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Male Reproductive Health Disorders and the Potential Role of Exposure to Environmental Chemicals

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Commissioned by CHEM Trust

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List of abbreviations

AF	amniotic fluid
AGD	anogenital distance.The distance between the anus and genitals, which is longer in men.
AH	aryl hydrocarbon
AR	androgen receptor
BBzP	butylbenzyl phthalate
CG	chorionic gonadotrophin or human chorionic gonadotrophin (hCG)
CIS	carcinoma in situ cells, cells which are precursor cells to cancer
DBP	di-n-butyl phthalate
DDE	1,1-bis-(4-chlorophenyl)-2,2-dichloroethene
DDT	1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethane
DEHP	di(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DES	diethylstilboestrol
ECs	environmental chemicals
ED	endocrine disruptor
HCB	hexachlorobenzene
HCE	heptachloroepoxide
β-HCCH	β-hexachlorocyclohexane
LH	luteinising hormone
MBP	mono-n-butyl phthalate
MBzP	mono-benzyl phthalate
MEHHP	mono(2-ethyl-5-hydroxy-hexyl) phthalate
MEHP	mono(2-ethylhexyl) phthalate
MEOHP	mono(2-ethyl-5-oxo-hexyl) phthalate
MMP	mono-methyl phthalate
PAHs	polycyclic aromatic hydrocarbons
PBDE	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
PFOS	perfluorooctane sulfonate- a pefluorinated chemical
PFOA	perfluorooctanic acid – a perfluorinated chemical
POPs	persistent organic pollutants
TCDD	2,3,7,8-tetrachlorodebenzo-p-dioxin
TDS	testicular dysgenesis syndrome
TGCT	testicular germ cell tumours
WHO	World Health Organisation

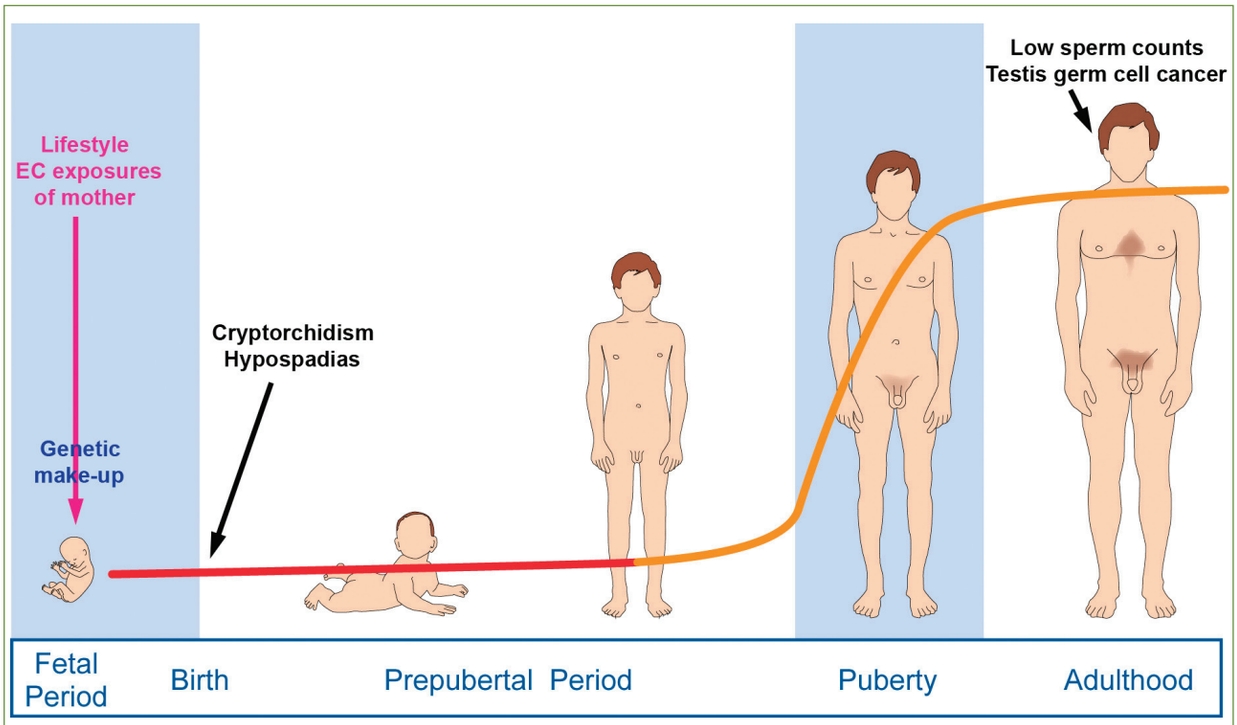


Diagram to illustrate potential TDS effects due to in-utero exposure

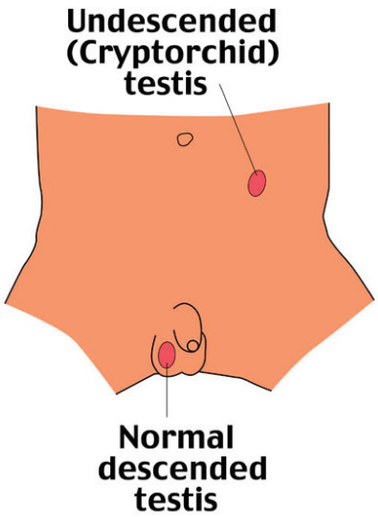


Diagram to illustrate cryptorchidism
(undescended testes)

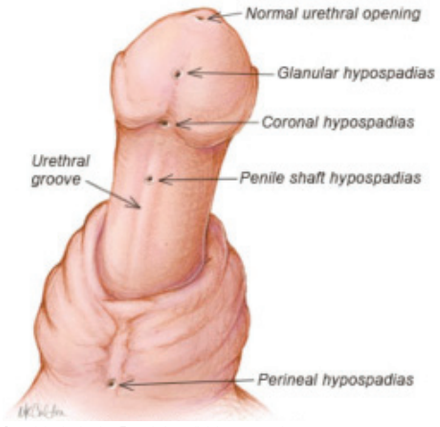
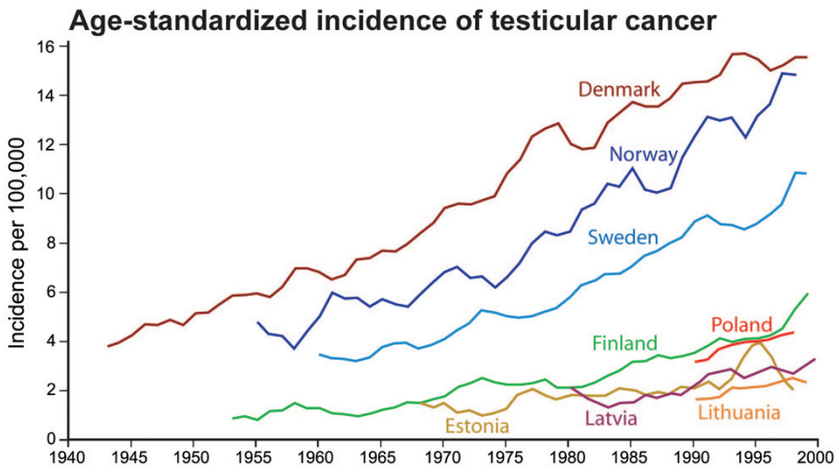


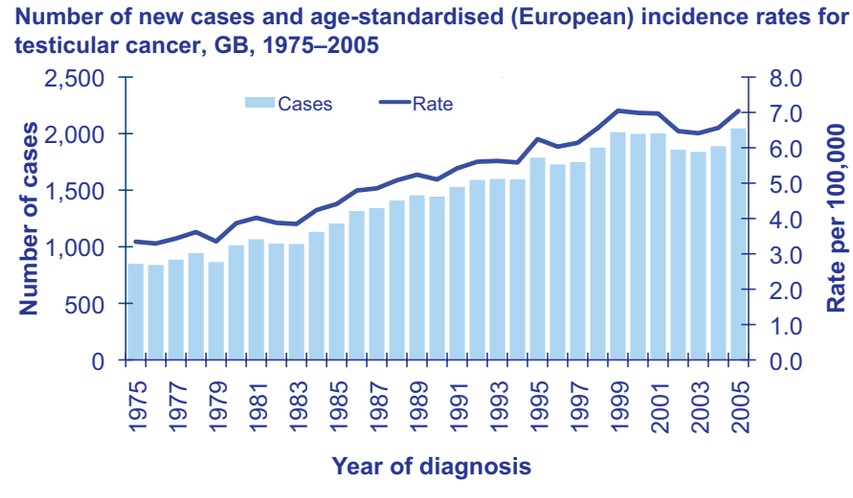
Diagram to illustrate four types of hypospadias



From: Richiardi et al (2004) *Cancer Epidemiol Biom & Prev.* **13;** 2157-2166

Graph to show increase in incidence of testicular cancer from 1950s-2000 in several EU countries.

This graph shows the rapid increase in testicular cancer in a number of EU countries over time.



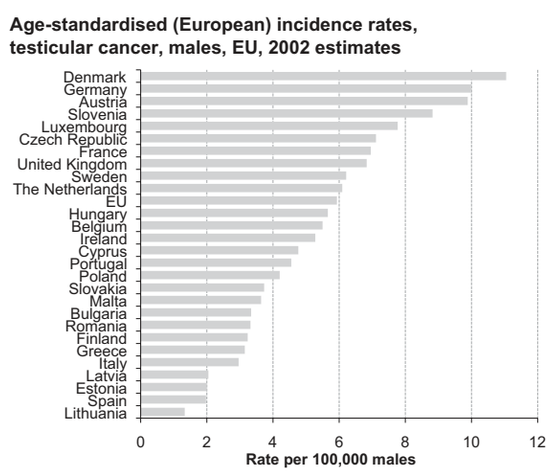
From: Cancer Research UK, http://info.cancerresearchuk.org/cancerstats/types/testis/incidence/

Graph to show increase in incidence of testicular cancer from 1975-2005 in Britain

This graph shows the approximate doubling of the incidence of testicular cancer in Britain over the last 25 years.

Age-standardised cancer incidence rates per 100,000 men, testicular cancer, by EU country 2002 estimates

EU Country	Incidence
Lithuania	1.3
Spain	2.0
Estonia	2.0
Latvia	2.0
Italy	3.0
Greece	3.1
Finland	3.2
Romania	3.3
Bulgaria	3.3
Malta	3.6
Slovakia	3.7
Poland	4.2
Portugal	4.6
Cyprus	4.8
Ireland	5.3
Belgium	5.5
Hungary	5.7
EU	5.9
The Netherlands	6.1
Sweden	6.2
United Kingdom	6.8
France	7.0
Czech Republic	7.1
Luxembourg	7.8
Slovenia	8.8
Austria	9.9
Germany	10.0
Denmark	11.0



From: Cancer Research UK, http://info.cancerresearchuk.org/cancerstats/types/testis/incidence/

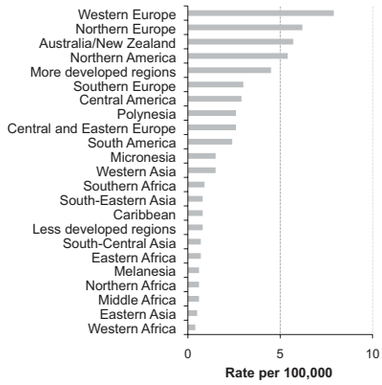
Bar chart to show differing incidence of testicular cancer in several EU countries

This bar chart shows the differing incidence of testicular cancer in various EU countries, with Denmark having the worst rates and Lithuania having the least incidence of testicular cancer.

TESTICULAR CANCER

	Incidence
Western Africa	0.4
Eastern Asia	0.5
Middle Africa	0.6
Northern Africa	0.6
Melanesia	0.6
Eastern Africa	0.7
South-Central Asia	0.7
Less developed regions	0.8
Caribbean	0.8
South-Eastern Asia	0.8
Southern Africa	0.9
Western Asia	1.5
Micronesia	1.5
South America	2.4
Central and Eastern Euro	2.6
Polynesia	2.6
Central America	2.9
Southern Europe	3
More developed regions	4.5
Northern America	5.4
Australia/New Zealand	5.7
Northern Europe	6.2
Western Europe	7.9

Figure 1.2: Age-standardised (World) incidence rates for testicular cancer, world regions, 2002 estimates



From: Cancer Research UK, http://info.cancerresearchuk.org/cancerstats/types/testis/incidence/

Bar chart to show differing incidence of testicular cancer worldwide

This bar chart shows that testicular cancer is more common in the developed world, with incidence rates around six times those found in developing countries

Summary

This review critically assesses the evidence that common and ubiquitous man-made environmental chemicals (ECs) contribute to human male reproductive disorders that manifest at birth (cryptorchidism, hypospadias) or in young adulthood (impaired semen quality or testicular germ cell tumours – hereafter referred to as TGCT). These disorders share risk factors and are hypothesized to comprise a testicular dysgenesis syndrome (TDS) with a common fetal origin, perhaps involving mild deficiencies in androgen production/action during fetal masculinisation.

A number of ECs, including pesticides, chemicals in consumer products and persistent organic pollutants (POPs) have been shown in animal studies to inhibit androgen production/action in fetal life; in addition, certain phthalates to which humans are widely exposed have been shown to induce a TDS-like collection of disorders in male rats following fetal exposure. Oestrogenic ECs have also been implicated in TDS disorders.

To provide background and to place the human studies in perspective, two overviews are initially presented to evaluate (1) the prevalence, and evidence for changing incidence, of human TDS disorders; and (2) the range of TDS-like effects of ECs and EC mixtures in animal studies, together with new understanding about when and how androgens regulate development of the male reproductive system and how this may relate to TDS disorders.

The aim is to provide a critical review of studies in humans which have investigated whether ECs contribute causally to male reproductive disorders that comprise TDS. The reason for this focus is that TDS disorders are common, some at least have increased in incidence in a time-frame that implicates environmental causes, and experimental animal and wildlife studies suggest that TDS-like disorders are induced by, or associated with, fetal exposure to certain ECs.

