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# Effect of environmental and pharmaceutical exposures on fetal testis development and function: a systematic review of human experimental data

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**BACKGROUND:** Overall, the incidence of male reproductive disorders has increased in recent decades. Testicular development during fetal life is crucial for subsequent male reproductive function. Non-genomic factors such as environmental chemicals, pharmaceuticals and lifestyle have been proposed to impact on human fetal testicular development resulting in subsequent effects on male reproductive health. Whilst experimental studies using animal models have provided support for this hypothesis, more recently a number of experimental studies using human tissues and cells have begun to translate these findings to determine direct human relevance.

**OBJECTIVE AND RATIONALE:** The objective of this systematic review was to provide a comprehensive description of the evidence for effects of prenatal exposure(s) on human fetal testis development and function. We present the effects of environmental, pharmaceutical and lifestyle factors in experimental systems involving exposure of human fetal testis tissues and cells. Comparison is made with existing epidemiological data primarily derived from a recent meta-analysis.

**SEARCH METHODS:** For identification of experimental studies, PubMed and EMBASE were searched for articles published in English between 01/01/1966 and 13/07/2018 using search terms including 'endocrine disruptor', 'human', 'fetal', 'testis', 'germ cells', 'testosterone' and related search terms. Abstracts were screened for selection of full-text articles for further interrogation. Epidemiological studies involving exposure to the same agents were extracted from a recent systematic review and meta-analysis. Additional studies were identified through screening of bibliographies of full-texts of articles identified through the initial searches.

**OUTCOMES:** A total of 25 experimental studies and 44 epidemiological studies were included. Consistent effects of analgesic and phthalate exposure on human fetal germ cell development are demonstrated in experimental models, correlating with evidence from epidemiological studies and animal models. Furthermore, analgesic-induced reduction in fetal testosterone production, which predisposes to the development of male reproductive disorders, has been reported in studies involving human tissues, which also supports data from animal and epidemiological studies. However, whilst reduced testosterone production has been demonstrated in animal studies following exposure(s) to a variety of environmental chemicals including phthalates and bisphenol A, these effects are not reproduced in experimental approaches using human fetal testis tissues.

**WIDER IMPLICATIONS:** Direct experimental evidence for effects of prenatal exposure(s) on human fetal testis development and function exists. However, for many exposures the data is limited. The increasing use of human-relevant models systems in which to determine the effects of environmental exposure(s) (including mixed exposures) on development and function of human tissues should form an important part of the process for assessment of such exposures by regulatory bodies to take account of animal–human differences in susceptibility.

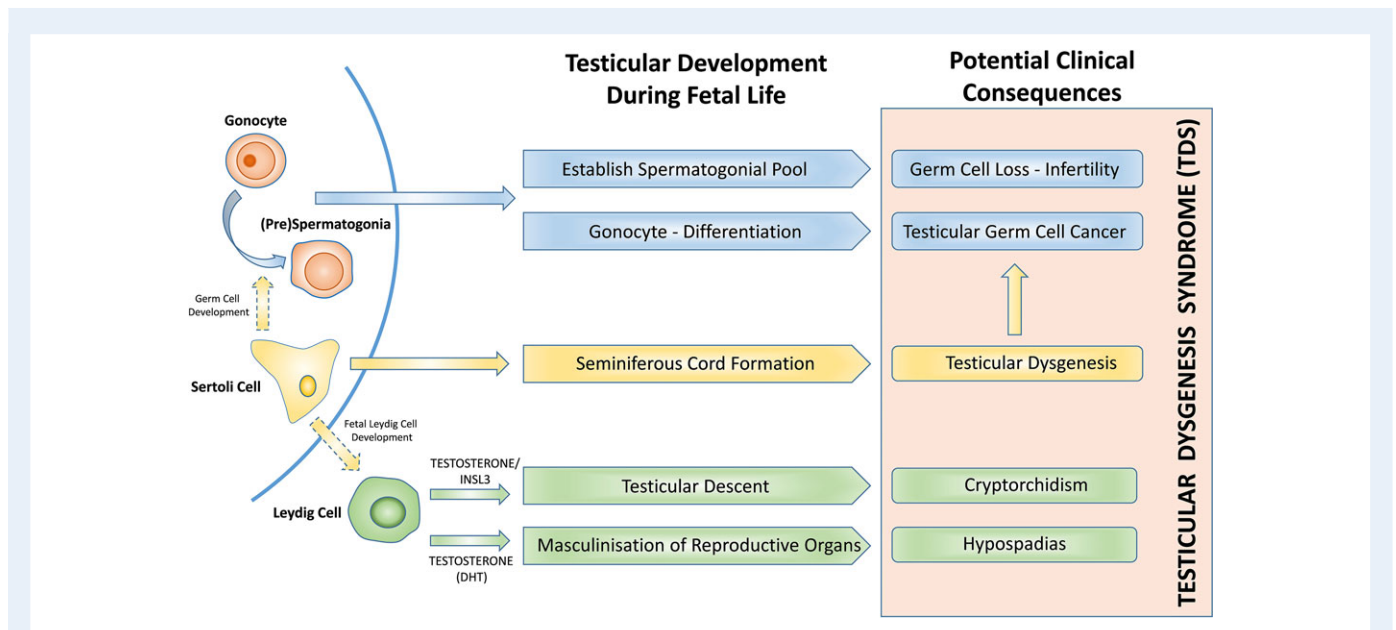
**Key words:** human fetus / testis / endocrine disruptor / environmental chemical / pharmaceutical / testosterone / germ cell / Leydig cell / Sertoli cell / steroidogenesis

## Introduction

Development of the male reproductive system and its subsequent function is impacted by events that occur *in utero*. Perturbations in testicular development or function during fetal life may result in male reproductive disorders that present postnatally (van den Driesche *et al.*, 2017). This includes anatomical abnormalities identified at birth, such as cryptorchidism and hypospadias, or disorders presenting in adulthood, including testicular cancer or infertility (Sharpe and Skakkebaek, 2008). These associated disorders are collectively referred to as the testicular dysgenesis syndrome (TDS). The development of TDS has been shown in rats to be influenced by a reduction in androgen production or action during a key period of fetal life, known as the masculinization programming window (MPW) (Welsh *et al.*, 2008; van den Driesche *et al.*, 2017). The increasing incidence of TDS disorders over recent decades, highlights the potential importance of environmental impacts in their etiology (Skakkebaek *et al.*, 2016). Environmental factors that have been proposed to affect fetal testis development and predispose to TDS disorders include environmental chemicals (e.g. plasticizers and pesticides), pharmaceuticals (e.g. analgesics, metformin and diethylstilboestrol) and lifestyle factors (e.g. diet, alcohol and smoking) (Habert *et al.*, 2014; Kilcoyne and Mitchell, 2017).

In order to understand how *in-utero* exposures might disrupt fetal development and result in postnatal testicular disorders, it is important to consider the normal development of the germ and somatic cell populations in the human fetal testis (Fig. 1). During fetal life, germ cells migrate into the developing gonad (4–5 weeks in human) where they undergo differentiation from gonocytes to spermatogonia. This transition takes place during fetal and early postnatal life and involves the loss of expression of pluripotency factors (e.g. POU5F1) and gain of differentiated germ cell-specific protein expression (e.g. MAGEA4) (Mitchell *et al.*, 2008). Failure of gonocyte differentiation can result in the development of pre-malignant germ cell neoplasia *in-situ* cells (GCNIS), which results in the development of testicular germ cell cancer (TGCC) in adulthood (Rajpert-De Meyts *et al.*, 2016) whilst loss of germ cells as a result of *in-utero* events can also potentially impact future fertility.

Germ cell development during fetal life is supported by somatic cells that form the germ-stem cell niche. Sertoli cells surround the gonocytes, forming seminiferous cords, at ~6–7 gestational weeks (GW) in the human (O'Shaughnessy and Fowler, 2011; Heeren *et al.*, 2015). Sertoli cells are fundamental for germ cell development (Bitgood *et al.*, 1996), regression of the Müllerian ducts (AMH, anti-Müllerian hormone) and initiation of fetal Leydig cell differentiation (Pierucci-Alves *et al.*, 2001; Yao *et al.*, 2002; Griswold and Behringer, 2009).



**Figure 1** Testicular development and function during fetal life and reproductive disorders associated with testicular dysgenesis syndrome. DHT, dihydrotestosterone.

Fetal Leydig cells are present in the interstitium of the testis from six GWs in humans and are responsible for the production of hormones involved in testicular descent. *Insl3* is involved in the transabdominal phase of testicular descent, whilst testosterone is required to enable the testis to traverse the inguinal canal (Hughes and Acerini, 2008). Leydig cell-derived testosterone is also converted to dihydrotestosterone (DHT) in peripheral tissues for masculinization of the fetus, which includes development of external genitalia. Therefore, perturbations to the function of fetal Leydig cells can predispose to the development of male reproductive disorders including TDS and some disorders of sex development (DSD) (van den Driesche et al., 2017).

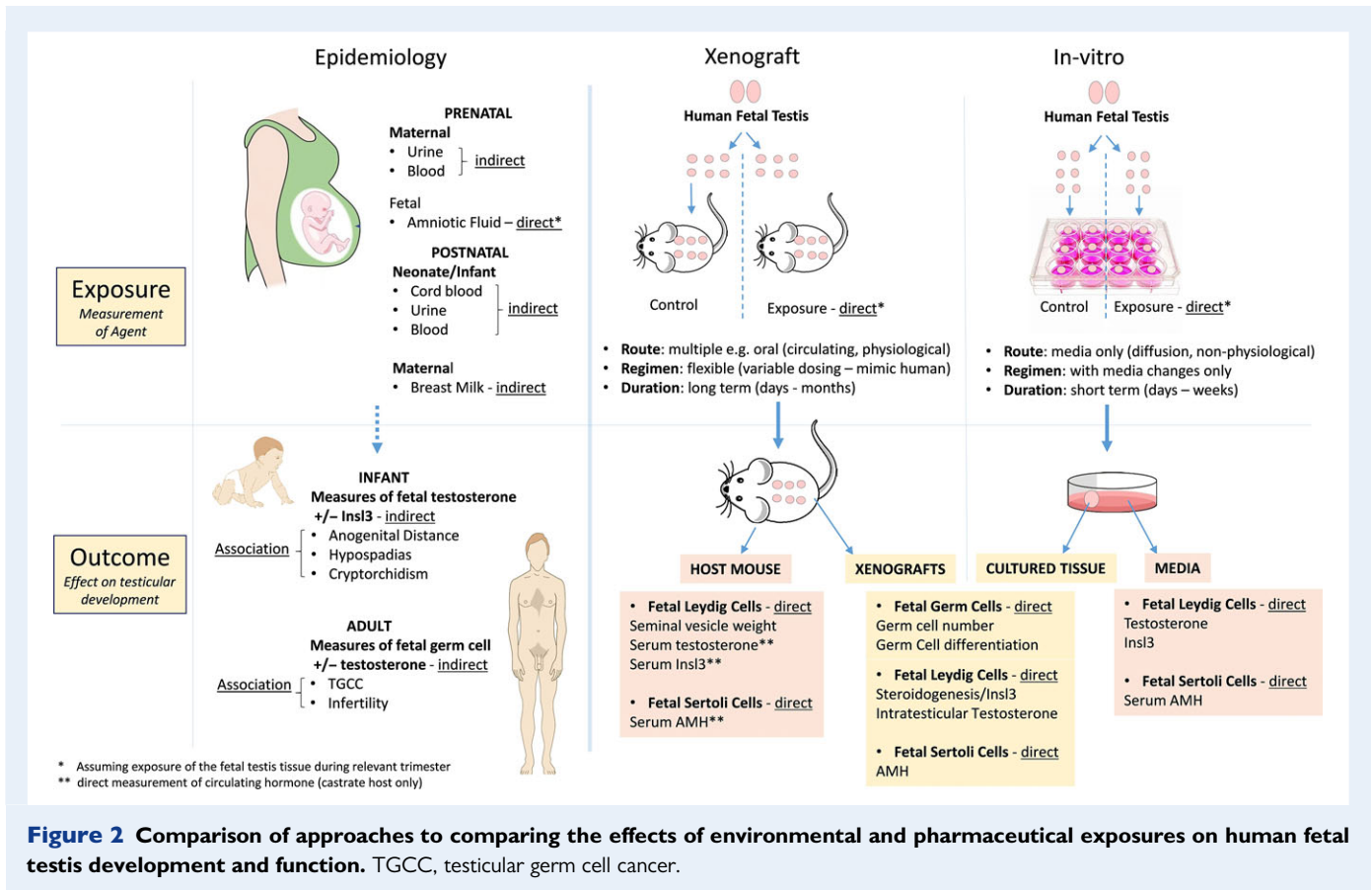
The majority of experimental studies investigating the effects of environmental exposures on fetal testis development and function involve rodents. These studies have provided a large amount of valuable information highlighting the potential for effects of in-utero exposure to a wide variety of environmental chemicals on male reproductive development. However, there are important differences in fetal testicular development between rodent and human in terms of germ cell development (McKinnell et al., 2013) and steroidogenesis (Scott et al., 2009). Furthermore, the exposures used for these studies may not reflect the levels of exposure that are directly relevant to humans. Assessment of experimental studies using experimental animal models must take account of the variations in model systems (*in-vitro* versus *in-vivo*), exposure regimen and drug metabolism, whilst also accounting for the impact of species differences for each of these parameters (Kilcoyne and Mitchell, 2017). As a result, animal studies often report findings based on relative exposures which exceed human-relevant exposures, often by several orders of magnitude.

