A novel biomarker for anti-androgenic activity in placenta reveals risks of urogenital malformations

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Abstract

It has been hypothesized that the rise in male reproductive disorders over recent decades may at least be partially attributable to environmental factors, including chemical exposures, but observed associations with single chemicals were rather weak. The aim of this case–control study was to explore the relationship between exposure to mixtures of (anti-)androgenic chemicals during pregnancy and the risk of cryptorchidism and/or hypospadias in offspring, using the total effective xenobiotic burden of anti-androgens (TEXB-AA) as a biomarker. A subsample of 29 cases (16 of cryptorchidism, 12 of hypospadias, and one of both disorders) and 60 healthy controls was nested in a cohort of male newborns recruited between October 2000 and July 2002. The (anti-)androgenic activity of placenta samples collected at delivery was assessed using TEXB-AA biomarker, combined with a bioassay-directed fractionation protocol that separated endogenous hormones from most (anti-)androgenic chemicals by normal-phase HPLC. The bioassay measures the androgen-induced luciferase activity and the inhibition of this pathway by (anti-)androgens. First, we collected 27 HPLC fractions in each placenta extract, which were all tested in the bioassay. The multivariable statistical analyses indicated a statistically significant positive dose–response association between the potent anti-androgenic activity of the HPLC fraction collected during minutes 1–2 (F2) and the risk of malformations (odds ratio: 2.33, 95% CI: 1.04–5.23). This study represents a novel approach for the estimation of combined effects of the total anti-androgenic load and the associations suggest an effect of environmental pollutants on the development of fetal reproductive tract.

Free Spanish abstract: A Spanish translation of this abstract is freely available at http://www.reproduction-online.org/content/149/6/605/suppl/DC1.


Introduction

Over the past few decades, most western countries have experienced an increase in male reproductive disorders, including cryptorchidism (absence of one or both testes from the scrotum), hypospadias (abnormally placed urinary meatus), poor semen quality, and testicular cancer (WHO/UNEP 2012). Although the etiology of these disorders is not known in most cases (Bay et al. 2011), it has been hypothesized that these unfavorable disease trends might be due not only to improvements in clinical detection but also to a combination of factors such as alcohol consumption, low birth weight, premature birth, and diets lacking in protein. However, these well-established risk factors alone cannot explain the current disease trends. Skakkebaek et al. (2001) hypothesized that cryptorchidisms and hypospadias are part of a syndrome termed testicular dysgenesis syndrome (TDS) that arises from insufficient androgen action in fetal life and also comprises poor semen quality and testicular germ cell cancers. The TDS hypothesis proposes that exposures to anti-androgenic chemicals are an etiological factor (Main et al. 2010).

Fetal androgens are key factors in the proper differentiation of the male urogenital tract, which takes place before the inguino-scrotal phase, during a so-called ‘common early programing window’ (Amann & Veeramachaneni 2007). The initial phase of testis descent is mediated by the Leydig cell hormone insulin-like peptide 3 (INSL3), while testosterone plays a role in controlling gubernacular growth and reorganization in the later phase (Bay et al. 2011). Severely or moderately
impaired androgen action during this period can result, respectively, in disorders of sexual differentiation (Hughes & Deeb 2006, Welsh et al. 2008), or a set of milder TDS-related disorders, such as cryptorchidism and hypospadias, with a reported prevalence of up to 9% of male newborns (Boisen et al. 2004, 2005). Cryptorchidism and hypospadias have also been implicated as potential risk factors for testicular germ cell tumors (Trabert et al. 2013).

Previous studies have identified a large group of environmental pollutants with endocrine-disrupting potential (endocrine-disrupting chemicals, EDCs) that are able to interact with the human androgen receptor (hAR) and therefore exert both androgenic and anti-androgenic effects (Wilson et al. 2008, Orton et al. 2011, 2012, Jimenez-Diaz et al. 2013). These chemicals include organochlorine pesticides, alkylphenols, parabens, brominated flame retardants, and polychlorobiphenyls, to which the general population is exposed on a daily basis (Arrebola et al. 2009, 2010, Orton et al. 2012). Interestingly, it has been reported that multicomponent mixtures of very low doses of these chemicals can produce biological effects at concentrations that would produce negligible effects on their own (Orton et al. 2014).

