A negative correlation between insulin-like peptide 3 and bisphenol A in human cord blood suggests an effect of endocrine disruptors on testicular descent during fetal development

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STUDY QUESTION: Does a relationship exist between insulin-like peptide 3 (INSL3) and selected environmental endocrine disruptors (EEDs) in human cord blood (cb)?

SUMMARY ANSWER: In the whole population (cryptorchid and control boys) cb INSL3 correlated negatively with cb free bisphenol A (BPA) providing indirect evidence for an impact of EEDs on fetal Leydig cell INSL3 production.

WHAT IS KNOWN ALREADY: INSL3 is a major regulator of testicular descent. This hormone has been shown to be decreased in cord blood from boys with idiopathic cryptorchidism, the most frequent male malformation. Fetal exposure to several EEDs has been suspected to be involved in the occurrence of idiopathic cryptorchidism.

STUDY DESIGN, SIZE, DURATION: Correlations between cb INSL3 or testosterone and cb free bioactive BPA and maternal milk polychlorinated biphenyls (PCB153), dichlorodiphenyldichloroethylene (DDE), and monobutyl phthalate (mBP) were assessed in newborn boys in a prospective case–control study. All boys (n = 6246) born after 34 weeks of gestation were systematically screened at birth for cryptorchidism over a 3-year period (2002–2005), and a diagnosis of cryptorchidism confirmed by a senior paediatrician.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We studied 52 cryptorchid (26 transient, 26 persistent) and 128 control boys born at two hospitals in southern France. INSL3 was assayed in CB by a modified validated enzyme-linked immunosorbertent assay. Testosterone was measured in CB after diethyl-ether extraction by means of ultra-pressure liquid chromatography-tandem mass spectrometry. Free cbBPA was measured after an extraction step with a radioimmunoassay validated after comparison of values obtained by high-pressure liquid chromatography–mass spectrometry. The xenobiotic analysis in mothers’ milk was performed after fat extraction by gas chromatography–mass spectrometry.

MAIN RESULTS AND THE ROLE OF CHANCE: EED concentrations were not increased in the cryptorchid versus control group although a trend for increased mBP (P = 0.09) was observed. In the whole study population, cb levels of BPA correlated negatively with INSL3 (P = 0.01; R² = 0.05) but not with testosterone. No other EED correlated with INSL3 or with testosterone.

LIMITATIONS, REASONS FOR CAUTION: The levels of BPA and INSL3 in cb may not reflect chronic fetal exposure to EEDs. The deleterious impact of EEDs on fetal testicular descent during specific windows of development has yet to be demonstrated.