In order to gain information on the potential for *in-utero* environmental exposures to impact development of male reproductive disorders in humans, epidemiological studies can be employed. A recent systematic review has described the epidemiological evidence for

associations between prenatal exposures and male reproductive disorders in humans (Bonde et al., 2016). A number of important considerations must be applied when assessing epidemiological evidence for such associations. These include, but are not limited to (i) the relevance and/or size of the population group, (ii) measurement of the exposure (direct/indirect) (iii) biological plausibility for the exposure alone causing the effect and elimination of potential confounders (reviewed in Foster et al., 2017) (Fig. 2 and Table I).

While human epidemiological and animal experimental studies are extremely informative, there remains a large gap in our understanding of how specific environmental exposures may directly affect the human fetal testis. Therefore, development of model systems using human fetal tissues and human-relevant doses can bridge the gap between direct evidence from animal experimental models and indirect evidence based on epidemiological data. A number of recent studies have utilized *in-vitro* or xenograft approaches using human fetal testis tissues to determine the effect of environmental and pharmaceutical exposures. As with epidemiological studies, there are a number of key considerations when interpreting the results of these studies relating to model system, exposure regimen and biological relevance of the measured outcome (Fig. 2 and Table I).

A comprehensive review of the experimental evidence for effects of environmental exposures on fetal testicular development and function using human cells or tissues has not previously been reported. This systematic review will detail the experimental evidence for impacts of environmental chemicals, pharmaceuticals and lifestyle factors on human fetal testis development and function. For each class of exposure for which human experimental evidence exists, we will first summarize the findings of animal studies and then provide a critical review of the epidemiological evidence. We will then describe in detail the evidence to support or refute these findings based on experimental models using human fetal testis tissues.



## Methods

The study was designed as a systematic review of the published literature relating to the effects of *in-utero* exposures on human fetal testis development and function in experimental models. The study followed the principles of the PRISMA guidelines for reporting systematic reviews (Moher et al., 2009). The protocol for searching and assessing the literature was determined prior to the start of the literature search. It is not currently possible to register laboratory experimental studies with PROSPERO.

### Information sources

We performed an online search of PubMed and EMBASE (13/07/2018) to identify all experimental studies relating to testicular effects of fetal exposures to environmental, pharmaceutical and lifestyle factors, limited to studies utilizing human fetal tissues or cells. For identification of relevant epidemiological studies, we included the publications identified in a recent systematic review and meta-analysis of associations between prenatal exposures and male reproductive disorders (Bonde et al., 2016). Additional epidemiological studies were identified from the reference lists of the screened articles.

### Eligibility for inclusion

We performed a systematic search of original publications according to the following criteria for inclusion: English language articles published between 01/01/1966 and 13/07/2018; experimental studies on exposure of human fetal testis tissue or cells to a clearly defined environmental, pharmaceutical or lifestyle factor; and outcomes including effects on

testicular hormone production (e.g. testosterone, *InsI3*, AMH), germ or somatic (Sertoli, Leydig) cell development.

### Exclusion criteria

We excluded studies according to the following criteria: exposure of tissues or cells representative of a period other than fetal life; exposure of non-testicular tissues or cells; outcomes other than those described above; and review articles.

### Search and study selection

We searched the databases using a combination of medical subject headings and generic terms relating to effects of exposures on human fetal testis development (Supplementary Table S1). We identified 3229 hits. Both authors screened the titles and/or abstracts independently to assess eligibility. Full texts were requested for studies that included in the abstract the use of human fetal testis tissue or cells and the effect of exposure to environmental, pharmaceutical or lifestyle factors. Full texts for 40 studies were obtained and a total of 25 publications were included in the review of experimental evidence (Supplementary Table S2; Fig. 3). A total of 15 studies were excluded (Supplementary Table S3). A further 44 publications were included in the review of epidemiological evidence (Supplementary Table S4).

### Summary measures

Studies included *in-vitro* and *ex-vivo* (xenograft) approaches and results were assessed primarily for effects on testosterone secretion and on germ cell number (both expressed as % change compared to vehicle

**Table 1** Key considerations for the assessment of human studies using epidemiological, or experimental (xenograft or *in-vitro*) approaches.

	Epidemiology	Xenograft	In vitro
<b>Population</b>	Potential limitation—population/tissue used may be unrepresentative of target population/tissue <ul style="list-style-type: none"> <li>Is the population studied representative of the target population (e.g. pregnant women)?</li> </ul>	unrepresentative of target population/tissue <ul style="list-style-type: none"> <li>Is the tissue representative of the target population (e.g. fetal tissue)?</li> </ul>	unrepresentative of target population/tissue <ul style="list-style-type: none"> <li>Is the tissue representative of the target population (e.g. fetal tissue or cells)?</li> </ul>
<b>Agent</b>	Potential limitation—agent under investigation may be not be representative of the exposure under investigation (e.g. metabolite) or there may be additional confounding agents <ul style="list-style-type: none"> <li>Is the relevant agent being measured in the population?</li> <li>Is there potential for confounding by other/similar exposures?</li> </ul>	not be representative of the exposure under investigation (e.g. metabolite) or there may be additional confounding agents <ul style="list-style-type: none"> <li>Is the investigated agent relevant to potential human exposure?</li> <li>Is it metabolized in the host animal to an active/inactive form (e.g. DBP–MBP)?</li> </ul>	not be representative of the exposure under investigation (e.g. metabolite) or there may be additional confounding agents <ul style="list-style-type: none"> <li>Is the investigated agent relevant to potential human exposure?</li> <li>Is the active agent or metabolite added to the medium?</li> </ul>
<b>Exposure</b>	Potential limitation—assessment of exposure in epidemiological studies or regimen used in experimental studies may not accurately reflect true human exposure <ul style="list-style-type: none"> <li>Is the exposure measured in the subjects (e.g. indirect measurement of fetal exposure through maternal serum/urine) or indirectly assessed (e.g. self-report) and does this accurately capture actual fetal exposure?</li> </ul>	assessment of exposure in epidemiological studies or regimen used in experimental studies may not accurately reflect true human exposure <ul style="list-style-type: none"> <li>Is the dose, frequency, duration and route of exposure representative of human exposure (e.g. pharmaceuticals)?</li> </ul>	assessment of exposure in epidemiological studies or regimen used in experimental studies may not accurately reflect true human exposure <ul style="list-style-type: none"> <li>Is the concentration of agent placed in the media representative of human levels (e.g. maternal serum, amniotic fluid, fetal serum) and/or human dosing regimen?</li> </ul>
<b>Timing</b>	Potential limitation—timing of assessment of exposure in epidemiological studies or developmental stage of tissue used in experimental studies may not accurately reflect the relevant stage <ul style="list-style-type: none"> <li>Is exposure measured at the appropriate developmental stage (e.g. trimester of fetal life or MPW)?</li> <li>Does timing and frequency of measurement accurately reflect internal exposure?</li> </ul>	timing of assessment of exposure in epidemiological studies or developmental stage of tissue used in experimental studies may not accurately reflect the relevant stage <ul style="list-style-type: none"> <li>Is the transplanted tissue at the same developmental stage (e.g. trimester of fetal life or MPW)?</li> <li>Does the experimental system maintain tissue development and function?</li> </ul>	timing of assessment of exposure in epidemiological studies or developmental stage of tissue used in experimental studies may not accurately reflect the relevant stage <ul style="list-style-type: none"> <li>Is the tissue cultured at the same developmental stage (e.g. trimester of fetal life or MPW)?</li> <li>Does the experimental system maintain tissue development and function?</li> </ul>
<b>Effect</b>	Potential limitation—the effects of exposure may be measured directly or through association and direct effects of exposure may not result in clinical consequences <ul style="list-style-type: none"> <li>Is there a direct clinical association with fetal exposure (e.g. cryptorchidism) or is it a surrogate marker for clinical effects (e.g. AGD)?</li> <li>What is the magnitude of effect and is it statistically significant?</li> <li>Is there a plausible mechanism?</li> </ul>	the effects of exposure may be measured directly or through association and direct effects of exposure may not result in clinical consequences <ul style="list-style-type: none"> <li>Is the effect clinically relevant (e.g. potential for reduced testosterone to induce cryptorchidism)?</li> <li>What is the magnitude of effect and is it statistically significant?</li> <li>Has the mechanism for the effect been defined?</li> </ul>	the effects of exposure may be measured directly or through association and direct effects of exposure may not result in clinical consequences <ul style="list-style-type: none"> <li>Is the effect relevant to <i>in-vivo</i> situation?</li> <li>Is the effect clinically relevant (e.g. potential for reduced testosterone to induce cryptorchidism)?</li> <li>What is the magnitude of effect and is it statistically significant?</li> <li>Has the mechanism for the effect been defined?</li> </ul>

control). Effects on additional testicular hormones, AMH (produced by SC) and *Insl3* (produced by LC) are also reported.

## Results

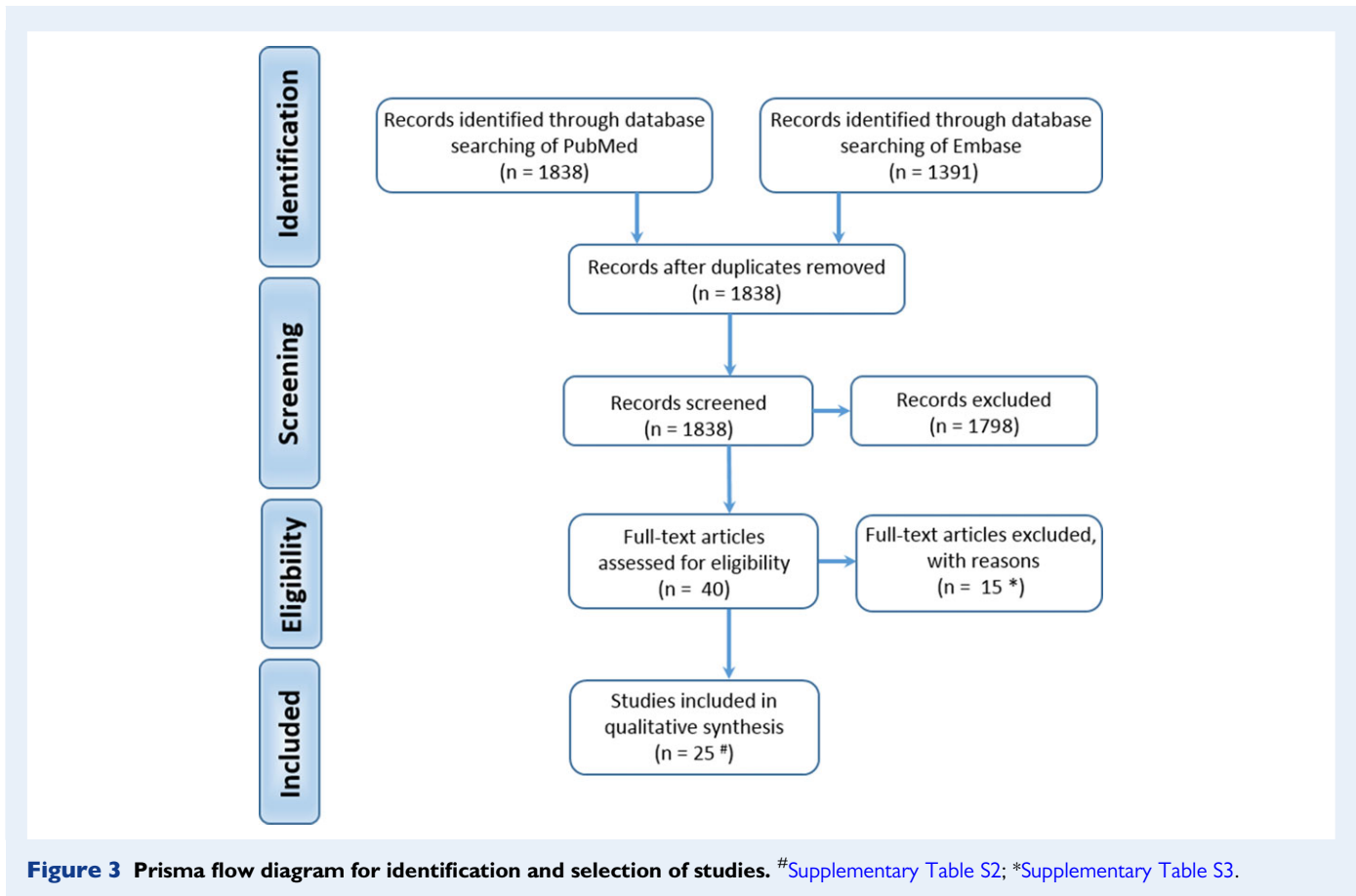
The distribution of studies based on exposure type and year of publication is shown in Fig. 4. The majority of the studies were published from 2007 to 2018. The earlier studies primarily investigated phthalates, pesticides and smoking, whilst more recent studies have mainly focused on bisphenols and analgesics.

