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Topic 3.4

Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research*

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Abstract: This review addresses whether there is a secular increasing trend in male reproductive developmental disorders (cryptorchidism, hypospadias, testis cancer, low sperm counts), and highlights the limitations of available data and how these issues are being addressed. These disorders are considered to represent a syndrome of disorders [testicular dysgenesis syndrome (TDS)] with a common origin in fetal life, and in which "endocrine disruption" plays a central role. The potential involvement of environmental estrogens in the etiology of these disorders is reviewed in light of new understanding about the pathways and dose–effect relationships of estrogen action on male reproductive development. Several new pathways of estrogen action have been identified, including suppression of the production of testosterone and insulin-like factor-3 by fetal/neonatal Leydig cells and suppression of androgen receptor expression in androgen target tissues. It is tentatively concluded that identified environmental chemicals are unlikely to activate these pathways because of their intrinsically weak estrogenicity. However, chemicals that may alter endogenous estrogen production, bioavailability, or inactivation represent a new focus of concern. Additionally, environmental chemicals that alter endogenous levels of androgens in the rat fetus (certain phthalates) induce a similar collection of disorders to TDS. Whether human exposure to such compounds might contribute to TDS remains to be shown, but studies in animals should help to define susceptible pathways for induction of TDS.

INTRODUCTION

World-wide interest in male reproductive disorders probably stems from the publication in 1992 of the paper by Carlsen et al. [1] suggesting that sperm counts in human males might have declined by nearly half during the previous 50 to 60 years. This interest became more intense when we published our hypothesis paper in the *Lancet* in the following year [2], in which we argued that the fall in sperm counts, increase in incidence of testis cancer, and in other reproductive abnormalities (cryptorchidism, hypospadias) in the human male might be related to increased estrogen exposure in utero. In our paper, we

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went on to identify several mechanisms by which this increased exposure could have occurred—one of which was exposure to environmental chemicals, in particular to environmental estrogens. The coincidence of this publication with an explosion of interest in environmental estrogens meant that a link between human exposure to environmental chemicals and the occurrence of male reproductive disorders was widely discussed [3]. There is still nothing other than circumstantial evidence to support this possibility. Though this may mean that environmental chemicals are not important in this context, it is becoming increasingly obvious that provision of definitive proof, one way or the other, is a demanding task. As this review will show, there have been many problems to overcome that relate to both the quality of the data and to our lack of understanding of the underlying biology. However, it is reassuring that these deficits have been recognized (though not by all!) and appropriate responses and changes made to overcome these shortcomings. These are helping to accurately define the reproductive health problem, identify how the search for "causes" may be addressed, and to redefine the causal role (if any) that environmental chemicals might play.

An important development in recent years, and one that forms the core around which this review is built, is the realization that the human male reproductive disorders of concern—namely cryptorchidism, hypospadias, testis germ cell cancer, and low sperm counts—may form a syndrome of interconnected disorders with a common origin in fetal life. This has been termed "testicular dysgenesis syndrome" (TDS) [4]. It is argued that there may be more than one cause of this syndrome and that its manifestation may include one or more of the aforementioned disorders. Most importantly, from the perspective of this review, is the recognition that "endocrine disruption" (altered androgen and/or estrogen levels/action) is a central feature of this syndrome. Below, we discuss the current views on secular trends in male reproductive disorders, the pathways via which endocrine disruption may occur, and the possibility that environmental chemicals may impact these pathways.

MALE REPRODUCTIVE DISORDERS—SECULAR TRENDS AND THE ROLE OF ENDOCRINE DISRUPTION

There are two major issues concerning human male reproductive disorders (testicular germ cell cancer, cryptorchidism, hypospadias, low sperm counts), namely, the evidence that the disorders are increasing in incidence with time (secular trends) and, second, the role of endocrine disruptors in this (increase in) incidence. These two issues are clearly linked in many people's minds, but it is more balanced to consider them as separate problems *that may be linked*. As this short review will emphasize, the evidence that "endocrine disruption" plays a central role in the origin of these disorders is becoming ever clearer (Fig. 1). However, what factors can cause or trigger endocrine disruption remains unclear, but is by no means confined to exposure to environmental endocrine disruptors. Endocrine disruption (i.e., hormonal imbalance) underlies many of the most common human disorders, but this disruption can have many causes such as infection, diet/bodyweight, lifestyle, inherited genetics, or environmental exposures, though there is a paucity of data for the latter when compared with the other listed triggers [5]. Below, we consider what new pieces of evidence have emerged in recent years to help redefine both the concerns and the potential causes.

Sperm counts—secular trends

As already indicated, the evidence that sperm counts in Western countries might have fallen by approximately 50 % since the 1930s to 1940s [1] was a driving impetus for concerns about environmental effects on the male. The initial study by Carlsen and colleagues was subjected to much criticism and reanalysis, many of which attempts appeared contrived [reviewed in: 3,6–8]. Independent reanalysis [8] reached exactly the same conclusions as had the original study, and a recent updated analysis [9] that included semen analysis data up to 1996 (101 studies in all) again confirmed the trends and conclusions of the original study. However, these studies are all based on meta-analyses of retrospective data, and

can only raise the *possibility* that sperm counts have fallen. Numerous studies were prompted by the original findings, nearly all retrospective, and their results divided into two camps, those showing evidence of a secular trend related to year of birth and those showing no such trend [see 3,6–8]. From these studies, it also appeared that significant differences in sperm counts, both within and between countries, might exist. If these differences are real, global analysis of sperm counts to discern secular trends must consider these geographical differences, and this cannot be done retrospectively with any confidence. This reality prompted a major effort at the European level to obtain prospective data for sperm counts in various European countries using standardized methods of subject recruitment and semen analysis [10,11]. These studies have had three main aims:

1. establish robust methodology that could be used in all semen analysis laboratories;
2. establish a solid data platform from which future trends in sperm counts can be discerned and followed with confidence; and
3. enable a cross-sectional comparison of sperm counts in similar groups of men in European (and other) countries at the present time.

