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## **Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome**

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### **Serum BPA in PCOS**

Bisphenol A, polycystic ovary syndrome (PCOS), endocrine disrupting chemicals (EDC), reproductive endocrinology, environmental exposure, toxicology

## ABSTRACT

The aim of this study was to determine serum bisphenol A (BPA) concentrations using high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) in women with polycystic ovary syndrome (PCOS) (n=106, age range 18-40 yrs) and to evaluate its potential impact on their hormonal and metabolic profile. The control group consisted of age- and BMI-matched 80 eumenorrheic women with no clinical or biochemical hyperandrogenism. Our results showed that women with PCOS had significantly higher serum BPA concentrations than healthy controls (geometric mean and [95% CI]: 0.202 ng/mL [0.150; 0.255] vs. 0.154 ng/mL [0.106; 0.201],  $P=0.035$ ), which correlated positively with serum total testosterone (TST) ( $R=0.285$ ,  $P=0.004$ ) and the free androgen index (FAI) ( $R=0.196$ ,  $P=0.049$ ). There were no significant correlations between serum BPA and BMI, waist circumference, serum glucose, insulin and lipids. These results point to the potential role of BPA in the pathogenesis of ovarian hyperandrogenism in women with PCOS.

## INTRODUCTION

Polycystic ovary syndrome (PCOS) in recent years has been attracting enormous attention, not only among reproductive medicine specialists, but also endocrinologists, diabetologists as well as dieticians and mental health providers (psychological therapists), which point to the complex nature of this syndrome. Although its prevalence is estimated to be up to 16 % among women before menopause [1, 2], the exact incidence is difficult to estimate due to its heterogeneity and lack of consensus on its diagnostic criteria [3, 4]. PCOS is characterized by anovulation, androgen excess and insulin resistance [5], which lead to the development of obesity, hypertension, and type 2 diabetes mellitus (T2DM) [6]; therefore, increasing the risk of cardiovascular disease [7-9]. Due to ovulatory dysfunction, PCOS is also the major cause of fertility problems [10, 11]. Not only is the diagnosis of PCOS still debatable, but also its pathogenesis; hence, together with the genetic and metabolic predispositions [12, 13], environmental factors, such as endocrine disrupting chemicals (EDCs), may contribute to its development [14, 15]. EDCs have recently gained a lot of attention and have been pointed out as the potential cause of ovarian dysfunction [16, 17] observed in women with PCOS [18, 19]. Bisphenol A (BPA) is one of the most common industrial compound with endocrine disrupting potential, which is used to bind or harden plastic [20] in packaging, food cans, bottles, plastic water pipes, thermal paper (receipts), cosmetics and healthcare equipment, as well as toys and articles for children [21]. Human exposure to BPA includes various routes such as oral – mainly by consumption of food products, inhalation of the dust, and transdermal contact e.g. with plastic products or thermal paper [19]. Due to its phenolic structure, BPA has been shown to interact with estrogenic receptors. Therefore, exposure to this EDC may impact fertility [22], lead to the development of estrogen dependent neoplasms (i.e. breast and uterine cancer) [23, 24] and also several metabolic disorders such as insulin resistance, obesity, and T2DM [22, 25]. Therefore, the aim of our study was to determine

serum concentrations of BPA using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in women with PCOS and to study its potential impact on their hormonal and metabolic profile.

## **1. SUBJECTS AND METHODS**

### **1.1. *Subjects***

This cross-sectional study was performed between [the] 16<sup>th</sup> of January 2016 the and 21<sup>st</sup> of December 2017 and was approved by the Ethics Committee of the Medical University of Gdańsk (permission number NKBBN/198/2012). In total, 304 female participants (age range: 18-40 yrs) were enrolled into the study. Women were recruited at the endocrinology outpatient practice by a specialist in internal medicine and endocrinology (D.R.), where they were referred by other clinical practitioners mostly due to clinical signs of hyperandrogenism (hirsutism, acne, androgenic alopecia) and/or menstrual disturbances and/or fertility problems. They were all informed about the purpose of the study and signed a written consent to participate. Women, who were taking oral contraceptives, anti-androgens (e.g. spironolactone) or glucocorticosteroids, were excluded from the study. The control group consisted of healthy women with regular menses and no biochemical or clinical signs of hyperandrogenism, which were referred to the same practice in order to exclude some of the common endocrine disorders (e.g. hypothyroidism, hyperprolactinemia).