## Environmental chemicals

### Phthalates

Phthalates are a class of industrial chemicals used mainly to soften polyvinyl chloride-based products and are found in a wide array of

general plastic products. Exposure to phthalates may occur via inhalation, ingestion or direct contact with items including packaging, oils, food storage and personal care products. Phthalates are not stored in the body but are instead rapidly metabolized into monoesters with a urinary excretion half-life of <24 h (Koch *et al.*, 2012). Commonly used plasticisers over recent decades include di-(2-ethylhexyl) phthalate (DEHP) which is converted to its monoester metabolite, mono-2-ethylhexyl phthalate (MEHP) and di-*n*-butyl phthalate (DBP), which is metabolized to mono-*n*-butyl phthalate MBP. In 2015, DEHP was banned from general use under EU law under the REACH restriction (Registration, Evaluation, Authorization and Restriction of Chemical Substances) and has been replaced by di-isononyl phthalate (DiNP), and more recently di-isononyl cyclohexane-1,2-dicarboxylate (DINCH), both of which are deemed less biologically active (Gray *et al.*, 2000). Recent biomonitoring data has shown that DiNP metabolites measured in human urinary samples are increasing in both America (Silva *et al.*, 2013) and Europe (Goen *et al.*, 2011).



### Animal studies

The majority of published experimental studies investigating the effects of *in-utero* exposure to certain phthalates (primarily DEHP or DBP) have used rodent models and have demonstrated a disruption of normal fetal testis development and subsequent development of male reproductive disorders, resulting from decreased fetal testosterone production in male offspring (Parks *et al.*, 2000; Mylchreest *et al.*, 2002; Habert *et al.*, 2014; Kilcoyne *et al.*, 2014; Fisher *et al.*, 2016). Moreover, effects primarily result from exposure during the MPV (Welsh *et al.*, 2008; Kilcoyne *et al.*, 2014; van den Driesche *et al.*, 2017) which is believed to equate to ~8–14 weeks gestation in humans (Scott *et al.*, 2009; Fowler *et al.*, 2011).

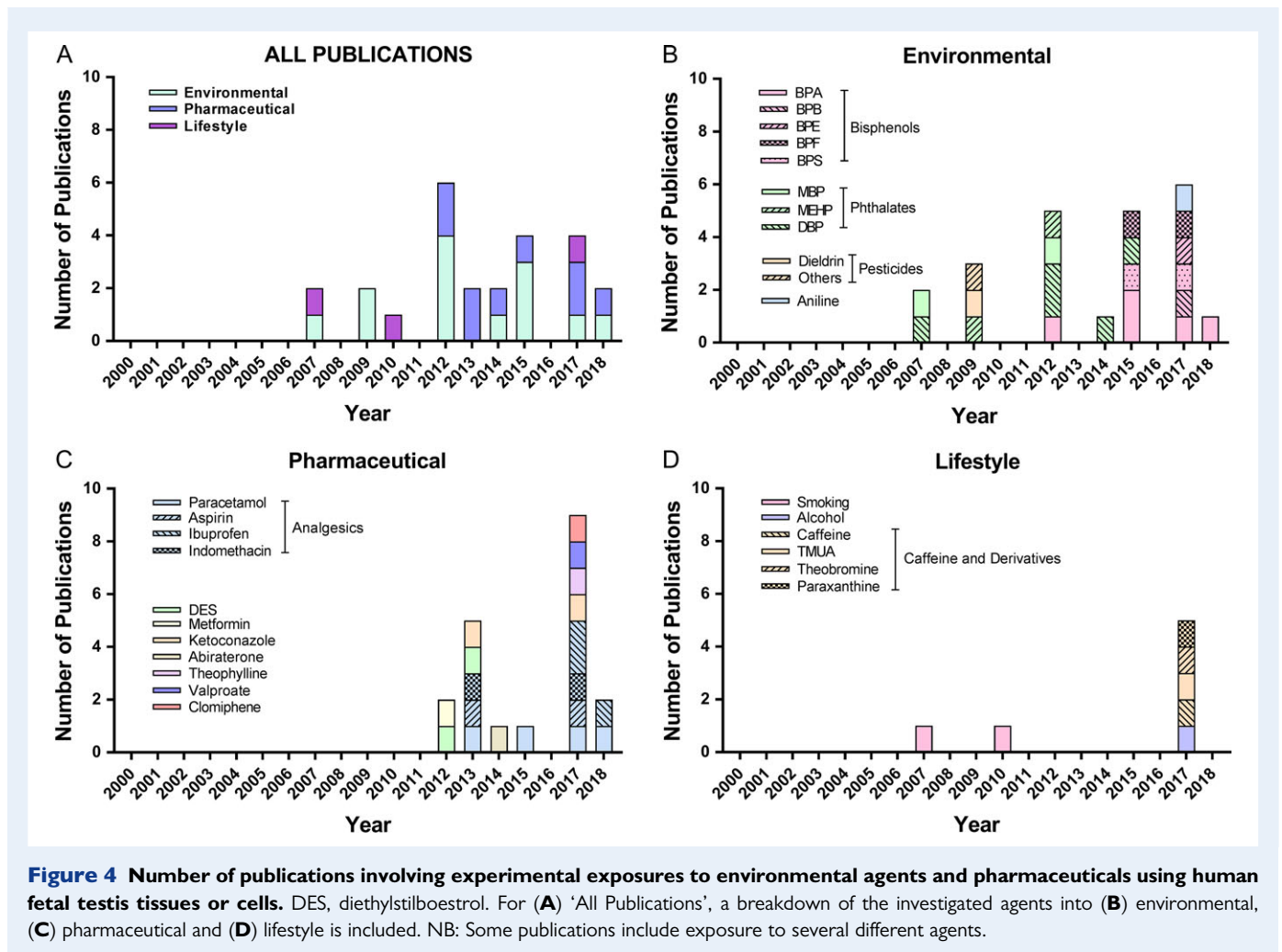
### Epidemiology - DBP/MBP

In humans, DBP is rapidly metabolized (primarily to MBP) and excreted (Koch *et al.*, 2012). Therefore measuring the effects of phthalate exposure on fetuses in pregnant women is challenging. Despite this, urinary concentrations of several phthalate metabolites (including MBP) have been shown to be positively correlated with the incidence of male developmental abnormalities in the newborn, such as cryptorchidism and shortened anogenital distance (AGD; a biological readout of fetal testosterone exposure) (Marsee *et al.*, 2006). Further indirect evidence for effects of maternal MBP/DBP exposure on steroidogenesis in the human fetal testis has also been reported in retrospective association studies (Marsee *et al.*, 2006; Swan, 2008). These studies demonstrate a negative correlation between maternal

urinary MBP concentration and AGD in males, although the changes are minor. However, a separate small prospective study failed to find any correlation (Huang *et al.*, 2009). Furthermore, whilst an association between urinary MBP during second and third trimester and AGD has been demonstrated (Swan *et al.*, 2005), a similar study has shown no such association when urinary MBP is measured during the first trimester (Swan *et al.*, 2015).

Several phthalate monoesters have been detected in maternal breast milk over a large concentration range (1.5–1 410 µg/L) and these have been correlated with infant hormone levels (Main *et al.*, 2006). Despite the reported association between phthalates (including MBP) in maternal breast milk and altered testosterone levels of infant males (Main *et al.*, 2006), a recent study did not detect significant associations between MBP concentrations in breast milk and either testosterone or INSL3 in newborns (Chevalier *et al.*, 2015). Importantly, this study found no association between MBP in breast milk and the presence of cryptorchidism, consistent with maternal MBP concentrations postnatally not being associated with human fetal testosterone production.

Phthalates, including DBP, have also been used in the enteric coating of several commonly used medications including proton pump inhibitors (e.g. omeprazole) and anti-epileptics (e.g. valproate, carbamazepine) (Kelley *et al.*, 2012; Jamieson and McCully, 2015). This can result in an increase in urinary phthalate levels in the region of 50–100 times, compared with the general population (Hernandez-Diaz *et al.*, 2009; Seckin *et al.*, 2009; Hait *et al.*, 2014). As a result, it



may be expected that the regular use of these medications during pregnancy may be associated with an increased risk of male reproductive disorders in the offspring of exposed mothers. DBP has been used in the coating of mesalazine (a 5-aminosalicylic acid drug), an anti-inflammatory agent used to treat inflammatory bowel disease (IBD). Several studies have reported the pregnancy outcomes for women taking 5-ASA drugs during pregnancy and a meta-analysis of 2200 pregnant women with IBD has reported no increased incidence of congenital abnormalities as a result of exposure to mesalazine during pregnancy ( $n = 642$ ), in comparison to non-exposed mothers with IBD ( $n = 1158$ ) (Rahimi *et al.*, 2008). It is important to point out that a limitation of the studies included in the meta-analysis is that they were not specifically designed to identify associations with male reproductive disorders; however, it is reassuring to note that none of the studies to date have reported an increase in the development of these disorders. Prospective studies designed to answer this specific question would be desirable, although conducting such studies will be challenging due to the widespread removal of DBP from these medications.

#### Epidemiology - DEHP/MEHP

DEHP has been detected in fetal cord blood samples (of  $1.1 \times 10^{-8}$  M) and cord blood of newborns (Latini *et al.*, 2003; Lin *et al.*,

2011), demonstrating that fetal exposure to DEHP can begin from an early stage. MEHP, measured in the urine of pregnant women ( $n = 111$ ; 9–40 weeks gestation) has been associated with a significant reduction in AGD in male offspring (Suzuki *et al.*, 2012). DEHP metabolites in pregnant women during the first trimester have been shown to be significantly and inversely related with AGD in male newborns ( $n = 366$ ) (Swan *et al.*, 2015), whereas no such association was identified for maternal urinary MEHP in second or third trimesters (Swan *et al.*, 2005). A similar inverse relationship between the anoscrotal distance (AGDAs) in newborn males ( $n = 168$ ) and maternal DEHP metabolite concentrations specifically during the first trimester, has also been described (Martino-Andrade *et al.*, 2016). These findings are in contrast to a Swedish study which found no significant association between first trimester concentrations of DEHP metabolites in mothers and AGDAs in 196 males aged 21 months (Bornehag *et al.*, 2015). Interestingly, this is despite the authors reporting a significant association between AGDAs (4% reduction) and some DiNP metabolites (Bornehag *et al.*, 2015).

The timing of measurement of exposure may be important given that whilst an association between first trimester maternal urine concentrations of DEHP and AGDAs have been described, no association was found for second or third trimester DEHP concentrations (Martino-Andrade *et al.*, 2016). Another recent study measured



**Table II** Summary of experimental studies investigating effects of phthalate exposure in human fetal testis tissue.

Model and regimen		Results										
Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	INSL3	Germ cells	Study	Comments
DBP	Xenograft	14–20	500 mg/kg/d	Oral	4 or 21 days	hCG						van den Dreische (2015b)
	Xenograft	14–20	500 mg/kg/d	Oral	4 or 21 days	hCG	↔					Mitchell et al. (2012)
	Xenograft	16–22	500 mg/kg/d	Oral	14 days	hCG	↔					Spade et al. (2014)
	Xenograft	10–24	100–500 mg/kg/d	Oral	24–72 h	Nil			↔			Heger et al. (2012)
MBP	<i>In-vitro</i>	15–20	10 <sup>-3</sup> M	Media	24–48 h	Basal/hCG/22 R-OH	↔					Hallmark et al. (2007)
	Xenograft	14–20	500 mg/kg/d	Oral	4 or 21 days	hCG	↔					Mitchell et al. (2012)
	<i>In-vitro</i>	15–20	10 <sup>-3</sup> M	Media	24–48 h	Basal/hCG/22 R-OH	↔					Hallmark et al. (2007)
MEHP	<i>In-vitro</i>	7–12	10 <sup>-4</sup> M	Media	72 h	LH	↔					Lambrot et al. (2009)
	<i>In-vitro</i>	7–12	10 <sup>-5</sup> M	Media	72 h	LH	↔					Lambrot et al. (2009)
	<i>In-vitro</i>	7–12	10 <sup>-6</sup> M	Media	72 h	LH	↔					Lambrot et al. (2009)
	<i>In-vitro</i>	7–12	10 <sup>-5</sup> M	Media	72 h	Nil						Muczynski and Cravedi et al. (2012)

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green. MNG, multinuclear gonocytes; GC, germ cells; 22 R-OH, 22 R-hydroxycholesterol; SV, seminal vesicle.

urinary phthalates (including DEHP metabolites) concentration in pregnant women during late second and third trimester and reported no association between prenatal phthalate exposure and AGD in male offspring ( $n = 273$ ) at 3 months of age (Jensen et al., 2016). Furthermore, a study involving measurement of 5cx-MEPP (a DEHP metabolite) in amniotic fluid during the second trimester found no association with cryptorchidism or hypospadias (Jensen et al., 2015).