The initial group chosen in which to analyze sperm counts were "recently fertile" men whose partners were currently pregnant, recruitment taking place via parentcraft classes. Some 250 to 350 men were recruited from four centers in Denmark, Finland, France, and Scotland, and their semen analyzed using methods standardized throughout the four centers. Sperm counts were then standardized for a 30-year-old man with an abstinence period of four days in each country by allowing for known confounders such as period of abstinence. Median sperm counts were found to be 34 % higher in Finland than in Denmark with Scotland and France intermediate between these two extremes [11]. A seasonal difference in sperm counts was also evident in all four countries, sperm counts being approximately 30 % lower in summer than in winter, confirming other studies [reviewed in ref. 12]. Although not all relevant studies have reported such a seasonal difference in sperm counts, season, as well as age, abstinence, and geographical location should be considered when undertaking studies related to secular trends in sperm counts.

For the reasons outlined above, retrospective studies of sperm count data, which have been extensively reviewed [3,6,7], cannot be considered definitive. We will therefore not consider this data further here. There is, however, one new approach that is relevant to the secular trends in sperm counts issue, and that is the analysis of sperm counts in young men aged 18 to 20 years. These were prompted by the demonstration in several of the retrospective studies in older men that sperm counts appeared to decline in relation to when a man was born rather than according to when the semen sample was actually provided, consistent with fetal origins of the problem [3,6]. If later year of birth is associated with declining sperm counts, then analysis of sperm counts in the latest birth cohort, namely young men, should provide evidence to support or refute this thinking. If the young men have normal sperm counts (i.e., typical of those reported in the literature) then a secular decrease appears unlikely, whereas if they have "low" sperm counts a secular decrease in sperm counts would appear more likely. For solidity and comparability of these studies, they have been undertaken in conjunction with the European multicenter study of recently fertile men, outlined above, and have used the same standardized methods. Semen analysis studies are always plagued by "biased" recruitment, so the principal approach has been to undertake the studies in countries in which there is still compulsory military service for young men. To date, only data for Danish young men are available, and these indicate a median sperm count of only 41 million/ml) [13], a value that is substantially lower than in older men from the same country [11]. With such low sperm counts, effects on time to achieve a pregnancy become more likely [14–16]. This finding, which has been confirmed by other as yet unpublished studies in Denmark, supports the hypothesis of a secular decrease in sperm counts related to later year of birth. It remains to be shown if this is a change specific to Denmark, and as similar studies in other European countries on 18- to 20-year-old men are nearing completion, it is prudent to wait for these results before conclusions are drawn. In this regard, a recent study of 408 young (18 years of age) men from the Czech Republic re-

ported median sperm counts of 44 million/ml [17], though it is emphasized that this study was not part of the European initiative outlined above, but was a study of the impact of seasonal air pollution.

Sperm counts—role of endocrine disruption

Though sperm are not made until later in puberty, capacity to make sperm is determined by the numbers within the testes of Sertoli cells, and as these cells proliferate in fetal, neonatal (in particular), and peripubertal life [18], inhibition of Sertoli cell proliferation during any of these phases in life could affect sperm counts in adulthood. This was the thinking in the original "estrogen hypothesis" in which it was proposed that perinatal estrogen exposure could reduce Sertoli cell proliferation, probably by suppressing follicle-stimulating hormone (FSH) secretion from the pituitary gland [2]. Studies in rats have since shown that neonatal estrogen exposure does indeed reduce Sertoli cell number, testis size, and sperm production in adulthood dose-dependently [19,20]. Though a direct effect of estrogens on the Sertoli cells is possible [20], it is likely that suppression of FSH secretion is the most important mechanism behind the reduction in sperm counts (unpublished data). However, whether estrogen suppression of FSH levels perinatally in humans would result in a *permanent* reduction in Sertoli cell numbers is questionable. This is because studies in the marmoset have shown that although suppression of FSH levels neonatally does reduce Sertoli cell proliferation/number by approximately 30 % at the end of the neonatal period, by the time such treated animals have reached adulthood Sertoli cell number has recovered to normal [21]. As the marmoset appears similar to man in terms of when Sertoli cell proliferation occurs [18,20], this might account for the rather modest changes in semen quality reported in men exposed to DES in utero [22] and the associated absence of any change in fertility [23]. Note that this explanation does not rule out the possibility that DES exposure in utero did reduce Sertoli cell numbers (which is highly likely based on data from the rat), only that if such a reduction did occur it was probably compensated for at some time postnatally.

Testis cancer—secular trends

Testis cancer has continued to increase in incidence in Caucasian men in many countries [22,24–27]. Numerous studies have demonstrated that later year of birth is associated with a progressively increasing risk of developing testis cancer [22,28,29], implicating environmental, as opposed to genetic, factors in the etiology of this increase. New data has also confirmed that men with testis cancer exhibit reduced fertility prior to the occurrence of their tumor [30,31]. This reinforces the hypothesis that there may be a causal link between occurrence of testis cancer and low sperm counts due to abnormal germ cell and/or Sertoli cell development in fetal life (see Fig. 1 and below).

