolites demonstrated substantially lower levels (2-OH-FLU: 0.08 ng/mL; 3-OH-FLU: non-detectable). Similar compositions were found in human urine, with OH-NAP being the predominant OH-PAHs<sup>[4,41,47]</sup>. Inhalation represents a probable major exposure route for PAHs in this non-smoking cohort, with likely environmental sources including petrochemical combustion, vehicular emissions, and secondhand smoke exposure. Inhalation may represent a significant route of human exposure to PAHs. Since none of the study participants were habitual smokers, potential sources of PAHs likely include incomplete petrochemical combustion, vehicle emissions, and secondhand smoke<sup>[48,49]</sup>.

### Association of various chemicals with population hormones and infertility form

As shown in Figure 1, the study assessed correlations between demographic characteristics (age and BMI), hormone levels, infertility form, and follicular fluid concentrations of chemicals, including mPAEs, bisphenols, parabens, and OH-PAHs. Based on literature defining advanced maternal age as  $\geq 35$  years, participants were categorized into advanced ( $\geq 35$  years) and non-advanced ( $< 35$  years) groups for analysis<sup>[50]</sup>. No significant differences were observed in EDC exposure relative to age or BMI [Figure 1A and B]. However, variations in EDC metabolite concentrations were detected in follicular fluid across different Basal\_T levels, with Basal\_T levels exhibiting an increasing trend alongside higher EDC exposure, particularly for bisphenols ( $P = 0.030$ ) [Figure 1C]. EDCs, such as bisphenols, modulate testosterone levels by inhibiting steroidogenic enzymes and disrupting negative feedback in the hypothalamic-pituitary-testicular (HPT) axis. Studies showed that BPA directly impairs Leydig cells, hindering cholesterol conversion to testosterone, while chronic low-dose exposure suppresses LH and FSH secretion, perpetuating testosterone decline<sup>[51,52]</sup>. Conversely, short-term high-dose exposure may transiently elevate testosterone, as demonstrated by positive correlations between urinary BPA levels and total testosterone in





**Figure 1.** Differences in the levels of bisphenols, OH-PAHs, parabens, and mPAEs in follicular fluid based on demographic characteristics. (A) Age; (B) BMI; (C) Basal\_T; (D) Infertility form. The lower and upper limits of the box denote the 25th and 75th quartiles, respectively. The horizontal line within the box represents the median value. The length of the whiskers is 1.5 times the interquartile range. The rhombus symbol denotes the 99th percentile. The small rectangular boxes represent average values, and the star symbol indicates that there are significant differences between groups [Kruskal–Wallis H and Mann–Whitney U tests were used.  $P < 0.05$  (2-tailed)]. OH-PAHs: Hydroxyl polycyclic aromatic hydrocarbon; mPAEs: phthalate metabolites; BMI: body mass index; Basal\_T: basal testosterone.

Italian men, and between serum BPA and total/free testosterone in women with PCOS<sup>[53,54]</sup>. Additionally, bisphenols may dysregulate genes involved in ovarian steroidogenesis, indirectly increasing testosterone<sup>[55]</sup>. Furthermore, higher testosterone levels (in PCOS) may contribute to impaired folliculogenesis<sup>[56]</sup> and adverse cognitive effects<sup>[57]</sup>. Hence, bisphenol exposure may pose a health risk to the female reproductive system by causing high levels of testosterone.

Epidemiological studies on infertility covered primary infertility (defined as the inability to conceive in nulligravid women) and secondary infertility (characterized by parous women's failure to conceive or carry a pregnancy to term, irrespective of previous outcomes)<sup>[58]</sup>. As shown in Figure 1D, the investigation of EDC metabolite concentrations in follicular fluid revealed a potential association between PAE exposure and secondary infertility development ( $P = 0.037$ ). Follicles are the functional units of the ovary. The process of folliculogenesis enables the maintenance, development, and maturation of oocytes<sup>[59]</sup>. Epidemiological

evidence demonstrates that PAE exposure directly impairs ovarian function by restricting sinus follicle growth and development, thereby contributing to diminished ovarian reserve (DOR) and infertility<sup>[44]</sup>. An *in vitro* study has shown that DEHP exposure significantly suppresses primordial follicle aggregation, leading to reduced primordial follicle counts and increased oocyte apoptosis<sup>[60]</sup>. Furthermore, PAE exposure has been shown to disrupt ovulation by modifying oocyte biochemical markers<sup>[61]</sup>. Therefore, these findings collectively indicate that PAEs can compromise fertility through ovarian dysfunction and may particularly contribute to secondary infertility development.

