**ORIGINAL ARTICLE**

**Influence on the adult male Leydig cell biomarker insulin-like peptide 3 of maternal exposure to estrogenic and anti-androgenic endocrine disrupting compound mixtures: A retrospective study**

Richard Ivell\(^1\) | Anne Marie Vinggaard\(^2\) | Hiroaki Soyama\(^1\) | Ravinder Anand-Ivell\(^1\)

\(^1\)School of Bioscience, University of Nottingham, Sutton Bonington, UK  
\(^2\)National Food Institute, Technical University of Denmark, Lyngby, Denmark

**Correspondence**  
Ravinder Anand-Ivell, School of Bioscience, University of Nottingham, Sutton Bonington, LE12 5RD, UK.  
Email: ravinder.anand-ivell@nottingham.ac.uk

**Funding information**  
National Defence Medical College, Saitama, Japan

**Abstract**  
Insulin-like peptide 3 (INSL3) is a peptide biomarker secreted specifically by the mature Leydig cells of the testes. It is constitutive, has low within-individual variance, and effectively measures the functional capacity of Leydig cells to make testosterone. In young adult men there is a large 10-fold range of serum INSL3 concentration, persisting into old age, and implying that later hypogonadal status might be programmed in early life. To determine whether maternal exposure to environmental endocrine disrupting compounds (EDCs) influences adult serum INSL3 concentration, using a retrospective paradigm, INSL3 was measured in young adult male rats (80–90 days) from the F1 generation of females maternally exposed to varied doses of bisphenol A (BPA), butylparaben, epoxiconazole, and fludioxonil as single compounds, as well as estrogenic and anti-androgenic mixtures of BPA and butylparaben, and di(2-ethylhexyl) phthalate and procymidone respectively. A mixture of BPA and butylparaben significantly reduced circulating INSL3 concentration in adult male progeny. The remaining compounds or mixtures tested, though sufficient to induce other effects in the F1 generation were without significant effect. Maternal exposure to low concentrations of some EDCs may be a contributing factor to the variation in the Leydig cell biomarker INSL3 in young adulthood, though caution is warranted translating results from rats to humans.

**KEYWORDS**  
endocrine disrupting chemicals, gestational exposure, INSL3, insulin-like peptide 3, Leydig cell

**1 | INTRODUCTION**

In adult male mammals, the male gonadal steroid testosterone is directly or indirectly involved in supporting a range of key physiological functions, including bone and muscle health, cognitive development, cardiovascular health, and sexual function. Its deficit in hypogonadism, especially in ageing men, is similarly associated with reduced function in these physiologies, as well as age-related morbidities, such as type 2 diabetes, kidney disease, and frailty (Ahern & Wu, 2015; Dwyer & Quinton, 2018; Heidelbaugh, 2016; Nieschlag, 2020; Traish & Zitzmann, 2015). Ninety-five per cent of the circulating testosterone in men derives from the Leydig cells...
within the testes, whose development and steroidogenesis are acutely regulated by the pituitary gonadotropin LH.

Adult-type Leydig cells are produced from resident stem cells in the testes as a result of proliferation and differentiation during pubertal development under the influence of LH (Teerds & Huhtaniemi, 2015). These stem cells appear to be discrete from those in the foetus, which give rise to the foetal Leydig cell population, though are presumably present in the foetus and responsive to maternal influences. Once the final population of adult-type Leydig cells is established at the end of puberty, these cells—like nerve cells—appear subsequently neither to divide nor die to any great extent. Thus, these Leydig cells of young adulthood are the ones which are going to be providing testosterone for the remainder of adult life. In the human, there are on average some 250 million Leydig cells in young adulthood (Petersen et al., 2015) which are therefore responsible for supporting most androgen-dependent physiology in later life.

The mature Leydig cells in the adult mammal also produce the hormone insulin-like peptide 3 (INSL3), which is secreted in a constitutive fashion into the circulation, where it may have a role in gametogenesis or supporting bone metabolism (De Toni et al., 2018; Ivell & Anand-Ivell, 2009). In the male, the Leydig cells are the only source of circulating INSL3 (Ivell & Anand-Ivell, 2009). Because of its constitutive expression, INSL3 changes very little within an individual over time and thus serves as an ideal biomarker to measure Leydig cell functional capacity, that is, the product of total Leydig cell numbers and their differentiation status (Ivell & Anand-Ivell, 2009; Ivell, Wade, & Anand-Ivell, 2013).