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**Introduction**

Testicular descent depends on anatomical and hormonal factors. It occurs in two successive phases, abdominal (10–23 weeks of gestation in humans), and inguino-scrotal (from 28 weeks of gestation through birth) (Hughes and Acerini, 2008; Hudson et al., 2013). Fetal tests secrete the two hormones controlling this two-phase descent, insulin-like peptide 3 (INSL3) and testosterone (Bay and Anderson, 2011; Hudson et al., 2013), as demonstrated in humans diagnosed with a congenital defect of the pituatary-testicular axis or in genetically modified rodents (Nef and Parada, 1999; Zimmermann et al., 1999; Yuan et al., 2010). INSL3, a peptide hormone belonging to the relaxin family, acts through the relaxin family peptide receptor 2 (RXFP2), which is developmentally expressed in the gubernaculum (Feng et al., 2007). INSL3 classically regulates the abdominal phase (Forestia et al., 2008; Hughes and Acerini, 2008), but experimental data also support its participation, along with testosterone, during the inguino-scrotal phase (Yuan et al., 2010). The role of testosterone during the inguino-scrotal phase, involving the regression of the gubernaculum, has been well characterized in animal models by gene invalidation (Yuan et al., 2010) or after anti-androgen exposure (Gray et al., 2001). Cryptorchidism, the most frequent congenital malformation in males, occurs in 2–5% of full-term male births (Toppari et al., 2001; Virtanen et al., 2007; Brucker-Davis et al., 2008; Foresta et al., 2008), and is recognized as a risk factor for infertility and testicular cancer in young men (Hadziselimovic, 2002; Cook et al., 2010). The failure for the tests to descend in the scrotum, usually during fetal life, remains largely unexplained and undescended tests (UDTs) is generally considered to be a multifactorial disease with anatomical, genetic and environmental risk factors (Virtanen et al., 2007; Foresta et al., 2008; Main et al., 2009; Hudson et al., 2013). Anatomical factors could explain that most idiopathic UDT cases are unilateral (Hudson et al., 2013). Genetic causes such as mutations of INSL3, testosterone or their receptors genes (Feng et al., 2004; Ferlin et al., 2008; Foresta et al., 2008) are rare in ‘idiopathic’ UDT. Environmental factors, including in-utero exposure to environmental endocrine disruptors (EEDs), have been proposed as co-factors for the occurrence of idiopathic UDT and other male reproductive developmental abnormalities (Main et al., 2009). This environmental hypothesis is supported both by epidemiological studies showing, for example, temporal (Chilvers et al., 1984) or geographical differences (Boisen et al., 2004) and by experimental data in rodents, showing that exposure to several EEDs with estrogenic or anti-androgenic effects during fetal life disturbs testicular descent by inhibiting INSL3 gene expression (Nef et al., 2000; Mckinnell et al., 2005) and/or testosterone production or action (Gray et al., 2001). However, in human, a direct link between fetal exposure to EEDs and impaired secretion and/or action of fetal Leydig cell hormones has not been reported.

We have recently shown, in agreement with data from Bay et al. (2007), that cbINSL3 concentrations, but not testosterone, were lower in human idiopathic UDT, especially in transient forms, compared with a control group (Fénichel et al., 2014). In order to test the environmental hypothesis, we went further by studying the relationship between INSL3 and several EEDs with estrogenic and/or anti-androgenic effects, including BPA and phthalates, in cord blood (cb) and maternal milk.

**Materials and Methods**

**Study design**

The research was approved by the ethic committee of our institution. Between 2002 and 2005, 6246 consecutive boys who were born alive after 34 weeks of gestation in the maternity wards of Nice University Hospital and the nearby Grasse General Hospital, France, were screened for cryptorchidism in a 3-year prospective study (Brucker-Davis et al., 2008; Wagner-Mahler et al., 2011). Neonatal examination was standardized and diagnosis of cryptorchidism was accepted after at least two concordant examinations by senior paediatricians before hospital discharge. Testicular position was determined according to the Scorer criteria, completed by Hack et al. (2003), after firm, but unforced traction of the tests to the most distal position along the pathway of normal descent. After parental consent was obtained, boys with non-palpable, inguinal, supra-scrotal, high scrotal tests, were included in the UDT group. Retractile tests were excluded from both the cryptorchid and control groups. All the boys were examined again at 3 and 12 months by the same team to determine whether cryptorchidism was persistent or transient.

**Population**

Over the 3-year period, 102 out of 6246 eligible newborn boys were diagnosed with cryptorchidism, and 95 of them were included after parental consent was obtained (Brucker-Davis et al., 2008). Two control boys, born on the same ward at about the same time, were recruited for each case; they were matched for gestational age, birthweight and, when possible, parental geographical origin. Samples with sufficient blood volumes were used for INSL3, testosterone and free bisphenol A (BPA) measurements (52 of the 95 cryptorchid newborns and 128 of the 188 controls). The boys in this study did not differ from the previously described boys (Brucker-Davis et al., 2008; Wagner-mahler et al., 2011), in terms of gestational age (>37 weeks of gestation in >92% of cases and controls), birthweight or other parameters. All 52 newborns were non-syndromic, isolated cases of cryptorchidism with normal karyotype, including 26 persistent and 26 transient forms, 48 unilateral and 4 bilateral. Considering the position of the undescended testes following Scorer classification, there were 24 palpable
and 28 non-palpable testes. Clinical characteristics of the boys are shown in Table I.