#### Experimental evidence from human studies

Investigating phthalate exposure directly using human fetal testis is challenging; however, several studies have utilized *in-vitro* cell or tissue culture and/or *in-vivo* xenograft models to explore potential human effects (Table II).

**DBP/MBP - Hormones.** Effects of exposure to DBP/MBP have been investigated in human fetal testis tissues using *in-vitro* or xenograft approaches. *In-vitro* exposure of second trimester human fetal testis explants to MBP had no effect on (basal or hCG stimulated) testosterone production after 48 h (Hallmark et al., 2007), whilst the same study demonstrated a reduction in testosterone production in fetal rat testis explants after hCG stimulation (Hallmark et al., 2007). Use of an *ex-vivo* xenograft system may represent a more physiological approach to investigating potential effects of phthalates on the human fetal testis. Despite several studies xenografting first and/or second trimester human fetal testes into different host species, and at different locations (rat; renal subcapsular space) (Heger et al., 2012; Spade et al., 2014) (mouse; subcutaneous tissue) (Mitchell et al., 2012), no effects on steroidogenic gene expression, seminal vesicle weight or circulating testosterone levels were identified at levels of exposure equivalent to those used in rat pregnancy studies, which are far in excess of human exposure. This included varying duration of DBP exposure for 4 or 21 days (Mitchell et al., 2012) or for 14 days (Heger et al., 2012; Spade et al., 2014). Furthermore, substituting MBP instead of DBP for 21 days also had no effect on testosterone production from xenografts (Mitchell et al., 2012). Exposure of human fetal testis xenografts to DBP at varying doses (100, 250, 500 mg/kg) over a more limited time-window (24, 48, 72 h) did not alter mRNA expression of Leydig cell genes including several steroidogenic genes and the hormone Insl3 (produced by Leydig cells and involved in testicular descent) (Heger et al., 2012).

Further support for the lack of effect of exposure to DBP/MBP on testosterone production and development of male reproductive disorders in primates is evident following *in-utero* exposure of marmoset monkeys to MBP (McKinnell et al., 2009). Pregnant marmosets ( $n = 5–6$ ) were dosed with 500 mg/kg MBP from ~7 to 15 weeks gestation and testicular and male reproductive effects were assessed in the neonate or adult. No effects on testicular morphology or germ cell number were identified at birth or adulthood following *in-utero* MBP exposure and testosterone levels at birth were unaffected. Importantly, there were no cases of hypospadias, cryptorchidism, impaired spermatogenesis or focal testicular dysgenesis in any of the exposed animals (McKinnell et al., 2009).

**DBP/MBP - Germ cells.** Despite the lack of effect of DBP exposure on testosterone production or steroidogenic gene expression in the human fetal testis, abnormal morphology of germ cells within the seminiferous cords has been described following DBP exposure in

the xenograft model, including an increase in the number of multi-nucleated gonocytes (MNGs) (Heger *et al.*, 2012). Furthermore, DBP-exposure (500 mg/kg) for 21 days in a similar xenograft model reduced the number of gonocytes and induced a higher proportion of MNGs in human fetal testes (van den Driesche *et al.*, 2015b). These effects of DBP exposure, namely reduced gonocyte number and increased MNGs, is in keeping with findings from rodent studies using comparable DBP exposure (Ferrara *et al.*, 2006; Jobling *et al.*, 2011; van den Driesche *et al.*, 2015b).

**DEHP/MEHP - Hormones.** *In-vitro* exposure of human first trimester testis to a range of doses of MEHP ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  M) using an organotypic culture system resulted in unchanged testosterone (basal or LH-stimulated) production compared to control after 72 h of culture (Lambrot *et al.*, 2009). In keeping with a lack of effect on Leydig cell function, there was also no change in gene expression of steroidogenic enzymes or *Ins3* (Lambrot *et al.*, 2009). These results are in keeping with similar *in-vitro* (Hallmark *et al.*, 2007) and xenograft (Heger *et al.*, 2012; Mitchell *et al.*, 2012; Spade *et al.*, 2014) studies involving DBP/MBP exposure of human fetal testis tissue.

In addition to assessing effects of MEHP exposure on testosterone production in the human fetal testis, exposure of first trimester testis (7–12 weeks gestation) to  $10^{-4}$  M MEHP for 72 h resulted in a reduction in AMH mRNA, although Sertoli cell-derived AMH protein expression was not affected (Lambrot *et al.*, 2009).

**DEHP/MEHP - Germ cells.** Exposure of human first trimester testis tissue to MEHP ( $10^{-5}$  M) *in-vitro* via an organotypic culture system resulted in an approximately 40–50% increase in apoptotic gonocytes compared to control testis tissue (Muczynski and Cravedi *et al.*, 2012). *In-vitro* studies using mouse fetal testis tissue conducted in parallel, demonstrated similar effects with a 30% reduction in gonocyte number and a 4-fold increase in the rate of apoptotic gonocytes (Muczynski and Lecureuil *et al.*, 2012). These results support the findings of a previous study in first trimester human fetal testes exposed to a range of doses of MEHP ( $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  M) for 72 h. Using a similar *in-vitro* system, MEHP-exposure resulted in a 40% reduction in germ cell number via increased apoptosis (without altering GC proliferation) at both  $10^{-4}$  and  $10^{-5}$  M MEHP under basal conditions, whilst  $10^{-5}$  M MEHP resulted in a reduction in gonocyte number under basal and LH-stimulated conditions (Lambrot *et al.*, 2009). As described for DBP/MBP, the germ cell effects were similar between MEHP-exposed human and mouse fetal testes, as gonocyte number was also significantly reduced in mouse fetal testes after MEHP exposure, via an increase in apoptotic gonocytes (Chauvigne *et al.*, 2009; Lehraiki *et al.*, 2009). However, *in-vitro* organ culture of fetal rat testes (at embryonic Day 13 or 18) with MEHP showed no effect on the mitotically quiescent germ cells (Li and Kim, 2003).

#### Summary—phthalates

Exposure to DBP/DEHP has consistently been shown to reduce fetal testosterone production resulting in a high frequency of TDS disorders in rats, whereas epidemiological studies have reported inconsistent associations between maternal phthalate exposures and indirect measures of fetal testosterone production, namely AGD, and no studies have reported an association between phthalate exposure and either cryptorchidism or hypospadias. In addition, no effects of

phthalates on testosterone production have been described in experimental models using *in-vitro* culture or xenografting of human fetal testis tissues and a single *in-vivo* study in a non-human primate. This is despite administration of phthalate doses that far exceed environmental/human exposure levels and includes exposure during the proposed MPW. Furthermore, the *in-vivo* study involving *in-utero* exposure of marmoset monkeys failed to demonstrate the development of male TDS disorders that result from a reduction in testosterone. Taken together the results indicate that exposure to environmental levels of DBP/DEHP are unlikely to result in effects on fetal testosterone production in humans. Interestingly, whilst no effects of phthalate exposure have been demonstrated in human fetal testes, anti-androgenic effects have been shown to occur in adult human testis following *in-vitro* culture, suggesting that the effect of exposure is dependent on the developmental stage of the testis (Albert and Jegou, 2014).

However, for germ cells effects, the results of experimental studies in rodents and human fetal tissues are more consistent, with a reduction in gonocyte number and an increase in multi-nucleated gonocytes being reported in several studies following phthalate exposure. The implications of these findings in terms of future fertility are uncertain.

## Bisphenols

Bisphenols are synthetic chemicals, widely used in the manufacture of hard plastic products. Bisphenol A (BPA) is also a component of epoxy-resins, used as the inner coating of metallic food and beverage cans. BPA has weak estrogenic properties, albeit several orders of magnitude less potent than endogenous  $17\beta$ -oestradiol (Leffers *et al.*, 2001).

#### Animal studies

*In-vivo* rodent studies have reported conflicting results regarding the effect of *in-utero* BPA exposure on testosterone production by the fetal testis. Exposure of pregnant rats to high concentrations of BPA throughout pregnancy was associated with a reduction in testosterone production in male fetuses around birth (Tanaka *et al.*, 2006), whilst other studies have reported no effect of *in-utero* exposure to BPA on AGD in male offspring (Kobayashi *et al.*, 2002; Howdeshell *et al.*, 2008). *In-vitro* studies have demonstrated a reduction in testosterone production in mouse and rat fetal testis following exposure to high concentrations of BPA (N'Tumba-Byn *et al.*, 2012). Given the conflicting results of animal studies involving BPA, a large collaborative study (CLARITY-BPA) is currently being undertaken in rodents to determine the effects of BPA on a range of body systems including reproductive organs (Schug *et al.*, 2013). A recent publication from the consortium reported no effects of combined *in-utero* and post-natal exposure on several testicular morphometric and histological endpoints, including adult testis weight, except at very high doses that far exceed environmental exposure (Dere *et al.*, 2018).

#### Epidemiology

A limited number of epidemiological studies have investigated the relationship between human pregnancy exposure to BPA and male reproductive disorders. In a study involving measurement of cord blood in 52 newborn boys with cryptorchidism and 126 controls, no

association was found between BPA exposure and testosterone; however, a significant negative correlation between BPA and *Ins3* was identified (Chevalier *et al.*, 2015). Given that *Ins3* and testosterone are both involved in testicular descent, it is interesting to note that there was no association between BPA levels in cord blood and cryptorchidism (Fenichel *et al.*, 2012; Chevalier *et al.*, 2015). Similarly, in another case-control study, no association was identified between BPA exposure, as measured in maternal urine, and cryptorchidism in the male offspring (Chevrier *et al.*, 2012). The relationship between BPA and cryptorchidism has also been investigated in 98 boys with unilateral cryptorchidism aged 1–4 years. Serum BPA was measured prior to surgery and a significant association was found between total BPA and cryptorchidism in boys compared to 57 healthy controls (Komarowska *et al.*, 2015). However, no association was found between free (unconjugated) BPA and cryptorchidism and it is of interest to note the wide variation and high degree of overlap between the BPA levels of the two groups. An association between urogenital abnormality (cryptorchidism or hypospadias) and free BPA levels in term placenta was also described in a study involving 79 boys (Fernandez *et al.*, 2016). In this study, placental BPA was significantly higher in the cases compared to controls; however, an association between BPA and cryptorchidism/hypospadias was only apparent for the upper tertile of BPA exposure which included just 26 (12 cases and 14 controls) boys (Fernandez *et al.*, 2016). In addition, this study was not able to assess BPA exposure during fetal life which temporally separates the measurement of the exposure from the development of the disorder. An association has been described between maternal occupational exposure to BPA and AGD in 56 exposed male offspring when compared to 97 unexposed controls (Miao *et al.*, 2011). Care must be taken when interpreting this data as the exposure to BPA was determined by personal air sampling with extrapolation to estimate past exposure for the index pregnancy.

#### Experimental evidence from human studies

Several experimental studies have investigated the effect of BPA exposure using human fetal testis tissue and cells (Table III). A key consideration when assessing the results of experimental studies involving effects of exposure(s) on the human fetal testis is the concentration/doses to which the tissue is exposed. For BPA, human internal exposure to unconjugated BPA has been reported to be in the range  $10^{-2}$  M to  $10^{-3}$  M, including in pregnant women (Vandenberg *et al.*, 2010) and mean cord blood levels have been reported closer to  $10^{-3}$  M (Fenichel *et al.*, 2012). However, a recent study involving 48 samples from 30 healthy pregnant women, which accounted for potential post-sampling contamination, reported serum BPA concentrations below the LOD apart from 12 (25%) samples which ranged from  $10^{-9}$  to  $10^{-12}$  M, demonstrating that exposures may be substantially lower than previously reported (Teeguarden *et al.*, 2016).