Testis cancer—role of endocrine disruption

It has long been established that disorders of sexual differentiation, a hormonally mediated process in fetal life, are associated with an exceptionally high risk of developing testis cancer in young adulthood [32]. Other studies had suggested an increased risk of testis cancer in men exposed in utero to DES or other estrogens, though not all such studies concurred [33]. A meta-analysis of published studies concluded that estrogen exposure in the first trimester of pregnancy was associated with a small but significant increase in risk of developing testis cancer [22]. Three new studies have readdressed this issue and reached somewhat opposing conclusions [34–36], consistent with earlier data. Overall, it seems fair to conclude that exposure to *exogenous* estrogens in early pregnancy results in only a modest increase in risk of developing testis cancer. However, two new studies also raise the possibility that an increased risk of testis cancer might stem from increased exposure to *endogenous* (maternal) estrogens. These showed that twins of brothers who had developed testis cancer had a 12- to 37-fold increased risk of developing testis cancer themselves [37,38]. Twin pregnancies are associated with higher estrogen levels

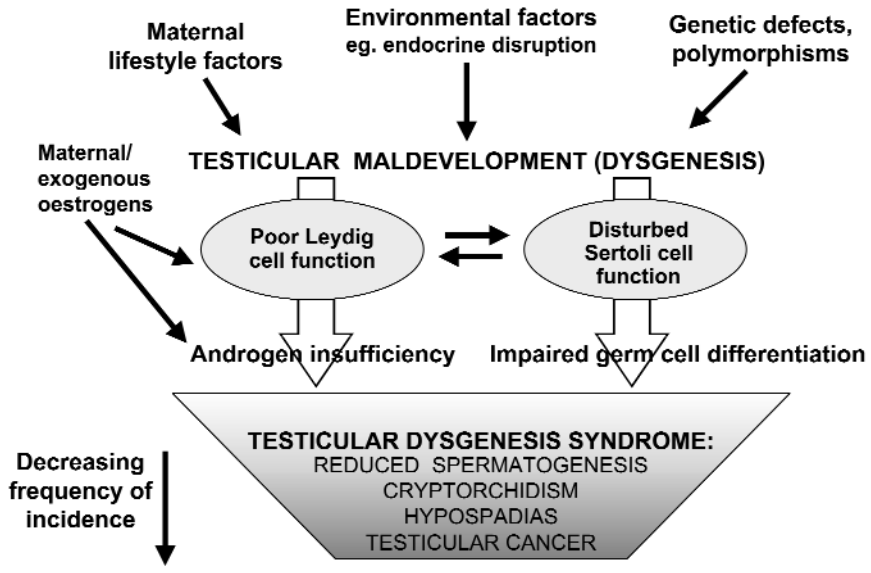

Fig. 1 TDS in the human. Schematic diagram to illustrate the central features and general pathways via which the disorders that may comprise this syndrome are likely to arise. Note that although abnormal testicular cell differentiation appears to be at the heart of this syndrome of disorders, numerous pathways might lead to this occurrence, including genetic, environmental, and lifestyle factors. It is tentatively suggested that any effect of maternal or exogenous estrogens on the induction of TDS is most likely to result from impairment of testosterone production or action, though impairment at an earlier step (e.g., cellular differentiation) cannot be ruled out. Note also that the disorders comprising TDS occur with differing frequency, varying from quite common (reduced sperm production, cryptorchidism) to rare (testis germ cell cancer). Note also that some of the disorders may occur for reasons other than TDS (e.g., low sperm counts).

than in single pregnancy and this is especially so for dizygotic twin pregnancies—accordingly, testis cancer risk is 50 to 100 % higher in dyzgotic than in monozygotic twins [38,39]. Of course, increased risk of testis cancer in twins might also indicate evidence of "genetic predisposition" to testis cancer, though there is a dearth of evidence to support such a view. Indeed, as the main identified risk factors for testis cancer all relate to fetal/pregnancy factors (see Table 1), it is most logical to explain the "twin data" on the basis that twins and siblings share a common intrauterine environment and that maternal/placental estrogens are one component of this, though not necessarily the most important component (Fig. 1).

It has become ever clearer that the premalignant germ cells from which testis cancer arises, namely carcinoma in situ (CIS) cells [40–42], themselves have their origins in fetal life [42–44]. It is presumed that the CIS cells arise because of failure of normal differentiation of fetal germ cells, but how and why this should occur remains unclear (Fig. 1). However, based on the identified risk factors, it is clear that subnormal androgen exposure [42–44] and/or increased estrogen exposure (see above) are potentially important factors (see Table 1). As these hormonal changes are also key risk factors for cryptorchidism (see below), it is noteworthy that cryptorchidism has been reconfirmed as the numerically most important risk factor for testis cancer [35], as well as being an important risk factor for low sperm counts, infertility, and hypospadias (Table 1).

Table 1 Common risk factors for testis germ cell cancer, cryptorchidism, hypospadias, and low sperm counts/infertility, based on numerous epidemiological studies. These support the suggestion that these four disorders may form a syndrome (testicular dysgenesis syndrome) with a common origin during the period of testicular/sexual differentiation in fetal life.

Testis germ cell cancer	Cryptorchidism	Hypospadias	Low sperm counts/ infertility
Cryptorchidism	Cryptorchidism in a sibling	Cryptorchidism	Cryptorchidism
Hypospadias	Hypospadias	Hypospadias in a sibling	
Low sperm count/ infertility	Low sperm count/ infertility		Low sperm count/ infertility in father
Testis cancer in a sibling			Testis cancer
Low birthweight (IUGR)	Low birthweight (IUGR)	Low birthweight (IUGR)	Low birthweight (IUGR)
First pregnancy	First pregnancy		
Impaired androgen production/action in fetal life[a]	Impaired androgen production/action in fetal life[a]	Impaired androgen production/action in fetal life[a]	Impaired androgen production/action in fetal life[a]
Increased DES exposure during mother's pregnancy	Increased DES exposure during mother's pregnancy	Increased DES exposure during mother's pregnancy	Increased DES exposure during mother's pregnancy

IUGR = intra-uterine growth restriction; DES = diethylstilboestrol
[a]Includes genetic disorders of androgen insensitivity.