### The correlations between chemical mixtures and hormones

To investigate potential associations between chemical mixtures and hormones, a series of systematic correlation analyses were conducted. As shown in Table 3, EDC metabolites showing statistically significant associations with hormone levels (HCG\_E2 and Basal\_P) were initially identified ( $P < 0.05$ ). While significant correlations between EDC metabolites and these two hormones were observed, the correlation coefficients remained relatively low. The correlation heatmap revealed not only strong intra-category chemical correlations, such as between MBP and MeP ( $r = 0.91$ ) and between MOP and MEHHP ( $r = 0.55$ ) [Supplementary Figure 2], but also notable inter-category associations, including BPS and MMP ( $r = 0.34$ ) and BUP and MBP ( $r = 0.22$ ). These findings support a hypothesis that these chemicals may collectively influence hormone levels through mixture effects.

Therefore, EDC metabolites demonstrating significant associations with either HCG\_E2 or Basal\_P ( $P < 0.05$ ) were selected for subsequent analysis of potential relationships between EDC mixture concentrations in follicular fluid and hormone levels. Consequently, two distinct correlation groups were established for further investigation: Group I comprising HCG\_E2 with chemical group A (BPS, BPAP, BPZ, MeP, and MBP), and Group II consisting of Basal\_P with chemical group B (BPS, BPP, and BUP).

To more comprehensively and accurately assess the correlation between chemical mixtures and hormone levels (the aforementioned groups), a multivariate nonlinear regression model was employed. However, the model's performance proved suboptimal. Consequently, hormone levels were stratified into quartiles, and classification models were subsequently implemented for simulation analysis [Supplementary Table 3]. Model accuracy metrics indicated correlation values exceeding 0.4 - substantially higher than the coefficients observed for individual chemical-hormone correlations. These findings demonstrate that chemical mixtures exhibit significantly stronger associations with hormone levels compared to single compounds. Collectively, these results warrant further investigation into the effects of chemical mixtures on HCG\_E2 and Basal\_P levels.

### The effect of chemical mixtures on hormones

To elucidate the effects of chemical mixtures on hormone levels, both BKMR and Qgcomp models for comprehensive analysis were employed for in-depth analysis, with results presented in Figure 2. The analysis revealed a significant downward trend in HCG\_E2 and Basal\_P levels when chemical mixture concentrations were at a higher quartile ( $> 55\%$ - $65\%$ ) [Figure 2A]. The PIP of each compound in the model was presented in Table 4. Regarding HCG\_E2, BPS demonstrated the highest PIP value (0.720), followed by MeP (0.637) and MBP (0.428), indicating their predominant influence relative to other compounds. For Basal\_P, BPP showed the strongest association (PIP = 0.587), with BUP (0.401) and BPS (0.292) following, suggesting BPP and BUP as primary contributors. Exposure-response relationships exhibited nonlinear decreasing trends between HCG\_E2 and specific chemicals (BPS, BPAP, and BPB; Figure 2B), with analogous patterns observed for Basal\_P in relation to BPS, BPP, and BUP [Figure 2C]. Notably, no significant associations emerged between individual chemical concentrations and either HCG\_E2 or Basal\_P levels [Figure 2B and C], suggesting that the observed negative trends predominantly stem from

**Table 3. The correlations between the chemicals and hormones [ $^{\circ}$   $P < 0.05$ ;  $^{**}$   $P < 0.01$  (2-tailed)]**