**Hormones.** A number of studies have investigated the effect of exposure to BPA on testosterone production by human fetal testis tissue using an *in-vitro* system (N'Tumba-Byn *et al.*, 2012; Ben Maamar *et al.*, 2015; Eladak *et al.*, 2015). Exposure of human fetal testis explants (6–11 GW) to BPA did not affect testosterone production at a concentration of  $10^{-12}$  M; however, exposure to higher

concentrations ( $10^{-8}$  to  $10^{-5}$  M) resulted in a reduction of 20–50% (N'Tumba-Byn *et al.*, 2012). Further studies have compared the effect of BPA exposure under basal and gonadotrophin supplemented conditions (Ben Maamar *et al.*, 2015; Eladak *et al.*, 2015). Exposure of human fetal testis explants (7–12 GW) to  $10^{-5}$  M BPA for 72 h resulted in a significant reduction in testosterone production under basal (70%), hLH- (30%) and hCG-stimulated (60%) conditions (Ben Maamar *et al.*, 2015; Gaudriault *et al.*, 2017). However, exposure to lower concentrations did not result in significant differences except for  $10^{-8}$  M under basal conditions in which a 30% reduction was reported (Ben Maamar *et al.*, 2015). Similar results were obtained in another study in which exposure to BPA for 72 h under basal conditions resulted in a significant reduction in testosterone production by human fetal testis tissue (6–11 GW) across a range of concentrations ( $10^{-8}$  to  $10^{-5}$  M), whereas reduction in testosterone only occurred at the highest concentration ( $10^{-5}$  M) under hLH supplemented conditions (Eladak *et al.*, 2015). Two separate studies using the xenograft system have also investigated the effect of BPA on testosterone production using first and second trimester human fetal testis tissue (Eladak *et al.*, 2015; Eladak *et al.*, 2018). Exposure of host mice to  $10^{-5}$  M BPA in drinking water for 5 weeks did not affect testosterone production from xenografted tissue, as measured by host mouse seminal vesicle weight or serum testosterone. Importantly, the authors were able to demonstrate that the plasma levels of unconjugated and total BPA were significantly higher in the BPA exposed host mice compared to the vehicle controls. Similarly, for second trimester xenografts, daily oral gavage of host mice with 0.5 or 50  $\mu$ g/kg BPA for 5 weeks did not impact on testosterone production (Eladak *et al.*, 2018). In both xenograft studies, hCG was administered to the host mice to mimic the hormonal environment of pregnancy as previously described (Mitchell *et al.*, 2010).

The use of gonadotrophin supplementation in the *in-vitro* and *ex-vivo* systems is a key consideration. Supplementation of media with hCG was able to maintain testosterone levels across the culture period, as opposed to hLH or basal conditions in which testosterone levels declined during culture under control (no BPA) conditions (Ben Maamar *et al.*, 2015). The demonstration that BPA effects are largely eliminated in the human fetal testis by addition of hLH in the media, indicates that under physiological conditions of pregnancy, in which gonadotrophin (hCG and/or LH) levels are extremely high, environmental exposure to BPA is unlikely to affect testosterone production in the human fetal testis (Eladak *et al.*, 2015). Moreover, a recent study has investigated the effects of alternative bisphenols that have been proposed as replacements for BPA (Eladak *et al.*, 2015). For unstimulated conditions, similar effects to those of BPA occurred following exposure to high concentrations of BPS, BPE and BPF, although the effects tended to be of lower magnitude and towards the higher concentrations compared to BPA (Eladak *et al.*, 2015; Gaudriault *et al.*, 2017).

AMH production from Sertoli cells appears to be unchanged as a result of BPA exposure in the human fetal testis (Ben Maamar *et al.*, 2015).

*Ins3* is reduced following *in-vitro* exposure of human fetal testis to BPA (Ben Maamar *et al.*, 2015). This occurred only at the highest ( $10^{-5}$  M) and lowest ( $10^{-8}$  M) concentrations of BPA tested and also only under basal conditions. However, levels of *Ins3* were generally much lower in these basal conditions than in media supplemented with

**Table III Summary of experimental studies investigating effects of bisphenol exposure in human fetal testis tissue.**

Model and regimen							Results					
Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	Insl3	Germ cells	Study	Comments
BPA	<i>In-vitro</i>	6–11	10 <sup>-5</sup> M	Media	24–72 h	LH	↓ (40–50%)				Eladak et al. (2015)	
	<i>In-vitro</i>	6–11	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	24–72 h	Nil	↓ (20–50%)*				Eladak et al. (2015)	*(10 <sup>-8</sup> not significant at 24 h)
	<i>In-vitro</i>	6–11	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	24–72 h	LH	↔				Eladak et al. (2015)	
	<i>In-vitro</i>	6–11	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	24–72 h	Nil	↓ (20–50%)				N-Tumba Byn (2012)	
	<i>In-vitro</i>	6–11	10 <sup>-12</sup> M	Media	24–72 h	Nil	↔				N-Tumba Byn (2012)	
	<i>In-vitro</i>	6–12	10 <sup>-5</sup> M	Media	72 h	LH				↓	Eladak et al. (2018)	70% ↑ apoptotic gonocytes
	<i>In-vitro</i>	6–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	LH				↔	Eladak et al. (2018)	
	<i>In-vitro</i>	7–12	10 <sup>-5</sup> M	Media	72 h	Nil	↓ (70%)	↔	↓		Ben Mamaan (2015)	
	<i>In-vitro</i>	7–12	10 <sup>-5</sup> M	Media	72 h	hCG	↓ (60%)	↔	↔		Ben Mamaan (2015)	
	<i>In-vitro</i>	7–12	10 <sup>-5</sup> M	Media	72 h	LH	↓ (30%)	↔	↔		Ben Mamaan (2015)	
	<i>In-vitro</i>	7–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72h	LH	↔	↔	↔		Ben Mamaan (2015)	
	<i>In-vitro</i>	7–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↔	↔	↔		Ben Mamaan (2015)	
	<i>In-vitro</i>	7–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	Nil	↔**	↔	↔***		Ben Mamaan (2015)	**30% ↓ (10 <sup>-8</sup> ), ***70% ↓ (10 <sup>-8</sup> ) and 60% ↓ (10 <sup>-5</sup> )
	<i>In-vitro</i>	10–12	10 <sup>-5</sup> M	Media	72 h	hCG	↓ (60%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-6</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
Xenograft	9–11	10 <sup>-5</sup> M	Drinking Water	5 weeks	hCG	↔				↓ (19%)#	Eladak et al. (2018)	#19% ↓ gonocytes (1spermatogonia)
Xenograft	14–18	0.5, 50 ug/kg	Oral Gavage	5 weeks	hCG	↔					Eladak et al. (2018)	
BPB	<i>In-vitro</i>	10–12	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↓ (25–90%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
BPE	<i>In-vitro</i>	10–12	10 <sup>-4</sup> M	Media	72 h	hCG	↓ (80%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
BPF	<i>In-vitro</i>	6–11	10 <sup>-8</sup> M	Media	72 h	Nil	↔				Eladak et al. (2015)	
	<i>In-vitro</i>	6–11	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	72 h	Nil	↓ (30–40%)				Eladak et al. (2015)	
	<i>In-vitro</i>	10–12	10 <sup>-4</sup> M	Media	72 h	hCG	↓ (60%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
BPS	<i>In-vitro</i>	6–11	10 <sup>-8</sup> to 10 <sup>-7</sup> M	Media	72 h	Nil	↔				Eladak et al. (2015)	
	<i>In-vitro</i>	6–11	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	72 h	Nil	↓ (50%)				Eladak et al. (2015)	
	<i>In-vitro</i>	10–12	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↓ (20–85%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green.

hLH or hCG. For gonadotrophin supplemented cultures, BPA did not affect InsI3 production at any of the concentrations tested, similar to the results already described for testosterone (Ben Maamar *et al.*, 2015).

*Germ cells.* A recent study has investigated the effects of BPA exposure on germ cells in the human fetal testis. For first trimester testis (6–12 GW) using the *in-vitro* approach, exposure to  $10^{-5}$  M BPA resulted in a significant increase in the number of apoptotic gonocytes after 72 h, although there was no effect on apoptosis at lower concentrations (Eladak *et al.*, 2018). In xenografts, long-term (5 weeks) exposure to high concentrations of BPA ( $10^{-5}$  M) in drinking water resulted in a modest (–19%) reduction in germ cells/mm<sup>2</sup>, indicating that a sustained increase in apoptosis of germ cells could reduce their number. Analysis of the individual germ cell populations demonstrated that there was a significant reduction in the proportion of germ cells expressing the gonocyte marker (AP2 $\gamma$ ) with a reciprocal increase in pre-spermatogonial (MAGEA4) population. These findings are consistent with a reduction in gonocytes or alternatively an acceleration in the normal germ cell differentiation from gonocyte to pre-spermatogonium following BPA exposure (Eladak *et al.*, 2018).

#### Summary—bisphenols

Animal studies investigating effects of BPA exposure on fetal testis development have reported inconsistent results for indicators of testosterone production and germ cell development. Similarly, results of epidemiological studies investigating association between BPA and clinical indicators of reduced fetal testosterone (cryptorchidism and hypospadias) are inconsistent. For experimental studies involving human testis tissue, whilst the results of *in-vitro* experiments indicate the potential for BPA to reduce testosterone production from the fetal testis, this has been under basal conditions and xenograft studies have failed to demonstrate similar effects in either first or second trimester human fetal testis under gonadotrophin stimulation, as occurs in normal pregnancy. Furthermore, typical human exposure is likely to be well below the concentrations used for experimental studies involving animal(s) or human tissues based on meta-analysis and latest data on human BPA exposure using LC/MS/MS (Teeguarden *et al.*, 2016). Whilst germ cell effects have been described in one small study involving human fetal testis xenografts and relatively high BPA exposure (Eladak *et al.*, 2018), future studies should focus on exposures within the human-relevant range and on potential effects during different periods of gestation in order to determine the potential for effects on germ cells and future fertility in humans.

## Pesticides and fungicides

Pesticides and fungicides represent a heterogeneous group of chemicals widely used in agriculture. Many of these agents (e.g. Vinclozolin, Procymidone and prochloraz) are known to have anti-androgenic properties (Albert and Jegou, 2014). Whilst reproductive effects of many of these agents have been investigated in animal models (Taxvig *et al.*, 2013) and epidemiological studies (Bonde *et al.*, 2016), relatively few have been studied in experimental models involving the use of human tissues.

#### Animal studies

Several pesticides and fungicides have been proposed to be anti-androgenic with potential to impact testicular development and

function in animal studies (Taxvig *et al.*, 2013). This includes *in-vivo* studies demonstrating effects of *in-utero* exposure to the fungicides Procymidone (Ostby *et al.*, 1999) or Prochloraz (Vinggaard *et al.*, 2005) on fetal testosterone production in experimental animals. Given that these agents are known to act as anti-androgens via interfering directly with the androgen receptor (Robitaille *et al.*, 2015), it can be hypothesized that effects occurring in rodent models might be more likely to be translated into human effects, as opposed to other agents (e.g. phthalates) in which the mechanism of action is less clear.

#### Epidemiology

Whilst a number of epidemiological studies have reported associations between *in-utero* exposure to selected pesticides and the development of male reproductive disorders (Bonde *et al.*, 2016), the majority of these studies involve different pesticides or exposure periods (e.g. outside the fetal period) to those investigated in experimental studies using human tissues. For studies in which human experimental data is available, epidemiological data exists only for Dieldrin (Damgaard *et al.*, 2006; Shen *et al.*, 2008). Higher concentrations of Dieldrin have been reported in the placentae of Danish compared to Finnish women. This correlates at the population level with the higher prevalence of cryptorchidism in Denmark than in Finland (Shen *et al.*, 2008); however, a study of Danish subjects from the same cohort did not identify a significant association between Dieldrin in breast milk and the development of cryptorchidism, when compared to controls (Damgaard *et al.*, 2006).

#### Experimental evidence from human studies

Experimental studies investigating the effects of exposure to pesticides on human fetal testicular development and function are limited. To date, only two studies have reported the effects of pesticide exposure on human fetal testis tissue/cells (Table IV). This includes a study investigating the effects of Dieldrin, an insecticide, on second trimester (14–16 GW) human fetal testis in an *in-vitro* system.

*Hormones.* Exposure to concentrations of Dieldrin relevant to environmental and maternal serum levels in pregnancy ( $10^{-12}$  M to  $10^{-9}$  M) did not affect testosterone production under basal conditions; however, testosterone (–30%) and gene expression of the steroidogenic enzyme ‘steroidogenic acute regulatory protein’ (StAR) were significantly reduced under LH-induced conditions (Fowler *et al.*, 2007). No effect of Dieldrin exposure was found on AMH production from Sertoli cells. A more recent study investigated the effect of a wide variety of chemicals, including pesticides, on testosterone production in human fetal testis using an *in-vitro* culture system (Gaudriault *et al.*, 2017). This study identified dose-dependent reductions in testosterone as a result of exposure to a range of pesticides including imazalil, propiconazole, bitertanol, prochloraz, chlordecone, and for the majority of these chemicals, exposure to  $\sim 10^{-5}$  M resulted in a 50% reduction in testosterone production compared to basal. For glyphosate and atrazine, there was no clear reduction in testosterone across the entire dose range and for ortho-phenylphenol, a reduction in testosterone was only demonstrated at the highest concentration ( $10^{-4}$  M).