Cryptorchidism—secular trends

The evidence for a secular increase in the incidence of cryptorchidism has been limited by inherent problems related to diagnosis and reporting [22]. Indeed, it is now accepted that registry data is unreliable and if any secular trends are to be discerned, then a structured and standardized diagnostic approach is essential and the studies should be prospective [45]. Such studies were initiated several years ago in Europe and involve a structured comparison of incidence of cryptorchidism at birth and at 3 months in consecutive series of babies born in various European countries. This data is likely to confirm that differences between countries in incidence of cryptorchidism are as marked as differences in incidence of testis cancer [22,24] and sperm counts [11], and will provide a sound baseline from which to monitor future trends in incidence. Despite the reservations about the accuracy of registry data for cryptorchidism, it is clear that this remains by far the most common congenital abnormality (2 to 4 % incidence at birth) in babies of either sex and surgical correction is required in 30 to 50 % of affected boys.

Cryptorchidism—role of endocrine disruption

Boys born to women treated with DES in early pregnancy have an increased incidence of cryptorchidism [see 22 for references], and new pathways via which cryptorchidism can be induced by estrogens in rodents have now been identified (see below). Nevertheless, it appears that exposure to hormones other than DES in early pregnancy, including those in the oral contraceptive pill, is not associated with increased risk of either cryptorchidism or hypospadias, according to meta-analysis [46].

Hypospadias—secular trends

Data from the United States Birth Defects Monitoring Program has shown a rate of increase of 2 to 3 % per year in incidence of hypospadias in all regions [47]. As with earlier studies that suggested similar trends [22], this is based on registry data, raising questions as to whether this increase is real or might be explained by reporting differences [48]. However, the study showed that the most severe forms of hypospadias (those requiring surgery), and which are less likely to go unreported, showed the same or even a larger temporal trend than did minor cases. A recent prospective study from the Netherlands that used a structured diagnostic procedure, reported a 4- to 6-fold *higher* incidence (0.7 % of boys at birth) than did the official registry data for the same region [49]. Remarkably, the ratio of severe to minor cases of hypospadias was 3:1 in the prospective study and the mirror image (1:3) in the registry data! This study therefore confirms that registry data for hypospadias is highly unreliable [45], and more importantly suggests that it underestimates the true incidence, especially for severe cases. Further evidence for an increasing incidence of hypospadias comes from neonatal intensive care units in the United States, which reported a 10-fold higher incidence (4 vs. 0.4 %) of hypospadias in 2000 compared with 1987 [50]. Though these new studies fuel concerns about the accuracy of registry data for hypospadias, and thus cast doubt on the accuracy of secular trends based on such data, they also suggest strongly that hypospadias is far more common than is generally perceived. If the new data from the Netherlands is representative, it suggests that hypospadias is the second most common congenital abnormality in children of either sex, after cryptorchidism [49].

Hypospadias—role of endocrine disruption

It was recently pointed out [51] that the widely disseminated belief that DES exposure during pregnancy led to an increased incidence of hypospadias in humans, was incorrect as the original study [52] did not specify hypospadias but "urethral abnormalities". In fact, these arguments have in turn been refuted, as a recent cohort study from the Netherlands has shown that boys born to mothers treated with DES during pregnancy show a massive 20-fold increased incidence of hypospadias [53]. Additionally, new studies in pregnant rats have uncovered the mechanism via which this might occur as DES treatment results in gross suppression of fetal testosterone levels (see below), which would be expected to increase risk of hypospadias (Table 1). In contrast, a meta-analysis of studies in which pregnant women were exposed to estrogens (other than DES) or other hormones in early pregnancy found no increased risk of hypospadias in the offspring [46], raising the possibility that induction of hypospadias is specific to DES, rather than estrogen, exposure per se (i.e., DES is an atypical estrogen). This seems unlikely based on two new pieces of data from animal studies. First, it has been shown in mice that a functional ERα receptor is essential for DES to induce reproductive tract abnormalities in either males or females [54,55]. Second, several of the abnormalities of development that are induced in animals treated in utero with DES, such as cryptorchidism and other abnormalities of the reproductive tract, are also induced in a transgenic mouse that overexpresses aromatase [56].

The apparent conflict between the increased risk of reproductive tract abnormalities in boys exposed in utero to DES and the lack of risk in those exposed to other hormones, including other synthetic estrogens, requires explaining, as it has important implications with regard to assessment of risk from endocrine disruptors. One obvious possibility is that the conflict relates to the dose and/or duration of estrogen exposure. When DES was administered to pregnant mothers in early gestation, extraordinarily high doses were used (80–>2000 μg per kilogram per day [μg/kg/day]; see ref. [22]). In contrast, in studies in which pregnant women were exposed to "hormones" other than DES during pregnancy, this usually involved either brief or single exposure to high levels during hormone administration as a pregnancy test or inadvertent exposure to oral contraceptives before the pregnancy was diagnosed [46], most of which involved exposure to lower doses of "estrogen" or for relatively shorter periods than occurred with DES exposure. In studies in rats and mice in which adverse effects on male

reproductive tract development have been induced by DES, ethinyl oestradiol, or oestradiol, very high doses of estrogen (>50 μg/kg/day) have been used [20]. Indeed, studies in neonatal rats suggest that only doses of estrogen that induce associated reductions in testosterone levels and in androgen receptor expression will cause reproductive developmental abnormalities, and for this to happen doses in excess of 100 μg/kg/day are required [20,57]. This suggests that only exposure to very high estrogen levels poses a major risk to male reproductive tract development, though this interpretation requires formal testing.