Population hormone	Chemical a1	Chemical a2	Chemical a3	Chemical a4	Chemical a5	Chemical group	Related group
HCG_E2 <sup>a,b</sup>	BPS	BPAP	BPZ	MeP	MBP	A	I
R <sup>c</sup>	-0.169 <sup>*</sup>	0.175 <sup>*</sup>	-0.168 <sup>*</sup>	0.242 <sup>**</sup>	0.226 <sup>**</sup>		
Basal_P <sup>a,d</sup>	BPS	BPP	BUP			B	II
R	0.249 <sup>**</sup>	-0.231 <sup>**</sup>	0.270 <sup>**</sup>				

<sup>a</sup>Levels of the hormone from another ongoing study were used with permission from our team; <sup>b</sup>HCG\_E2: estradiol on HCG trigger day; <sup>c</sup>R: correlation coefficient; <sup>d</sup>Basal\_P: basal progesterone. BPS: Bisphenol S; BPAP: bisphenol AP; BPZ: bisphenol Z; MeP: methylparaben; MBP: mono-n-butylphthalate; BPP: bisphenol P; BUP: butylparaben.

**Table 4. PIPs for the chemical-related group incorporated into the HCG\_E2 and Basal\_P models, utilizing the BKMR model**

Chemical	PIP	
	HCG_E2	Basal_P
BPS	0.720	0.292
MBP	0.428	0.587
MeP	0.637	0.401
BPZ	0.359	
BPAP	0.313	

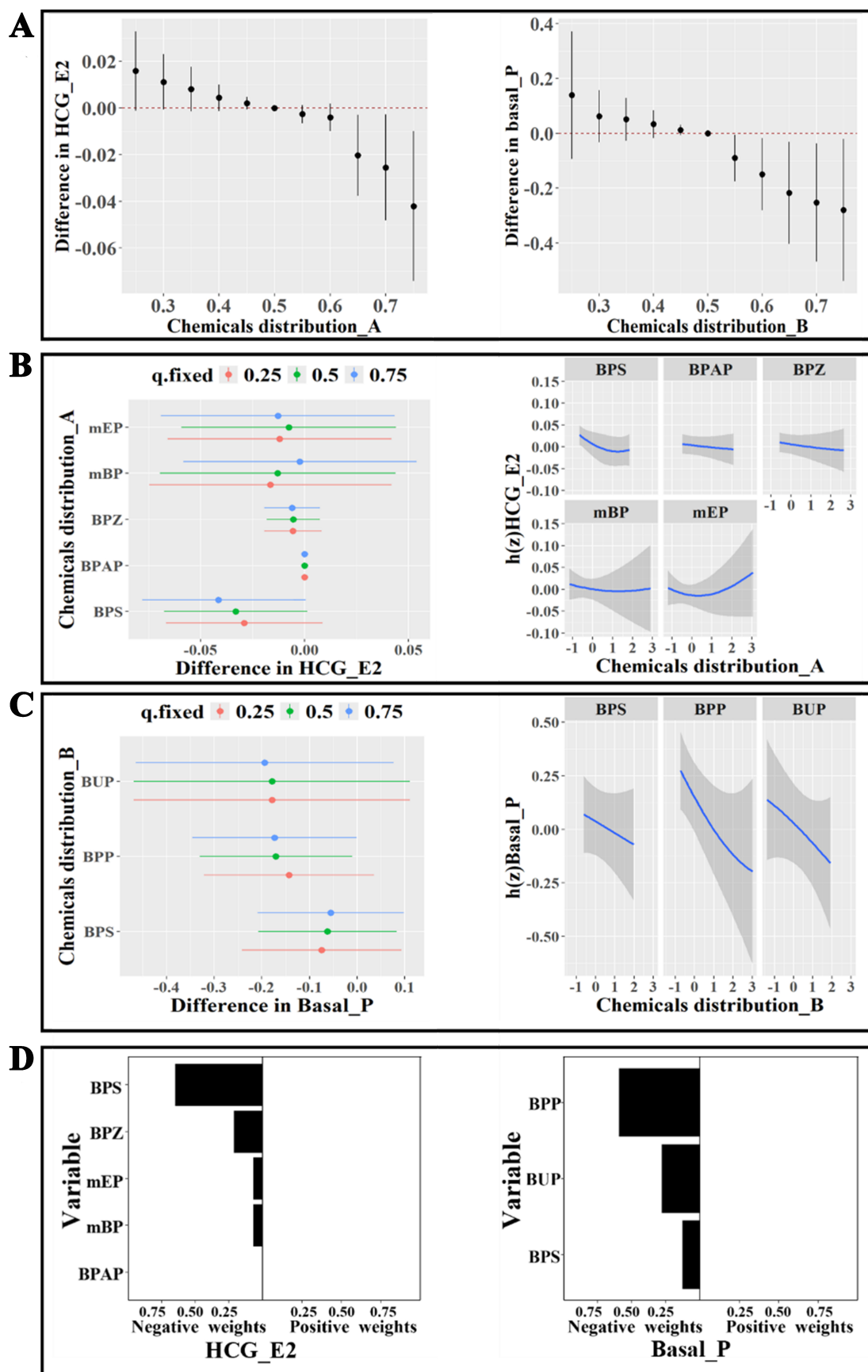
PIPs: Posterior inclusion probabilities; HCG\_E2: estradiol on HCG trigger day; Basal\_P: basal progesterone; BKMR: Bayesian kernel machine regression; BPS: bisphenol S; MBP: mono-n-butylphthalate; MeP: methylparaben; BPZ: bisphenol Z; BPAP: bisphenol AP.