### **1.2. *Gynecological examination and ultrasonography***

All the participants were also examined by a specialist in gynecology and endocrinology (A.K.). Gynecological examination, together with a transvaginal or transabdominal ultrasonography, was performed using the Accuvix V20 Ultrasound Machine (Medison, Seoul, South Korea), 4-9 MHz and 4-8 MHz, respectively. The ovarian volume was



calculated and the total number of antral follicles was counted. The presence of an ovulatory follicle (>10 mm) was also noted.

### **1.3. Study protocol**

An interview-based medical form was used to acquire the information regarding gynecological and obstetric history (*menarche*, menstrual regularity, time of last period, number of pregnancies and oral contraceptive use). Then a physical examination was performed where the height, body weight, waist circumference and blood pressure were measured. Also the presence of hirsutism, acne or androgenic alopecia was recorded.

### **1.4. Sample collection and preparation**

Venous blood was obtained during the follicular phase (days 6-10) of a spontaneous or a progestin induced menstrual cycle (oral dydrogesterone: 10 mg twice daily for 10 days). All the hormonal and biochemical analyses were performed at the clinical diagnostic laboratory (ALAB-Bruss Laboratories, Gdynia, Poland), which possesses current ISO certificates and has an accreditation in the field of medical laboratory diagnostics. The blood was collected into three polypropylene tubes: one on the clot, one containing sodium citrate for the analysis of glucose concentrations and another containing EDTA for the evaluation of total blood count. In order to analyze serum BPA, blood was additionally collected into a glass tube with no additives and was centrifuged within 30-90 minutes at the speed of 2500 rpm for 15 min. After the centrifugation, serum samples for the BPA analyses were collected using a disposable transfer (Pasteur) pipette made of BPA-free low-density polyethylene (LDPE) with precautions intended to minimize the risk of sample contamination with BPA and were then stored at -70°C for further analysis.

### 1.5. *Biochemical and hormonal analyses*

Total blood count was evaluated automatically using XE-2100 D analyzer (Sysmex, Kobe, Japan). Serum C-reactive protein (CRP), glucose, triglycerides (TG), HDL-Cholesterol (HDL-C) and total cholesterol (TCh), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin (TSH), prolactin (PRL), 17 $\beta$ -estradiol (E2), total testosterone (TST), dehydroepiandrosterone sulphate (DHEA-S), progesterone, 17OH-progesterone (17OH-P), and insulin concentrations were measured in an electrochemiluminescence immunoassay (Roche Diagnostics – Cobas E601, Mannheim, Germany).

The intra-assay coefficients of variation (CVs) for CRP, glucose and TG were: 3.5 %, 1.5 % and 2 %, whereas the limits of detection (LODs) were 0.3 mg/L, 0.11 mmol/L and 9 mg/dL, respectively. The intra-assay CVs for HDL-C and TCh were 2 % and LODs were 4 mg/dL and 3 mg/dL, respectively. The intra-assay CVs for gonadotropins (FSH and LH), TSH and PRL were 2 % and LODs for these parameters were 0.1 mIU/mL, 0.005  $\mu$ IU/mL, 1.0  $\mu$ IU/mL, respectively. The intra-assay CVs for E2, TST and DHEA-S were 3.5 %, 3.0 % and 3.5 % and LODs were 18.5 pmol/L, 0.087 nmol/L and 0.1  $\mu$ g/dL, respectively. The intra-assay CVs for progesterone, 17OH-P and insulin were 2.1 %, 4.6 % and 3.5 %, whereas LODs were 0.159 nmol/L, 0.11ng/mL and 0.2 mIU/L, respectively. Serum sex hormone binding globulin (SHBG) concentrations were measured in an immunoassay (Access 2 Immunoassay System, Beckman Coulter, Brea, United States) with the intra-assay CV of 4.15 % and LOD of 0.35 nmol/L. Serum androstenedione concentrations were evaluated using the chemiluminescent immunoassay (CLIA) method on a Liaison analyzer (Diasorin, Saluggia, Italy), with the intra-assay CV of 3.6 % and LOD 0.3 mg/mL. The inter-assay CVs for: CRP, glucose, TG, HDL-C, TCh, FSH, LH, TSH, PRL, E2, TST, DHEA-S, progesterone, 17OH-P, insulin, SHBG and androstenedione were 2.0 %, 2.5 %, 1.4 %, 1.8 %, 1.7 %, 1.9 %, 1.9 %, 1.0 %, 1.8 %, 1.0 %, 2.5 %, 3.2 %, 2.1 %, 4.6 %, 1.8 %, 1.2 %, 3.6 %, respectively.