MECHANISMS VIA WHICH ALTERED HORMONE EXPOSURE (ENDOCRINE DISRUPTION) COULD INDUCE MALE REPRODUCTIVE DISORDERS

Adequate androgen production and action are essential prerequisites for normal male reproductive tract development. Gene mutations or other factors that interfere with such processes result inevitably in abnormalities of reproductive development (Fig. 1). At its most extreme, in "complete androgen-insensitivity syndrome", complete failure of phenotypic masculinization occurs, despite the fact that the affected individuals have testes. In such cases, the testes are abdominal and are at high risk of testis cancer [32]. Though this has long been established, it is worth restating as it emphasizes the central role that androgens play in normal male development and perhaps questions the logic behind the focus on environmental estrogens, or indeed even on potent estrogens such as DES. However, perusal of Table 1 clearly shows that *reductions* in androgen production or action or *increases* in estrogen exposure during male development are both associated with increased risk of all of the disorders listed, and the same is true for experimental studies in animals [20]. This similarity suggests a link, and recent data from animal studies appears to have uncovered four mechanistic links that may explain this phenomenon.

Suppression of androgen production

Administration of potent estrogens such as DES or ethinyl estradiol to the rat *in high doses* (>100 μg/kg/day), drastically suppresses Leydig cell function and, as a result, testosterone levels in both the testis and/or blood are dose-dependently suppressed. Treatment of pregnant females in this way results in gross suppression of testosterone levels in the male fetus [58], and direct administration to male rat pups neonatally has similar effects [20] that may persist through to adult life [19]. The pathways via which estrogens reduce testosterone production may involve suppression of expression of steroidogenic factor-1 [59] and 17α-hydroxylase-$C_{17\text{-}20}$-lyase [60].

Suppression of androgen receptor expression

Coincident with suppression of testosterone production, neonatal treatment of rats with DES or ethinyl estradiol also grossly suppresses expression of the androgen receptor (AR) in the testis and throughout the developing reproductive tract [20,57,61]. This suppression only occurs at very high doses (>100 μg/kg), similar to the effects on suppression of testosterone levels reported above. However, the suppression of testosterone production and loss of AR expression are separate effects as experimental suppression of testosterone levels by other mechanisms (switching off pituitary gonadotrophin secretion by treatment with a GnRH antagonist) is unable to induce loss of AR expression [20,57,61]. Dose–response studies indicate that estrogen-induction of testicular and reproductive tract abnormalities is coincident with suppression of AR expression, so this change is presumably involved in the aetiology of these disorders [57].

Distortion of the androgen–estrogen balance

Though the findings in 1 and 2 described above imply that estrogens may simply function as effective anti-androgens, at least at very high doses, other findings suggest that the relationship between androgens and estrogens is more complex than this. For example, it has been shown that most, and perhaps all, of the adverse effects induced by high doses of DES on male reproductive development when administered neonatally, can be prevented by coadministration of testosterone, even though the particular adverse efects induced by DES cannot be induced by simply suppressing testosterone production or action [57]. More recently, it has been shown that administration of a 100-fold lower dose of DES, which is largely ineffective on its own, can induce a similar spectrum of reproductive tract abnormalities if testosterone production or action is suppressed at the same time [62]. These findings have been interpreted as evidence that it is the balance between androgens and estrogens that is important for normal reproductive tract development rather than the absolute levels of either hormone [20,62]. If this is true, it has considerable implications for the assessment of mixtures of endocrine disruptors, especially mixtures of compounds with anti-androgenic and estrogenic activity. However, in the study by Rivas et al. [62], administration of a high dose of bisphenol A was still unable to induce any "estrogenic" reproductive tract abnormalities when testosterone production was inhibited at the same time.

Suppression of secretion of insulin-like factor-3 by fetal Leydig cells

In the last few years, a completely new pathway involved in testicular descent has been discovered and has been shown to be suppressible by estrogens, namely production of insulin-like factor-3 (InsL3), also termed relaxin-like factor, by fetal Leydig cells. InsL3 acts on the gubernaculum of the testis, which plays an important role in guiding the testis during its phase of transabdominal descent. Administration of estrogens to pregnant rats during the time of sexual differentiation of the male fetus results in suppression of InsL3 expression and failure of testis descent [63,64]. The importance of InsL3 was confirmed by transgenesis, as mice homozygous for a knock-out of the InsL3 gene exhibit bilateral cryptorchidism [65,66]. Indeed, in transgenic mice that overexpress InsL3, ovarian "descent" is induced in the female offspring [67]. These findings demonstrate that production of InsL3 by fetal Leydig cells plays a key role in the transabdominal phase of testis descent, and that InsL3 secretion can be suppressed by estrogens. It remains unknown what relationship, if any, there is between estrogen-induced suppression of testosterone and InsL3 production, or what physiological role estrogens might play in regulating InsL3 secretion.