**Hormone and EED measurements**

CB was drawn immediately after delivery through a metallic needle and a glass syringe into two 10-ml glass tubes. Maternal milk was collected between Days 3 and 5 post-delivery into two 10-ml glass containers. Samples were then centrifuged (cb), aliquoted, frozen and stored at −80°C within hours of sampling and immediately after centrifugation in supplier-certified BPA- and phthalate-free tubes.

**Hormone measurements**

INSL3 was assayed in CB by a modified validated enzyme-linked immunosorbent assay using Phoenix reagents (Phoenix Pharmaceuticals, Belmont, CA, USA) (Cabrol et al., 2011; Hirsch et al., 2013). Sensitivity was 11 pg/ml. Intra-assay coefficients of variation at the levels of 36 and 115 pg/ml were 9.7 and 5%, respectively. Testosterone was measured in CB after diethyl-ether extraction by means of ultra-pressure liquid chromatography-tandem mass spectrometry using Waters Acquity-Quattro Premier equipment (Saint-Quentin en Yvelines, France). The limit of quantification at 20% coefficient of variation was 0.09 nmol/l. The intra-assay coefficients of variation at levels 1.5, 8.9 and 35.9 nmol/l were 6.3, 3.9 and 2.9%, respectively. The inter-assay coefficients of variation at levels 1.62, 8.67 and 34.7 nmol/l were 5.2, 4.1 and 5.0%, respectively.

**Xenobiotics measurements**

Experimental and epidemiological studies have shown that some pollutants can be involved in the occurrence of cryptorchidism (Virtanen et al. 2007; Brucker-Davis et al., 2008). The xenobiotic analysis in milk included polychlorinated biphenyls (PCB 153), monobutyl phthalate (mBP) and dichlorodiphenylchloroethylene (DDE). These xenobiotics may have estrogenic (PCB153), and/or anti-androgenic properties (DDE, PCB 153, mBP). Furthermore, we also decided to study BPA, which is an interesting candidate for its estrogenic and anti-androgenic effects; in addition, it is a compound we have studied previously (Bouskine et al., 2009; Chevalier et al., 2012a), and for which there is a large body of literature. Special care was taken to avoid BPA or phthalate contamination from polycarbonate equipment by using certified BPA- and phthalate-free material.

BPA was measured in cord blood with a radioimmunoassay (RIA) described in Kaddar et al. (2009) after an extraction step with ethyl acetate to minimize matrix effects. The antibody used has demonstrated negligible cross-reactivity with most analogous BPA phenolic structures and no cross-reactivity with endogenous steroids (Kaddar et al., 2009). Recovery after loading test was 96 ± 4%, and dilution tests had a linear profile (R² > 0.93) (Kaddar et al., 2009). The limit of detection of this BPA RIA was 0.08 ng/ml. The intra- and inter-assay coefficients of variation were 5.6 and 8.6%, respectively, at a BPA concentration of 0.7 ng/ml and 6.9, and 5.7% at a BPA concentration of 1.3 ng/ml. On a random subsample of cbs of normal boys (n = 40), RIA values were compared with those obtained by HPLC-MS, as described in Kaddar et al. (2009), with a good correlation (R² = 0.72; y = 1.321x + 0.2379). All cb BPA values were above the limit of detection.

The xenobiotic analysis in milk was performed as described previously (Brucker-Davis et al., 2008) by gas chromatography–mass spectrometry at the Laboratoire de l’Environnement de l’Agglomération Niçoise, a laboratory accredited by the French Ministry of Health and the French Ministry of the Environment. In brief, for milk specimens, fat was first extracted according to standard procedures with a median percentage of fat of 2% (range 0.5–4.8%). Then, the extracts were processed as described by Brucker-Davis et al. (2008). Results were expressed in nanogram per gram of milk for phthalates, and in nanogram per gram of fat (recommended for lipophilic compounds) for PCB and DDE, to allow comparison with other studies. Quantification and detection thresholds were, respectively, 0.1 and 0.03 ng/g of milk or ng/g of fat. The percentage of recovery varied from 75 to 96%. Inter-assay coefficients of variation varied from 5.1% for DDE to 13.5% for PCB153. Intra-assay coefficients of variation varied from 3.9% for PCB153 to 8.4% for BPA. All analyses were carried out blinded for cryptorchid status, and cases and controls were analysed within the same run.