TDS disorders are best placed in perspective by considering some basic facts. Cryptorchidism (undescended testes) is probably the commonest congenital malformation of babies (of either sex) at birth. Hypospadias, in which the urethral opening on the penis is misplaced, is also remarkably common. Impaired semen quality is the most common TDS disorder and robust data collected from thousands of young men in prospective studies have established that, across western Europe, more than 1 in 6 have an abnormally low sperm count (<20 million sperm/ml) which will compromise their fertility. TGCT is the most common cancer of young men

and has doubled in incidence in many western countries – ~every 25 years over the past 60 years. Whether the other TDS disorders have increased in incidence is unclear due to lack of robust data – but some studies suggest this is the case.

The evidence from experimental studies in rats has established unequivocally that a growing number of ECs can inhibit androgen production/action and cause TDS-like disorders. The most human-relevant data comes from studies in rats using EC mixtures as these show that such ECs have additive effects at levels at which the individual component ECs are without significant effect. Fetal exposure of rats to certain phthalates has shown induction of a TDS-like syndrome that involves suppression of fetal testis androgen production. A key finding in rats has been identification of a 'male programming window' within which androgens must act to set up later correct development of the male reproductive system. Cryptorchidism, hypospadias and reduced testis and penile size all arise if there is deficient androgen action in this window – and this is also reflected for life by reduced anogenital distance (AGD). It is reckoned that the equivalent time window in humans is 8-12 weeks' gestation, and it is likely that EC action only within this time-frame could affect male development via an anti-androgenic mechanism. Oestrogenic ECs have been implicated in TDS because of evidence from diethylstilboestrol (DES)-exposed women in pregnancy and similar rodent studies. However, species differences in testicular oestrogen effects, and rather weak evidence for DES/oestrogen induction of TDS disorders in humans, makes oestrogenic ECs less likely than anti-androgenic ECs as causal agents, although recent evidence for effects of bisphenol A on germ cells merits further study in relation to TGCT.

Proof that ECs/EC mixtures cause TDS disorders in humans requires demonstration of exposure (at the relevant fetal time) linked to a mechanistic effect (reduced androgen production, for example) which is then linked to an outcome disorder(s). There are huge practical and ethical obstacles to being able to do this definitively, and this has to be taken into account (Table 1, p25). In particular, linking EC exposure in pregnancy to adult-onset TDS disorders is problematical for a number of reasons. Geographical differences in TDS disorders are established, suggestive of ethnic/genetic differences in susceptibility to TDS disorders (which may confound studies looking for EC associations with TDS) and/or reflecting differences in environmental impacts.

The best evidence has come from prospective studies focused specifically on TDS disorders in which EC exposure has been

measured directly rather than deriving it (from questionnaires, for example). Such studies have shown small but significant associations between specific ECs or EC groups and occurrence of cryptorchidism and/or hypospadias and TGCT, although the ECs identified are not always the same – they are mainly POPs, perhaps because it is easier to measure such chemicals in the body long after exposure. However, the most ubiquitous of these persistent pollutants (DDT, PCBs) were infrequently identified as being important in this context.

Phthalate exposure in pregnancy has been associated in one study with cryptorchidism in male offspring and with reduced AGD (indicative of reduced fetal testosterone exposure) in a US and a Mexican, but not in a Taiwanese, study. Other studies suggest that phthalates may reduce neonatal testosterone production in three-month boys and neonatal marmosets. On the other hand, two in vitro studies have failed to show any inhibitory effect of specific phthalate monoesters (MBP, MEHP) on testosterone production by human fetal testis explants. Therefore, the role that phthalates may play in TDS in humans is at present uncertain. If phthalate exposure does reduce fetal testosterone levels in vivo in humans to cause reduced AGD, this occurs at levels of exposure common to the general population and at lower doses (of individual phthalates) than those which induce this effect in rats. This might be explained by the fact that humans (but not laboratory rats) are exposed to other ECs in addition to phthalates.

Alternatively, the association of maternal phthalate exposure with adverse changes in boys may be fortuitous and result from connected lifestyle or other factors in the mother – in other words, it is the lifestyle that is causal but this lifestyle also happens to increase phthalate exposure of the mother (for example, by heavy use of personal care products). Further human studies to resolve the potential role phthalates may play in TDS are an urgent priority. No study has examined fetal EC exposure and sperm counts in adulthood, except for those that have shown a robust and major inhibitory effect of maternal smoking in pregnancy on sons' sperm counts; this may also increase the risk of cryptorchidism and hypospadias, but not TGCT.

It is concluded that EC exposure may contribute causally to TDS disorders, but there is presently no clear evidence that any single EC or EC class of compound is a major cause of TDS. The evidence points more towards the likelihood that EC effects on the risk of TDS results from the combined small effects of individual ECs (i.e. a 'mixtures' effect), which is challenging and expensive to evaluate; this risk is likely to be influenced by genetic predisposition. The role of EC mixtures in human TDS is likely to become clearer over the next few years as new studies in both humans and laboratory animals address this in more detail. Arguably the most urgent issue that needs to be resolved is whether or not phthalates – which are the most ubiquitous ECs and some of which can clearly cause TDS disorders in rats – contribute to the risk of TDS in humans, because present evidence is equivocal.

Overall, data suggest that exposure to EC mixtures probably accounts for a proportion of cases of cryptorchidism and hypospadias.

Introduction

Over the last 20 or so years, there has been a continuing debate as to whether exposure to common environmental chemicals (ECs) may cause male reproductive disorders in humans. This review will outline the strength of evidence for changing trends in male reproductive health, and highlight the difficulties inherent in establishing the relationships between these disorders and EC exposures – in particular the enormous practical issues and costs involved in trying to establish this in a rigorous, scientific manner.

Understanding these uncertainties and difficulties is essential when evaluating the degree to which ECs contribute to male reproductive disorders, and for decision-makers in determining the most appropriate policy. However, for the majority of human disease, it is accepted that interactions between the genetic make-up of the individual and his/her exposure to environmental and lifestyle factors is what determines whether or not disease will occur. This applies also to male reproductive health disorders, and has to be taken into account when considering the potential impact of ECs.

Aims, perspectives and limitations of this review

The aim is to provide a critical review of studies in humans which have investigated whether ECs contribute causally to male reproductive disorders that comprise testicular dysgenesis syndrome (TDS; see below for details). The reason for this focus is that TDS disorders are common: indeed, some have increased in incidence in a time-frame that implicates environmental causes, and experimental animal and wildlife studies suggest that TDS-like disorders are induced by, or associated with, fetal exposure to certain ECs. There are innumerable studies involving experimental exposure of laboratory animals to ECs, but these are not reviewed in detail and are only described when they are of direct relevance to the human TDS disorders, and a brief overview of such studies is provided to set the scene. This review also does not evaluate evidence for all chemical effects on human reproductive function, only those that are of relevance to TDS disorders. This necessarily imposes limitations on the scope of this review.

Two overviews are used to set the scene for the review. First, an assessment of the latest evidence on the prevalence of human TDS disorders and whether this is increasing. Second, an overview of recent studies in animals showing that individual ECs may cause TDS-like disorders, and in particular the growing evidence for effects of EC-mixtures in this context. An in-depth review of all relevant animal studies is not provided, as it is accepted that exposure to a number of ECs at high enough doses will cause one or more TDS-like disorders in experimental animals.

A particular emphasis of this review will be phthalates, because human exposure to them is ubiquitous and some have been shown to induce a TDS-like spectrum of disorders in rats. Moreover, there are several emerging studies in humans that have specifically investigated the potential link between exposure to phthalates and evidence for their anti-androgenic effects perinatally. In critically reviewing the relevant studies in humans, account has to be taken of the difficulties inherent in establishing ‘cause and effect’ for EC involvement in human TDS disorders. This involves considering the strengths and weaknesses of the approaches used in the various studies; most emphasis has been attached to prospective, specifically designed studies that have involved direct measurement of EC exposure.

Overview of prevalence and trends in male reproductive health disorders

The reproductive disorders that will be considered here affect males either at birth or in young adulthood; other disorders that manifest in older age such as prostate disease/cancer are not considered. For the diseases of interest here, there is surprisingly little visible public interest, probably because they are mostly not life-threatening and because of the embarrassing nature of the defects. Nevertheless, these disorders are remarkably common and pose considerable health problems for affected individuals.

Interest has focused primarily on four disorders which are thought to be interconnected (see below). These are: low sperm counts and testicular germ cell tumours (TGCT), which present in young adulthood, and incomplete testicular descent (cryptorchidism) and misplacement of the opening (meatus) of the urinary tract on the penis (hypospadias), which present at birth. There are probably other connected disorders (Sharpe & Skakkebaek 2008), but these will not be discussed because at present there is little in the way of hard data.

Low sperm counts/male infertility

An abnormally low sperm count (<20 million/ml; the WHO cut-off for normal) is extremely common in men, with a prevalence of 4-8% according to textbooks (Irvine 1998), although this is almost certainly an underestimate based on most recent studies (as detailed below). A low sperm count considerably increases the likelihood of the male being infertile, especially if his female partner also has low or reduced fertility (Irvine 1998). Concern about low sperm counts was raised dramatically in 1992 with publication of a meta-analysis of published studies for sperm counts in men without fertility problems that had been reported over the preceding ~50 years (Carlsen et al 1992). This showed that average sperm counts had fallen by approximately half in this time period. This finding, which has been reinforced by further analysis of even more studies (Swan et al 2000), has attracted controversy and debate (see Jouannet 2001). Without going into the details of this debate, the bottom line is that it is uncertain whether sperm counts

really have fallen as these studies indicate, nor if they have, what the magnitude of this fall has been. Nor is it clear in which countries these declines have occurred or not.

This uncertainty may be surprising, but needs to be placed in context. Most men will never know what their sperm count is, because they will never need to have it measured – whereas most men who do have their sperm count measured are experiencing couple fertility problems and this measurement forms part of their clinical work-up (e.g. Irvine 1998; WHO 1999). Therefore, most of the information on sperm counts derives from men with potential fertility problems – and even when supposedly fertile men are recruited for studies, there are often concerns whether they are truly representative of the normal population.

It is also well established that sperm counts in an individual can fluctuate enormously over time, even descending into the abnormal range for some periods (WHO 1999). Additionally, sperm counts not only show remarkably high variation between individual healthy men, but there are also huge errors associated with measuring sperm counts, even in reputable, experienced laboratories (e.g. Irvine 1998; WHO 1999). Despite these issues, data from some countries, including the UK and France, that show a significant decline in sperm count according to later years of birth (Auger et al 1995; Irvine et al 1996) is consistent with a fall in sperm counts over time; other evidence also points to this (see below).

Other factors contribute to sperm count variation. There have been several well-controlled studies in the last 15 years which have shown marked geographical differences in sperm counts between normally fertile men either within a country (France, US) or between different north-European countries (Auger et al 1997; Jorgensen et al 2001, 2002; Swan et al 2003a); additionally, there may be ethnic differences such as between Asian and western men (Johnson et al 1998). This and the other factors outlined above have raised questions about the comparability of data for sperm counts reported over the past few decades, and cast doubt as to whether they really have fallen. Therefore, based on the available scientific evidence, the issue of 'falling sperm counts' must be considered as unresolved. However, no rational explanation has been put forward to explain why sperm counting errors, variability in sperm counts or geographical influences should have pushed them in a single, downward direction rather than simply increasing variability, and a mean decrease of ~50% across all of the studies is difficult to explain away rationally. In Europe, it was recognized that the only alternative approach to this issue was to establish in a robust fashion what sperm counts were in young men. The thinking involved was that if sperm counts really have fallen, then men who have been born most recently should have low average sperm counts.

A series of coordinated studies in seven European countries (Germany, Denmark, Sweden, Norway, Finland, Estonia, Lithuania) have thus been undertaken prospectively over the last 10 years, involving thousands of young men and carefully standardized techniques; this avoids criticisms about comparability of measurements levelled at retrospective sperm count studies. The studies have focused mainly on military conscripts aged 18-25 years, who are considered representative of the general young male population. Across all of these studies, the average sperm counts in young men has turned out to be remarkably low (~40-65 million/ml) – and, even more worryingly, a remarkably high proportion (20-25%) of these men have an abnormally low sperm count (<20 million/ml) (see Jorgensen et al 2002, 2006; Richthoff et al 2002; Carlsen et al 2005; Paasch et al 2008). These findings are exactly what would have been predicted from the 'falling sperm count' data (Carlsen et al 1992), and can be viewed as the closest that it is possible to get to proving this hypothesis.

Notwithstanding the difficulty of being sure whether or not sperm counts have really fallen, it is clear that, at least in much of Europe, low sperm counts in young men are extremely common. Similar studies have not yet been

undertaken in the same age group in countries outside Europe, but as the Baltic countries in Europe (including Finland) have generally higher sperm counts than in more western European countries (Vierula et al 1996; Jorgensen et al 2001, 2002, 2006; Tsarev et al 2005), a high prevalence of low sperm counts in young men may not be a completely generalized phenomenon. Even so, this raises concerns about the future fertility of western European young men, as sperm counts for many of them are at or below the level that affects couple fertility (Bonde et al 1998). Such effects will be exacerbated by the trend among women to delay having their first babies, as female fertility is already on the decline at age 30. Evidence for such effects may already be apparent in Denmark – the country with the lowest reported sperm counts in young men, and where 7% of all live births in 2007 were attributable to some form of assisted conception (www.fertilitetsselskab.dk), a rate that has increased progressively over the past decade or so (see Skakkebaek et al 2007; Andersson et al 2008).