Recent cohort studies in humans have shown that both in ageing (Anand-Ivell et al., 2006, 2022) and in young men at 18 years of age (Anand-Ivell et al., 2021), adult Leydig cell functional capacity, as represented by circulating INSL3 concentration, varies considerably by more than 10-fold between apparently healthy individuals. Moreover, INSL3 concentration within an individual remains relatively constant over several years at least (Anand-Ivell et al., 2022). Thus, since Leydig cells appear neither to die nor to divide during the adult lifespan, commencing adulthood with a low Leydig cell functional capacity will have marked negative consequences for testosterone production in later life and hence probably also morbidity. We understand very little about the source of this large, between-individual quantitative variation, particularly in young men after puberty. One study has suggested that childhood obesity may play a role (Laakso et al., 2018), and a study in cattle has indicated that prepubertal nutrition may impact adult INSL3 levels (Anand-Ivell et al., 2019). Whilst both smoking and elevated BMI correlate negatively with INSL3 levels in adult cohort studies (Anand-Ivell et al., 2021, 2022; Atlantis et al., 2009), the degree of correlation is relatively minor, suggesting that other factors during development are likely to be important. These could include genetics, since adults of different breeds within the same species may show quite different levels of circulating INSL3 (Anand-Ivell et al., 2009). Alternatively, epigenetics and/or environmental exposures might also explain the high adult variance in INSL3 that is observed in human cohorts.

Studies in rats have suggested that maternal exposure to environmental endocrine disrupting chemicals (EDCs), such as phthalate esters, oestrogens, or anti-androgenic pesticides, may influence diverse parameters of the male reproductive system. These EDCs may derive from plastics, personal care products, food packaging materials, pesticides, fungicides, flame retardants and other common sources (Gore et al., 2015) and appear to affect oestrogen- or androgen-sensitive targets such as anogenital distance, testicular descent, spermatogenesis, or sexual behaviour (Gore et al., 2015; Hass et al., 2012, 2016; Ivell et al., 2014; Jacobsen et al., 2012). Pubertal dynamics may also be altered by such substances (Heng et al., 2012; Hunter et al., 2021; Parent et al., 2015), though to date no studies have specifically addressed potential impacts on the final adult-type Leydig cell population and its functional capacity. The present retrospective investigation was therefore undertaken to redress this deficit. The specific Leydig cell biomarker INSL3 was measured in the adult male

![Idealised hormone profiles for luteinizing hormone (LH; upper panel), testosterone (T, middle panel), and insulin-like peptide 3 (INSL3) (lower panel) during puberty in the rat. LH is represented as a range of maximum and minimum values since it is highly pulsatile during puberty (based on Ivell et al., 2014). The sampling period used for the present study is indicated by the dashed box.](image-url)
rat offspring resulting from five independent studies applying material exposure to a diverse range of EDCs and their mixtures; these included both oestrogens such as bisphenol A, as well as anti-androgenic pesticides. In rats, circulating INSL3 increases during puberty to a peak level at around PND42, before declining to a more stable lower level at around PND60-80, when the strong LH pulses driving Leydig cell differentiation have subsided (Anand-Ivell et al., 2009; Ivell et al., 2014). Here we have specifically selected a time-point for blood collection which represents early adult physiology in rats (PND80-90), after the peak in circulating INSL3 at the end of puberty at PND42 (Anand-Ivell et al., 2009), and reflecting the final, established population of Leydig cells to be retained throughout adulthood (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Animal experimentation

For this retrospective study, full experimental details of the animal experiments have already been published (Boberg et al., 2016; Christiansen et al., 2014, 2020; Hass et al., 2012, 2016; Jacobsen et al., 2012; Mandrup et al., 2016; Overgaard et al., 2013; Scholze et al., 2020). These were all carried out within the same institute, following essentially similar protocols, and are summarised here.