**Statistical analysis**

Because of sample volume limitations, an exhaustive study of the whole cohort (Brucker-Davis et al., 2008) was not possible. However, the subgroup analysed here did not differ from the whole cohort for clinical parameters. Simple logistic regressions (using a binomial distribution of errors and a first-level risk α = 0.05) were used to test the correlation between variables and cryptorchidism in each of the sub-samples. A general linear model with a Gaussian link function was used to test the correlation between INSl3 or testosterone and xenobiots. Both direct effects and indirect effects (second-order only) were studied. Statistical regressions and tests were performed using the environment for statistical computing R 3.0.1 (2013) (R Foundation for Statistical Computing, Vienna, Austria). A P < 0.05 was considered significant.

**Results**

**Population studied**

There was no difference in clinical parameters, including gestational age, birthweight and percentage of delivery after Caesarian section, between the group of cryptorchid and control boys (Table I). cbINS3L, but not

| Table I: Clinical characteristics of cryptorchid and control boys in the study. |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Cryptorchid                              | Controls        |                |
| Transient                                | Persistent      | Total           |                |
| N = 26                                   | N = 26          | N = 52         | N = 128        |
| Gestational age (weeks)                  | 38.5 ± 1.7      | 39.1 ± 1.7      | 38.8 ± 1.7     | 39.1 ± 1.4      | NS              |
| Birthweight (g)                          | 3145 ± 513      | 3173 ± 540      | 3159 ± 526     | 3220 ± 494      | NS              |
| C section %                              | 24.4            | 18.7            | 21.5           | 20.1c           | NS              |

Results are expressed as mean ± SEM.

C section % corresponds to the percentage of delivery after Caesarian section.

*Gestational age at birth.
testosterone was significantly decreased in the cryptorchid group compared with the control group (Table II).

**Xenobiotics and cryptorchidism**

_cbBPA in cryptorchid boys was not significantly increased (Table II) when compared with controls (P = 0.1). There was no difference either, when comparing permanent and transient forms of cryptorchidism: 1.19 ± 1.03 ng/ml versus 1.31 ± 1.2 ng/ml (P = 0.64). Comparison of palpable versus non-palpable cryptorchid groups revealed that the non-palpable group exhibited cbBPA levels higher than the palpable group, though not significantly: 1.61 ± 1.51 ng/ml versus 1.01 ± 0.71 ng/ml (P = 0.19). In addition, mean values of milk xenobiotics concentrations were systematically, but not significantly, higher in the UDT group, compared with control group (Table II), with a trend for mBP (P = 0.09). Analysis of the interactions between xenobiotics (data not shown) indicated that cbBPA was positively correlated with maternal milk PCB153 in the whole population (P = 0.019; R² = 0.065).

**Xenobiotics and hormones**

In the whole population (cases and controls taken together) there was no significant relationship between INSL3 and either the linear combination of xenobiotics (adjusted R² = 0.025; P = 0.44) or the second-order xenobiotic combinations (P = 0.95) and tests using cb testosterone concentrations gave identical results (P = 0.49 and P = 0.25, respectively). On the other hand, when testing individual linear correlations between INSL3 and xenobiotics, we found a weak (R² = 0.05) but significant (P = 0.01) inverse correlation with BPA (Fig. 1). PCB153 was not correlated with INSL3 (P = 0.26), either alone or in interaction with BPA (P = 0.9), nor was mBP (P = 0.45). In addition, testosterone did not correlate with any of the xenobiotics assessed (Table III).