An important question that arises from the low sperm count issue is what determines sperm counts in an individual man? Unlike most animals, men do not store sperm, so their sperm count is largely a reflection of how many sperm are being produced, coupled with their ejaculatory frequency. The major factor determining sperm count in an individual is the number of Sertoli cells in his testes: these control the process of spermatogenesis and each Sertoli cell can only support a fixed number of germ cells through development into sperm (Sharpe et al 2003). Sertoli cell numbers in men vary just as widely as do sperm counts (Johnson et al 1984; Sharpe et al 2003) – and as is outlined below, the number of Sertoli cells may be affected by events in fetal life, which could be vulnerable to effects of ECs.

Testicular germ cell tumours (TGCT)

TGCT is the commonest cancer of young men, peaking at 25-30 years; this is unusual, as most cancers affect older people. TGCT in young men arises from precursor cells (termed CIS cells) which have their origins in fetal life. The details of this evidence are beyond the scope of this review but can be found elsewhere (see Rajpert-de Meyts 2006; Cools et al 2006; Rajpert-de Meyts & Hoei-Hansen 2007). TGCT incidence has increased progressively over the past 50-60 years in European and several other countries across the world, at least among Caucasian men (Richiardi et al 2004a; Purdue et al 2005; Bray et al 2006a, b). Because of the rapidity of this increase, it must have environmental/lifestyle causes. TGCT is six times more common in developed compared with developing countries, although this may reflect lower susceptibility to TGCT among non-Caucasians (Bray et al

2006a). About 500,000 new cases of TGCT were diagnosed worldwide in 2002 (Bray et al 2006a). Although curable in most cases, it has significant morbidity and men who develop TGCT are likely to have lower fertility than normal (Richiardi et al 2004b; Baker et al 2005; Raman et al 2005; Dieckmann et al 2007). A history of cryptorchidism is the most important risk factor for development of TGCT (Dieckmann & Pichimeier 2004; Kaleva & Toppari 2005), increasing risk by ~8-fold, although most boys born with cryptorchidism do not go on to develop TGCT.

An important source of variation in the incidence of TGCT is geographical location. Denmark and Norway have about a four-fold higher incidence of TGCT than does Finland, with Sweden intermediate (Richiardi et al 2004a). In the US, there is a similar magnitude of difference in incidence of TGCT between Caucasians and Afro-Americans (McGlynn et al 2005; Shah et al 2007). The latter suggests differences in genetic predisposing factors to TGCT as these ethnic groups share broadly the same environment – although, interestingly, recent data indicate that the incidence of TGCT in Caucasian men in the US may have plateaued (Shah et al 2007), whereas in Afro-American men it is increasing (McGlynn et al 2005; Shah et al 2007).

A similar trend is perhaps also emerging in Europe, as TGCT incidence is increasing in Finland (where the incidence has been low) but appears to be plateauing or even declining in Denmark (Moller 2001; Jacobsen et al 2006). Therefore, although the differences in TGCT incidence between ethnic groups/ Scandinavian countries could reflect genetic differences in predisposition, an alternative view is that environmental factors may be more important and that they may have been experienced differently by ethnic groups or different Scandinavian countries. Strong support for this interpretation comes from the study of migrants from Finland, with a low risk of TGCT, who move to a country such as Denmark with a high risk, or vice versa. These show that first-generation immigrants have the same incidence of TGCT as in their country of origin, whereas second-generation immigrants (i.e. those born in the country to which their parents have emigrated) have a similar risk to those native to that country (Montgomery et al 2005; Giwercman et al 2006; Myrup et al 2008). This indicates that environmental factors are important determinants of the risk of TGCT. Nevertheless, familial factors are also important (Richiardi et al 2007; Walschaerts et al 2007), so gene-environment interactions are almost certainly involved in determining risk of TGCT.

Cryptorchidism

This is arguably the commonest congenital malformation at birth in children of either sex. It is generally accepted to affect 2-4% of boys at birth according to registry data (Toppari et al 2001; Virtanen et al 2007), although recent prospective, non-registry based studies in Denmark suggest that the incidence may be considerably higher (9%) (Boisen et al 2004) and a recent prospective study in the UK suggested that incidence at birth may be over 6% (Hughes & Acerini 2008). Cryptorchidism can affect either or both testes but most cases usually involve one testis (Foresta et al 2008). By around three months of age, the incidence is usually more than halved due to spontaneous descent of the originally cryptorchid testis (Berkowitz et al 1993; Virtanen et al 2007). This 'delayed' testicular descent has perhaps coloured perceptions of the disorder as simply representing a somewhat late variation of normal (delayed descent) as opposed to an abnormality per se. An alternative view is that even where the condition is self-resolving, it may indicate that there has been malfunction of the normal reproductive development process in that individual, even though this may be relatively subtle (Skakkebaek et al 2001; Kaleva & Toppari 2005).

Normal testis descent into the scrotum from its point of origin by the kidney occurs in two phases – descent within the abdomen into

the pelvis, then through the pelvis (inguinal canal) into the bottom of the scrotum where it should remain fixed for life (Amann & Veeramachaneni 2007; Foresta et al 2008). The trans-abdominal phase of testes descent occurs early in gestation (11-17 weeks) whereas the second (trans-inguinal) phase is a late event (27-35 weeks). It is the second phase of testicular descent that is thought to be most androgen-dependent and its failure may therefore indicate deficiencies in androgen production/action, as detailed below.

It is also significant that it is the second phase of testicular descent that most commonly occurs in boys who present with cryptorchidism at birth, and the high frequency of self-resolution of these cases is often attributed to the high levels of testosterone produced by most boys in the first three to five months after birth (Toppari et al 2001; Virtanen et al 2007). In contrast, regulation of the intra-abdominal phase of testis descent depends to an extent on another hormone produced by the fetal testis, insulin-like factor 3 (Insl3), and deficiencies in the production or action of this hormone can result in failure of testis descent (Foresta et al 2008). However, such deficiencies do not appear to be a common cause of cryptorchidism in humans and, as already mentioned, deficiencies in the second, androgen-dependent, phase of testis descent is the most common in boys at birth (Foresta et al 2008). Nevertheless, one reason for interest in Insl3 is that in animal studies its production can be inhibited by fetal over-exposure to oestrogens, and thus potentially by oestrogenic ECs, and it can also be inhibited by exposure to certain phthalates; these aspects are discussed briefly below.

Despite the recent studies suggesting a high incidence of cryptorchidism at birth in some countries, it remains unclear if the incidence has changed in recent decades across Europe and elsewhere (Paulozzi 1999; Toppari et al 2001; Virtanen et al 2007; Hughes & Acerini 2008). This uncertainty is due to several factors. First, diagnosis of cryptorchidism is not straightforward and the exact position of the testis is not always recorded and reported. This means that the use of registry data (in some, but not all, countries cryptorchidism has to be registered as a birth anomaly) is unreliable and is therefore difficult to compare between countries and across time intervals (Paulozzi 1999; Virtanen et al 2007). The added complication is that because spontaneous resolution of cryptorchidism occurs in many cryptorchid boys in the first three months of life, standardization of the time of diagnosis of cryptorchidism is important. Therefore, the most reliable data on incidence trends is that which has been collected in prospective studies that have targeted normality of testicular descent and have defined this using standardized criteria. Studies which have used

these approaches in the UK (see Paulozzi 1999; Toppari et al 2001; Hughes & Acerini 2008) and in Denmark and Finland (Boisen et al 2004) have produced data suggesting an increased incidence of cryptorchidism over the past few decades. Overall, however, evidence of any generalized increase with time is lacking (Paulozzi 1999), although this could be due to the unreliability of registry data.

Another important finding from careful prospective studies was that newborn boys in Denmark have a 4.4-fold higher incidence of cryptorchidism at birth than do boys born in Finland – a difference that reduces to 2.2-fold at three months of age (Boisen et al 2004); the difference is of similar magnitude to that found for TGCT between Denmark and Finland (Richiardi et al 2004a). However, in comparing Afro-Americans and Caucasians in the US, evidence suggests that the incidence of cryptorchidism in these boys is not substantially different and certainly does not show the same magnitude of difference as is found for TGCT in these populations (McGlynn et al 2006a). Nevertheless, the Danish-Finnish difference suggests that, like TGCT, cryptorchidism may differ geographically in incidence, and this should be kept in mind when evaluating results. It should be remembered that cryptorchidism is the most important risk factor for development of TGCT.

Hypospadias

After cryptorchidism, hypospadias is the commonest congenital abnormality in boys and reportedly affects 0.2-0.7% of boys at birth depending on the study and country (Paulozzi 1999). Hypospadias varies considerably in its severity (Willingham & Baskin 2007). Many cases are relatively mild with the urethral meatus being misplaced to the edge of the glans or to the top of the penile shaft. In moderate cases the meatus is located lower down the shaft and in severe cases lower still and perhaps even in the perineal region, the latter often being associated with other malformations of the penis (Willingham & Baskin 2007). Moderate and severe cases need surgical correction and may involve several operations. In terms of mechanistic causes of hypospadias, it is established from human and animal experimental studies that interference with androgen production or action is critically important in ensuring normal location of the urethral meatus as a result of closure of the urethral folds over the urethra during fetal development of the penis (Baskin et al 2001). Though mild androgen deficiency provides a potential explanation for some cases of hypospadias, direct cause is usually not established.

As with cryptorchidism, data for incidence of hypospadias largely derives from registry information which is widely accepted as unreliable (Paulozzi 1999; Toppari et al 2001). This is due to several reasons, such as under-diagnosis (especially in mild cases) and under- or incomplete reporting. This uncertainty makes it difficult to establish whether or not there is an increase in incidence of hypospadias, but data in the literature for several European countries (England, Finland, France, Denmark and Norway) (Paulozzi et al 1999; Pierik et al 2002) as well as for the US (Paulozzi et al 1997; Paulozzi 1999; Nelson et al 2005), Australia (Nassar et al 2007) and China (Wu et al 2005) all appear to indicate an increased incidence of hypospadias in recent decades. Whether this increase has continued over the past 10-20 years is less certain, especially in the US (Paulozzi 1999; Carmichael et al 2003; Porter et al 2005). There may also be between-country differences – notably between Denmark and Finland, with the former having a substantially higher incidence of hypospadias than the latter (Boisen et al 2005), as was found also for cryptorchidism (Boisen et al 2004) and TGCT (Richiardi et al 2004a). This comparison derives from carefully designed prospective studies and is therefore reliable. Data from the USA is also consistent with Caucasian boys having a higher incidence of hypospadias than Afro-American boys (Carmichael et al 2003; Nelson et al 2005; Porter et al 2005) but this derives from registry-based studies and is therefore not as reliable as the Danish-Finnish comparison.

Testicular Dysgenesis Syndrome (TDS)

Based on epidemiological studies, the four disorders outlined above are risk factors for each other and share other pregnancy-related risk factors (Skakkebaek et al 2001; Sharpe & Skakkebaek 2003). Developmentally, it is understandable how maldevelopment of the early fetal testis could lead to functional changes in the testis, notably in hormone production, which would then increase the risk of developing one or more of the described disorders (Skakkebaek et al 2001; Sharpe & Skakkebaek 2008). As a consequence, it has been suggested that the disorders represent a syndrome, termed 'testicular dysgenesis syndrome' (TDS), with a common origin in fetal life (Skakkebaek et al 2001). The shared common origin is a hypothesis, although it is now widely accepted as a reality in view of the strong support from human epidemiological data (Skakkebaek et al 2008), from experimental animal research (see below) and the growing recognition in medicine of the key importance of fetal events in determining risk of adult disease (Gluckman & Hanson 2005).

Nevertheless, even assuming that the TDS hypothesis is correct, it does not mean that every case of each of the component TDS disorders will arise as part of this syndrome, except perhaps for cases of TGCT. For example, low sperm counts can result from a number of factors that include genetic mutations/disorders, or factors that may impact on the adult testis and which do not involve any events in fetal life. In this regard, it remains unclear what percentage of cases of low sperm counts in young men might have their origins in fetal life as part of TDS (Sharpe & Skakkebaek 2008), as there is currently no way of identifying such individuals definitively. It is also certain that some cases of cryptorchidism and hypospadias will arise for reasons other than TDS (both are common in various syndromes due to chromosomal disorders/mutations, for example), but again the percentage of cases arising because of TDS remains uncertain.

Even if it is accepted that many cases of TDS disorders have their origins in fetal life, identifying the causes of TDS remains difficult for two reasons. First, the fact that adult-onset TDS disorders are separated from their cause in fetal life by 20-40 years or more makes establishing causal links very difficult. Second, the time period in fetal life when TDS disorders are thought to be induced (8-15 weeks gestation – see below), is largely inaccessible for evaluation of the fetus and of the fetal testis, even if study of the mother (her

EC exposure, for example) is a possibility.

Nevertheless, there are several lines of evidence that support the idea that environmental exposure of the baby in the womb could contribute causally to TDS. First, it is beyond dispute that incidence of TGCT has increased progressively in Caucasian men in recent decades (see above) and this increase must have environmental/lifestyle causes that affect the germ cells in the fetal testis. Second, there is abundant evidence from wildlife that reproductive development, including of the gonads and genitalia, can be affected adversely by EC exposures of one or more types (Lyons 2008). Third, and perhaps most convincingly, a TDS-like syndrome can be induced experimentally in laboratory rats by fetal exposure to certain phthalate esters such as dibutyl phthalate (DBP) or diethylhexyl phthalate (DEHP). Exposure of pregnant rats to high levels of such phthalates results in a spectrum of disorders in the male offspring similar to TDS disorders in humans (Gray et al 2000, 2006; Mylchreest et al 2000; Fisher et al 2003; Mahood et al 2007), also termed 'phthalate syndrome' (Foster 2006). For example, DBP exposure results in increased incidence of cryptorchidism and hypospadias of varying severity and impairment of sperm production and fertility in adulthood (Fisher et al 2003; Mahood et al 2007). Some causes of these changes are established and revolve around inhibition of testosterone and/or Insl3 production by the fetal testis, which then leads to downstream disorders (Parks et al 2000; Fisher et al 2003; Foster 2006; Mahood et al 2007), a change predicted by the original TDS hypothesis (Skakkebaek et al 2001). Additionally, focal dysgenesis of the testis occurs in DBP/DEHP-exposed fetal rats (Mahood et al 2005, 2007) and similar testicular changes can be observed in some adult patients with TGCT (Sharpe 2006).