Serum LDL-Cholesterol (LDL-C) concentrations were calculated using the Friedewald equation taking into the account its limitations (e.g. serum TG > 4.5 mmol/L). Free androgen index (FAI) was calculated according to the formula:  $FAI = TST \times 100 / SHGB$ . Insulin resistance was assessed using the Homeostasis Model of Assessment-Insulin Resistance (HOMA-IR) according to the formula:  $\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose } (\text{mmol/L}) / 22.5$ .

### **1.6.***Diagnosis of PCOS*

The diagnosis of PCOS was made according to the criteria of the Androgen Excess and PCOS (AE&PCOS) Society where apart from the ovulatory dysfunction (OvD) or the polycystic ovarian morphology (PCOM) the presence of clinical or biochemical hyperandrogenism (HA) is mandatory [26].

Biochemical HA was defined as having TST, DHEA-S and/or androstenedione and/or FAI above the 95th percentile of the values recorded among the women from the control group (n=80). The threshold levels for TST, DHEA-S, androstenedione, and FAI were 1.54 nmol/L, 8.75  $\mu\text{mol/L}$ , 13.65 nmol/L, and 3.32, respectively.

OvD was defined as having menstrual cycles lasting for less than 25 or more than 35 days or anovulation. Therefore, in women who had apparently regular menses, serum progesterone concentrations were measured 7 days before the end of their menstrual cycle. The threshold value for the presence of ovulation was > 5 ng/mL. PCOM was defined as an antral follicle count (AFC) of  $\geq 12$  in 2-9 mm diameter and/or ovarian volume of  $\geq 10$  ml at least in one ovary [27]. When an ovulatory follicle was present, the volume of that ovary was not taken into consideration. In order to exclude other causes of ovulatory dysfunction or in the case of severe signs of hyperandrogenism, serum concentrations of TSH and PRL as well as 17OH-P, were measured respectively. Women with overt thyroid dysfunction (n=36),



hyperprolactinemia (n=14) or a suspicion of non-classical congenital adrenal hyperplasia (serum 17OHP > 2 ng/dL – n=3) were excluded from further analysis. Women who were morbidly obese (body mass index (BMI)>40 – n=5), underweight (BMI<18.5 – n=2), had laboratory signs of an infection (serum CRP > 15 mg/L – n=2) or were diagnosed with T2DM (n=2) were also excluded from the study. Some women were also excluded due to the lack of some laboratory results (n=36), or the lack of TV USG (n=18). After these exclusions, the results from 186 subjects were taken into consideration. Among them 106 women were diagnosed with PCOS and 80 were considered healthy (the control group) [26]. The AE&PCOS Society diagnostic criteria yield three separate PCOS phenotypes (A, B and C). Phenotype A includes all the three features (HA, OvD and PCOM) whereas phenotype B and C only two (HA and OvD or HA and PCOM, respectively). The prevalence of the A, B and the C phenotype among women with PCOS in our cohort was 38 %, 33 % and 29 %, respectively.

### 1.7. *Serum BPA analyses*

The evaluation of serum BPA concentrations were performed using high performance liquid chromatography method with tandem mass spectrometry (HPLC-MS/MS) on the Shimadzu triple quadrupole LC-MS/MS system (LCMS-8060; Shimadzu, Japan) equipped with an electrospray ionization source (ESI) working in the negative multiple reaction mode (MRM). Detailed description of the whole method has been already published by our group elsewhere [28]. This method was chosen as it provides extra precision and higher sensitivity in comparison to MS [29]. Additionally, HPLC-MS/MS is believed to be a selective and sensitive method to determine phenols concentration in serum samples [30]. The limit of quantification (LOQ) and LOD of BPA in sera was 0.028 ng/mL and 0.0093 ng/mL, respectively.