However, only few epidemiological studies have thus far demonstrated associations between single anti-androgenic chemicals and TDS disorders. This complicates an assessment of the TDS hypothesis, not least because only a limited range of chemicals have been investigated. Studies of paternal and maternal pesticide exposures in agricultural occupational settings have reported associations with cryptorchidisms and hypospadias, but due to their design, could not pinpoint specific chemicals (Pierik et al. 2004, Carbone et al. 2006, Gaspari et al. 2012). There is evidence for associations of diethylstilbestrol (DES; Palmer et al. 2009) and polybrominated diphenyl ethers (Carmichael et al. 2010) with the risk of developing cryptorchidisms. It would appear that it is difficult to explain current disease trends solely on the basis of exposures to known anti-androgenic chemicals (Kortenkamp et al. 2014).

However, there is epidemiological evidence that human exposure to low doses of EDCs might be associated with urogenital tract malformations (Fernandez et al. 2007, Gaspari et al. 2011, 2012). In this regard, Main et al. (2007) found a statistically significant association between the risk of cryptorchidism and a sum parameter of polybrominated diphenyl ether levels in maternal milk that was not evident when the analysis considered individual congeners alone. Damgaard et al. (2006) found significant relationships between the sum of eight prevalent pesticides and cryptorchidism. Similarly, Fernandez et al. (2007) observed an increased risk for male urogenital malformations due to the combined effect of environmental estrogens in placenta.

This study, which was conducted as part of the EU-funded CONTAMED project (grant agreement no. 212502), was motivated by the current difficulties in convincingly ascribing exposures to combinations of anti-androgenic chemicals to male reproductive disorders. To make advances in studying the relationship between exposure to mixtures of anti-androgenic EDCs during pregnancy and the risk of cryptorchidism and/or hypospadias in offspring, it was necessary to develop a biomarker representative of a biological effect measure of cumulative anti-androgen exposures in fetal life. Instead of seeking associations with measured levels of individual known anti-androgenic chemicals, we conducted a nested case–control study in Southern Spain in which we assessed the combined (anti-)androgenic activity of placenta extracts using the total effective xenobiotic biomarker (TEXB-AA) as a biomarker, based on a steroid hormone receptor cell bioassay.

Materials and methods

Study population

Subjects were sampled from a previous case–control study nested in a human birth cohort (Fernandez et al. 2007). Briefly, from October 2000 to July 2002, male newborns registered at the San Cecilio University Hospital (one of the two reference public hospitals serving Granada province in Southern Spain) were recruited at birth, excluding delivering mothers with serious chronic diseases (e.g., diabetes, hypertension, or thyroid disease), those who developed any pregnancy complication that could affect fetal growth or development, and nonresidents in the hospital referral area. All boys with cryptorchidism and/or hypospadias born in the study period were included.

All boys in the cohort were examined at birth (within 2 days), and those diagnosed with cryptorchidism and/or hypospadias were reexamined at 1 month of age. Only boys with these congenital malformations at reexamination were considered cases. The examination technique and definition of cryptorchidism and hypospadias followed the recommendations of a Danish–Finnish study (Boisen et al. 2004; developed by: SCORER (1964)). All examinations were carried out with the child in supine position. Testicular position was recorded after manipulation of the testis to the most distal position along the pathway of normal descent using firm traction. For each case, two matching controls were selected for gestational age (±1 week), date of birth (±7 days), and parity (primiparous/multiparous).

Although the original nested case–control study consisted of 48 cases and 114 paired controls, adequate biological sample was available for 29 cases and 60 controls and selected for the final study. Comparison of the cases and controls in the final sample with those in the original cohort found no statistically significant differences in maternal age, BMI, smoking habit, weeks of gestation, water consumption, residence, occupation, or newborn weight at delivery. However, there was a larger proportion of mothers reporting historical (pre-pregnancy) use of oral contraceptives in the selected vs non-selected cases (21% vs 53%, P=0.034), but not in the selected vs non-selected controls (37% vs 42%, P=0.686).
Information on potential confounding variables related to parents, pregnancy, and delivery was gathered from a structured face-to-face interview with the mother within the first 48 h after delivery. The interviewer was blinded to the case or control status of the child.