These four new pieces of data from animal studies go a long way toward explaining why suppression of androgen production/action or overexposure to estrogens may have very similar effects on the developing male reproductive system [20], and probably explain the coincidence of these two factors as risk factors for reproductive developmental disorders in humans (Table 1, Fig. 1). They also have other important implications. First, estrogen exposure *on its own*, either during fetal or neonatal life, only appears to cause adverse effects that impact health/function when administered at very high doses (doses at which effects on androgen production/action occur). At face value, this implies that weakly estrogenic compounds will be incapable of inducing such effects, at least because of their intrinsic estrogenicity. In this regard, it is emphasized that studies showing effects of extremely low doses of estrogens or estrogenic compounds (so-called "low-dose effects") on male reproductive development have used endpoints that are fundamentally different from those being discussed here (see below).

RELEVANCE OF ENVIRONMENTAL COMPOUNDS WITH ESTROGENIC OR ANTIANDROGENIC ACTIVITY TO HUMAN MALE REPRODUCTIVE DEVELOPMENTAL DISORDERS

In view of the improvement in understanding the mechanisms via which estrogens can induce male reproductive abnormalities, and the demonstration that only very high doses of (potent) estrogens are apparently able to activate these pathways, it is possible to make a fairly radical reassessment of the hazard posed by estrogenic and anti-androgenic chemicals. For example, it is clear that all of the identified "environmental estrogens" possess weak or very weak intrinsic estrogenic activity when measured by conventional in vitro and in vivo assays for estrogenicity [68–70], with the possible exception of zeranol (which is used as a growth promoter in livestock in the United States [71]). Based on intrinsic estrogenic potency of these chemicals, it seems unlikely that any of the identified environmental compounds could induce either cryptorchidism, hypospadias, or testis germ cell cancer, and only a tiny possibility that such compounds could affect sperm counts/sperm production.

We reason this conclusion as follows. For induction of male reproductive tract abnormalities in *male* animals, exposure to doses of DES higher than or equal to 50 μg/kg/day are required, and these are the doses that also induce major suppression of androgen production/androgen receptor expression [20,58]. Similarly high amounts were administered to pregnant women and were associated with increased incidence of reproductive developmental abnormalities (Table 1), whereas exposure to lower estrogen doses or to high doses for only short periods did not induce such effects (at least not cryptorchidism or hypospadias) [46]. Based on estrogenic potency, human exposure to the most potent environmental estrogens would need to be >1,000-fold higher than 50 μg/kg/day for adverse effects relevant to the human male to be induced, and such levels of exposure are remote or impossible [69]. Zeranol, in contrast to other identified environmental estrogens, is considerably more potent based on in vitro tests [71], but even in this case it is inconceivable that exposure in this range due to residues in meat could occur, as the available evidence points to levels of exposure that are in the pg/kg/day range, or at the very most the low ng/kg/day range [72,73]. Consistent with this interpretation, we have been unable to induce "DES-like" reproductive tract abnormalities in rats treated neonatally with zeranol at a high dose of 300 μg/kg/day (unpublished data) or with either of two environmental estrogens, bisphenol A or octylphenol, even when these were administered at levels approximating 37 to 150 mg/kg/day [20,61,74].

In contrast, other studies have reported adverse effects of various environmental estrogens such as bisphenol A and octylphenol on male rodents. These effects have included impaired development/proliferation of germ cells and altered steroidogenesis [75–78]. In most studies, relatively high doses that are probably not relevant to human exposure were used to induce such effects. However, extremely low levels were used in the studies by vom Saal et al. [79] in which sperm production and prostate weight were both altered significantly (sperm production downwards) in adulthood in mice after in utero exposure to levels of bisphenol A in the range 2 to 20 μg/kg. Such "low-dose" effects can also be induced by 0.02 to 2 μg/kg/day ethinyl estradiol in this model system [80], though it should be noted that comparable and more detailed studies have been unable to repeat these findings using doses of bisphenol A in the range 0.2 to 200 μg/kg/day [81,82; reviewed in ref. 70]. In the studies by vom Saal and colleagues [79,80], it is impossible to account for the effects of bisphenol A in terms of its intrinsic estrogenic potency, so an estrogen-independent mechanism of action needs to be invoked, and this remains to be identified [70]. However, it must be kept firmly in mind that estrogenic or anti-androgenic environmental chemicals may exhibit other biological activities that might still be relevant to human health, and some examples of this are discussed below.

Though the disparate findings just described are difficult to reconcile, it is clear that none of the disorders that comprise the "testicular dysgenesis syndrome" in humans, with the possible exceptions of reduced sperm counts, have been reported in any study in which an environmental estrogen has been administered to rats or mice. As has been suggested above, it may be that in humans, exposure to very

high levels of potent estrogens during a relatively long period of time may induce congenital abnormalities such as cryptorchidism and hypospadias, and the animal data are largely consistent with this hypothesis. Therefore, based on present understanding, it seems unlikely that altered human exposure to weak estrogenic compounds can account for the possible increasing incidence of male reproductive-tract disorders, though this must be considered a tentative conclusion. Nevertheless, this does not mean that exposure to environmental chemicals can be ruled out as being involved etiologically in "testicular dysgenesis syndrome", as in utero exposure of rats to certain phthalates has been shown to induce a remarkably similar constellation of disorders [83–85]. These effects are attributable to impaired testosterone production by fetal Leydig cells in phthalate-exposed rats [86]. Such findings, together with the understanding that lowered androgen production/action (or altered androgen-estrogen balance) are involved in DES-/estrogen-induction of similar disorders, suggests strongly that in the search for environmental causes of human male reproductive developmental disorders, the focus should be very much on factors that can lower endogenous androgen production/ action or on factors that can elevate endogenous (potent) estrogens in the fetus. This assumes that exposure to exogenous estrogens will be insufficient, for reasons argued above. Any factor(s) that can both lower androgen production/action and elevate endogenous estrogens, thereby drastically altering the androgen–estrogen balance, would be of greatest concern.