### 1.8. *Statistical analyses*

All the statistical analyses were performed using the GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, La Jolla, California USA, [www.graphpad.com](http://www.graphpad.com)). The D'Agostino–Pearson test was used to determine the distribution of the measured variables. Non-normally distributed variables were log-transformed before the analyses and are presented as geometric means and their 95 % confidence interval (CI). Normally distributed variables are presented as arithmetic means  $\pm$  SD. Due to the possibility of serum sample contamination with BPA during the procedures of sample preparation and analysis, BPA results were also analysed for outliers using the ROUT method with the Q value set at 1 %. Differences between the groups were compared using an unpaired Student's t-test. Serum BPA concentrations among the three PCOS phenotypes (A, B and C) were compared using the one-way analysis of variance (ANOVA). Correlation analyses between the selected variables and serum BPA concentrations in women with PCOS were performed using Pearson's correlation calculations where in the case of large samples ( $n > 100$ ) the assumption that both X and Y variables are sampled from populations that follow a Gaussian distribution is not too important. P value of less than 0.05 was considered statistically significant.

## 2. RESULTS

Clinical, biochemical, and metabolic characteristics of women with PCOS and healthy controls are presented in Table 1. There were no significant differences in age, BMI, waist circumference, serum glucose and insulin concentrations or HOMA-IR among the studied groups. Women with PCOS had also significantly higher serum LH concentrations ( $P=0.007$ ) and greater LH/FSH ratio ( $P=0.044$ ). Serum concentrations of E2 were lower, and TST, androstenedione, and DHEA-S along with FAI were significantly higher in women with



PCOS ( $P<0.001$ ). Compared with the control subjects, women with PCOS had significantly higher serum LDL-C concentrations ( $P=0.008$ ). There were no differences in serum TCh, HDL-C nor TG concentrations between the studied groups.

Table 1. Clinical, hormonal, and metabolic characteristics of women with PCOS (n=106) and healthy controls (n=80)

Variable	PCOS (n=106)	Control group (n=80)	P-value
Age (yrs)	26.9 ± 5.2	28.2 ± 5.7	0.123
BMI <sup>1</sup>	24.8 (23.9; 25.7)	24.1 (23.1; 25.1)	0.273
Waist circumference (cm)	88.2 ± 11.9	85.6 ± 12.2	0.270
Fasting plasma glucose (mmol/L) <sup>1</sup>	4.83 (4.76; 4.9)	4.79 (4.71; 4.87)	0.483
Serum Insulin (mIU/L) <sup>1</sup>	8.6 (6.8; 10.4)	7.8 (7.0; 8.6)	0.097
HOMA-IR <sup>1</sup>	1.84 (1.43; 2.25)	1.67 (1.47; 1.86)	0.094
Serum LH (IU/L) <sup>1</sup>	8.1 (7.3; 9.0)	6.6 (5.7; 7.4)	0.007
Serum FSH (IU/L) <sup>1</sup>	6.7 (6.2; 7.2)	6.4 (5.9; 6.8)	0.284
LH/FSH <sup>1</sup>	1.22 (1.08; 1.35)	1.0 (0.90; 1.18)	0.044
Serum E2 (pmol/L) <sup>1</sup>	200.6 (174.4; 226.7)	250.7 (184.4; 317.1)	0.005
Serum PRL (mIU/L)	18.5 ± 6.8	18.0 ± 7.2	0.623
Serum DHEA-S (μmol/L) <sup>1</sup>	297.1 (275.1; 319.1)	194.3 (178.7; 209.8)	<0.001
Serum androstenedione (nmol/L) <sup>1</sup>	11.2 (10.4; 12.1)	7.1 (6.5; 7.7)	<0.001
Serum total TST (nmol/L)	1.79 ± 0.5	1.08 ± 0.3	<0.001