Observations such as those just described provide strong support for the TDS hypothesis in humans as well as providing an animal model in which some of the mechanistic pathways leading to TDS disorders can be explored further (Sharpe & Skakkebaek 2008). One example of such a development has been the discovery that androgen action is essential within the fetal testis to increase Sertoli cell proliferation in fetal life (Scott et al 2007), this being of importance because it is final Sertoli cell numbers that determine sperm-producing capacity in adulthood and thus determine sperm count in an individual man (Sharpe et al 2003). DBP exposure of the rat in utero results in reduced Sertoli cell numbers at birth as a consequence of reduced androgen production/action (Scott et al 2007, 2008). This provides a potential explanation of how reduced androgen action in fetal life could lead to reduced sperm counts in adulthood in humans. Whether this is truly the case is, however, questionable: recent follow-up studies in these animal models have shown that even substantial reductions in Sertoli cell numbers at birth can be compensated for postnatally (presumably by increased Sertoli cell proliferation) (Hutchison et al 2008; Scott et al 2008), and such compensatory mechanisms are likely also to operate in primates (Sharpe et al 2000).

Despite the similarities between 'phthalate syndrome' in rats and TDS disorders in humans, caution should be exercised when extrapolating from the rat to the human. For example, one recent study has shown that DBP has no effect on steroidogenesis by the fetal mouse testis as it does in the rat, despite causing similar germ cell changes to those observed in fetal rats (Gaido et al 2007). Some of the evidence for humans, reviewed below, suggests that the human fetal testis might respond in a similar way to the mouse rather than the rat. Another study has shown that different strains of rats (Sprague-Dawley and Wistars) can respond differently to DBP/DEHP exposure in terms of resulting disorders (Wilson et al 2007), perhaps analogous to the ethnic differences in incidence of TDS disorders in humans described above.

Male programming window

Another important development from experimental studies in rats has been the discovery of what is termed the 'male programming

window'. These studies have established that when the fetal testis first forms and begins to produce testosterone, it is the actions of androgen at this stage in development that are responsible for setting up later normal development of the entire reproductive tract, including the genitalia (Welsh et al 2008). This is referred to as a programming window because the time at which androgens have this effect is not manifest by obvious morphological changes in the target organs, which remain essentially the same in males and females at this fetal stage. However, androgen action within this time-frame is essential if the reproductive organs are to develop later in gestation and in the postnatal period. This applies to the internal reproductive organs, penile development and testicular descent.

Arguably the most important aspect of this discovery is that cryptorchidism and hypospadias can only be induced by deficient androgen action within the male programming window (Welsh et al 2008). Blockade of androgen action during the period when the penis is forming or when testis descent is being completed has no effect. Another important factor is that it is the second phase of testicular descent (the androgen-dependent phase) which is affected by this programming, and this phase is most commonly affected in human cryptorchidism.

These findings have considerable implications for EC-induction of TDS-like disorders in rats via anti-androgenic mechanisms, because the same timing windows for androgen action will apply, as indeed is the case for phthalates (Wolf et al 2000; Carruthers & Foster 2005; Scott et al 2008). The latest evidence shows that phthalates such as DBP only exert modest suppression of testosterone production by the fetal rat testis during the male programming window, and its major suppressive effects on steroidogenesis occur after this time window (Shultz et al 2001; Scott et al 2008). Thus, DBP and other phthalates may be relatively ineffective in causing TDS disorders as a result of androgen suppression, and this presumably explains why DBP/DEHP exposure has rather small negative effects on endpoints such as anogenital distance (AGD; see below) (Mylchreest et al 2000; Carruthers & Foster 2005; Scott et al 2008). This has implications for human studies, discussed later. In contrast, ECs that inhibit androgen action by blocking the androgen receptor (AR) will do so with equal effectiveness within and outside the male programming window (Wolf et al 2000; Foster & Harris 2005; Welsh et al 2008), but their effectiveness in causing TDS disorders will be directly related to exposure during the period of the window. For example, two studies have shown that fetal exposure of rats to 2,3,7,8-tetrachlorodebenzo-p-dioxin (TCDD) commencing at the start of the male programming window (e15.5) reduces AGD,

prostate weight and penis length (Ohsako et al 2001, 2002), all of which are predicted outcomes of inhibiting androgen production or action within the male programming window (Welsh et al 2008).

AGD is normally about 1.7 times as long in males as in females in rats (Gray et al 1999, 2001) and humans (Huang et al 2008; Swan 2008), and it is also programmed by androgen action within the male programming window (Ema et al 2000; Carruthers & Foster 2005; Foster & Harris 2005; Welsh et al 2008). Although AGD is of minimal biological significance, it is fixed for life after androgen action in the programming window and thus (in most instances) provides a lifelong 'readout' of peripheral androgen exposure of the fetus during this period. This potentially provides a non-invasive insight into this hidden period of fetal life and may prove clinically useful.

Studies in rats have shown that AGD length predicts the incidence and severity of cryptorchidism and hypospadias (Welsh et al 2008), penile length (Welsh et al 2008) and size of the testes (Scott et al 2008) at all ages from birth through to adulthood. The latter observation is important as it suggests an integral connection between androgen action within the male programming window and subsequent capacity to make sperm in adulthood. It was anticipated that this relationship involved programming of Sertoli cell number, but this has proved not to be the case (Scott et al 2008). Studies in humans suggest that, as in rats, a similar relationship exists between AGD in babies and the occurrence of hypospadias (Hsieh et al 2008) and cryptorchidism (Swan et al 2005; Hsieh et al 2008). These observations reinforce the idea that early production and action of androgens by the male fetus is important in determining normality of reproductive development and function throughout life and that deficiencies in androgen production/action, irrespective of the cause, is likely to lead to one or more TDS disorders (Sharpe & Skakkebaek 2008). ECs that can reduce androgen action or, especially, its production by the human fetal testis, would therefore be logical candidates for causing or contributing to TDS disorders in humans, assuming a sufficient level of exposure of the fetus.

Overview of experimental animal studies involving environmental chemical (EC) induction of 'TDS-like' disorders

Anti-androgenic ECs and TDS

Humans are exposed to a considerable number of ECs with potential endocrine disrupting (ED) activity. These include chemicals with anti-androgenic activity (see Toppari et al 1996; Gray et al 1999, 2001; Wilson et al 2008) and those with oestrogenic activity (Toppari et al 1996; Vos et al 2000). Phthalates such as DBP, which have been discussed above in the context of TDS models, are one example of an anti-androgenic chemical to which there is substantial human exposure (see below), but humans are exposed to a range of anti-androgenic chemicals which, in experimental animals, induce their effects via different mechanisms (Gray et al 2001, 2006; Wilson et al 2008).

For example, several pesticides and fungicides (such as Vinclozolin, DDE and Procymidone) exert their anti-androgenic effects by binding to the AR – and then instead of activating it, sit there and block it and thus prevent activation of that receptor by endogenous androgens (Gray et al 2006; Wilson et al 2008). Such chemicals are referred to as AR antagonists and they mimic some of the therapeutic drugs, such as flutamide, which were developed specifically for their anti-androgenic properties. In animal experimental studies, such compounds have been shown to cause dose-dependent disruption of male reproductive development and to induce disorders such as hypospadias, cryptorchidism and reduced AGD. Some compounds, such as Linuron and Prochloraz (both pesticides), exert anti-androgenic effects by both inhibiting testosterone production by the fetal rat testis (Wilson et al 2008, 2009) and by binding to and blocking the AR (Wilson et al 2008), and these will also induce some of the TDS disorders.

Other widely distributed ECs, such as PCBs and PBDEs, can affect adult testis size and/or spermatogenesis/fertility when administered to rats and may also be able to affect steroidogenesis in adulthood (Hany et al 1999;Kaya et al 2002; Kuriyama et al 2005), although such compounds have not really been considered as anti-androgens. However, one study (Lilienthal et al 2006) has shown reduced AGD in male rats after in utero exposure to PBDE (indicating inhibition of fetal testosterone production) as well as reduced testosterone levels at puberty and in adulthood. The list of anti-androgenic ECs is continuing to grow as more ECs are evaluated by screening assays (Araki et al 2005). With the high prevalence of TDS disorders in humans and the likely role that deficient androgen production/action may play in their aetiology (Sharpe & Skakkebaek 2008), an obvious question is whether the anti-androgenic ECs that cause TDS-like disorders in rats also cause or contribute to these disorders in humans. This review addresses that very question.

Oestrogenic ECs and TDS

Oestrogenic ECs have also been considered as having the potential to cause TDS disorders in humans and in animal studies (see Toppari et al 1996; Sharpe 2003; Hotchkiss et al 2008). The main impetus for this was the evidence for reproductive disorders in human males whose mothers had been treated during pregnancy with high doses of diethylstilboestrol (DES), the potent synthetic oestrogen, to prevent threatened miscarriage (Toppari et al 1996). This resulted in increased incidence of cryptorchidism and 'urethral abnormalities' (but not hypospadias) in exposed males and also evidence for adverse testicular effects (small testes) and an increased frequency of low sperm counts (Toppari et al 1996; Baskin et al 2001), although fertility was unaffected (Wilson et al 1995). This was reinforced by studies showing that experimental exposure of fetal rats and mice to DES or ethinyl oestradiol could reduce AGD (Howdeshell et al 2008), and induce cryptorchidism, hypospadias and testicular and other reproductive tract abnormalities in >30% of male offspring (Vorherr et al 1979; Toppari et al 1996).

These results are readily explained by further studies showing that DES exposure in pregnancy suppresses both testosterone (Haavisto et al 2001; Delbes et al 2006) and Insl3 production (Nef et al 2000; Sharpe 2003) by the fetal rat/mouse testis. The discovery that numerous ECs also have (weak) oestrogenic activity (Toppari et al 1996; Hotchkiss et al 2008) raised the obvious possibility that such compounds could cause similar effects to DES, especially as exposure to some of these compounds had been associated with intersex or masculinisation disorders in a range of animals (reviewed in Hotchkiss et al 2008; Lyons 2008).

The apparent similarity of animal and human findings with regard to DES raised the possibility that human exposure to oestrogenic ECs might contribute causally to human TDS disorders, in particular to cryptorchidism and hypospadias. However, there is a fundamental species difference that effectively rules this out, at least via any direct oestrogenic effect on fetal Leydig cells. The DES-induced suppression of testosterone and Insl3 production by fetal rodent Leydig cells is mediated via oestrogen receptor-α (ERα), as these effects do not occur in ERα knockout mice (Cederroth et al 2007). In the human, ERα is not expressed in fetal or postnatal Leydig cells (Gaskell et al 2003), unlike in rats and mice (Fisher et al 1997), and steroidogenesis appears unaffected by ethinyl oestradiol (Kellokumpu-Lehtinen et al 1991). Therefore, oestrogen-mediated inhibition of fetal steroidogenesis is unlikely in humans, and it seems equally unlikely that any (direct) effect on Insl3 will occur for the same reason. This means that there is no straightforward explanation for the DES-induced disorders in humans (for the increased incidence of cryptorchidism, for example), although it perhaps explains why serious masculinisation disorders were infrequent (especially in comparison with rodent studies), despite the exceedingly high DES exposure (Toppari et al 1996).

There is a potential Leydig cell-independent mechanism via which DES or oestrogenic ECs might adversely affect development of male reproductive tissues, such as the penis, and this is via direct effects on the target organ. In rats, DES exposure neonatally can induce complete loss of AR protein expression in the testis, penis, epididymis and prostate, thus blocking androgen action, and this effect is also ERα-mediated (McKinnell et al 2001; Rivas et al 2002; Goyal et al 2007). It is not known whether a similar effect can also occur in the human, but an obvious question is whether oestrogenic ECs might also activate this mechanism. This seems unlikely as this effect has only been shown to occur after exposure to extremely high doses of potent oestrogens, such as DES, and not after high dose exposure (~4mg/kg) to a weak environmental oestrogen, bisphenol A (Rivas et al 2002). Overall, the absence of convincing evidence that DES or other potent oestrogen/hormone exposure in early pregnancy can induce hypospadias or other TDS disorders in humans (Raman-Wilms et al 1995; Toppari et al 1996; Martin et al 2008) makes it rather unlikely that exposure to oestrogenic ECs will be major players in causing TDS disorders, at least those involving an ER-mediated mechanism.

It is still possible that oestrogens, or certain oestrogenic ECs, could exert effects via an ER-independent mechanism. In this regard, a recent study has shown that environmentally relevant levels of bisphenol A can increase the proliferation of a human TGCT (seminoma) cell line via an ER-independent, membrane-mediated mechanism (Bouskine et al 2009). Furthermore, ER-mediated oestrogen action on the same cells (presumably via ERα) antagonized this effect of bisphenol A, and similar inhibitory effects of oestrogens on fetal germ cell proliferation have been found in rodent studies (reviewed in Delbes et al 2006). Whether bisphenol A might stimulate proliferation of fetal human germ cells, from which the seminoma cells derive via CIS, is unknown but seems likely. However, even if this occurred, it is not obvious how this might relate to the formation of CIS cells or their development into TGCT.

No single study of sons of DES mothers has shown a significant increase in testicular cancer, but a meta-analysis of available studies concluded there was an overall increase of approximately two fold which was just statistically significant (Toppari et al 1996), but there are no relevant data for bisphenol A. Studies in rats have shown no effect of fetal exposure to bisphenol A, in a wide range of doses (2 - 40,000μg/kg/day), on AGD or the occurrence of TDS-like disorders in male offspring (Kobayashi et al 2002; Tinwell et al 2002; Howdeshell et al 2008). Therefore, compared with anti-androgenic ECs, it appears that oestrogenic ECs are probably not important players in the origins of human TDS disorders, although whether they might exacerbate effects of anti-androgenic ECs in mixtures is an interesting possibility that has yet to be investigated.