Alteration of fetal androgen production/action during human pregnancy

As has already been discussed, and as illustrated in Table 1, gene mutations or chromosomal disorders that result in lowered androgen production or action increase the risk of male offspring having one or more of the disorders that comprise testicular dysgenesis syndrome (Fig. 1). Other than rare genetic changes, no other causes of lowered androgen production or action in the human fetus have been identified. However, one important observation is that African-American males have a substantially lower risk of testis cancer than do Caucasian Americans [27], and there is also a reported three-fold lower incidence of cryptorchidism in African-American babies compared with Caucasian babies [87]. The demonstration that pregnant African-American women in early gestation have 55 % higher blood levels of testosterone than do pregnant Caucasian women in the United States, when matched for gestational stage and body weight [88], has therefore been mooted as potentially playing a role in ensuring "high androgen status in the male fetus" [88]. One study in the United Kingdom has also suggested a link between low maternal testosterone levels during early gestation and the risk of cryptorchidism in the male offspring [89]. However, it is not clear what relationship, if any, there is between maternal and fetal androgen levels during early gestation, though it is clear that overproduction of androgens by the mother can partially masculinize the female fetus. The factors that might affect maternal androgen levels, other than SHBG (see below) remain to be defined.

A new possibility is that exposure of the pregnant mother to certain phthalate esters might lower fetal production of testosterone, based on their ability in studies of rats to do this and to cause a syndrome of disorders that are similar to those of concern in the human [83–85]. Human exposure to phthalates is extensive, and recent data (based on measurement of urinary phthalate metabolites), has identified that a subset of humans may have very much higher levels of exposure than the majority [90]. These are mainly women of reproductive age, and the source of their high exposure remains to be defined. In this subset, exposure ranges up to 160 μg/kg/day [91] whereas the effects in rats have been demonstrated in the range 100 to 750 mg/kg/day. This suggests that the risk to humans from phthalates is perhaps more theoretical than real, unless the human male fetus is more susceptible to the testosterone-lowering effects of phthalates than is the fetal rat; or differences in the ability of individuals to absorb, metabolize, or excrete phthalates makes them unusually susceptible to effects of phthalates. Studies that assess the relative sensitivity in vitro of the rat and human fetal testis to phthalate monoesters should help to address this possibility, and such studies are in progress.

Alteration of fetal exposure to endogenous estrogens during human pregnancy

An obvious way in which exposure of the human fetus to potent estrogens could be increased is via increased bioavailability and, thus, increased transplacental transfer of the enormously high estradiol levels that occur during normal human pregnancy. This is more than a theoretical concern, as it is established that first pregnancies or twin pregnancies, when estradiol levels are accepted to be unusually high, are risk factors for testis cancer and cryptorchidism (Table 1). The bioavailability of estradiol is determined by three factors:

1. level of production;
2. levels of sex hormone-binding globulin (SHBG) to which most estradiol and testosterone are bound; and
3. rate of metabolism/excretion of estradiol.

All three factors are potentially modulable, but the available evidence points to the second and third possibilities as likely to be the most susceptible to environmental/lifestyle influence. For example, insulin is a powerful, physiological suppressor of SHBG production, which will increase the bioavailability of estradiol and alter the androgen–estrogen balance [92]. The Western trend toward obesity and associated insulin resistance (= raised insulin levels) in women could therefore be an increasingly influential factor [93] to consider.

Reduced ability to inactivate or excrete estrogens could be another mechanism via which "pregnancy estrogens" might get transferred to the fetus in abnormally high amounts. One possible mechanism is altered inactivation of estradiol via estrogen sulphotransferase (SULT1E1), which is the primary mechanism via which estradiol is inactivated and excreted [94]. Polychlorinated biphenyls (PCBs) have been shown to suppress activity of SULT1E1 [95], while another study has shown even more potent suppressive effects of a range of environmental polyhalogenated hydrocarbons [96]. Human exposure to PCBs has been widespread, as is that to polyhalogenated hydrocarbons, so these new findings identify at least one route via which environmental chemicals could affect sex steroid levels or balance in the fetus, independent of there being any intrinsic hormonal activity in the compounds in question. It will prove of interest to see whether human exposure to such compounds during early pregnancy can be accurately determined and related to the occurrence of cryptorchidism or hypospadias in the offspring.

CONCLUSIONS AND FUTURE PERSPECTIVES

Though some of the data regarding the incidence and time trends in human male reproductive developmental disorders are less than robust, the widest perspective indicates that such disorders are extremely common and are becoming more common, at least in certain countries. Male reproductive health receives far less attention and healthcare investment than does female reproductive health, yet cryptorchidism and hypospadias are the two most common congenital malformations (affecting 2 to 5 % of boys, many of whom will require surgery), testis cancer is the most common cancer of young men (lifetime risk 0.3 to 0.8 % in most countries), and poor semen quality is the biggest defined cause of couple infertility and affects 6 to 8 % of men. The recognition that all of these disorders may form a hitherto unrecognized syndrome of disorders, "testicular dysgenesis syndrome", with a common origin in fetal life [4], has also emerged recently and provides an important focus for research to identify the pathways involved (Fig. 1). The fact that each of the component disorders of this "syndrome" are important risk factors for each other (Table 1), and that a similar "syndrome" can be induced in rats by phthalate exposure, strongly supports this contention. It is becoming increasingly clear that lowered androgen production/action is of central importance in "testicular dysgenesis syndrome", but it should also be kept in mind that lowered androgen production/action may itself be only a downstream manifestation of an earlier change, for example incomplete differentiation of Sertoli, Leydig, peritubular,

and/or fetal germ cells with consequent impairment of function. More than one path (cause) may lead in this direction (Fig. 1).