2.1.1 | Bisphenol A (BPA; purity >99.5%, CAS no. 80-05-7; Sigma-Aldrich, Bronby, Denmark)

Nulliparous time-mated pregnant Wistar rats (HanTac:WH, SPF, Tacnic Europe, Ejby, Denmark) were received on gestation day 3 (GD3) of pregnancy. The detection of a vaginal plug was referenced as GD1 and irrespective of the actual day of birth, the expected GD23 was considered to be postnatal day 1 (PND1). Rats were held under standard conditions with 12 h light/12 h dark cycle, 55% humidity, and at 21°C. In pairs until GD17 and alone thereafter in semi-transparent polysulfone (PSU) type III cages with aspen woodchip bedding (Tapvei, Gentofte, Denmark), EnviroDry nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (aspen wood) shelters (Brogaarden). Animals were fed a standard diet of Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tapwater in PSU bottles was given ad libitum. The dams were gavaged once daily, except for the day of parturition, from GD7 to PND22 with 2 ml/kg bodyweight per day, a period which covers the most sensitive stages of reproductive development during gestation and lactation, using the following doses of BPA in corn oil (Sigma-Aldrich): 0 (control), 0.025, 0.25, 5.0, or 50 mg/kg bodyweight/day. The resulting pups were extensively analysed for a range of anatomical, physiological, and behavioural parameters as detailed in (Christiansen et al., 2014; Hass et al., 2016; Mandrup et al., 2016). One subset of male offspring was allowed to mature to PND80, when blood was collected for INSL3 assessment (see below). None of these animals had received any prior treatments additional to the BPA dosing likely to influence the INSL3 concentration in the blood.

2.1.2 | Butylnparaben (purity >99%, CAS no. 94-26-8; Sigma-Aldrich)

Time-mated pregnant Wistar rats were treated exactly as above and similarly gavaged with 0 (control), 10, 100, or 500 mg/kg bodyweight/day of butylparaben dissolved in corn oil, before submitting to a range of tests, as previously described in detail (Boberg et al., 2016). As above, a subset of the male offspring was allowed to mature to PND90, when blood was collected for INSL3 assessment (see below).

2.1.3 | Epoxiconazole (technical quality; CAS no. 106325-08-8; VWR—Bie & Berntsen, Herlev, Denmark)

Time-mated pregnant Wistar rats were treated exactly as above and similarly gavaged with 0 (control), 15, or 30 mg/kg bodyweight/day of epoxiconazole dissolved in corn oil. As above, a subset of the male offspring was allowed to mature to PND90, when blood was collected for INSL3 assessment (see below).

2.1.4 | Fludioxonil (purity >99.9%, CAS no. 131341-86-1; Sigma-Aldrich)

Time-mated pregnant Wistar rats were treated as above and similarly gavaged with 0 (control), 20, 60, or 180 mg/kg bodyweight/day of fludioxonil dissolved in corn oil, here from GD7 until PND16, before submitting to anatomical examination as described previously (Scholze et al., 2020). As above, a subset of the male offspring was allowed to mature to PND98, when blood was collected for INSL3 assessment (see below).

2.1.5 | Estrogenic and anti-androgenic mixtures

BPA and butylparaben in a mixture were investigated as above and combined to allow low and high dose estrogenic mixtures (Emix low: 0.25 mg/kg BPA/100 mg/kg butylparaben; Emix high: 0.5 mg/kg BPA/200 mg/kg butylparaben). Also, procymidone (purity >99.9%, CAS no. 32809-16-8; Sigma-Aldrich) and diethyl phthalate (DEHP; purity 99%, CAS no. 117-81-7; Merck, Darmstadt, Germany) were combined to create low and high dose anti-androgenic mixtures (Amix low: 10 mg/kg procymidone/30 mg/kg DEHP; Amix high: 20 mg/kg procymidone/60 mg/kg DEHP). Finally, a mixture of both estrogenic and anti-androgenic substances was made, combining the low and high concentration mixes as above (Totalmix low: 140.25 mg/kg total; Totalmix high: 280.5 mg/kg). Time-mated Wistar rats were gavaged once daily from GD7 to PND22 with 2 ml of the respective mixtures
in corn oil, as previously and subjected to a range of anatomical measurements (Christiansen et al., 2020). A subset of the male offspring was allowed to mature to PND 83–87, when animals were euthanised and blood collected for INSL3 assessment (see below).