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**Table II** Cord blood hormone and xenobiotic levels in cryptorchid and control boys.

<table>
<thead>
<tr>
<th></th>
<th>Cryptorchid N = 52 mean ± SEM</th>
<th>Controls N = 128 mean ± SEM</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insl3 pg/ml</td>
<td>225.7 ± 19.3</td>
<td>271.4 ± 18.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.92 ± 0.25</td>
<td>2.73 ± 0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Xenobiotics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPA ng/ml</td>
<td>1.26 ± 0.17</td>
<td>1.14 ± 0.13</td>
<td>0.1</td>
</tr>
<tr>
<td>PCB153 ng/g</td>
<td>88.3 ± 11.16</td>
<td>65.5 ± 23.2</td>
<td>NS</td>
</tr>
<tr>
<td>DDE ng/g</td>
<td>213.6 ± 54.2</td>
<td>139.9 ± 28.1</td>
<td>NS</td>
</tr>
<tr>
<td>mBP ng/g</td>
<td>33.2 ± 9</td>
<td>11.3 ± 3.7</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*P*-value corresponds to the significance level for the logistic regression test. BPA was measured in cord blood (ng/ml) and the others in maternal milk. Concentrations of mBP in milk is given in ng/g of milk and the others in ng/g of fat in milk.

**Table III** Correlation between hormones and xenobiotics.

<table>
<thead>
<tr>
<th></th>
<th><strong>INSL-3</strong></th>
<th><strong>Testosterone</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>P</strong></td>
<td><strong>r²</strong></td>
</tr>
<tr>
<td>PCB153</td>
<td>0.26</td>
<td>0.018</td>
</tr>
<tr>
<td>mBP</td>
<td>0.45</td>
<td>0.022</td>
</tr>
<tr>
<td>DDE</td>
<td>0.64</td>
<td>0.003</td>
</tr>
<tr>
<td>BPA</td>
<td><strong>0.01</strong></td>
<td>0.05</td>
</tr>
</tbody>
</table>

BPA was measured in cord blood (ng/ml) and the other environmental endocrine disruptors in maternal milk.

*P*-value corresponds to the significance level for the linear regression test. Bold font underlines the significant parameters.

**Discussion**

In a prospective, case–control study on human isolated, idiopathic UDT, we have recently reported, in agreement with data from Bay et al. (2007), that INSL3, a major regulator of testicular descent, was significantly decreased in cb, particularly in transient forms (Fénichel et al., 2014). We show here that in the whole study population, cbINS3 correlated negatively with free cbBPA, an EED with suspected estrogenic effects. This is the first time that a concomitant link is established in human, in vivo, between a hormonal regulator of testicular descent and fetal exposure to one EED. While the participation of BPA in this decrease remains moderate (R² = 0.05), the statistical link is significant and consistent (negative effect at low dose) with the recently reported decrease of INSL3 fetal production in vitro by NTumba-Byn et al. (2012) on human explanted fetal testes, cultured with low doses of BPA. From a mechanistic point of view, it is also in agreement with what is known from experimental data on both the BPA disrupting effect and INSL3 gene expression regulation. INSL3 gene expression is negatively regulated by estrogens, as...
shown in Leydig cells in vitro (Lague and Tremblay, 2009), and positively by androgens (Lague and Tremblay, 2008). In mice, maternal exposure to xenoestrogens, including the potent synthetic estrogen diethylstilbestrol (DES), results in down-regulation of INSL3 (but not testosterone) mRNA expression level in Leydig cells (Emmen et al., 2000; Nef et al., 2000), and is associated with intra-abdominally located testes. In humans, an increased risk of cryptorchidism has been reported after fetal exposure to DES given as maternal treatment to prevent miscarriages (Palmer et al., 2009). BPA, like DES, was initially designed as a synthetic estrogen, but it rapidly came to be widely used in the manufacture of plastics and epoxy resins. BPA leaches out from food and beverage containers and has been found in human fluids at concentrations similar to those shown to have an impact in an experimental setting (Vandenbergen et al., 2010). Because of its low affinity for the classical nuclear estrogen receptors (ER)α and ERβ (Matthews et al., 2001), the classification of BPA as a xenoestrogen has also been debated (Sharpe, 2010). To explain the mechanisms of action, other receptors have been proposed such as androgen receptor (Lee et al., 2003), ERRγ (Takayanagi et al., 2006) or membrane non-classical estrogen receptors (Bouskine et al., 2009; Chevalier et al., 2012a,b). N’Tumba-Byn et al. (2012), in reporting the negative effect of BPA on INSL3 Leydig cell secretion during human fetal testis culture, were able to exclude the ERα pathway by gene invalidation, and they suggested the participation of non-classical ERs. We have recently identified, in human testis, including Leydig cells, one of these receptors, GPR30/GPER (G protein coupled ER) for which BPA has a high affinity (Bouskine et al., 2009; Chevalier et al., 2012a). An anti-androgenic effect of BPA (Lee et al., 2003) has also been reported which could interfere with the positive regulation of testosterone on INSL3 gene expression (Lague and Tremblay, 2008).