Risk assessment of ECs and EC mixtures

In general, the effects of the types of ECs mentioned above have been demonstrated in rodents at levels of exposure (for individual ECs) which are thought to be considerably higher than the comparable level for humans, although often there is a paucity of accurate human exposure data (see below for phthalates). This is too large and complex an area to be reviewed here, but it is a key issue. If humans are only exposed to levels of an EC that are 100,000 times lower than the lowest dose that causes TDS effects in rats, it might be considered safe to conclude that, although the chemical poses a potential hazard, it does not pose a risk at normal human exposure levels.

However, accurate risk assessment depends on knowing the range of human exposure (especially in vulnerable groups such as children and particularly the unborn child), the true no-observed effect level (NOEL) in rats and what sort of assessment factors should be included to guard against species differences and other differences in individuals within the species to be protected – for example, in metabolism. These issues are handled by government regulatory agencies which then decide on an acceptable level of exposure consistent with no effect (a safe level of exposure or an acceptable daily intake). Such risk assessments are largely performed on a chemical by chemical basis. However, a series of recent studies involving exposure of fetal rats to mixtures of anti-androgenic ECs have shown that this individual chemical method of safety assessment may not be adequate and will need to be rethought (Kortenkamp 2008). This is because in reality, humans and wildlife are exposed to many chemicals simultaneously, both from the environment around them and from chemicals already stored in their bodies – meaning that a judgment on the acceptable level of exposure to the mixture is a necessity.

The aforementioned studies have shown additive effects of mixtures of 'anti-androgenic' ECs in causing adverse male reproductive changes such as hypospadias and reduced AGD at doses at which the individual component ECs have minimal or no effect. Such effects have been shown for mixtures of 2 (Howdeshell et al 2007; Hsu et al 2008) or 5 (Howdeshell et al 2008) phthalates, for a mixture of 2-3 non-phthalate anti-androgenic ECs (Hass et al 2007; Metzdorf et al 2007; Christiansen et al 2008) or a mixture of 1 phthalate + 1 non-phthalate anti-androgenic ECs (Hotchkiss et al 2004) or, in the biggest study of all, a mixture of 3 phthalates + 4 non-phthalate anti-androgenic ECs (Rider et al 2008). Essentially comparable results were found in all studies, with the anti-androgenic effects being concentration-additive, although the endpoints assessed were not identical in every study. One study showed that exposure to a mixture of five phthalates additively suppressed testosterone levels/production in the fetal rat testis (Howdeshell et al 2008).

There are numerous implications of these new findings (Kortenkamp et al 2007; Kortenkamp 2008), the most important being that risk assessment of anti-androgenic ECs for humans has to consider not the toxicity and level of exposure of the individual ECs, but the sum of exposure to all ECs with anti-androgenic activity. Indeed, a recent report from the US National Academies concluded that cumulative risk assessment should be applied to chemicals that cause common adverse outcomes (www.nap.edu/catalog/12528.html). To achieve this effectively means that we must know the full range of such ECs, the level of human exposure to each EC and their dose-response anti-androgenic effect; in the context of TDS the latter has to involve in vivo studies. These new findings also have important implications for both the design and interpretation of epidemiological studies in humans to assess the involvement of EC exposure in TDS disorders. Such studies need to measure and take account of multiple EC exposures, and it is reassuring to see that this is becoming the case (see below). This will also necessitate careful design of statistical analytical methods to avoid confounding from multiple measures.

Causes of TDS disorders in humans

Genetic causes/ predisposition

The original TDS hypothesis postulated that any event that resulted in maldevelopment of the fetal testis was likely to lead to malfunction of the somatic cells of the testis and thus lead potentially to TDS disorders (Skakkebaek et al 2001). Deficient androgen production by the Leydig cells was suggested as one such malfunction. The hypothesis was framed in this way because it was established that genetic disorders leading to testicular dysgenesis (Skakkebaek et al 2001) as well as inactivating mutations, such as partial inactivation of the AR (Cools et al 2006; Looijenga et al 2007), could lead to increased risk of TDS disorders. Consequently, there is unlikely to be a single cause of TDS disorders, but rather multiple causes which interact with each other. The fact that TDS disorders occur with different frequency in different countries (Denmark versus Finland, for example) indicates that either there are unique environmental factors that differ between countries or that there are genetic differences between populations in different countries that either predispose to, or protect from, TDS disorders. For example, in normal boys at birth, testis development appears to be more advanced in Finns than in Danes (Main et al 2006b, c), which could be consistent with the Finns being relatively protected against factors that impact negatively on testis development and function in fetal life, with the converse applying to Danes. An alternative interpretation is that these fundamental differences reflect differences in environmental exposures and/or lifestyle between Danes and Finns.

Low sperm counts/infertility (Mak & Jarvi 1996), TGCT (Hemminki & Chen 2006), cryptorchidism (Weidner et al 1999) and hypospadias (Bauer et al 1981; Kallen et al 1986) all have a familial component: they are more common than would be expected in brothers, fathers or near-relatives. This can be viewed as evidence of genetic predisposition, but increased risk in brothers could also reflect a common uterine environment and exposures therein. The fact that increased risk of TGCT in a male is nine-fold when a brother has had TGCT but only four-fold for a father (Hemminki & Li 2004) supports this interpretation. However, the different prevalence in some (but not all) TDS disorders between Caucasian and Afro-American individuals is more likely explained by predisposing (genetic) factors that are protective in the black population compared with the white, as both groups share the same general environment. Therefore, in searching for causes of TDS, in particular environmental and lifestyle causes, it should be kept in mind that such factors

will interact with genetic factors to determine whether or not TDS disorders occur. This is fundamentally important as it implies, for example, that similar environmental/lifestyle exposures in Finns and Danes might have no effect in the Finns but induce TDS in some of the Danes; such an interaction could confound studies searching for relationships between EC exposures and TDS disorders.

Evidence that environmental factors, such as ECs, can cause TDS in humans

The clearest, and irrefutable, evidence that something in the environment is impacting on risk of TDS is the dramatic and progressive increase in incidence of TGCT over the past 60+ years, as detailed above. This can only have an environmental, as opposed to a genetic, explanation. However, there is no reason to suppose that there is only a single cause such as exposures to ECs: the lifestyle and diets of western nations have also changed dramatically over this time and may therefore play a role. When considering the published evidence that ECs may contribute causally to human TDS disorders, several factors need to be taken into account, and these are listed in Table 1. What this sets out are some of the difficulties that stand in the way of establishing definitively whether EC exposure contributes to TDS. These factors come in various forms, but relate to problems associated with either (a) accurate measurement of EC exposure of the fetus in the appropriate time-frame (early in gestation), (b) determination of the mechanism via which ECs cause an effect within the fetal testis or in the reproductive tract, or (c) accurate determination of the occurrence of the endpoint TDS disorder as well as establishing a clear relationship to EC exposure. These will be outlined and discussed when considering the results of human studies below.

Table 1. Some of the inherent difficulties in establishing if human exposure to ECs is associated causally with TDS (testicular dysgenesis syndrome) disorders.

Chemical exposure	Mechanism of effect	Endpoint (TDS) disorders
• Measurement issues o Availability of relevant samples o Availability of sensitive detection system, cost o Parent compound or active metabolites o Metabolism needs to be known o Environmental contamination • Access to relevant population o Retrospective, less definitive o Prospective, more definitive o High cost of prospective studies o Ethics • Fetal exposure issues o Inaccuracy of inference from maternal levels o ?Amniotic fluid o ?Meconium o ?Fetal blood o ?Fetal testis o ?Relevant age • Chemical mixtures o Multiplies the complexities o Similarly acting, feasible based on animal studies o Dissimilarly acting, no animal data	• Difficulties in distinguishing environmental from genetic effects for developing fetus (twin/sibling studies) • Difficulties in establishing mechanism due to poor understanding of early fetal events • The mechanism needs to be measurable, but probably not possible because of access and ethical issues • Problems in relating fetal events to endpoint disorders, especially for adult-onset ones • Cause and effect difficult to distinguish from non-causal associations	• Cryptorchidism and hypospadias easiest to relate accurately to fetal exposure o Inconsistently ascertained (unreliable registry data) o Prospective studies very expensive o Causes other than TDS • Low sperm counts – most common TDS disorder o Adult-onset makes cause and effect difficult to pin down o Causes other than TDS, and causes not confined to fetal life • Testis germ cell cancer (TGCT) – most definitive TDS disorder o Rarest of TDS disorders o Adult-onset makes cause and effect difficult to pin down • Other TDS disorders (e.g. low adult testosterone levels) o Understanding of causes too poor o No definitive evidence yet for fetal origins

Note also that genetic make-up may affect exposure to a chemical(s) (e.g. difference in metabolic activity of a relevant enzyme) or predispose towards an effect (e.g. lower androgen levels according to genotype) or via other mechanisms.

Each of the factors mentioned in Table 1 is important and, viewed together, illustrate how complex the situation can be. This may make it difficult to unequivocally establish cause and effect so that at some point a holistic 'weight of evidence' approach needs to be taken which makes due allowance for these complexities. It should be remembered that unequivocal proof that an EC (or a mixture of ECs) causes increased risk of a TDS disorder in humans requires establishment of a clear relationship and timeline between exposure, mechanistic effect and outcome disorder, effectively compounding the difficulties outlined under each of the headings in Table 1 (shown on page 25).

Recognising the difficulties of proving cause and effect of any particular EC in humans, regulation often relies solely on animal data. Realistically, unless an individual chemical (or chemical class) is the major causal factor in a substantial proportion of TDS cases, establishing clear cause, mechanism and effect for a specific EC is a monumental task. This is at its most demanding when considering the relationship between fetal EC exposure and adult-onset TDS disorders. These difficulties have to be taken into account when evaluating published data as otherwise the lack of cause-effect in the various epidemiological studies could easily be wrongly interpreted as ruling out involvement of ECs in TDS. In critically considering the evidence, studies of the involvement of ECs in TDS disorders evident at birth are considered first, as these should be the 'easiest' in which to establish cause and effect, compared with the adult onset disorders.

EC exposure and cryptorchidism and/or hypospadias

These disorders are often studied together because they are evident at birth and occurrence of either can be triggered by subnormal androgen action. However, there is a huge difference in their relative prevalence at birth (cryptorchidism is ~10-fold more common than hypospadias), and one or other disorder might be more susceptible to EC-induction, although there is no clear evidence for this from the studies discussed below. Before considering published studies, it is important to delineate some of the weaknesses and problems that can affect the quality of studies investigating the potential causes of cryptorchidism and hypospadias.

Quality assessment of the various studies and of the data obtained: The first major issue with both cryptorchidism and hypospadias is the accuracy of their diagnosis, as several factors impinge on this (Table 1). As mentioned earlier, 60% of cases of cryptorchidism evident at birth resolve themselves without treatment by ~3 months of age. It is therefore important that incidence at birth is used

to identify cases or that cases at birth and at three months or later are identifiable and not mixed up. Milder cases of cryptorchidism (where the testis has descended into the top but not into the bottom of the scrotum, for example) and of hypospadias (where the urethral opening is displaced towards the edge of the glans penis) are likely to go undiagnosed unless these disorders are specifically being looked for in a systematic way (and in a prospective study).

Many studies have used registry data to identify cases of cryptorchidism and hypospadias and there is agreement that such data is unreliable. Commonsense would argue that severe cases of both disorders (those requiring surgical treatment) are more likely to be accurately diagnosed, and therefore some studies have used the incidence of surgical correction of cryptorchidism (orchidopexy) as an indicator of cryptorchidism rate (e.g. Richiardi et al 2008); this will miss all cases that self-resolve. In any case, in a careful study of registry-based and direct evaluation of hypospadias incidence in one area in the Netherlands, it was found that severe cases were inexplicably under-reported (Pierik et al 2002). Ideally, studies should be prospective with cases of cryptorchidism and hypospadias diagnosed at birth using rigorous criteria in which all relevant personnel have been trained; these are the most time-consuming and expensive studies as the vast majority of baby boys in the study will not have the disorders. Finally, the larger the number of boys studied, the more accurate the study is likely to be; conversely, small studies are far more likely to identify spurious associations, especially when multiple factors (such as from questionnaires) are being investigated.

Another major issue, which applies to all studies of EC involvement in TDS disorders, is the accuracy of measurement of EC exposure of the pregnant mother and fetus (Table 1, p25). Direct measurement of fetal exposure is the most accurate but this is rarely possible except for measurements in umbilical cord blood or in extracts of placenta or meconium at birth or more rarely in amniotic fluid, usually from 1st or 2nd trimester. The latter is not routinely collected and is usually only from problem pregnancies or to diagnose chromosomal disorders. Most studies have therefore used maternal samples after completion (colostrum/breastmilk) or during (blood, urine) pregnancy, the latter usually being collected late in gestation (3rd trimester). In view of the potential importance of the male programming window discussed above, such measurements will assess exposure considerably later than when this is thought to be in the human (8-12 weeks' gestation; 1st trimester). Where the study has focused on persistent, usually lipophylic ECs – for example persistent organochlorine pollutants (POPs) such as DDT/DDE, or PCBs – measurements made in the mother in late gestation or at birth, or even years later, are likely to reflect levels of exposure during the 1st trimester, but where non-persistent, non-cumulative compounds are concerned (phthalates, for example) this is less certain to be the case. Nevertheless, irrespective of the sample collected it should still provide a more direct indication of fetal exposure than indirect (questionnaire) measures, even if uncertainties may remain as to how accurately this reflects the actual level of fetal exposure.