mixture effects rather than single compounds.

Consequently, the Qgcomp model was implemented for additional analysis, with age, BMI, and therapeutic regimen adjusted as covariates. The analysis demonstrated negative weight values for chemical mixtures in the model [Figure 2D]. Regarding HCG\_E2, BPS exhibited the strongest negative association (weight = 0.651), followed by BPZ (0.213), MeP (0.069), and MBP (0.068). For Basal\_P, BPP showed the most pronounced effect (weight = 0.593), with BUP (0.280) and BPS (0.127) displaying weaker associations. Collectively, these results indicate an inverse relationship between follicular fluid chemical mixture concentrations and hormone levels, with BPS and BPP emerging as the primary contributing factors for HCG\_E2 and Basal\_P, respectively.

The concordance between BKMR and Qgcomp model results mutually reinforces the reliability of the conclusions, demonstrating consistent negative associations between chemical mixtures and hormone levels. These findings suggest that elevated follicular fluid concentrations of chemical mixtures may substantially decrease estradiol and progesterone levels. Similar findings have been reported in the previous study, including Shi *et al.*, who similarly reported model consistency in demonstrating positive associations between various mPAEs and abdominal adiposity in women<sup>[39]</sup>. Regarding female reproductive physiology, estradiol and progesterone serve critical functions. As Panagopoulos *et al.* documented, both hormones, i.e., estradiol and progesterone, are indispensable for uterine development<sup>[62]</sup>. Specifically, estradiol serves as a predictive biomarker for mature oocyte yield in ART cycles<sup>[20]</sup>, while progesterone is fundamental for terminal follicular maturation and ovulation<sup>[63]</sup>. Clinically, depressed levels of these hormones may promote failure of mature follicle development, irregular menstruation, and elevated miscarriage risk<sup>[21,63]</sup>.

Previous studies established that PAEs exhibit reproductive toxicity by interfering with ovarian estradiol production through inhibition of follicular estradiol synthesis<sup>[63-65]</sup>. BPA, structurally analogous to 17- $\beta$



**Figure 2.** Joint association (95%CI) of the mixture on hormone levels. (A) Observe the combined effect (95%CI) of the selected chemical group and HCG\_E2/Basal\_P through the BKMR model; (B and C) The correlation between a single chemical and HCG\_E2 and Basal\_P when all other exposed compounds are fixed at the 25th, 50th, or 75th percentile of exposure. The univariate exposure-response function (95%CI) of the selected chemical concentration on HCG\_E2 and Basal\_P.  $h(Z)$  is interpreted as the relationship between the chemical and the potential continuous outcome; (D) The Qgcomp model is used to reveal a bar chart of the relative weight of each chemical and its influence on hormone levels. HCG\_E2: Estradiol on HCG trigger day; Basal\_P: basal progesterone; BKMR: Bayesian kernel machine regression.