*Germ cells.* Potential effects of exposure to pesticides and antifungals on germ cell development in the human fetal testis have been

**Table IV** Summary of experimental studies investigating effects of pesticide exposure in human fetal testis tissue.

Model and regimen							Results					Study	Comments
Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	INSL3	Germ cells			
4-Octylphenol	<i>In-vitro</i>	6–12	10 <sup>-5</sup> M	Media	3 weeks	Nil				↓	Bendsen <i>et al.</i> (2001)		
Atrazine	<i>In-vitro</i>	10–12	10 <sup>-9</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
Bitertanol	<i>In-vitro</i>	10–12	10 <sup>-7</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↓ (50–75%)				Gaudriault <i>et al.</i> (2017)		
Chlordecone	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↓ (50–90%)				Gaudriault <i>et al.</i> (2017)		
Dieldrin	<i>In-vitro</i>	14–16	10 <sup>-12</sup> to 10 <sup>-9</sup> M	Media	24 h	Nil	↔	↔			Fowler <i>et al.</i> (2009)		
	<i>In-vitro</i>	14–16	10 <sup>-12</sup> to 10 <sup>-9</sup> M	Media	24 h	LH	↓ (30%)	↔			Fowler <i>et al.</i> (2009)		
Glyphosate	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
Imazalil	<i>In-vitro</i>	10–12	10 <sup>-6</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↓ (50–100%)				Gaudriault <i>et al.</i> (2017)		
Ortho-PhenylPhenol	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-4</sup> M	Media	72 h	hCG	↓ (40%)				Gaudriault <i>et al.</i> (2017)		
Prochloraz	<i>In-vitro</i>	10–12	10 <sup>-8</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↓ (20–75%)				Gaudriault <i>et al.</i> (2017)		
Propiconazole	<i>In-vitro</i>	10–12	10 <sup>-7</sup> M	Media	72 h	hCG	↔				Gaudriault <i>et al.</i> (2017)		
	<i>In-vitro</i>	10–12	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↓ (40–75%)				Gaudriault <i>et al.</i> (2017)		

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green.

investigated in one study (Bendsen *et al.*, 2001). The effects of 4-octylphenol, a surfactant used as a component of several pesticides, were investigated in prolonged (3 weeks) culture of first trimester (6–12 GW) human fetal testis tissue. Exposure resulted in a significant decrease in the mitotic index and in the number of spermatogonia per unit area compared to vehicle-exposed controls (Bendsen *et al.*, 2001).

#### Summary—pesticides and fungicides

Evidence for effects of several pesticides have been described in a limited number of experimental studies using human fetal testis tissues. The majority of the agents investigated thus far have demonstrated a reduction in testosterone production following short term *in-vitro* culture. However, the relationship between the concentrations used for each individual agent and measures of human exposure remain to be elucidated. Furthermore, the possibility of correlating the results of epidemiological and experimental studies is hindered by the variation in agents investigated by each approach.

## Pharmaceuticals

Over recent years, there has been increasing interest in the potential effects of pharmaceutical exposure during pregnancy on reproductive development and subsequent reproductive function in the offspring. The use of pharmaceuticals during pregnancy results in direct exposure to mother and the developing fetus. Therefore exposure to relatively high concentrations of the circulating drug may result, which contrasts with the very low level exposure to environmental chemicals such as those described above. In addition, for some pharmaceuticals (e.g. analgesics) the frequency of use during pregnancy may be high resulting in sustained exposures.

### Analgesics

Analgesics are the most commonly used medications worldwide. Several of these are available without prescription and it has been reported in many countries that 50–90% of women will use an analgesic at some stage during pregnancy (Kristensen *et al.*, 2016). Paracetamol (acetaminophen) is the most commonly used analgesic, whilst non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen are also used by up to 15% of pregnant women (Kristensen *et al.*, 2016).

#### Animal studies

Paracetamol exposure reduced fetal androgen production in rodents in studies using both *in-vivo* and *in-vitro* approaches (Kristensen *et al.*, 2011, 2012; Axelstad *et al.*, 2014; Holm *et al.*, 2015; van den Driesche *et al.*, 2015a), similar to findings for aspirin exposure following *in-vivo* (20% reduction in AGD) (Gupta and Goldman, 1986) or *in-vitro* (70% reduction in testosterone) (Kristensen *et al.*, 2012) exposure. For indomethacin exposure in mice, results are conflicting in terms of fetal testosterone production, with one *in-vivo* study describing a 20% reduction in AGD (Gupta and Goldman, 1986), whilst rat studies described either no effect *in vivo* (Dean, Mungall *et al.*, 2013), or a 30% reduction in fetal testosterone production *in vitro* (Kristensen *et al.*, 2012).

#### Epidemiology

The majority of studies that investigated associations between maternal analgesic exposure and cryptorchidism in male offspring have reported a significant positive association (Berkowitz and Lapinski, 1996; Jensen *et al.*, 2010; Kristensen *et al.*, 2011; Snijder *et al.*, 2012), although this is not a consistent finding (Philippat *et al.*, 2011). The timing of maternal exposure may be a key factor as most associations are reported to occur following prolonged exposure (>4 weeks) (Jensen *et al.*, 2010) or during the second trimester (Hurtado-Gonzalez and Mitchell, 2017), which would coincide with at least part of the postulated critical human MPW period (8–14 GW) (Welsh *et al.*, 2008). A reduction in fetal testosterone production, as demonstrated in experimental studies described above, could provide a mechanistic explanation for paracetamol-induced cryptorchidism in male offspring, although proving this in humans is challenging. However, measurement of the AGD in offspring can provide an indirect read-out of fetal androgen production, linking the reported association with the proposed mechanism (Dean and Sharpe, 2013). A recent study has shown an association between paracetamol exposure (inclusive of the MPW) and reduced AGD in boys up to 24 months, independent of body size (Fisher *et al.*, 2016). Nevertheless, for these epidemiological studies, extrapolation of results to direct clinical effects should be considered with caution, primarily due to the lack of direct analgesic measurements and the reliance on retrospective questionnaires for exposure classification which may involve a degree of recall bias.

#### Experimental evidence from human studies

Several recent studies have investigated the effect of analgesics on human fetal testis using experimental models (Table V).

**Paracetamol - Hormones.** *In-vitro* studies using first trimester testis (8–12 GW) exposed to paracetamol ( $10^{-5}$  M) for 1–3 days did not alter testosterone production, compared with vehicle controls (Mazaud-Guittot *et al.*, 2013). This was also the case for the paracetamol metabolite AM404 ( $10^{-5}$  M) (Mazaud-Guittot *et al.*, 2013). A recent study using an organotypic *in-vitro* culture system of first trimester human fetal testes explants showed that exposure to paracetamol in a dose range of  $10^{-8}$  to  $10^{-6}$  M increased testosterone by 25%; however, higher doses of  $10^{-5}$  M and  $10^{-4}$  M did not have any effect on testosterone production (Gaudriault *et al.*, 2017).

The conflicting results between rodent and human *in-vitro* studies may relate to the stage of testis development (i.e. timing within the MPW) or differences in the experimental system (Mazaud-Guittot *et al.*, 2013). Caution should be exercised when relating effects using *in-vitro* models to the *in-vivo* situation in humans, as the former cannot directly recapitulate normal pharmacokinetics, including *in-vivo* peak and trough concentrations. To circumvent some of the potential limitations of the *in-vitro* approaches for the human fetal testis, subsequent studies have utilized an *ex-vivo* approach involving subcutaneous xenografting of human fetal testis tissues ( $n = 5$ ; 14–20 GW) into host castrate nude mice. Oral exposure of these mice to a human-relevant regimen of paracetamol (20 mg/kg; three times daily) for 1 week significantly reduced (–45%) host serum testosterone and seminal vesicle (androgen dependent organ) weight (–18%), unlike a single daily exposure which had no effect on either parameter (van den Driesche *et al.*, 2015a). Further confirmation of the human relevance of

**Table V** Summary of experimental studies investigating effects of analgesic exposure in human fetal testis tissue.

Model and regimen							Results					
Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	INSL3	Germ Cells	Study	Comments
Paracetamol	<i>In-vitro</i>	8–11	10 <sup>-5</sup> M	Media	7d	Nil				↓	Hurtado-Gonzalez et al. (2018)	
	<i>In-vitro</i>	8–12	10 <sup>-5</sup> M	Media	24–72 h	hCG	↔		↓*	↔	Mazaud-Guittot et al. (2013)	*Dose response ↓
	<i>In-vitro</i>	10–12	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↑ (25%)				Gaudriault et al. (2017)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	7d	hCG	↓				van den Driesche et al. (2015a)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	7d	hCG				↓	Hurtado-Gonzalez et al. (2018)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	1d	hCG	↔				van den Driesche et al. (2015a)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	1d	hCG				↓	Hurtado-Gonzalez et al. (2018)	
Ibuprofen	Xenograft	14–17	60 mg/kg/d	Oral Gavage	7d	hCG	↔	↔			Ben Mamaan (2017)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	1d	hCG				↓	Hurtado-Gonzalez et al. (2018)	
	Xenograft	14–20	60 mg/kg/d	Oral Gavage	7d	hCG				↓	Hurtado-Gonzalez et al. (2018)	
	<i>In-vitro</i>	7–8	10 <sup>-5</sup> M	Media	72 h	hCG	↔	↓			Ben Mamaan (2017)	
	<i>In-vitro</i>	8–10	10 <sup>-5</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↓	↓	↔	↔	Ben Mamaan (2017)	
	<i>In-vitro</i>	8–10	10 <sup>-7</sup> to 10 <sup>-6</sup> M	Media	72 h	hCG	↔	↓**	↔		Ben Mamaan (2017)	**10 <sup>-7</sup> only
	<i>In-vitro</i>	8–11	10 <sup>-5</sup> M	Media	7d	Nil				↓***	Hurtado-Gonzalez et al. (2018)	***Gonocytes only
	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-4</sup> M	Media	24–72 h	hCG	↔	↔*	↔*		Ben Mamaan (2017)	*Dose response ↓
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
Aspirin	<i>In-vitro</i>	10–12	10 <sup>-4</sup> M	Media	72 h	hCG	↑				Gaudriault et al. (2017)	
	<i>In-vitro</i>	8–10	10 <sup>-7</sup> M	Media	72 h	hCG	↔				Mazaud-Guittot et al. (2013)	
	<i>In-vitro</i>	8–10	10 <sup>-6</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↑	↑#		↔	Mazaud-Guittot et al. (2013)	#Only tested at 10 <sup>-5</sup> (8–12 GW)
	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↔		↔##		Mazaud-Guittot et al. (2013)	##Only tested at 10 <sup>-5</sup>
Indomethacin	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-4</sup> M	Media	72 h	hCG	↔		↔###	↔	Gaudriault et al. (2017)	
	<i>In-vitro</i>	8–12	10 <sup>-5</sup> M	Media	72 h	hCG	↑ (20%)			↔###	Mazaud-Guittot et al. (2013)	###(10–12 GW)
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
Aniline	<i>In-vitro</i>	10–12	10 <sup>-4</sup> M	Media	72 h	hCG	↑ (20%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	96 h	hCG	↔####				Gaudriault et al. (2017)	####↓ 20% (10 <sup>-7</sup> )

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green.



paracetamol exposure is evident from the finding that 1 h after the final dose in host mice, plasma paracetamol concentrations were significantly lower than post-therapeutic levels reported in pregnant women (Rayburn *et al.*, 1986). However, it must be considered that circulating paracetamol levels in pregnant women may not be a direct indicator of intra-testicular levels in the developing fetus.

InsI3 hormone production was significantly reduced in first trimester human fetal testis cultures exposed to paracetamol, in which a clear dose–response relationship with increasing paracetamol exposure (at  $10^{-7}$  M to  $10^{-4}$  M) was demonstrated (Mazaud-Guittot *et al.*, 2013).

**Paracetamol - Germ cells.** An *in-vitro* study using first trimester human fetal testis (8–12 GW) exposed to paracetamol ( $10^{-5}$  M) for 1–3 days found no alteration in germ cell number (Mazaud-Guittot *et al.*, 2013). However, a more recent *in vitro* study with a longer period (7 days) of exposure showed that similar paracetamol concentration ( $10^{-5}$  M) significantly reduced (–28%) gonocyte number (Hurtado-Gonzalez *et al.*, 2018). The differing findings in these studies may reflect the longer period of exposure in the latter study or may be the result of differences in the culture systems. To further investigate the potential effect of paracetamol exposure on the human fetal testis, a xenograft approach was also used alongside the *in-vitro* model (Hurtado-Gonzalez *et al.*, 2018). Xenografted second trimester tissue (14–20 GW) exposed to paracetamol using a human-relevant exposure regimen (20 mg/kg, three times daily) resulted in a reduction in gonocyte number after 7 days exposure (–32%). Interestingly, a reduction in gonocyte number (–17%) was also demonstrated after just 1 day of exposure to paracetamol (Hurtado-Gonzalez *et al.*, 2018).

Whilst most of circulating paracetamol in humans comes from use of paracetamol-containing medications, an alternative source has also been described. The industrial chemical aniline, which is found in a wide variety of manufactured products, such as rubber, pharmaceuticals, cosmetics and cigarette smoke, is rapidly metabolized to paracetamol inside the body (Modick *et al.*, 2014, 2016). Furthermore, *in-vivo* studies in which male mice exposed *in utero* to aniline have shown similar fetal anti-androgenic effects to those described for exposure to paracetamol (Holm *et al.*, 2015). Only one study has investigated the effect of aniline on the human fetal testis (Gaudriault *et al.*, 2017). *In-vitro* exposure of first trimester (10–12 GW) human fetal testis to aniline for 96 h had no effect on testosterone production across a range of doses ( $10^{-8}$  M to  $10^{-5}$  M) apart from a small reduction (–20%) for an intermediate concentration ( $10^{-7}$  M) (Gaudriault *et al.*, 2017).