The lack of correlation between BPA and testosterone concentrations is not completely surprising since INSL3 and testosterone have been shown to be differentially regulated at the Leydig cell level. INSL3 secretion is dependent on the pituitary axis in a less acute way than testosterone (Bay et al., 2011) and both genes are also distinctly regulated (Lague and Tremblay, 2008).

Our data are strengthened by the fact we used a new validated immunoassay for INSL3 (Cabrol et al., 2011; Hirsch et al., 2013), as well as the reference method for testosterone measurement (chromatography coupled to mass spectrometry). There are, however, some limitations in our work concerning the measurement of BPA in cord blood. In human, BPA, which mostly comes from the diet, is rapidly metabolized by the liver into more hydrophilic conjugated compounds before its elimination into the urine (Dekant and Volkell, 2008). Measurement of conjugated forms in urine is now recommended as a better assessment of 24 h exposure, avoiding the risk of contamination of free BPA from plastic devices (Calafat et al., 2013). However, urine samples were not available in our cohort because at the time of the design of the study (2002), the optimal conditions for collection were unknown and also because urine from the delivering mother or the newborn are difficult to collect. Thus, since cb was available, we chose to measure cb free BPA as a marker of fetal exposure to BPA at the end of pregnancy. Sensitive measures had been taken to avoid sources of contamination, as already detailed (Féniel et al., 2012). The assay we used (RIA method after extraction) had been validated (Kaddar et al., 2009; Féniel et al., 2012) by correlation with the reference method gas chromatography–mass spectrometry. Due to a short half-life, blood levels of bioactive, unconjugated BPA are usually low when compared with conjugated urinary metabolites (Vandenbergen et al., 2010; Koch et al., 2012; Yeh et al., 2012), but values in cord blood are higher than in adult blood (Mielke and Gundert-Remy, 2009; Vandenbergen et al., 2010; Zhang et al., 2013). Indeed, maternal cbBPA easily crosses the placenta (Schönfelder et al., 2002; Balakrishnan et al., 2010), and will be less easily conjugated and cleared by the fetus because of immature hepatic glucurononyltransferase enzymes (Coughtrie et al., 1988; Matsumoto et al., 2002) and active placental or fetal glucuronidases or sulfatases (Coughtrie et al., 1988). While it has been clearly shown that maternal exposure to estrogenic or anti-androgenic EEDs could induce cryptorchidism in rodents, it remains uncertain whether such an environmental factor is operating in human idiopathic UDT (Virtanen and Adamson, 2012). The tendency for the four pollutants reported here to be systematically higher in cryptorchid boys suggests that the mother may be more exposed to environmental pollutants or have less capacity to metabolize them. As in our previous report (Féniel et al., 2012), there was no significant increase of cbBPA in boys with UDT when compared with controls. However, mean levels of BPA were higher in the cryptorchid group, and importantly even more in the non-palpable versus palpable subgroups, suggesting a link with the degree of migration defect. Interestingly, we have already reported a similar trend for cbINSL3 levels (Féniel et al., 2012), compatible with a potential link between these parameters. On the other hand, a single blood or spot urine BPA or conjugates test reflects short-term exposure and not chronic exposure (Lassen et al., 2013). Therefore, although exposure through diet is likely to be continuous, it cannot be concluded from this study, performed at the time of delivery, whether chronic fetal exposure to maternal BPA could disturb testicular descent at the time when INSL3 is most likely to be acting directly on the testis, in the first phase of testicular descent (gestational week 12–16). However, our data support the hypothesis that INSL3 is a target of endocrine disruption. Anand-Ivell and Ivell (2014) have even proposed recently that INSL3 could be a ‘monitor of endocrine disruption’.