In many studies, EC exposure is not assessed directly but is inferred from questionnaire or from place of residence (within a certain distance from an incinerator or waste dump, for example, or within an agricultural area in which pesticide use is high). Questionnaires may ask about lifestyle, occupation, use of or exposure to ECs from pesticides, solvents, paints, glues etc, about types and quantities of food consumed and then use data available for specific contaminants of the various foodstuffs to determine 'exposure'. By their nature, questionnaires do not provide a direct measure of exposure but may allow calculation of exposure or, more commonly, enable identification of sub-groups of individuals with different levels of 'relative exposure' to groups/classes of ECs. The inherent inaccuracy of the questionnaire approach may be further compounded if it is

applied retrospectively – as for example when used to establish if mothers whose sons went on to develop TGCT had different diets or EC exposures during the relevant pregnancy. Furthermore, recall of events 25-40 years previously must obviously reduce accuracy.

From the foregoing discussion it must be recognized that 'not all studies are equal' in terms of their quality, so more attention or weighting should be given to studies that are as close as possible to being optimally designed. The ideal would be a prospective study that collected fetal blood or amniotic fluid in the 1st trimester for EC measurement and then ascertained occurrence of cryptorchidism and hypospadias at birth. No such study has been undertaken, presumably for ethical and practical reasons (invasive samples of no proven benefit to most of the individuals concerned). The prospective studies that come closest have used maternal blood or urine from mid-pregnancy or these media or breastmilk collected at or soon after birth to determine likely fetal EC exposure.

Evaluation of published studies: Several studies have shown that residence in a highly industrial area (Bianca et al 2003) or in an agricultural area with high pesticide usage (Bianca et al 2003; Carbone et al 2006), or its proximity to hazardous landfills (Dolk et al 1998; Elliott et al 2001) can increase the risk of hypospadias by two- to three-fold, though not all studies agree (Morris et al 2003). Others have shown an increased risk of cryptorchidism in boys born to mothers living in the vicinity of an acrylonitrile factory (Czeizel et al 1999), working in farming/gardening (Weidner et al 1998; Carbone et al 2007) or in areas of high pesticide use (Garcia-Rodriguez et al 1996; Andersen et al 2008), though again other studies did not find this (Vrijheid et al 2003; Pierik et al 2004). Most of these studies were registry-based and all were questionnaire-based, so the level of proof that they offer regarding EC exposure and the disorders is relatively poor. Nevertheless, the best designed of these studies – in that it was prospective and involved standardized diagnosis (Andersen et al 2008) – also found that pesticide exposure was associated with decreased testis volume, decreased penile length and decreased blood levels of testosterone, all of which might be expected to associate with cryptorchidism, though only the reduction in penile length was individually significant.

Even where well-designed prospective studies with direct measurement of EC exposure have been conducted, results have not shown dramatic associations. There have been eight such studies (seven prospective, one retrospective), all case-control design with reasonable numbers of cases (50-219) and in which EC exposure was evaluated using cord or maternal blood (3rd

trimester) or placental extracts or breastmilk. A variety of ECs were measured, most commonly PCBs, DDE, PBDEs and certain phthalate metabolites, although not all were assessed in every study. With the exception of one study (Fernandez et al 2007), only occasional and mildly significant associations were found, for example between cryptorchidism and total PCBs (Brucker-Davis et al 2008a), the sum of seven PBDEs (Main et al 2007), trans-chlordane and the eight most abundant POPs (Damgaard et al 2006), with other studies showing (statistically non-significant) 'trends' for MBP (Brucker-Davis et al 2008a, 2008b).

Most studies that measured DDT/DDE found no significant association (Bhatia et al 2005; Damgaard et al 2006) or only a near-significant trend (Brucker-Davis et al 2008a) with cryptorchidism and no significant association with hypospadias (Flores-Luevano et al 2003; Bhahia et al 2005), similar to results from a large (~200 cases of both disorders) retrospective case-control study (Longnecker et al 2002). In the largest of the prospective studies no association was found between maternal blood levels of HCE (heptachloroepoxide), HCB (hexachlorobenzene) or ß-HCCH (beta-hexachlorocyclohexane) and cryptorchidism (Pierik et al 2007). In contrast, one small study measured fat levels of various POPs in boys with or without cryptorchidism and found an association of this condition with HCE and HCB but not with PCBs or DDT (Hosie et al 2000), while a study of 196 Faroese boys also found no association between cryptorchidism and PCB levels in cord blood (Mol et al 2002).

In contrast to these other studies, one prospective study found quite marked associations between the placental levels of DDT, Lindane, Mirex and Endosuphan alpha and hypospadias/cryptorchidism, although cases of these disorders were not distinguished from each other (Fernandez et al 2007). It is also worth mentioning that cryptorchidism and hypospadias are both more common in boys from first, compared with subsequent, pregnancies (Akre et al 1999), as is also the case with TGCT (see below), and maternal POP levels decline from first to later pregnancies (Schade & Heinzow 1998; Shen et al 2008) as mothers pass on a greater proportion of their body burden of POPs to their first born in the womb and when breastfeeding.

The overall conclusion from these studies is that no single EC examined to date shows a strong, consistent association with cryptorchidism or hypospadias and, in general, where significant associations do occur, it is with groups (mixtures) of ECs – although even here the effects are not dramatic (increased risk of 10-25% for the relevant disorder). Nevertheless, the fact that significant associations are found between ECs and cryptorchidism or hypospadias in the majority of these (best designed) studies suggests that the associations have some substance and, taking into account the inherent difficulties (Table 1, p25), the data suggest that exposure to EC mixtures probably accounts for a proportion of cases of cryptorchidism and hypospadias.

As maternal smoking has been associated with reduced sperm counts in their sons in adulthood (see below), its association with cryptorchidism or hypospadias is of interest and several questionnaire-based studies have addressed this. Two large studies showed increased risk of cryptorchidism with maternal smoking (Thorup et al 2006; Jensen et al 2007b) while three smaller studies did not (Pierik et al 2004; Damgaard et al 2007; Mongraw-Chaffin et al 2007). One study found an association of paternal smoking with hypospadias (Pierik et al 2004) and another with cryptorchidism (Kurahashi et al 2005). One study found an association between maternal smoking and risk of hypospadias in sons (Brouwers et al 2007) and a similar trend was found in another study (Pierik et al 2004). There is also inconsistency in studies of maternal alcohol consumption and cryptorchidism, with two showing a positive association (Carbone et al 2007; Damgaard et al 2007) and two others not (Jensen et al 2007a; Mongraw-Chaffin et al 2007).

EC exposure and hormone levels

In view of the potential importance of fetal androgen levels/exposure in relation to risk of TDS disorders, evidence that EC exposure could alter hormone levels in humans, especially testosterone levels at birth or in the first three months, would provide support for their involvement in TDS. One prospective study found a relationship between pesticide exposure of mothers (greenhouse workers) and reduced testosterone levels in their sons, though this was not statistically significant (Andersen et al 2008). Another prospective study of 90 mother-infant pairs (boys+girls) involved measurement of POPs in 3rd trimester maternal blood followed by measurement of hormones, testis size (by ultrasound) and stretched penile length in their sons at 3 and 18 months of age (Meijer et al 2008). A number of significant correlations were found between individual POP exposure and testosterone levels, testis size and penile length. However, more POPs were correlated positively than negatively with these parameters and, with the exception of PCB-153, no compound was associated negatively with both testosterone and penile length, and even for PCB-153 the effect was modest.

As compounds in the same chemical class exhibited both positive and negative relationships to the measured parameters in this study, the safest conclusion is that the associations detected were incidental (it was a small study) and not biologically meaningful. A similarly designed study of much the same size evaluated dioxins+PCBs in 3rd trimester maternal blood and milk (at birth) and found no effect on cord blood levels of testosterone in boys (but they were reduced in girls) – although an association was found with reduced oestradiol levels (Cao et al 2008). This confirms other data showing that boys exposed to dioxins either via their mothers in pregnancy or directly in infancy, in the 1976 Seveso incident, also had significantly reduced oestradiol levels in adulthood (Moccarelli et al 2008). Interestingly, a similar reduction in oestradiol levels was shown in adult men in relation to exposure to the insecticide chlorpyrifos (Meeker et al 2008). The health implications of these reductions in oestradiol are unknown.

In contrast, a study of boys exposed prenatally to PCBs in the Yucheng rice-oil contamination incident reported significantly higher oestradiol, and lower testosterone, levels at puberty, in comparison with a control cohort (Hsu et al 2005). As puberty embraces wide variations in timing and hormone levels in boys, it is as likely that such changes reflect this variation rather than PCB exposure. This conclusion would fit with the absence of any effect at puberty in a larger study of boys from the Faroe islands who exhibited a wide range of exposure to PCBs, based on cord blood levels (Mol

et al 2002). Finally, one in vitro study has shown that human fetal testes exposed to environmentally relevant concentrations of dieldrin show a significant reduction in LH-stimulated testosterone production (Fowler et al 2007), but there are no relevant in vivo data for this compound.

Human exposure to phthalates: In view of the strong evidence that fetal exposure of rats to certain phthalates results in reduced fetal testosterone levels leading to reduced AGD and increased incidence of cryptorchidism and hypospadias, this class of compounds is of interest for human studies. There is now extensive data showing widespread human exposure to a range of phthalates, based on the measurement of common primary metabolites (e.g. MEHP, MBP) in urine (Silva et al 2004a; Wittasek et al 2007), although it has more recently been recognized that such measurements may underestimate exposure due to secondary or other metabolism of some phthalates (Koch et al 2006: Heudorf et al 2007).

Based on such measurements, daily internal exposure doses have been derived which show generally higher exposures in infants than in adults and in women versus men. In the context of TDS, it is exposures in women that are most relevant and on average these fall into the range 0.3-2.5 µg/kg/day for phthalates (DEHP, BBzP, DBP) that have been shown to cause TDS-like disorders in rats when administered at high oral doses (>100mg/kg/day). Maximum exposure levels in women are typically 1.5-15 µg/kg/day (Wormuth et al 2006: Heudorf et al 2007), although consumption of some pharmaceutical drugs which contain phthalates in their enteric-coating may result in considerably higher levels of exposure (Hernandez-Diaz et al 2008).

The urinary phthalate levels reported in pregnant women in association with reduced AGD in their male offspring (Marsee et al 2006; see below) are within the range reported in the general population. These exposure levels are inordinately lower than those used in experiments in rats to induce TDS disorders (100-750mg/kg/day administered orally), although this superficial comparison may be misleading as it is comparing administered (oral) dose in rats with urinary metabolite levels in humans. This will embrace numerous aspects of uptake and metabolism as well as species differences.

The important comparison is the relative exposure of the fetus in these two situations: measurements made in fetal amniotic fluid (AF) in DBP-treated rats (Calafat et al 2006) and in normal humans (Silva et al 2004b; Huang et al 2008) allows such a comparison. Dosing of rats with 100mg/kg DBP is a useful point of comparison as this has been shown to result in significant suppression of fetal testosterone levels (Lehmann et al 2004; Mahood et al 2007) and to cause some male reproductive disorders, though not severe. AF levels of MBP in rats 24 hours after this dose of DBP resulted in average levels of 1400 ng/ml compared with reported median levels in human AF of either 5.8 ng/ml (US; N=54) or 83 ng/ml (Taiwan; N=64), values that are 241- and 17-fold lower than in the rats, respectively. If the maximum reported MBP values in human AF are used (170 and 264 ng/ml in Taiwan and US studies respectively), the magnitude of difference from rat AF levels drops to 8.2- and 5.3-fold respectively. Of course this comparison is relatively meaningless if there is a fundamental difference in sensitivity (higher or lower) of the human fetal testis to phthalates when compared with the rat.

Although the foregoing comparison suggests there may still be a margin of safety for DBP exposure of women on average, this comparison takes account of only one phthalate. Humans are exposed to several phthalates (see above) that can induce TDS-like disorders in rats, and it is well established that additive effects of these compounds occur when administered together to pregnant rats (Howedeshell et al 2007, 2008). Therefore, it is the combined exposure of humans to relevant phthalates that is important and this will erode the relatively small margins of safety derived as above (Sharpe 2008). This conclusion presupposes (i) that the same phthalates will induce similar TDS disorders in humans as in rats,

and (ii) that this occurs at similar levels of exposure (of the fetal testis). It could be that the human is more sensitive or more resistant to phthalate effects or, as some recent studies suggest, completely unaffected in terms of steroidogenic effects (see below), which would render these considerations redundant.

Phthalate effects in the human: Three studies have assessed whether phthalate exposure might affect fetal testosterone production in humans, though the endpoint measurement used (AGD) is indirect. The first was a cross-sectional study of 85 boys aged 2-36 months in which AGD corrected for bodyweight was shown to negatively correlate with urinary levels in pregnancy of certain phthalate metabolites, including MEP and MBP (Swan et al 2005). A subsequent expansion of this study to include a total of 106 boys, plus repeated measurement of AGD in some boys, confirmed that smaller AGD was associated with maternal (urinary) levels of MEP, MBP, MEHP and the further MEHP metabolites MEHHP and MEOHP (Swan 2008). Moreover, boys with shorter AGDs were significantly ($p<0.00001$) more exposed to multiple phthalate metabolites than were boys with longer AGDs (Swan 2008), consistent with additivity of phthalate effects as shown in rat studies (Howdeshell et al 2008).

Similar to rat studies using flutamide (Welsh et al 2008), AGD correlated with penile volume/length (Swan et al 2005; Swan 2008) and the proportion of boys with cryptorchidism (Swan et al 2005), and DEHP metabolite exposure was also significantly associated with cryptorchidism (Swan 2008). Another study of 73 pregnant Mexican women in a hospital-based cohort investigated the association between exposure to MEHP, monobenzyl phthalate (MBzP), MEP and MBP during pregnancy and AGD in male newborns (Bustamente-Montes et al 2008). This study found a statistically significant association between MEP exposure and reduced AGD, and also between MBzP exposure and reduced penis length and width. These studies are consistent with gestational phthalate exposure inhibiting testosterone production in the male fetus (during the male programming window), resulting in reduced AGD and penile volume/length, as well as inhibiting normal testis descent. While these findings are in broad agreement with what might be predicted from experimental studies in rats, there are some fundamental differences.