**Ibuprofen - Hormones.** Exposure of first trimester human fetal testis explants to ibuprofen using an organotypic culture system did not affect testosterone production (Gaudriault *et al.*, 2017). However, another *in-vitro* study reported a reduction in steroidogenic enzyme expression across a similar range of concentrations ( $10^{-4}$  M,  $10^{-5}$  M). This effect was only evident for early first trimester testes (8–9 GW), as there was no effect at any other gestational time-point examined (<8 GW or >10 GW) (Ben Maamar *et al.*, 2017). Similarly, there was no effect of exposure to ibuprofen on testosterone production in host mice carrying xenografts of second trimester human fetal testis tissue (Ben Maamar *et al.*, 2017).

Ibuprofen exposure for 3 days in an *in-vitro* model reduced AMH in first trimester human fetal testes at 7–8 GW ( $10^{-5}$  M), and at 8–10 GW ( $10^{-4}$  M to  $10^{-5}$  M); however, no significant difference in AMH was found after 7 days of exposure of host mice carrying second trimester human fetal testis xenografts (Ben Maamar *et al.*, 2017).

InsI3 production was not affected in early first trimester (8–10 GW) human fetal testis following *in-vitro* culture with ibuprofen, whilst for late first trimester (10–12 GW) testis, an overall dose response reduction was demonstrated (Ben Maamar *et al.*, 2017). The fact that ibuprofen exposure affects testosterone and InsI3 production only during specific periods of human fetal testis development, has implications for the potential of this analgesic to impact testis descent, i.e. cryptorchidism (which is under the control of these two hormones).

**Ibuprofen - Germ cells.** *In-vitro* culture and exposure of first trimester human fetal testis to ibuprofen for 7 days resulted in a reduction in gonocyte number (–22%). However, there were no significant changes to germ cell number following exposure of second trimester xenografted testis tissue (Hurtado-Gonzalez *et al.*, 2018).

**Aspirin - Hormones.** Aspirin exposure of first trimester testis explants in an organotypic culture system, did not alter testosterone production across a range of concentrations ( $10^{-4}$  to  $10^{-8}$  M) (Gaudriault *et al.*, 2017). However, in a separate *in-vitro* culture study, a significant dose–response relationship was reported whereby aspirin exposure for 3 days significantly increased the production of testosterone by early first trimester (8–9 GW), but not in late first trimester (10–12 GW) testes (Mazaud-Guittot *et al.*, 2013). *In-vitro* exposure of human fetal testis (10–12 GW) to aspirin for 3 days did not affect InsI3 production (Mazaud-Guittot *et al.*, 2013), whereas AMH production was significantly increased.

**Aspirin - Germ cells.** *In-vitro* exposure to aspirin did not affect germ cell number in first trimester (8–10 GW) human fetal testis tissue (Mazaud-Guittot *et al.*, 2013).

**Indomethacin - Hormones.** Similar to rodent studies, there are conflicting results of experimental studies using *in-vitro* culture of human fetal testis. Exposure of first trimester human fetal testis explants (10–12 GW) to indomethacin ( $10^{-4}$  M) for 72 h, reduced testosterone (–20%), whereas exposure at lower concentrations ( $10^{-5}$  M to  $10^{-8}$  M) had no effect (Gaudriault *et al.*, 2017). In contrast, a previous study found that indomethacin exposure ( $10^{-5}$  M) increased testosterone production (+20%), when first trimester (8–12 GW) testes were exposed *in-vitro* for a similar duration (Mazaud-Guittot *et al.*, 2013). *In-vitro* exposure of human fetal testis (10–12 GW) to indomethacin ( $10^{-5}$  M) for 2 days did not affect InsI3 production (Mazaud-Guittot *et al.*, 2013).

**Indomethacin - Germ cells.** *In-vitro* exposure to indomethacin ( $10^{-5}$  M) for 2 days did not affect germ cell number in first trimester (8–12 GW) human fetal testis cultures (Mazaud-Guittot *et al.*, 2013).

Summary—analgesics

Exposure to analgesics has been linked to abnormalities in testicular function and development of male reproductive disorders across a range of studies. This includes epidemiological and experimental studies using animal and human tissues. Results regarding testosterone production are not consistent and this may reflect differences between species, model systems or dose, timing and duration of exposure (Table 1 and Fig. 2). However, there is increasing evidence from human studies that paracetamol and ibuprofen can affect germ cell number in the fetal testis and evidence exists for similar effects on germ cells in the fetal ovary (Hurtado-Gonzalez et al., 2018). These studies involve exposure to human-relevant concentrations of these drugs and for xenograft studies they include comparable dosing regimens to those used therapeutically in humans. Whilst the evidence for effects of paracetamol and ibuprofen on germ cells appears robust, the potential for such exposures to impact subsequent male reproductive function and fertility are unexplored. Indeed, it is possible that there may be compensation later in gestation or during childhood that would rescue the effects of any fetal germ cell loss. Large-scale prospective epidemiological studies and longer-term experimental (e.g. xenograft) studies can help to address this particular question.

Diethylstilboestrol

Diethylstilboestrol (DES) is a synthetic estrogen that was used clinically to prevent spontaneous miscarriage and pre-term labor from the 1940s until the early 1970s (Marselos and Tomatis, 1992). DES was withdrawn from clinical use after the demonstration of a causal role in the development of vaginal carcinoma in girls born to exposed mothers (Herbst et al., 1971). In addition to the effects on female offspring, an association with structural abnormalities of the male reproductive tract was also described including epididymal cysts, microphallus and testicular hypoplasia (Toppari et al., 1996).

Animal studies

Animal studies involving *in-vitro* culture of rat and mouse fetal testis, have reported a reduction in testosterone production following exposure to DES (N'Tumba-Byn et al., 2012), similar to the results of previous *in-vitro* studies involving fetal mice (Delbes et al., 2005) and *in-vivo* studies in rats (Haavisto et al., 2001).

Epidemiology

For TDS disorders, which are linked to a reduction in androgen action during fetal life, there is conflicting evidence regarding their association with maternal DES exposure. Three studies have reviewed the literature relating to exogenous estrogen exposure and male reproductive disorders (Toppari et al., 1996; Storgaard et al., 2006; Martin et al., 2008). Whilst early studies reported that hypospadias was significantly associated with DES exposure (Henderson et al., 1976), it has subsequently been pointed out that this related to urethral abnormalities resulting from exposure to exogenous estrogens (including DES), which may have resulted from abnormalities in penile development rather than an effect on urethral formation as a result of reduced androgen exposure (Joffe, 2002). The meta-analysis of all available evidence revealed a significant association between DES exposure and hypospadias; however, it was concluded that any

Table VI Summary of experimental studies investigating effects of pharmaceutical exposure in human fetal testis tissue.

Model and regimen	Results												
	Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	INSL3	Germ cells	Study	Comments
DES	<i>In-vitro</i>	Xenograft	6–12	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	72 h	Nil	↔				N-Tumba Byn (2012)	
			15–19	100 ug	Subcut.	3x/wk for 35 d	hCG	↔*				Mitchell et al. (2013)	*↑ SV (60%)
Metformin	<i>In-vitro</i>	Xenograft	10–12	5 x (10 <sup>-5</sup> to 10 <sup>-3</sup> ) M	Media	72 h	n/a	↓ (30–70%)**				Tartarin et al. (2012)	**5 x 10 <sup>-5</sup> and 5 x 10 <sup>-3</sup> M
Abiraterone	<i>In-vitro</i>	Xenograft	16–22	75mg/kg/day	Oral	14 d	hCG	↓ (80%)*			↔	Spade et al. (2014)	**↑ SV (45%)
Ketoconazole	<i>In-vitro</i>	<i>In-vitro</i>	10–12	10 <sup>-7</sup> M	Media	96 h	hCG	↔				Gaudhault et al. (2017)	
			10–12	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	96 h	hCG	↓ (50–90%)				Gaudhault et al. (2017)	
	<i>In-vitro</i>	<i>In-vitro</i>	8–12	10 <sup>-5</sup> M	Media	24–72 h	hCG	↓ (20–90%)	↓ (65%)*#	↓ (90%)		Mazzaud-Guittot et al. (2013)	#72 h
Theophylline	<i>In-vitro</i>	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-5</sup> M	Media	96 h	hCG	↓ (30–40%)				Gaudhault et al. (2017)	
Valproate	<i>In-vitro</i>	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-6</sup> M	Media	96 h	hCG	↔				Gaudhault et al. (2017)	
			10–12	10 <sup>-5</sup> M	Media	96 h	hCG	↓ (65%)				Gaudhault et al. (2017)	
Clomiphene	<i>In-vitro</i>	<i>In-vitro</i>	10–12	10 <sup>-6</sup> to 10 <sup>-5</sup> M	Media	96 h	hCG	↓ (25–65%)				Gaudhault et al. (2017)	

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green.

effect of DES on hypospadias is likely to be small (Martin *et al.*, 2008). For cryptorchidism, an increased risk in association with DES exposure is reported; however, this was dependent on the statistical model used and was indicative of heterogeneity (Martin *et al.*, 2008). A subsequent cohort study has reported an association between *in-utero* exposure to DES and an increased risk of cryptorchidism, however, only for those in whom the initial exposure occurred prior to the 11th week of gestation with no significant association following exposure after 11 GWs (Palmer *et al.*, 2009). Studies have demonstrated no effect of prenatal DES exposure on sperm counts (Leary *et al.*, 1984) or fertility (Wilcox *et al.*, 1995); however, this is in contrast to a previous study demonstrating an association between prenatal exposure to DES and semen parameters in adult men (Gill *et al.*, 1979). Importantly, this study included analysis of men born to a large cohort of mothers who participated in an RCT involving DES exposure during pregnancy.

#### Experimental evidence from human studies

To date, only two studies have investigated the effect of DES exposure on the human fetal testis (Table VI). *In-vitro* organ culture of first trimester human fetal testis exposed to DES ( $10^{-5}$  to  $10^{-6}$  M) for 3 days did not alter testosterone production (N'Tumba-Byn *et al.*, 2012). Interestingly, this study compared effects of DES exposure in rodent and human fetal testis demonstrating contrasting results between species using an identical experimental system (N'Tumba-Byn *et al.*, 2012).

In a separate study using the xenograft model, exposure to DES (100 µg/kg, three times weekly) for 35 days resulted in no significant difference in testosterone production by second trimester (15–19 GW) testis tissue. Interestingly, host mouse seminal vesicles were significantly increased in weight, which was indicative of increased testosterone production from the xenografted tissue over the entire grafting period (Mitchell *et al.*, 2013). The reason for this unexpected increase in testosterone is unclear.

#### Summary—diethylstilboestrol

Whilst rodent studies have indicated a profoundly negative effect of DES exposure on testosterone production by the fetal testis (Haavisto *et al.*, 2001; Delbes *et al.*, 2005; N'Tumba-Byn *et al.*, 2012), experimental studies utilizing human fetal testis tissues have failed to identify similar effects (N'Tumba-Byn *et al.*, 2012; Mitchell *et al.*, 2013), which may relate to the presence of ESRI in rodent Leydig cells, and the absence of this estrogen receptor in human fetal testis (Mitchell *et al.*, 2013). Epidemiological data suggests that any potential effect of DES exposure on male reproductive development is likely to be of small magnitude. Taken together the results suggest an important species difference in terms of DES effects on fetal testosterone production which may explain why this frequently results in the development of male reproductive disorders in rodents, whilst associations between DES and subsequent male reproductive disorders in humans are rather modest. Whilst DES is unlikely to be used in pregnant women in the future, the findings of this study offer some reassurance regarding the potential of low-level exposure to environmental estrogens to affect human male reproductive development,

given their extremely low potency compared with DES and the high exposures that resulted from therapeutic use of DES.

## Metformin

Metformin is a biguanide used as an insulin sensitizer in the treatment of Type 2 Diabetes and obesity. Metformin may also be prescribed during pregnancy in individuals with pre-existing diabetes or those that have developed gestational diabetes during their pregnancy (<https://bnf.nice.org.uk/drug/metformin-hydrochloride.html#pregnancy>).

#### Animal studies

Exposure to metformin has been shown to reduce testosterone production in mouse fetal testis following *in-vitro* or *in-vivo* exposure. Exposure of pregnant mice to metformin (300 mg/gk/d) from embryonic day (e) 0.5 resulted in a significant reduction in testosterone at e16.5 (Tartarin *et al.*, 2012).

#### Epidemiology

To date, there has been no epidemiological data relating *in-utero* exposure to metformin to TDS disorders at birth, although one study found no association between prepubertal testicular volumes in offspring born to mothers who had received metformin, compared with insulin, for gestational diabetes (Terti *et al.*, 2016).