estradiol, functions as both an estrogen receptor agonist and antagonist, thereby disrupting endocrine and reproductive system function<sup>[66]</sup>. Although BPS was introduced as a presumably safer industrial alternative to BPA, emerging evidence indicates comparable adverse effects on human reproductive health<sup>[62]</sup>. For example, *in vitro* studies demonstrate BPS-induced suppression of progesterone and estradiol secretion in sheep granulosa cells<sup>[67]</sup>. These findings suggested that both PAEs and bisphenols may negatively affect the secretion of estradiol and progesterone, thereby causing adverse effects on female reproductive health. However, current literature primarily examines EDC effects on hormonal profiles through urinary biomarkers, revealing positive correlations between serum estradiol and mPAEs/1-OH-PHE, an inverse relationship between BPA and peak estradiol, and negative associations between MeP and LH/FSH ratios<sup>[24–28]</sup>. These findings underscore the need for mechanistic studies to elucidate how chemical mixtures influence female endocrine physiology.

### Limitations of the study

First, due to the cross-sectional design of our study, the observed associations cannot definitively establish causality. Future longitudinal studies, particularly those including clinical populations such as women with PCOS or DOR, are warranted to further investigate the effects of chemical mixtures on hormone levels. Second, the EDCs or their metabolites (mPAEs, bisphenols, parabens, and OH-PAHs) analyzed in the present study have relatively short half-lives<sup>[4,10,68]</sup>. Additionally, follicular fluid samples were collected only on the day of oocyte retrieval during ART procedures, which limits their temporal scope. Consequently, these measurements better reflect short-term rather than long-term exposure. Third, while our study emphasizes the combined effects of chemical mixtures, the inherent complexity of these mixtures, including potential interactions among different EDCs, merits further investigation. More studies are needed to elucidate the underlying mechanisms by which EDCs influence hormonal regulation.

### CONCLUSIONS

Analysis of 144 follicular fluid samples from women undergoing ART treatment detected measurable concentrations of mPAEs, bisphenols, parabens, and OH-PAHs, with mPAEs exhibiting the highest median concentration followed by parabens, bisphenols, and OH-PAHs. The study revealed a potential association between PAE exposure and secondary infertility, along with a positive correlation between follicular fluid bisphenol levels and testosterone concentrations. BKMR analysis indicated that EDC mixtures, rather than individual compounds, primarily influenced estradiol levels on HCG trigger day and basal progesterone concentrations, demonstrating an inverse dose-response relationship where higher mixture concentrations corresponded to lower hormone levels. The Qgcomp model further identified BPS as having the most pronounced negative effect on estradiol levels on HCG trigger day, while BPP showed the strongest impact on basal progesterone. These findings collectively suggest that EDCs may adversely affect female reproductive health through hormonal disruption. Given the complex mechanistic pathways underlying EDC effects on endocrine function and reproductive outcomes, additional research is clearly warranted to fully elucidate these relationships.

## DECLARATIONS

### Authors' contributions

Methodology, data analysis and draft preparation: Dou, X.

Sampling, methodology and data analysis: Li, X.

Data analysis: Huang, S.; Long, C.; Chen, C.

Design, reviewing and editing: Chen, X.; Yu, Y.

### Availability of data and materials

Information related to this article can be found in [Supplementary Materials](#). Further data are available from the corresponding authors upon reasonable request.

### Financial support and sponsorship

The study was supported by open research funds from the Affiliated Qingyuan Hospital (Qingyuan People's Hospital), Guangzhou Medical University (202301-104), Talent Project of Center for Disease Prevention and Control of Guangdong Province (2024D344), Guangdong Province Outstanding Youth Medical Talent Training Program (SRSP2022022), and Guangdong-Hong Kong-Macao Joint Laboratory for Contaminants Exposure and Health (2020B1212030008).

### Conflicts of interest

Yu, Y. is the Guest Editor of the Special Issue of "The Impact of Bisphenol Exposure on Human Health". Yu, Y. is not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, and decision making. The other authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

The study was approved by the Ethics Committee of Shunde Hospital of Southern Medical University (20210301). All donors involved in this study signed an informed consent form before the sample collection.

### Consent for publication

Not applicable.

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