Serum SHBG (nmol/L) <sup>1</sup>	54.0 (48.11; 59.85)	68.9 (62.87; 74.90)	0.002
FAI	3.97 ± 2.9	1.68 ± 0.8	<0.001
Serum TCh (mmol/L)	5.04 ± 0.97	4.82 ± 0.8	0.089
Serum HDL-C (mmol/L) <sup>1</sup>	1.67 (1.59; 1.75)	1.76 (1.66; 1.85)	0.170
Serum LDL-C (mmol/L) <sup>1</sup>	2.80 (2.63; 2.96)	2.47 (2.30; 2.63)	0.008
Serum TG (mmol/L) <sup>1</sup>	0.87 (0.78; 0.95)	0.83 (0.72; 0.95)	0.642
<p>Normally distributed variables are presented as arithmetic means ± SD</p> <p><sup>1</sup>Non-normally distributed variables are presented as geometric means (95% CI)</p> <p>BMI – body mass index, LH – luteinizing hormone, FSH – follicle-stimulating hormone, E2 – 17β-estradiol, PRL – prolactin, DHEA-s – dehydroepiandrosterone sulphate, TST – testosterone, SHBG – sex hormone binding globulin, FAI – free androgen index, TCh – total cholesterol, HDL-C – HDL-cholesterol, LDL-C – LDL-cholesterol, TG – triglycerides, HOMA-IR – Homeostasis Model of Assessment-Insulin Resistance</p>			

BPA was detectable in 99 % and 92 % of serum samples from the women with PCOS and controls, respectively. Outliers were excluded from further analyses (n=4 in PCOS group and n=5 in the control group). Women with PCOS had significantly higher serum BPA concentrations than the control subjects (geometric mean and [95% CI] – 0.202 ng/mL [0.150; 0.255] vs. 0.154 ng/mL [0.106; 0.201],  $P=0.035$ ) (Fig. 1). Moreover, in women with PCOS serum BPA concentrations correlated positively with FAI ( $R=0.196$ ,  $P=0.049$ ) and serum total TST concentrations ( $R=0.285$ ,  $P=0.004$ ) (Fig. 2). There were no significant differences in serum BPA concentrations between the A, B and C phenotypes within the PCOS group (geometric means and [95 % CI]: 0.136 ng/ml [0.101; 0.182] vs. 0.224 ng/ml [0.140; 0.358] vs. 0.185 ng/ml [0.116; 0.295] respectively,  $P=0.157$ ).

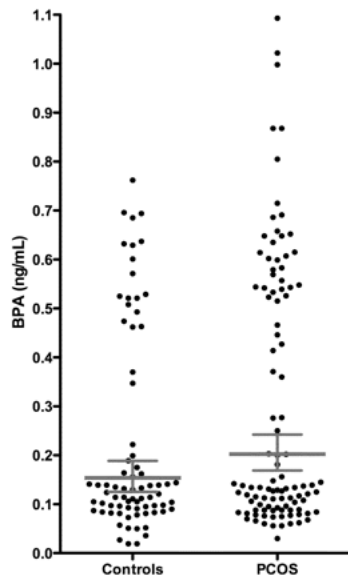


Fig 1. Serum BPA concentrations in studied groups. The horizontal lines represent geometric mean with whiskers as 95% confidence interval.

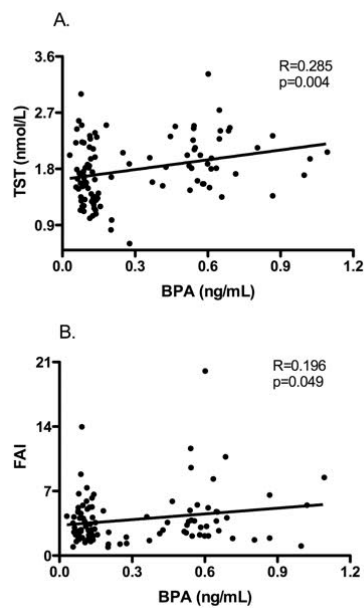


Fig 2. Correlations of BPA concentrations with total TST and FAI in women with PCOS.

Serum BPA concentrations in women with PCOS did not correlate with BMI ( $R=-0.072$ ,  $P=0.473$ ), waist circumference ( $R=0.039$ ,  $P=0.716$ ), serum insulin ( $R=0.033$ ,  $P=0.742$ ), glucose ( $R=0.047$ ,  $P=0.642$ ) or HOMA-IR ( $R=0.038$ ,  $P=0.710$ ). Furthermore, no correlations

were found between serum BPA and serum lipids in this group (TCh:  $R=-0.061$ ,  $P=0.545$ ; HDL-C:  $R=-0.019$ ,  $P=0.853$ ; LDL-C:  $R=-0.071$ ,  $P=0.480$ ; TG:  $R=0.065$ ,  $P=0.515$ ).

### 3. DISCUSSION

The aim of the present study was to evaluate serum BPA concentrations among women with PCOS and to determine, if they may have an impact on the hormonal and metabolic parameters in these patients.