First, in rats DEP (of which MEP is the main metabolite) is completely without effect on AGD and reproductive development at very high oral exposure levels (900mg/kg/day) of the pregnant mother (Gray et al 2000). This contrasts with its suggested effect on AGD in the human studies by Swan and colleagues. Second, and more important, the levels of phthalate exposure associated

with reduced AGD in these human studies are within the range of exposure of the general population (Marsee et al 2006), and are considerably lower than doses of individual phthalates shown to cause reduced AGD in rats. However, it should be kept in mind that in the human studies there will be exposure to multiple phthalates as well as to other ECs which may also contribute to the observed 'effect', namely reduced AGD. Administration of 100mg/kg/day DBP to pregnant rats results in MBP levels in maternal urine of 2,500,000 ng/ml (1,400 ng/ml in amniotic fluid; Calafat et al 2006) which is ~7,400-fold higher than the highest reported level of MBP in maternal urine (337ng/ml; median 16.2 ng/ml: Marsee et al 2006) in the human AGD studies, though this difference will be less for amniotic fluid levels (see earlier).

Administration of 100mg/kg/day DBP to pregnant rats has no significant effect on AGD (Barlow et al 2004), suggesting an even greater disparity from the human studies. This difference implies that steroidogenesis by the human fetal testis may be considerably more sensitive to phthalates, and to a wider range of phthalates (i.e. DEP), than is the case in rats. This difference could be explained, for example, by greater sensitivity to phthalate effects during the male programming window, as fetal steroidogenesis in rats is relatively unaffected by phthalates during this critical period (Scott et al 2008), as discussed earlier. However, such conclusions do not fit with results from the second study investigating AGD in boys at birth and which related this to phthalate exposure during the relevant pregnancy (Huang et al 2008).

This Taiwanese study was prospective and involved only 33 boys, but found no relationship between MBP or MEHP levels in pregnancy (measured in both amniotic fluid and urine) and AGD of the male offspring at birth – though inexplicably, a significant negative relationship was found between these two parameters in girls (Huang et al 2008). This study also demonstrated that Taiwanese pregnant women are surprisingly highly exposed to DBP, with highest urinary levels of 524 ng/ml (median 79.6 ng/ml), although there was notably lower exposure to MEHP and MEP. Though the method of AGD measurement was slightly different in the Taiwanese and US studies (Swan 2008), it is difficult to explain the contrasting results based on this difference.

Whilst the studies on the effects of phthalates on AGD are conflicting, the results from the Taiwanese study are consistent with two studies of the in vitro effects of MBP or MEHP on testosterone production by the human fetal testis. The first found no effect of MBP on basal or hCG-stimulated testosterone production by 2nd trimester human fetal testis explants in short-term culture (Hallmark et al 2007). Similarly, the second found no effect of MEHP on 1st trimester human fetal testis explants in the presence or absence of LH/CG, and also showed no effect on steroidogenic enzyme expression (Lambrot et al 2009). As the latter study showed that MEHP had significant negative effects on germ cells in the human fetal testis explants, as it did in rat fetal testis explants, the absence of its effect on testosterone production is more convincing. Nevertheless, there is always concern with explant cultures that negative results may stem from a failure of the in vitro system to replicate in vivo conditions, although both studies showed that steroidogenesis could be grossly inhibited by ketoconazole.

However, one of the cited studies failed to show an inhibitory effect of MBP in vitro on testosterone production using e19.5 rat fetal testes (Hallmark et al 2007), when clear inhibitory effects are demonstrable in vivo at this age. More recently, another group were able to demonstrate inhibitory effects of MEHP on testosterone production using explants of e14.5 fetal rat testes (Chauvigne et al 2008). Studies in adult men do not clarify the situation, as one study of men working in a PVC factory (and thus highly exposed to DEHP and DBP) found a weak negative correlation between MBP and MEHP levels and reduced free testosterone (Pan et al 2006), whereas a cross-sectional study of 295 men attending an andrology clinic and with lower levels of phthalate exposure (more akin to that of pregnant women) found no association

(Duty et al 2005). In any case, as there are differences in function between fetal and adult Leydig cells (they have separate origins), presence or absence of an effect of phthalate exposure on adult Leydig cell function would not provide definitive proof that the same applied to fetal Leydig cells. It should also be remembered that there are some fundamental differences between humans and rats/mice as to how fetal Leydig cells, and their steroidogenic functions, are regulated (LH/CG-dependent in humans, mainly LH/CG-independent in rodents).

Another human study has provided some support for phthalates negatively impacting on testosterone production by the newborn testis. It was a prospective study that investigated breastmilk levels of phthalate metabolites and related this to blood hormone levels collected on the same day from the corresponding boys and included normal and cryptorchid boys (with cryptorchidism assessed rigorously) at age 1-3 months (Main et al 2006a). Phthalate monoester levels showed no relationship to cryptorchidism (in contrast to Swan 2008) but levels of MBP were found to be associated with lower free testosterone levels – and it is free testosterone that is considered to be biologically active. In this study, MMP and MEP, as well as MBP, also showed a positive correlation with the LH:free testosterone ratio in the boys, which could indicate impaired testosterone production which has then been compensated for by increased LH..

In support of this finding, experimental studies in male marmosets of a corresponding age to the boys showed that MBP administration at a high dose (500mg/kg/day) caused initial inhibition of testosterone secretion followed by compensation, presumably driven by elevated LH secretion (Hallmark et al 2007). Although phthalate inhibition of testosterone production in the neonatal boys provides a plausible explanation for the observed hormone changes (Main et al 2006a), there is potentially another explanation. In the boys, a significant positive relationship was found between phthalate exposure and levels of sex hormone binding globulin (SHBG) to which most circulating testosterone is bound (and thus is not 'free'). SHBG is produced by the liver and is regulated by sex hormones and especially by insulin, and phthalates are also known to act on the liver. Increased levels of SHBG would decrease the level of free testosterone (without affecting testosterone synthesis) and would be expected to thus trigger a compensatory increase in LH levels, thus raising the LH:free testosterone ratio, as was found (Main et al 2006a). There is no easy way of distinguishing between these two interpretations.

Only one other study, in adult men, has looked at SHBG levels in relation to phthalate exposure

and found no effect (Duty et al 2005). Studies in rodents do not clarify this issue as they do not produce SHBG. If the association between breastmilk levels of phthalates and reduced free testosterone levels in breastfed infants is a causal relationship, it again points to high sensitivity to phthalates in infants based on calculated oral exposures (via breastmilk) of 0.8, 3.5 and 7.7 μg/kg/day for MEP, MBP and MEHP respectively (Main et al 2006a), consistent with the phthalate-AGD studies described above (Marsee et al 2006). An alternative explanation for the apparently greater sensitivity to phthalates in humans versus rats may be that in humans there is also exposure to other ECs that are inducing effects in addition to those induced by the phthalates, but this is speculative.

Uncertainties remain about whether phthalates affect steroidogenesis by the fetal or newborn human testis. But based on the evidence discussed above, one conclusion may be that if phthalates do have negative effects on fetal Leydig cell steroidogenesis in humans, this probably occurs with greater sensitivity than in rats. It would also suggest that much of the general (pregnant) human population is at risk of such effects – a point of considerable concern.

The alternative, and completely opposite, interpretation is that steroidogenesis by the fetal human testis, like the mouse testis, is unaffected by phthalates, which would suggest that phthalates are not a public health concern in this context. This would leave the phthalate-AGD relationship found in US and Mexican studies unexplained, unless phthalate exposure is a surrogate for some other exposure(s) or lifestyle. This is a distinct possibility, as phthalate exposure could relate to lifestyle. In this context, it should be remembered that there are some fundamental differences between Asian and US populations which could also be important. Asian men are 5- to 10-fold less at risk of TGCT than Caucasian US men (Purdue et al 2005), which could be indicative of a lower susceptibility to TDS/suppression of fetal testosterone; if so, this might explain the different results obtained in the US and Taiwanese studies, as >80% of the participants in the US study were Caucasian. These fundamental uncertainties need to be resolved, and urgently, as there are far-reaching implications of the differing interpretations offered above. New prospective studies – in particular further evaluation of the relationship between pregnancy exposure to phthalates and AGD, cryptorchidism and hypospadias – are paramount. Ideally, these should also take into account exposures to other similarly acting (anti-androgenic) compounds, as it is presumably the sum of such effects that will determine any effect, and its magnitude.

Do hormone levels at birth/neonatally reflect those in fetal life?: As discussed above, most studies of EC effects on testosterone levels perinatally in humans have evaluated testosterone at birth (cord blood) or in the first three months after birth, so an important question is whether such measurements can be used as a guide to levels earlier in pregnancy, in particular at 8-12 weeks' gestation, during the presumptive male programming window. There is no clear answer as no study has compared levels in individuals at these two time points, although this may be possible in future using AGD as a surrogate for early fetal androgen exposure (Welsh et al 2008). Nevertheless, the majority of boys presenting with hypospadias, especially when it is not associated with other disorders, have demonstrably normal testosterone levels and responsiveness to hCG stimulation at birth (Holmes et al 2004; Rey et al 2005). More extensive studies show the same in most cases of cryptorchidism (De Muinck Keizer-Schrama et al 1988), although some evidence of dysfunction may be indicated by an increased LH:testosterone ratio in some cases (Suomi et al 2006; Main et al 2006a, b). Therefore, if deficient androgen action is a common cause of these two disorders, this deficiency must be restricted to early fetal life and is either not evident or has been compensated for by around the time of birth.

EC exposure and low sperm counts

In the context of TDS, it is EC exposure in utero that is considered important as a potential cause of (later) low sperm counts, and this aspect is the focus of this review. However, low sperm counts can also occur as a consequence of lifestyle or environmental effects in adulthood, so EC effects on the adult testis are also worth considering (but are completely separate from TDS). Studies to establish whether EC exposures cause adverse effects on sperm production in adulthood has the merit that exposure and effect are concurrent, although such studies are still far from straightforward (see Bonde et al 1996; Tas et al 1996). In theory, studies of adult effects might give insights into which ECs can affect the fetal testis and thus might be of potential relevance to TDS. For example, adverse effects of phthalates on the pubertal/adult rat testis were discovered decades before the fetal effects were uncovered (Foster 2006). However, caution needs to be exercised in extrapolating from one to another as spermatogenesis does not occur in the fetal testis, so effects of ECs on the fetal testis may be fundamentally different from effects that occur in adulthood. EC effects on the adult testis are also likely to be inherently 'self-correcting' once exposure ceases, especially if they involve hormonal changes. The opposite is generally considered to be the case for fetal EC effects, as they are likely to be irreversible once caused, in particular for disruption of androgen action within the confines of the male programming window.

Fetal EC exposure and sperm counts in adulthood: There are few published studies, reflecting the difficulties in assessing EC exposure in pregnancy and relating this to subsequent sperm counts in male offspring. The most direct and reliable evidence comes from a study of the previously mentioned 1976 Seveso 'dioxin' accident, for which accurate data for exposed humans (including pregnant mothers) are available. This study (Moccarelli et al 2008) showed that men exposed perinatally/in infancy to dioxin had significantly lower sperm counts in adulthood, whereas those who were peripubertal at the time of exposure had significantly increased sperm counts and those exposed as adults showed no effect. Although such a 'pattern' of effect is often best interpreted as 'noise', these findings fit reasonably well with other data. For example, in rats exposed prenatally to dioxin, sperm counts are reduced in adulthood (Gray et al 1995), a change that might result from perturbation of androgen action via activation of the aryl hydrocarbon (Ah) receptor (Mutoh et al 2006), especially as AGD is reduced by fetal exposure to dioxin in rats (Ohsako et al 2002). In this regard, other studies in rats and mice have shown that fetal exposure to diesel exhaust also reduces sperm production

in adulthood, an effect partly explained by reduced Sertoli cell numbers (Takeda et al 2004; Watanbe 2005), and thought to be mediated via activation of the Ah receptor (Izawa et al 2007). There is no comparable data for humans, although there is evidence that occupational exposure to diesel exhaust/traffic fumes in adulthood can reduce motile sperm counts/ fertility (De Rosa et al 2003).

As there is widespread exposure to diesel/car exhaust fumes, and this exposure will have changed significantly over the past few decades, similar effects in pregnancy in humans to those in rodents are possible and could have contributed to the high incidence of low sperm counts in young men. Strong, if indirect, support for this possibility comes from studies of the effects of maternal smoking in pregnancy and their sons' sperm counts. Five such studies have been reported. In four of these (Storgaard et al 2003; Jensen et al 2004, 2005; Ramlau-Hansen et al 2007), each involving 316-1,770 males, marked reductions in sperm counts (~40% in three of the studies) were found in men whose mothers smoked heavily in pregnancy. In a much smaller study of young men (Richthoff et al 2008) no effect of maternal smoking on sons' sperm counts was found, although the study found a significant negative impact of current smoking on sperm counts. It is generally considered that such effects would be mediated via the polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke binding to Ah receptors, and then presumably working via a similar mechanism to dioxins and diesel fumes.

A study of 387 fertile US males reported that high beef consumption by their mothers during the relevant pregnancy (derived from questionnaire many years later) was associated with lower sperm counts in their sons (Swan et al 2007). As most beef cattle produced in the USA at the time were treated with anabolic steroids, including diethylstilboestrol (DES), to enhance growth, the effect on sperm counts could have resulted from increased fetal exposure to DES. However, recent reviews of evidence for adverse effects of pregnancy oestrogens on sons' sperm counts found little supporting evidence for adverse testicular effects (Storgaard et al 2006; Martin et al 2008). Alternatively, increased exposure to POPs in beef fat could be involved or effects via the Ah receptor due to cooking-related generation of PAHs. This uncertainty illustrates the difficulties inherent in such 'long-range' retrospective studies.