This study was approved by the Institutional Ethical Committee of the San Cecilio University Hospital, and all participating mothers signed informed consent.

**Sampling and extraction protocol**

Samples of placenta without decidua basalis and chorionic plate were collected at the time of delivery and sent to the Laboratory of Medical Investigations for analysis. They were examined and weighed, and a triangular portion, including maternal and fetal sides and central and peripheral parts of the placenta, was cut and mechanically homogenized. Finally, the samples were immediately frozen at $-70^\circ$C and stored until analyses.

The (anti-)androgenic activity of the placentas was assessed by developing a bioassay-directed fractionation protocol, based on a modification of a previously published methodology (Indiveri et al. 2014). Samples of 1.0 g of homogenized placenta were extracted twice with 4 ml of acetonitrile using a Sonoplus HD 2070 Ultrasonic probe (Bandelin, Berlin, Germany) at 10 W for 45 s. Each extract was vortexed for 45 s and centrifuged (1270 g, 5 min), and the two supernatants were then combined, concentrated to dryness under vacuum, and re-dissolved in 500 ml of acetonitrile. To remove the proteins from the solvent extract, it was sequentially filtered using 0.2 mm and 10 kDa pore size centrifuge filters (WhatmanVectaSpin Micro with Anopore filter membrane, and Millipore, Billerica, MA, USA, Amicon Ultra with Ultracel-10 membrane) at 15682 g for 7 and 15 min respectively. The solvent was removed under vacuum, and the residue was resolubilized in 200 μl of hexane with 20% hexane/methanol/isopropanol (50/25/25% v/v).

**HPLC fractionation**

In order to separate endogenous hormones from (anti-)androgenic EDCs, aliquots of placenta extracts were fractionated using a NP-HPLC protocol with an Agilent 1260 equipped with an analytical silica column (Waters Spherisorb, 3 μm particle size, 4.6 X 100 mm) and a guard column (5 μm, 4.6 X 10 mm). Three mobile phases were used: solvent A, hexane (0.2% v/v of acetic acid); solvent B, hexane/methanol/isopropanol, 50/25/25 (0.2% v/v of acetic acid); and solvent C, isopropanol (0.2% v/v of acetic acid). The separation was performed at 32°C with a flow rate of 1.5 ml/min, using a gradient of 0.0–4.0 min (98:2, A:B), 4.0–5.0 min (94:6, A:B), 5.0–17.0 min (94:6, A:B), 17.0–35.0 min (50:50, B:C), and 35.0–60 min (98:2, A:B).

Blank workup samples were fractionated to check for contamination. In order to identify the elution times of several families of both man-made and endogenous hormones, we injected spiked blank samples into the HPLC system. These families included organochlorine pesticides (e.g., p,p'-dichlorodiphenyldichloroethylene, hexachlorobenzene, and mirex), polychlorobiphenyls (congeners K138, K153, and K180), phthalates (bis-ethyl–hexyl–phthalate and dibutyl phthalate), fatty acids (docosahexanoic acid, docosatetranoic acid, and linoleic acid), parabens (ethylparaben and methylparaben), endogenous hormones (estrone, progesterone, estradiol (E2),

![Figure 1](image-url)
testosterone, and ethinyl E₂), and bisphenols (bisphenol A). The retention time of each chemical in the HPLC chromatogram is shown in Fig. 1.