#### Experimental evidence from human studies

A recent study has investigated the effect of metformin exposure on the human fetal testis using an *in-vitro* culture system (Tartarin *et al.*, 2012) (Table VI). Exposure to a range of metformin concentrations ( $5 \times 10^{-5}$  M to  $5 \times 10^{-3}$  M) resulted in a significant decrease in testosterone production from the testis. Importantly, the lowest concentration ( $5 \times 10^{-5}$  M) reflects the serum levels measured in humans following a therapeutic dose of metformin (Robert *et al.*, 2003).

## Azole antifungals and abiraterone

Azole antifungals (e.g. ketoconazole, fluconazole) and abiraterone are drugs known to inhibit key enzymes of the steroidogenic pathway including P450scc (CYP17A1; ketoconazole) and CYP17A1 & 3βHSD (abiraterone). As a result, exposure of the fetal testis to these agents could be predicted to affect testosterone production and potentially to result in the development of male reproductive disorders.

#### Epidemiology

Exposure to 'azole' antifungals that interfere with steroidogenesis may be relevant to pregnancy, given that they are frequently prescribed for the treatment of vaginal candidiasis. No association between maternal use of antifungals and hypospadias has been reported in two studies (Carter *et al.*, 2008) (Norgaard *et al.*, 2008), or for AGD after exposure to antifungals administered as vaginal tablets or as topical cream (Mogensen *et al.*, 2017). However, the latter study demonstrated a significant association between oral fluconazole and reduced AGD in the male offspring (Mogensen *et al.*, 2017). Importantly, for each of these three studies, the numbers of cases was small and larger prospective studies would be required to

**Table VII** Summary of experimental studies investigating effects of lifestyle exposures in human fetal testis tissue.

Model and regimen		Results										
Exposure	Model	Fetal age (weeks)	Dose	Route	Regimen	Supplemented	Testosterone	AMH	INSL3	Germ cells	Study	Comments
1,3,7 TMUA	<i>In-vitro</i>	10–12	10 <sup>-9</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔**				Gaudriault et al. (2017)	*↓ at 10 <sup>-7</sup> M
Alcohol	<i>In-vitro</i>	10–12	10 <sup>-8</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↑ (225–275%)				Gaudriault et al. (2017)	Ethanol
Caffeine	<i>In-vitro</i>	10–12	10 <sup>-9</sup> M	Media	72 h	hCG	↓ (20%)				Gaudriault et al. (2017)	
	<i>In-vitro</i>	10–12	10 <sup>-7</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
Paraxanthine	<i>In-vitro</i>	10–12	10 <sup>-9</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔				Gaudriault et al. (2017)	
Smoking	<i>In-vitro</i>	14–19	10 <sup>-6</sup> M	Media	24 h	Nil					Coutts et al. (2007)	DMBA-DHD
	<i>In-vitro</i>	7–11	10 <sup>-7</sup> to 10 <sup>-5</sup> M	Media	72 h	Nil	↔				Angenard et al. (2010)	Cadmium: **10 <sup>-6</sup> M only
Theobromine	<i>In-vitro</i>	10–12	10 <sup>-9</sup> to 10 <sup>-5</sup> M	Media	72 h	hCG	↔***				Gaudriault et al. (2017)	***↓ at 10 <sup>-7</sup> M

Significant effects associated with adverse outcomes are highlighted in red, no change or significant effects not expected to result in adverse outcomes are highlighted in green.

provide definitive evidence of associations between antifungals and indicators of fetal testosterone production.

#### Experimental evidence from human studies

Exposure to ketoconazole results in a significant reduction (50–90%) in testosterone following *in-vitro* exposure of first trimester human fetal testis for 96 h (Gaudriault et al., 2017) (Table VI). Similar results have been described for ketoconazole with a progressive reduction in testosterone production after 24 h (–20%), 48 h (–90%) and 72 h (–95%), compared to vehicle-exposed tissue (Mazaud-Guittot et al., 2013). This study also reported a ketoconazole-induced reduction in INSL3 (–100%) and AMH (–50%) after 72 h of culture.

Abiraterone (an anti-androgen used in prostate cancer) has also been shown to result in a reduction in testosterone production (–80%) in second trimester human fetal testis xenografts (grafted for 14 days), whilst no effect on germ cell number was demonstrated (Spade et al., 2014) (Table VI). Indeed, these agents may be considered as positive controls for studies investigating the effects of exposures on testosterone production in human fetal testes (Mazaud-Guittot et al., 2013; Spade et al., 2014).

#### Other pharmaceuticals

A recent study has described the effect of exposure to 27 different chemicals, including several additional pharmaceuticals, on the human fetal testis using an *in-vitro* system (Gaudriault et al., 2017) (Table VI). A dose dependent reduction in testosterone production was determined for clomiphene (an anti-estrogenic substance used to stimulate ovulation), theophylline (a methylxanthine drug which acts as a non-selective phosphodiesterase inhibitor used in asthma) and valproate (an anti-epileptic) following *in-vitro* culture of human fetal testis tissue for 96 h (Gaudriault et al., 2017). Interestingly, valproate has been associated with hypospadias in male offspring of exposed mothers (Veroniki et al., 2017).

#### Lifestyle

Exposures that relate to lifestyle may also impact on the development of male reproductive disorders. However, to date only a limited number of studies using human fetal tissues have been conducted in this area (Table VII).

#### Xanthines

Xanthines are a class of compounds that share a common structure and have stimulant properties, including caffeine, one of the most commonly used recreational drugs worldwide. Effects of several of these compounds have been investigated *in vitro* (Gaudriault et al., 2017).

#### Experimental evidence from human studies

No negative effects on testosterone production from cultured human fetal testis tissue were demonstrated following exposure to caffeine, paraxanthine, theobromine or 1,3,7 trimethyluric acid (TMUA), albeit there appeared to be a modest decrease in testosterone production for caffeine only at the lowest concentration (Table VII).

## Alcohol

Alcohol exposure during pregnancy is known to impact on fetal development, and its mechanism of action could occur centrally or in the placenta. Fetal alcohol syndrome is well described and can include neurodevelopmental disorders, facial dysmorphism and growth abnormality (Nash and Davies, 2017).

### Epidemiology

An epidemiological study involving a Danish–Finnish cohort (~2500 boys), demonstrated an association between maternal alcohol consumption and increased risk of cryptorchidism in sons (Damgaard *et al.*, 2007).

### Experimental evidence from human studies

The effect of exposure to alcohol on human fetal testis has only been investigated in one study (Table VII). Interestingly, exposure of first trimester human fetal testis to ethanol ( $10^{-8}$  M to  $10^{-5}$  M for 72 h) using *in-vitro* culture resulted in a significant increase in testosterone production across a wide dose range (Gaudriault *et al.*, 2017).

## Smoking

Maternal smoking is known to have many potentially harmful effects on the developing fetus including intra-uterine growth retardation and low birth weight (Abraham *et al.*, 2017).

### Epidemiology

Epidemiological studies have measured sperm counts in men exposed *in-utero* to maternal cigarette smoke, which demonstrated a reduction in sperm concentration (38–48%) in exposed- compared to unexposed-men (Storgaard *et al.*, 2003; Ramlau-Hansen *et al.*, 2007). Use of nicotine substitutes during pregnancy significantly increased the risk of cryptorchidism in male offspring (Damgaard *et al.*, 2008) and an association between maternal smoking during pregnancy and cryptorchidism in male offspring has also been demonstrated (Jensen *et al.*, 2007).

### Experimental evidence from human studies

**Hormones.** One study compared testosterone levels in plasma of human fetuses exposed to maternal cigarette smoke with those of non-smoking mothers and found no difference in testosterone between the groups despite a significant reduction in hCG (Fowler *et al.*, 2009).

**Germ cells.** The effects of maternal smoking on human fetal testis has been investigated in two experimental studies, both of which used exposure to components of cigarette smoke (Coutts *et al.*, 2007; Angenard *et al.*, 2010). *In-vitro* exposure of human fetal testis to DMBA-DHD, the active metabolite of polyaromatic hydrocarbons found in cigarette smoke, resulted in a significant increase in apoptosis in germ cells, which could be rescued by antagonism of the aryl hydrocarbon receptor (AHR), indicating that activation of the AHR is the likely mechanism for the effects of DMBA-DHD on germ cells (Coutts *et al.*, 2007). Cadmium (another component of cigarette smoke)-exposure of first trimester human fetal testis resulted in increased apoptosis in germ cells, without any effect on testosterone production (Angenard *et al.*, 2010).

## Mixtures

Over recent years, it has been increasingly recognized that the impact of environmental exposures depends not only on the individual agents, but also on the combination of agents. A number of animal studies have investigated the effects of 'mixtures'; however, to date such approaches using human fetal testis tissues is limited. A key aspect of this is whether the effects can be considered as additive or synergistic. The effect of four separate mixtures (all including BPA) has been investigated in a recent study involving *in-vitro* exposure of first trimester human fetal testis for 96 h (Gaudriault *et al.*, 2017). This included two mixtures (four agents each) of BPA + pharmaceuticals and two mixtures (eight agents each) which included additional environmental (pesticides and bisphenols) chemicals.

As expected, each mixture resulted in a reduction in testosterone production. Importantly, the authors compared the individual dose–response results to those predicted by the additive effect of the individual agents. There was a high correlation between predicted and actual response for each of the four mixtures indicating that these agents acted in an additive manner (Gaudriault *et al.*, 2017). This allows such experimental systems to use the results of exposure to individual agents for approximation of the combined anti-androgenic effect of multiple exposures, based on an assumption of dose-addition. This has important implications for informing regulation of environmental chemicals and pharmaceutical exposures.

## Discussion

Whilst a relatively large body of animal data exists for determining the impacts of *in-utero* exposures on fetal testicular development and male reproductive disorders, a limited number of experimental studies involving human tissues have been conducted. Animal models offer the potential to conduct more expansive studies involving exposures across multiple developmental periods and generation of dose response data. An additional advantage of animal studies is the possibility of conducting *in-vivo* fetal exposure studies in animals which is not possible for human studies which currently rely on *in-vitro* or *ex-vivo* (xenograft) approaches. However, this review has described several exposures for which the studies utilizing human tissues have demonstrated important differences to those found in animals. This may relate to differences in study design, dose administered or exposure regimen; however, it has also been shown that many of these differences appear to result from fundamental species differences in the effect of specific agents at human-relevant levels of exposure. Whilst epidemiological studies can to some extent bridge the gap between effects demonstrated in animal studies and human-relevance, such studies cannot demonstrate direct causation or elicit underlying mechanisms for effects; such studies are also prone to confounding. This highlights the importance of experimental models using human fetal tissues in determining the potential impact of *in-utero* environmental and pharmaceutical exposures in humans.

## Future perspectives

It is clear that understanding effects of *in-utero* exposures on male reproductive development will continue to rely on interpretation of a combination of animal studies, epidemiology and experimental studies

utilizing human tissues. Conducting co-ordinated studies that combine these methods represents an important approach. This may include combining experimental studies using *in-vitro* and *in-vivo* approaches (Kristensen *et al.*, 2011; Hurtado-Gonzalez *et al.*, 2018), studies comparing results in both rodent and human tissues (Ben Maamar *et al.*, 2015; Hurtado-Gonzalez *et al.*, 2018), or combined studies of epidemiological and experimental evidence (Kristensen *et al.*, 2011).

Epidemiological studies may be enhanced by developing large-scale prospective cohort studies. This is of particular importance to ensure that the timing of measurement of exposure coincides with the expected mechanism of effect (e.g. *in-utero* exposure and cryptorchidism/hypospadias identified in the neonatal period). Furthermore, for pharmaceuticals with relatively short half-lives, which are taken intermittently and do not accumulate in the body, obtaining accurate and detailed records of exposure during pregnancy is essential. This information is extremely difficult to obtain retrospectively and is prone to recall bias making prospective studies crucial for such exposures.

The future for determining effects of *in-utero* exposure(s) on male testicular development and reproduction is also likely to involve refinement of existing experimental approaches. Whilst recent development of *in-vitro* and xenograft approaches has allowed direct testing of environmental chemicals and pharmaceuticals on the human fetal testis, these models may be limited by tissue supply and heterogeneity between individuals. The recent development of organoids for a variety of organs and tissues may prove critical for future studies to test the effects of exposures in organoids generated from human testicular tissues (Alves-Lopes and Stukenborg, 2017). Computational and mathematical modeling may also be used to predict the effects of exposure(s) *in-silico* (Krysiak-Baltyn *et al.*, 2012), although this will be dependent on the robustness of the imputation of biological data.

The scientific and public interest in the effects of chemical exposures in humans is likely to continue to increase. Regulation of these agents will increasingly rely on models that can provide direct human-relevant data. Therefore, we propose that assessment of the experimental evidence from studies using human fetal tissues should be an integral part of informing regulatory policies in relation to the effects of environmental and pharmaceutical exposures on male reproductive development.

## Supplementary data

Supplementary data are available at *Human Reproduction Update* online.

## Authors' roles

R.T.M. developed the concept for the article. R.T.M. and K.K. performed the systematic search of the literature, assessment of eligibility, data extraction and tabulation of data. R.T.M. and K.K. wrote, revised and approved the final version of the article.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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