Women with PCOS had significantly higher indices of hyperandrogenemia (TST, DHEA-S, androstendione and FAI), which well characterizes the hormonal profile of these patients [31]. Although women with PCOS are usually characterized by atherogenic dyslipidemia (low HDL-C and high TG) [32, 33] there were no significant differences in serum TCh, HDL-C or TG concentrations between our studied groups. Nevertheless, women with PCOS had significantly higher serum LDL-C levels than healthy controls. This is consistent with the results of a study conducted by Couto *et al.* [34] where after adjusting for age and BMI, also serum LDL-C concentrations remained increased in women with PCOS. It's well known that many factors, including body composition, physical activity, intake of dietary macronutrients or the presence of the metabolic syndrome affect the cholesterol efflux and lipid metabolism [35, 36]. It is also believed that higher LDL-C concentrations in women with PCOS may be linked to [the] androgen excess [32]. Nevertheless, in our study serum LDL-C concentrations did not correlate with serum androgen levels (data not shown).

Serum BPA concentrations in women with PCOS were significantly higher than in age- and BMI- matched healthy controls. Analogous results were found in studies conducted by other researchers [37-39], even though serum BPA concentrations were measured using the immunoenzymatic assay and not the HPLC-MS/MS method. In the study of Vahedi *et al.* [40], serum BPA concentrations were also higher compared to the control subjects although

they were determined using the HPLC method only, without the concomitant use of MS. To our best knowledge, our study is the first one where this very reliable and sensitive method has been used for BPA quantification in sera of women with PCOS. Similarly to other studies, BPA was detectable in almost all of the analyzed serum samples (99 % in the PCOS and 92% in the control group), which point to the unavoidable exposure to this EDC in our daily life [41].

Moreover, likewise in other studies [37, 42] among women with PCOS, serum BPA concentrations correlated positively with both testosterone concentrations as well as FAI. The possible mechanism involves both the direct stimulation of the ovarian theca cells to the exaggerated androgen synthesis [43, 44] as well as the potential of displacing sex hormones from SHBG binding sites. On the other hand, BPA has been shown to possess estrogenic effects [45] and as such could stimulate the production of SHBG in the liver. However, studies from women who use estrogen replacement therapy show that only oral administration increases the hepatic production of SHBG, whereas transdermal regimen do not have such an effect [46]. Therefore, it might be possible that the exposure to BPA does not occur mainly through food consumption but through transdermal contact with materials containing that compound such as plastic furniture and decorations, receipts, and packages [21]. Nevertheless, it has been already shown *in silico* [47] and in a study using a competitive binding assay that BPA has a binding affinity to SHBG [48]. Hence, we might speculate that BPA by binding with SHBG may displace TST, and thus increase FAI. Nevertheless, in our study we did not find any correlation between serum BPA concentrations and the SHBG levels.

Our study design has some limitations. First of all, serum BPA concentrations may not necessarily reflect the actual exposure to this EDC. It has been shown that when present in blood, BPA is rapidly metabolized in the liver and has a half-life of several hours, and some

researchers suggest that urine might be a better material to determine the actual exposure risk [49]. Nevertheless, it is alarming that BPA was detectable in almost all of the serum samples from our studied subjects.

Another limitation of our study is that in our cohort serum testosterone concentrations were measured using an automated electrochemiluminescence immunoassay. Although, these measurements were performed in the clinical diagnostic laboratory, evaluating serum testosterone concentrations in women is inherently problematic [50]. The novel LC-MS/MS methods, which have been shown to be superior over the immuno-metric platforms, would probably be more appropriate [51]. Regrettably, we were not able to validate our serum testosterone measurements against this method. Nevertheless, the diagnosis of PCOS in our cohort was made according to the AE&PCOS Society criteria [26] where not only laboratory indices of hyperandrogenism were present but also clinical (i.e. hirsutism). Therefore, it is very unlikely that we have misdiagnosed these subjects.

In summary, the results of our study show that not only serum BPA concentrations in women with PCOS are significantly higher than in age- and BMI-matched controls but they also correlate positively with serum total TST and FAI which points to the potential role of this EDC in the pathogenesis of [the] ovarian hyperandrogenism.

## **CONFLICT OF INTERESTS**

The authors declare no conflict of interests related to the content of this manuscript

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