**TEXB-AA biomarker**

PALM cells were used to characterize the response of the interaction of HPLC fractions with the hAR. (Ant-)agonistic activity was tested in the presence of increasing dilutions of HPLC fractions. After HPLC fractionation, all 1-min fractions and pools of fractions were serially diluted with ethanol, and 10-µl volumes were transferred to 96-well white opaque tissue culture plates. The ethanol was evaporated at room temperature followed by the addition of PALM cells at a density of 1×10⁵ cells/well in 200 ml test culture medium (containing 5% stripped serum) with or without the agonist R1881 (0.2 nM), and the plates were incubated for 2 days at 37 °C. On each plate, alongside the test samples, serial dilutions of antagonist procymidone (with 0.2 nM R1881) and R1881 were included as positive controls and test culture medium alone or with solvent served as a negative control. At the end of incubation, the medium containing test compounds was removed and replaced by test culture medium containing 0.3 mM luciferin. Luminescence was measured in intact living cells for 2 s. Luminescence was normalized to R1881 co-exposure (100%) and solvent-only (ethanol) controls (0%). Data were expressed as a percentage of maximal luciferase activity. Finally, the percentage of luciferase activity of each fraction was referred to the maximal luciferase activity obtained with the R1881 or procymidone and transformed into R1881 equivalents or procymidone equivalent units by reading from dose–response curves of R1881 or procymidone standard dilution series included in each plate.

In order to identify the most informative HPLC fractions for (anti-)androgenic activity, we first performed an extensive profiling of ten placenta control samples, which were individually extracted and injected into the HPLC system following the protocol described earlier in this study. A total of 27 1-min fractions were recovered from each placenta, the solvent was removed, and the extract was re-dissolved in ethanol (100 µl). Aliquots were tested to estimate their androgenic and anti-androgenic activities in the PALM reporter gene assay.

**Figure 1** depicts the results of the extensive profiling of fractionated placenta extracts from ten control subjects, revealing several fractions with anti-androgenic activity. The most pronounced hAR antagonistic activity was found in the non-polar fractions (2–4 min), which eluted first, with comparatively little variation among placenta samples. This was followed by an array of fractions (5–11 min) with intermediate hAR antagonistic activity and some agonist activities, which widely varied among samples. Finally,

| Table 1 Characteristics of the study population (I). |
|----------------------------------|------------------|
|                                | **Total** | **Cases (n=29)** | **Controls (n=60)** |  
| Residence                       | n    | %    | n    | %    | n    | %    | P  
| Urban                           | 21   | 23.6 | 6    | 20.7 | 15   | 25.0 | 0.792  
| Rural                           | 68   | 76.4 | 23   | 79.3 | 45   | 75.0 | 0.054  
| Type of delivery                |       |      |      |      |      |      |  
| Spontaneous                     | 57   | 64.0 | 18   | 62.1 | 39   | 65.0 |  
| Cesarean                        | 20   | 22.5 | 10   | 34.5 | 10   | 16.7 |  
| Instrumental                    | 12   | 13.5 | 1    | 3.4  | 11   | 18.3 | 0.220  
| Past consumption of hormonal contraceptive = yes | 28   | 31.8 | 6    | 21.4 | 22   | 36.7 |  
| Received treatment for infertility = yes | 4    | 4.5  | 2    | 7.1  | 2    | 3.3  | 0.589  
| Received pharmaceutical treatment during pregnancy = yes | 36   | 40.4 | 15   | 53.6 | 21   | 35.0 | 0.110  
| Marital status = single         | 87   | 92.0 | 27   | 96.4 | 60   | 100.0 |  
| Occupation = Homemaker          | 24   | 27.3 | 7    | 25.0 | 17   | 28.3 | 0.589  
| Cleaner                         | 4    | 4.5  | 1    | 3.6  | 3    | 5.0  |  
| Agriculture/farming             | 6    | 6.8  | 4    | 14.3 | 2    | 3.3  |  
| Administrative                  | 25   | 28.4 | 8    | 28.6 | 17   | 28.3 |  
| Service sector                  | 12   | 13.6 | 3    | 10.7 | 9    | 15.0 |  
| Health worker                   | 2    | 2.3  | 1    | 3.6  | 1    | 1.7  |  
| Freelance                       | 15   | 17.0 | 4    | 14.3 | 11   | 18.3 |  
| Education = No studies          | 4    | 4.5  | 1    | 3.6  | 3    | 5.0  | 0.999  
| Primary                         | 42   | 47.7 | 14   | 50.0 | 28   | 46.7 |  
| Secondary                       | 20   | 26.1 | 7    | 25.0 | 16   | 26.7 |  
| University                      | 19   | 21.6 | 6    | 21.4 | 13   | 21.7 |  
| Water consumption = no consumption | 2    | 2.3  | 0    | 0.0  | 2    | 3.3  | 0.982  
| 1–3 glasses/week                | 15   | 17.0 | 6    | 21.4 | 9    | 15.0 |  
| >3 glasses/week                 | 71   | 80.7 | 22   | 78.6 | 49   | 81.7 |  