None of the adult animals assessed for INSL3 had received any prior treatments additional to the EDC dosing likely to influence the INSL3 concentration in the blood. All animal experimentation was approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation, authorization number: 2012-15-2934-00089) and by the in-house Animal Welfare Committee of the National Food Institute at the Technical University of Denmark.

2.2 | INSL3 measurement

All sera were stored at −80°C for up to 10 years within the National Food Institute at the Technical University of Denmark and had been subject to maximally one freeze–thaw cycle before analysis. Previous control studies had shown that INSL3 in serum is very stable, showing no significant change after several years of storage at −80°C and with up to three separate freeze–thaw cycles (Ivell & Anand-Ivell, 2009, and unpublished). Sera were transported to the Nottingham laboratory on dry ice. INSL3 was measured in the serum from the adult rats in each treatment group using a well-validated time-resolved fluorescent immunoassay specific for rodent INSL3 (Anand-Ivell et al., 2009). All retrospective samples were measured together in a single batch, also including internal and external serum controls as well as standards; samples were measured as singlets, with a few additional replicates to validate assay reproducibility. For this assay, sample volume was 50 μl. The limit of quantification was 20 pg/ml and the midrange within-assay and between-assay coefficients of variation were <0.3% and <3% respectively. There was no cross-reactivity with any other insulin-like peptides.

2.3 | Statistical analysis

All presented data were analysed by one-way analysis of variance followed by Tukey’s multiple comparisons test, as well as by a post-test for linear trend (pTrend) using GraphPad Prism (version 9.2.0; GraphPad Software, San Diego, CA).

3 | RESULTS

All test substances used were applied at dosing regimens which were below the no observed adverse effect levels for maternal weight and

![Figure 2](image-url)
offspring survival, but which also led to small but significant alterations in anogenital distance and some pubertal and adult parameters, such as prostate weight, or mammary gland development (Boberg et al., 2016; Christiansen et al., 2014, 2020; Hass et al., 2012, 2016; Jacobsen et al., 2012; Mandrup et al., 2016; Overgaard et al., 2013; Scholze et al., 2020).

3.1 | Application of known estrogenic and antiandrogenic EDCs as single compounds

Although bisphenol A at the lowest applied dose showed a small, though non-significant increase in INSL3 at PND80, all higher concentrations showed no effect (Figure 2a). Similarly, for butylparaben, there was no effect on the PND90 INSL3 level at all concentrations applied (Figure 2b). Also, for the recognised anti-androgenic pesticides epoxynzaol and fludioxidil (Figure 2c,d) there was no effect on the adult INSL3 concentration (PND90 and PND98 respectively) for any tested dose of the pesticides.

3.2 | Application of mixtures of estrogenic and antiandrogenic EDCs

Butylparaben and bisphenol A were applied as high and low concentration estrogenic mixtures (Figure 3a). Both dosages led to a reduction in the circulating adult INSL3 concentration (pTrend <0.05), though only for the higher dosage was this individual decrease statistically significant. A similar low and high dosage mixture of the anti-androgenic EDCs procymidone and DEHP indicated no effect on circulating adult INSL3 concentration (Figure 3b). Nor was there any effect of a low or high dose mixture of all four EDC compounds (Totalmix; Figure 3c).

4 | DISCUSSION

Maternal exposures to potential EDCs in the environment have been linked to several aspects of decreasing male reproductive function, including timing and dynamics of puberty (Gore et al., 2015; Heng et al., 2012; Ivell, Heng, et al., 2013; Parent et al., 2015), sperm counts (Gray, 1998; Istvan et al., 2021), prostate weight (Gore et al., 2015), incidence of cryptorchidism (Gore et al., 2015), and sexual behaviour (Gore et al., 2015; Hass et al., 2012, 2016; Hunter et al., 2021; Jacobsen et al., 2012). Whilst such symptoms can be associated with anti-androgenic or estrogenic influence during development, to date there have been no observed associations in humans between early EDC exposure and adult circulating levels of testosterone. This is probably due to continued compensation of lower testosterone by increased LH from the HPG axis as well as by the high biological variance observed in measuring testosterone. In contrast, INSL3 is a constitutive biomarker representing Leydig cell functional capacity (the product of Leydig cell numbers and their differentiation status) and shows very little within-individual biological variance (Ivell & Anand-Ivell, 2009). Moreover, it appears to exhibit wide interindividual variance even in young people (Anand-Ivell et al., 2021).
suggesting that some early factor(s) may be influencing Leydig cell development and maturation. Adult Leydig cells undergo proliferation and differentiation during puberty from early resident stem cells, which probably persist from the foetus and are relatively inactive in early postnatal life. With the activation of the HPG axis, these proliferate and differentiate under the influence of LH and paracrine factors from the testis, such as desert hedgehog (Teerds & Huhtaniemi, 2015), to become progenitor, precursor, immature, and finally mature adult-type Leydig cells at the end of puberty, when all proliferation effectively ceases. In rats and humans, we have shown that this pubertal process can be monitored by measuring serum levels of INSL3 (Anand-Ivell et al., 2009; Johansen et al., 2014).