Adult EC exposure and sperm counts: Adult EC effects on sperm counts are completely separate from low sperm counts that arise as part of TDS, and the causes are likely to be different: adult effects mostly target the process of spermatogenesis, whereas fetal effects will target some aspect of testis development. Numerous studies have investigated the impact of ECs on sperm counts/fertility of adult men, usually in an occupational context, and surprisingly few examples of major adverse effects have emerged, though a notable exception is exposure to the nematocide dibromochloropropane (DBCP) (Bonde et al 1996; Tas et al 1996; Sharpe et al 2000). In contrast, most studies of occupational exposure to pesticides have found either modest or no effects on sperm counts/fertility of men (Bonde et al 1996; Abell et al 2000). There is not space to consider this literature here and readers are referred to the cited reviews. However, mention will be made of studies that have investigated the potential adult impacts on the general population of exposure to ubiquitous and persistent ECs. In doing so, it should be noted that many of the practical problems that hinder assessment of the prenatal causes of low sperm counts in adulthood do not apply to adult exposure studies because sperm counts can be evaluated alongside concurrent exposures. Despite this, few studies have found clear or consistent associations between specific EC exposure and reduced sperm counts or effects on sperm function such as motility.

The most widely studied group of ECs has been POPs, including PCBs, DDT and other chlorinated pesticides. Six studies have focused on comparing various European groups (including east versus west Europe) and Inuits, who have an extremely high fat (blubber) intake and are therefore

likely to be highly exposed to lipophylic POPs (Bonefeld-Jorgensen et al 2006; Elzanaty et al 2006; Giwercman et al 2006; Kruger et al 2007; Toft et al 2006, 2007). However, in none of these studies was any major association found with sperm counts or sperm quality, and two reviews of this and other data concluded that although POPs may have some minor effects on reproductive function (Bonde et al 2008) they have no impact on fertility (Giwercman et al 2007; Bonde et al 2008) and do not cause any obvious endocrine disruption (Bonde et al 2008). Other studies of POPs (especially PCBs and DDT) have reported generally similar findings (Magnusdottir et al 2005; Tildo et al 2005), although in some studies there are trends towards reduced sperm quality and high POP exposures. For example, decreased sperm motility has been associated with high PCB exposure (Rignell-Hydbom et al 2004; Hauser 2006). Similarly, one study has suggested an association between high combined PFOS and PFOA levels in men and fewer normal sperm (Joensen et al 2009).

However, one study of US men (Swan et al 2003b) did find a highly significant association between urinary metabolite levels of three currently used pesticides (alachlor, atrazine, diazinon) and the occurrence of low sperm counts, but this study involved relatively small numbers, and as the men were partners of women who were currently pregnant, the presumption is that pesticide exposure did not affect fertility.

A number of mainly US studies have assessed whether phthalate exposure is associated with changes in sperm counts or quality in men from infertile couples (who may therefore not be representative of the normal male population). Three of these studies found significant or near significant associations between urinary levels of MBP and low sperm counts and/or motility and/or velocity (Duty et al 2003, 2004; Hauser et al 2006); similar trends were also found for some other phthalate metabolites such as monobenzyl phthalate (MBzP) in some of the studies. However, a similar study of young Swedish men who were military conscripts, and thus likely to be representative of the normal young male population, found no association between urinary phthalate metabolite levels and any sperm parameter (Jonsson et al 2005).

Based on the above and on earlier data, the present view is that there is no firm evidence that exposure of adult men to common ECs, whether persistent or not, has any biologically major impact on their fertility or semen quality (Hauser 2006), although further studies of perfluorinated chemicals, alachlor, atrazine and diazinon are warranted.

EC exposure and testicular germ cell tumours (TGCT)

Any assessment of studies attempting to link EC exposures (or lifestyle) to occurrence of TGCT has to recognize that the evidence is now overwhelming that these tumours originate from precursor cells (CIS cells) that themselves originate in fetal life. If ECs are involved in the aetiology of TGCT, then it is exposures during pregnancy (probably very early pregnancy) that are important. In practice, many published studies of risk factors for TGCT have investigated 'adult factors' such as occupation, lifestyle or EC exposure levels. A number of these studies have found significant associations – one recent study, for example, found increased risk among railway traffic supervisors, programmers, university teachers and electrical engineers (Guo et al 2005). Such results are now generally viewed as being spurious statistical associations as they fail to fit with any of the known biology of TGCT, so they will not be considered in detail here. Nevertheless, it seems biologically plausible that postnatal factors can affect risk of developing TGCT – not by inducing it, but by creating conditions that favour survival (and perhaps proliferation) of the CIS cells from which the TGCT will develop (Garner et al 2005; Richiardi et al 2007). Such factors might be influential during early postnatal life and during puberty, though this is largely speculative (Richiardi et al 2007), but it may be sensible to take some account of this possibility when considering studies of postnatal EC exposures. In terms of POPs, it is also likely that measurement of adult levels of such compounds will to an extent reflect perinatal exposure because of their persistence.

Unlike cryptorchidism and hypospadias, registry data for TGCT is highly accurate, so problems related to misdiagnosis or under-diagnosis are not relevant. However, as outlined earlier, there is strong evidence for inherited susceptibility to TGCT (Richiardi et al 2007; Walschaerts et al 2007), although linkage analysis to identify the gene loci responsible concluded that there are multiple susceptibility genes, each exerting weak effects (Crockford et al 2006). An example of this may be the AZFc region on the Y chromosome, deletion of which is the commonest known genetic cause of male infertility, but which also increases risk of TGCT (Nathanson et al 2005). Men with TGCT are known to be less fertile in general than men without (see earlier), consistent with common causes as proposed in the TDS hypothesis. As maternal smoking appears to have a clear negative impact on sons' sperm counts in adulthood and perhaps also on risk of cryptorchidism (above), an obvious question is whether the same applies to risk of TGCT. This is especially pertinent as female smoking frequency in the Nordic countries has increased in parallel with the increasing incidence of TGCT (Petersson et al 2004) and fetal human germ cells express the Ah receptor through which PAHs in cigarette smoke may act (Coutts et al 2007). There have been three such studies of varying design. The largest, population-based, study used female lung cancer as a surrogate measure of maternal smoking and searched for cases of TGCT among the offspring of these mothers and found nearly twice as many as expected by chance (Kaijser et al 2003). However, two direct case-control studies of reasonable size found no evidence of increased risk of TGCT as a consequence of maternal smoking (McGlynn et al 2006b; Petterson et al 2007), and current opinion is that there is no connection (Richiardi et al 2007).

Use and exposure to POPs parallels to a large extent the increase in incidence of TGCT in Caucasian men over the last 50 years, and because of their persistence (DDT may have a half-life in the body of >60 years) measurements in men with TGCT or, better still, in their mothers is likely to reflect perinatal exposure, thus to some extent sidestepping the problems of relating events two or more decades apart (Table 1, p25). A recent large study in which POPs were measured in blood from 754 adult men prior to diagnosis of TGCT showed a moderately increased risk in relation to DDE and chlordane exposure (McGlynn et al 2008). A smaller study in which maternal POP levels were measured (~30 years after the relevant pregnancy) confirmed the risk for chlordane

and also showed increased risk for PCBs and HCB but not for DDE (Hardell et al 2003); more recently, increased risk of TGCT was similarly identified for exposure to PBDE (Hardell et al 2006).

Other less direct data may support a role for certain POPs in TGCT. For example, risk of TGCT is increased up to two-fold in boys from first, compared with later, pregnancies (Weir et al 2000; Cooke et al 2008) and most studies show that POP levels in women decline with increasing number of pregnancies (Schade & Heinzow 1998; Nakagawa et al 1999; Shen et al 2008), presumably due to mobilization of POPs from fat stores. Experimental studies in rabbits have shown that DDT exposure in utero results in sporadic 'germ cell atypia' (Veeramachaneni et al 2007; Veeramachaneni 2008), which may have analogies with CIS cells in the human, but it is not known if this leads to later germ cell cancer. Finally, it has been shown that levels of certain POPs in placentae and breastmilk are consistently higher in Danes than in Finns (Shen et al 2008), corresponding with the incidence of TGCT in these two populations. Although the magnitude of the Danish-Finnish difference in POP levels was not huge (~1.5- to 2-fold), it also needs to be kept in mind that there may be greater genetic susceptibility of the Danes to induction of TDS disorders due to their slower perinatal testis development (see above), and this might mean that even similar levels of POP exposure would have a proportionately greater effect.

Overall, the level of association between individual ECs and increased risk of TGCT in the above mentioned studies is broadly similar to that found for cryptorchidism and hypospadias, consistent with certain ECs (especially some of the chlorinated POPs) causing a small but significant increased risk of TDS disorders, but not being the major sole influence explaining the majority of cases.

In view of the effects in rats of certain phthalates, in particular their effects on fetal testosterone levels (see above), investigation of risk of TGCT in relation to phthalate exposure in pregnancy would be informative. However, as phthalates are metabolised and cleared rapidly from the body, measurement of their levels in adulthood (in sons or mothers) cannot be used to predict levels of exposure during pregnancy. One case-control study reported a six-fold increase in risk of one type of TGCT in workers exposed occupationally to PVC in adulthood (Ohlson & Hardell 2000), and DEHP is an important ingredient of PVC. In view of what is known about the origins of TGCT, it is difficult to draw any useful conclusion from this study. Indeed, a follow-up study by the same authors failed to confirm the association (Hardell et al 2004).

Conclusions and future perspectives

1. Animal studies have established beyond doubt that certain ECs, and in particular mixtures of anti-androgenic ECs, can cause TDS-like disorders, though for individual ECs these occur at levels of exposure higher than is documented to occur in humans. Nevertheless, because it is the summation of effects of all ECs that is critical, and the number of such ECs that humans are exposed to is considerable, this provides the strongest possible incentive to minimize human exposure to all relevant ECs, especially in women planning a pregnancy, as it is obvious that the higher the exposure the greater the risk.

2. Environmental factors, including lifestyle, diet and ECs, are clearly responsible for the progressive increase in incidence of TGCT in recent decades.

3. Certain POPs are associated with small increased risks of TGCT and groups of some of these compounds are also associated with small increased risks of cryptorchidism and/or hypospadias, consistent with them playing a role in the origins of some cases of TDS. In view of the inherent difficulties in such studies (Table 1), this is more likely to under- than over- estimate the involvement of ECs in TDS disorders. Human exposure to some POPs is declining progressively while exposure to other ECs remains high (phthalates) or may be increasing (PBDEs). Therefore, changing effects of these particular compounds may occur and be reflected in altered incidence of TDS disorders (if there is a causal relationship).

4. There is particular concern about the potential contribution of phthalate exposure to human TDS disorders, but present data are conflicting in several respects, so no definitive conclusion can be reached. It is an urgent priority to clarify this uncertainty, as some of the data implies that humans could be more sensitive than rats to the effects of certain phthalates on fetal steroidogenesis. The conflicting data on phthalate effects could also indicate ethnic/genetic differences in susceptibility to phthalates in humans, much as there is between rats and mice. In light of this uncertainty it would be prudent to reduce exposure to phthalates, and particularly to reduce exposure in pregnant women.

5. Some evidence that mixtures of EC might cause TDS disorders in humans is beginning to emerge – but again, it is as yet unclear that these EC mixtures are likely to account for a substantial proportion of human TDS disorders. However, the complex mixtures of differently acting chemicals in the 'real world' have still to be evaluated.

6. Genetic/ethnic factors (predisposition or protection) are established as being important in TDS disorders, and more account needs to be taken of these in the context of EC effects. Non-ethnic, genetic differences in susceptibility to either TDS disorders or to EC effects may obscure effects of ECs in individuals in population/cohort studies. This would likely manifest as low-level significant

associations between the disorder and the particular EC/ECs, which is more or less what current studies are reporting. Such effects might emerge more obviously if different ethnic groups are being compared and one is more susceptible than the other; the Asian-US contrast in phthalate-AGD relationship could be such an example. Better understanding of normal and abnormal (TDS disorders) male reproductive development and its variation between countries and ethnic groups will allow identification of the genetic and mechanistic basis for such differences, which can then be allowed for in future studies of EC involvement.

7. Of the studies that have used indirect measures of fetal exposure, those directed at maternal smoking have provided convincing evidence for effects on sons' sperm counts and some evidence for increased risk of cryptorchidism and hypospadias, but no effect on risk of TGCT. This suggests that some ECs (in this case PAHs) may preferentially affect risk of some, but not all, TDS disorders. This conclusion adds to the substantial evidence already available, indicating that cessation of smoking by women planning a pregnancy is the single biggest investment they can make in the future wellbeing of their babies.

8. Prospective, hypothesis-driven studies involving biomarkers of exposure to ECs will provide the most persuasive evidence for or against their involvement in the origin of human TDS disorders. These are also by far the most expensive studies. These should be targeted so as to derive the maximum benefit and insight possible, examples being the series of studies that have compared Finnish and Danish birth cohorts.

9. Experimental studies in animals have proved to be the primary route via which ECs with potential involvement in TDS have been identified. As with human prospective studies, the most informative and conclusive animal studies are often the most expensive because of their robust and careful design (including adequate animal numbers). Recent studies with EC mixtures are such examples and their unequivocal results provide a solid foundation to guide researchers, and regulators, particularly as confirmation of effects in humans may take several years and could result in otherwise preventable occurrence of disorders.

10. Caution should be exercised when extrapolating from experimental studies in rodents to humans with regard to dose/level of exposure and mechanisms of (presumed) effect. While it may be acceptable to assume similarities initially, direct confirmation then needs to be sought for in humans in order to identify which chemicals, and mixtures of chemicals, may be damaging. Detailed mechanistic studies and evaluation of species-specific adsorption, distribution, metabolism and excretion of ECs are also needed.

11. No single abundant EC, or class of EC, whether persistent or non-persistent, plays a major individual causative role in human TDS disorders based on present evidence. It is therefore recommended that all future studies addressing this should take account of multiple EC exposures (mixtures).

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