*Fisher’s exact test. bMother’s characteristics.
intermediate hAR antagonistic activity was identified in the more polar fractions (21–24 min). The selection of the fractions of interest (F2 (minutes 1–2), F3 (minutes 2–3), F4 (minutes 3–4), F5–11 (minutes 5–11), and F21–24 (minutes 21–24)) was based on the clear variability shown in the bioassay and on the absence of toxicity.

**Statistical analysis**

As ANOVA assumptions were not always fulfilled, differences between continuous variables were assessed by Mann–Whitney's U test, and categorical data by Fisher's exact test. The significance level was set at α = 5% (two-sided).

Associations between the estimated TEXB-AA levels and the occurrence of cryptorchidism or hypospadias in male offspring were measured for each fraction (or pool of fractions) by an odds ratio (OR). ORs were estimated by conditional logistic regression, and their precision expressed as 95% CIs. TEXB-AA levels were used in logistic regression analysis either as a continuous variable (after a log10-transformation), or they were categorized using tertiles of their distribution. TEXB-AA levels below the first tertile were compared with TEXB-AA levels between the first and second tertiles, or compared with all TEXB-AA levels above the third tertile. ORs were estimated with and without adjustment. A confounder was considered as a variable significantly associated with both the outcome and the effect (i.e., birth weight). Statistical analyses were performed using SPSS 18.0 (IBM, Chicago, IL, USA) and SAS 9.3 (SAS Institute, Inc., Cary, NC, USA).

**Results**

Out of the 29 cases of urogenital tract malformations in this study, 16 (55.2%) had a diagnosis of cryptorchidism, 12 (41.4%) a diagnosis of hypospadias, and one (3.4%) had both disorders. The main clinical, socio-demographic, and lifestyle characteristics of cases and controls are summarized in Tables 1 and 2. Cases and controls differed in the type of delivery, with a higher prevalence of cesarean interventions in cases and of instrumental deliveries in controls (P = 0.054). The distribution of TEXB-AA levels in the study population is shown in Fig. 2. Fractions F5–11 were likely to contain androgen receptor agonist activity, which may have masked some of the antagonist activity measured in this pooled fraction.

Table 3 provides the results of the logistic regression analysis on the influence of the estimated TEXB-AA levels in the selected HPLC fractions on the occurrence of cryptorchidism and/or hypospadias.TEXB-AA levels were first analyzed as continuous variables (first row) and then as tertiles, setting the group with the lowest TEXB-AA levels as a reference. Only TEXB-AA levels in F2 showed a statistically significant positive dose–response association with the occurrence of cryptorchidism/hypospadias in the study population, with an OR of 4.31 (95% CI: 1.1–17.2) among subjects with high (third tertile) vs low (first tertile) TEXB-AA levels. The statistical
In this study, we have used a bioassay-directed fractionation methodology for the identification of anti-androgenic activity in five HPLC fractions of un-fractionated, crude placenta extracts. Two of these fractions/pools were significantly associated with the risk of urogenital tract malformations (95% CI: 0.0–0.5). The extensive profiling of fractionated placenta extracts revealed competing AR-antagonistic and AR-agonistic effects within the total anti-androgenic burden (Fig. 1). It therefore became clear that the testing of fractions of human placenta extracts, potentially via free access

![Distribution of TEXB-AA levels in the study population.](image)