Using this biomarker, we have shown that brief maternal exposure in rats to either the anti-androgen dibutyl phthalate or to the potent oestrogen, diethylstilbestrol, while affecting the rate of Leydig cell proliferation and differentiation during puberty, had no impact on the final adult level of INSL3 (Ivell, Wade, & Anand-Ivell, 2013). A similar result was observed using the model system of adult rats treated with ethane dimethyl sulfonate that kills all mature Leydig cells and causes a new Leydig cell population to regenerate with similar kinetics to that taking place in puberty; EDC treatment in vivo of the resident Leydig stem cells, whilst altering the dynamics of Leydig cell recovery, had no effect on the finally attained INSL3 expression (Heng et al., 2012).

In the present study, using a range of anti-androgenic and estrogenic EDCs, with one exception, we see no effect of gestational EDC exposure on the finally attained INSL3 concentration in adult male offspring and hence on the adult Leydig cell capacity to make testosterone. The one exception was provided by the higher dose of a mixture of bisphenol A and butylparaben, both considered as xenoestrogens. Since high doses of the single compounds, as well as of the oestrogen diethylstilbestrol (Ivell, Heng, et al., 2013), failed to show any influence on adult INSL3 concentration, this could indicate some toxicokinetic interactions between the two EDCs used here, for instance the absorption, distribution, metabolism, or excretion of one compound might be affected by the other compound, or may cause the formation of (a) new metabolite(s) or a changed target concentration. Alternatively, there may be a synergistic or potentiating effect of the compounds. One or other of the two compounds, bisphenol A and butylparaben, are likely acting not only at conventional oestrogen receptors, but may also be targeting additional molecular pathways. Notably, we know from the ToxCast program performed at the US-EPA that bisphenol A is targeting very many receptors and has a broad EDC profile (Reif et al., 2010). The potentially involved pathways are evidently different from any being targeted by the anti-androgens used here, DEHP and procymidone, since these compounds have no effect on INSL3 concentration, a result supported by the previous study using dibutylphthalate (Ivell, Wade, & Anand-Ivell, 2013). It should be noted, however, that the anti-androgenic mixture was able to counteract the effects of the estrogenic mixture, since their addition to the latter led to no effect on the INSL3 concentration (Figure 2c).

Since the compounds used here are relatively short-lived in rats, any effects in the adult offspring of maternal exposure must be due to impacts on the Leydig stem cells persisting from the foetal testes into the adult testes, presumably influencing later Leydig cell proliferation and differentiation events by means of long-term epigenetic changes. The results of this study, whilst suggesting that exposure to individual EDC compounds has little influence, exposure to estrogenic mixtures may indeed be effective to explain some of the inter-individual variation in INSL3 observed in young and older men, though differences between rats and humans in their responses to EDCs need to be recognised (Johnson et al., 2012).

**AUTHOR CONTRIBUTIONS**

Richard Ivell helped conceive the study and wrote the first draft. Anne Marie Vinggaard was responsible for all animal experimentation and edited various drafts of the manuscript. Hiroaki Soyama supported the project with experiments and advice. Ravinder Anand-Ivell jointly conceived the study, supervised the measurement of INSL3, and edited the manuscript.

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**CONFLICT OF INTEREST**

The authors declare nothing to disclose.

**DATA AVAILABILITY STATEMENT**

The datasets generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

**ORCID**

Richard Ivell https://orcid.org/0000-0001-6513-2109

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