Indeed, INSL3 could be influenced by fetal exposure to several estrogenic and/or anti-androgenic EEDs acting as a ‘cocktail’, as suggested by epidemiological studies in idiopathic UDT (Damgaard et al., 2006; Brucker-Davis et al., 2008; Virtanen and Adamson, 2012). Beside BPA, phthalates are among the strongest candidates for affecting the testis (Albert and Jegou, 2014). Indeed, there are robust data in rodents (Mckinnell et al., 2005), and more recently in humans (Desdoits-Lethimonier et al., 2012), supporting their deleterious effects on testicular descent and function. They may act as androgen antagonists, on steroid hormone production, or on INSL3 gene expression or action (Mckinnell et al., 2005; Desdoits-Lethimonier et al., 2012). The effects of phthalates on INSL3 are sometimes contradictory, with some data showing an impact (Mckinnell et al., 2005; Shono et al., 2005; Lague and Tremblay 2008), and others not (Desdoits-Lethimonier et al., 2012). This discordance is likely due to a differential effect according to time of exposure or species (Mckinnell et al., 2005; Desdoits-Lethimonier et al., 2012; Albert and Jegou, 2014). We report here a non-significant increase (P = 0.09) of milk monobutylphthalate (mBP) in a subgroup of boys with transient UDT, in agreement with the positive relation we have previously reported between cryptorchidism and the maternal self-reported exposure to phthalates (Wagner-mahler et al., 2011). Our limited data do not show an effect of fetal exposure to phthalates on INSL3 in human. As for conjugated BPA, measurement of oxidated phthalate metabolites in 24 h urine
samples, would be a better reflection of true exposure, ruling out the risk of post-sampling contamination (Koch et al., 2005). In order to approach fetal exposure during specific windows of development, the assessment of phthalates in amniotic fluid has also been recently proposed with, however, the well-known technical and/or ethical difficulties associated with such studies (Anand-Ivell and Ivell, 2014).

**Conclusion**

cbINSL3, a major regulator of testicular descent, is decreased in idiopathic UDT and inversely related, in the whole population of newborn males, to bioactive cbBPA concentrations. This negative correlation provides indirect evidence for an impact of endocrine disruptors on INSL3 Leydig production during fetal development. It strongly suggests that INSL3 is a possible target of fetal exposure to EEDs. However, the deleterious impact of EEDs on fetal testicular descent, via the disturbance of INSL3 pathway, has yet to be demonstrated directly. The challenge is to design prospective studies correlating INSL3 with the most appropriate EEDs or their metabolites, in the most appropriate fluids of the maternal–fetal unit, during the specific windows of development.

**Authors’ roles**

N.C. discussed and wrote the paper. F.B.-D. directed the prospective study and wrote the paper. N.L. performed INSL3 and testosterone assay. P.C. made the statistical study. M.P. supervised BPA assay. P.P. measured EEDs by GC/MS. P.P.-F. supervised collection, freezing, storage of cord blood and milk bank. P.F. conceived the study, analysed the results and wrote the paper.

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**Conflict of interest**

None declared.

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