**Figure 2** Distribution of TEXB-AA levels in the study population.

**Table 3** Odds ratios for cryptorchidism/hypospadias according to in vitro anti-androgenicity (procymidone-equivalent concentrations).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Cases</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>OR</td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Fraction 2 (proc eq/g)</td>
<td>60 29</td>
<td>2.56</td>
<td>1.16</td>
<td>5.66</td>
<td>2.33</td>
</tr>
<tr>
<td>&lt;1.34×10⁻⁷</td>
<td>23 38.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.34×10⁻⁷–4.40×10⁻⁶</td>
<td>21 35.0</td>
<td>1.79</td>
<td>0.48</td>
<td>6.74</td>
<td>1.65</td>
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<tr>
<td>&gt;4.40×10⁻⁶</td>
<td>16 26.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 3 (proc eq/g)</td>
<td>60 29</td>
<td>0.51</td>
<td>0.26</td>
<td>1.01</td>
<td>0.54</td>
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<tr>
<td>&lt;2.62×10⁻⁷</td>
<td>16 26.7</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>2.62×10⁻⁷–1.75×10⁻⁶</td>
<td>19 31.7</td>
<td>0.65</td>
<td>0.22</td>
<td>1.93</td>
<td>0.77</td>
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<tr>
<td>&gt;1.75×10⁻⁶</td>
<td>25 41.7</td>
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<tr>
<td>Fraction 4 (proc eq/g)</td>
<td>60 29</td>
<td>0.53</td>
<td>0.08</td>
<td>0.89</td>
<td>0.32</td>
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<tr>
<td>&lt;8.33×10⁻⁸</td>
<td>17 28.3</td>
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<tr>
<td>8.33×10⁻⁸–1.46×10⁻⁷</td>
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<td>0.15</td>
<td>1.38</td>
<td>0.42</td>
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<tr>
<td>&gt;1.46×10⁻⁷</td>
<td>20 33.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fractions 5–11 (proc eq/g)</td>
<td>60 28</td>
<td>0.62</td>
<td>0.27</td>
<td>1.39</td>
<td>0.60</td>
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<tr>
<td>&lt;5.86×10⁻⁷</td>
<td>19 31.7</td>
<td></td>
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<td></td>
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<tr>
<td>5.86×10⁻⁷–1.79×10⁻⁶</td>
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<td>0.21</td>
<td>2.07</td>
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<td>&gt;1.79×10⁻⁶</td>
<td>21 35.0</td>
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<td></td>
<td></td>
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<td>Fractions 21–24 (proc eq/g)</td>
<td>33 25</td>
<td>0.12</td>
<td>0.03</td>
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<tr>
<td>&lt;2.04×10⁻⁷</td>
<td>15 25.0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2.04×10⁻⁷–1.03×10⁻⁶</td>
<td>18 30.0</td>
<td>0.58</td>
<td>0.19</td>
<td>1.75</td>
<td>0.79</td>
</tr>
<tr>
<td>&gt;1.03×10⁻⁶</td>
<td>27 45.0</td>
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</table>

OR, odds ratio.

!Adjusted for birth weight (kg) (corresponding \(P\) values were between 0.07 and 0.20). !^Log_{10}-transformed concentrations. Bold values indicate statistically significant associations.
separate out various fractions in order to develop a meaningful biomarker of internal exposure that could be used in epidemiological studies. On the other hand, the profiles obtained suggested that it was not essential to test all 27 fractions but rather focus on the lipophilic fractions F2, F3, and F4, and the pool of fractions F5–11 (including endogenous steroid hormones) in addition to the pool of fractions F21–24 (more polar components).

It has been reported that human fetal testes produce testosterone from weeks 8 to 37 of gestation (Siiteri & Wilson 1974), with a peak during early gestation (weeks 12–16), which overlaps with the period acknowledged to be the critical stage for TDS development (8–14 weeks) (Welsh et al. 2008). Androgen levels during this ‘common early programing window’ are particularly relevant to the masculinization of the urogenital tract (Amann & Veeramachaneni 2007). Thus, previous research has suggested that human exposure to environmental pollutants with estrogenic and anti-androgenic effects during this ‘window’ can disturb the androgen balance and consequently affect genital differentiation (Welsh et al. 2008).

The positive association found between TEXB-AA levels in F2 and the risk of cryptorchidism/hypospadias in male offspring should be indicative of the combined effect of lipophilic chemicals on the fetal development. The testing of spiked samples showed that some of the chemicals eluting in F2 included organochlorine pesticides, polychlorobiphenyls, phthalates, and fatty acids. These chemicals might be candidates for explaining the anti-androgenic activity measured in F2, and they might contribute to the observed association with genital tract malformation risk, although this association probably results from complex interactions among the chemicals and with the biological environment. In addition, it has been suggested that the severity of male genital malformations is more dependent on exposure to a low-dose mixture of EDCs rather than to a high dose of a single chemical (Gaspari et al. 2011).

The present results are in agreement with previous findings that pointed to a key role for certain environmental pollutants in the development of TDS. In fact, our research group previously reported the presence of several pesticides (o,p'- and p,p'-dichlorodiphenyltrichloroethane, p,p'-dichlorodiphenyldichloroethylene, lindane, dieldrin, mirex, and endosulfan-α, among others) in the same placentas, with some of them associated with an increased risk of urogenital malformations at birth (Fernandez et al. 2007). Accordingly, Gaspari et al. (2011) found that parental occupational exposure to pesticides was related to a fourfold higher risk of cryptorchidism, hypospadias, and micropenis in the offspring. Other authors also observed that maternal exposure to organochlorine pesticides and/or polychlorinated biphenyls was significantly associated with the risk of urogenital tract abnormalities in male offspring (Longnecker et al. 2002, Bhatia et al. 2005, Damgaard et al. 2006, Brucker-Davis et al. 2008, Giordano et al. 2010, Krysiak-Baltyn et al. 2012). Evidence has also been published on the potential impact of phthalates on TDS development (Swan et al. 2005, Welsh et al. 2008).

We also observed a significant inverse association between TEXB-AA levels in F21–24 and the risk of urogenital tract malformations. F21–24 (minutes 21–24) eluted at the end of the HPLC run and should therefore be constituted by polar chemical compounds, but none of the spiked chemicals elute in this fraction; therefore, further research is needed to elucidate this putative association.

Other HPLC fractionation protocols have been successfully applied in epidemiological research on the combined effect of EDCs (Ibarluzea et al. 2004, Fernandez et al. 2007, Arrebola et al. 2012) but never on their anti-androgenic potential. Assessment of potential health outcomes based solely on chemical analysis might be an unrealistic task due to the large number of chemical residues to be measured and the wide structural diversity of chemicals with hormonal activity (Payne et al. 2001).

In this study, we estimated the anti-androgenic load in the fetus by analyzing the anti-androgenicity of placenta at delivery, i.e., after the critical stage for TDS development (Welsh et al. 2008). However, the placenta has been described as an ideal matrix for the estimation of the mother-to-son transference of environmental pollutants (Li et al. 2013). Several studies have previously found that a number of pollutants with endocrine disruptive potential, e.g., phthalates, organochlorine pesticides, or bisphenol A, are able to cross the placenta and enter the fetal circulation (Lopez-Espinosa et al. 2007, Shen et al. 2008, Jimenez-Diaz et al. 2011, 2013, Vela-Soria et al. 2011, Fernandez et al. 2012, Li et al. 2013).

Despite the limited sample size, statistically significant associations were found in the present study and warrant in-depth research in different populations. To the best of our knowledge, this epidemiological study represents the first attempt to use a biomarker of total anti-androgenic activity to assess the effect of prenatal exposure to endocrine disruptor chemicals on the risk of urogenital tract malformations, with promising results. However, further research is warranted to determine whether TEXB-AA can be used to identify subjects particularly at the risk of TDS-related disease. Our group is currently working on a thorough screening of the chemicals present in each HPLC fraction in order to ascertain their potential role in the activities found.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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