Research in experimental animals during the last decade has helped to redefine the risk to male reproductive health from estrogens, and the current perspective is that "environmental estrogens" at least probably pose little, if any, risk to human male reproductive development, based on their intrinsic estrogenicity. However, the very fact that an "estrogen hypothesis" was first proposed in the late 1970s to early 1980s as potentially explaining some cases of testicular cancer and cryptorchidism [33], and that it can still be restated today (though in somewhat different terms), emphasizes that it must contain a kernel of truth. The emergence of new data and new mechanisms via which estrogens can induce cryptorchidism (suppression of InsL3; suppression of androgen production/action) or hypospadias (suppression of androgen production/action) in experimental animals is exciting, and provides a path forward to even better understanding. This data, coupled with the findings from studies with phthalates, also emphasizes the important role that experimental studies in animals are likely to play in dissecting apart the pathways that lead to male reproductive developmental abnormalities. Identifying these pathways and their experimental manipulation will provide an important means of pinpointing potential environmental or lifestyle factors that might impact them, which can then be factored into human epidemiological and prospective clinical studies.

If the incidence of testicular cancer is a beacon, then it must be accepted that environmental and/or lifestyle changes must play an important role in the increasing prevalence of male reproductive developmental disorders. Although we argue that exposure to environmental estrogens are probably unimportant in this context, an important new focus should be on chemicals that alter endogenous hormone production, action, or inactivation. Perusal of any clinical endocrinology textbook shows the catastrophic effects when normal endogenous hormonal status of an individual is altered, and male reproductive development provides some prime examples. The discovery that the most ubiquitous and/or persistent environmental chemicals, DDT/DDE, PCBs, and phthalates, can all perturb endogenous hormones in this way, serves as a timely reminder that the possibility that environmental chemicals play a role in the aetiology of human male reproductive disorders cannot be dismissed, only redefined.

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REFERENCES

1. E. Carlsen, A. Giwercman, N. Keiding, N. E. Skakkebaek. *Br. Med. J.* **305**, 609–613 (1992).
2. R. M. Sharpe and N. E. Skakkebaek. *Lancet* **341**, 1392–1395 (1993).
3. R. M. Sharpe. *Pure Appl. Chem.* **70**, 1685–1701 (1998).
4. N. E. Skakkebaek, E. Rajpert-de Meyts, K. M. Main. *Hum. Reprod.* **16**, 972–978 (2001).
5. R. M. Sharpe and S. Franks. *Nature Cell Biol.* (2002). In press.
6. D. S. Irvine. *Bailliere's Clinics Obstet. Gynaecol.* **11**, 655–671 (1997).
7. B. Jégou, J. Auger, L. Multigner, C. Pineau, P. Thonneau, A. Spira, P. Jouannet. In *The Male Gamete: From Basic Science to Clinical Applications*, C. Gagnon (Ed.), pp. 446–454, Cache River Press, Clearwater, Florida (1999).
8. S. H. Swan, E. P. Elkin, L. Fenster. *Environ. Health Perspect.* **105**, 1228–1232 (1997).
9. S. H. Swan, E. P. Elkin, L. Fenster. *Environ. Health Perspect.* **108**, 961–966 (2000).
10. N. Jorgensen, J. Auger, A. Giwercman, D. S. Irvine, T. K. Jensen, P. Jouannet, N. Keiding, C. Le Bon, E. MacDonald, A. M. Pekuri, T. Scheike, M. Simonsen, J. Suominen, N. E. Skakkebaek. *Int. J. Androl.* **20**, 201–208 (1997).

11. N. Jorgensen, A.-G. Andersen, F. Eustache, D. S. Irvine, J. Suominen, J. H. Petersen, A. N. Andersen, J. Auger, E. H. Cawood, A. Horte, T. K. Jensen, P. Jouannet, N. Keiding, M. Vierula, J. Toppari, N. E. Skakkebaek. *Hum. Reprod.* **16**, 1012–1019 (2001).
12. R. M. Sharpe. *Baillieres Clinics Endocrinol. Metab.* **14**, 489–503 (2000).
13. A. G. Andersen, T. K. Jensen, E. Carlsen, N. Jorgensen, A.-M. Andersson, T. Krarup, N. Keiding, N. E. Skakkebaek. *Hum. Reprod.* **15**, 366–372 (2000).
14. J. P. Bonde, E. Ernst, T. K. Jensen, N. H. Hjollund, H. Kolstad, T. B. Henriksen, T. Scheike, A. Giwercman, J. Olsen, N. E. Skakkebaek. *Lancet* **352**, 1172–1177 (1998).
15. M. J. Zinaman, C. C. Brown, S. G. Selevan, E. D. Clegg. *J. Androl.* **21**, 145–153 (2000).
16. R. Slama, F. Eustache, B. Ducot, T. K. Jensen, N. Jorgensen, A. Horte, D. S. Irvine, J. Suominen, A. G. Andersen, J. Auger, M. Vierula, J. Toppari, A. N. Andersen, N. Keiding, N. E. Skakkebaek, A. Spira, P. Jouannet. *Hum. Reprod.* **17**, 503–515 (2002).
17. S. G. Selevan, L. Borkovec, V. L. Slott, Z. Zudova, J. Rubes, D. P. Evenson, S. D. Perrault. *Environ. Health Perspect.* **108**, 887–894 (2000).
18. R. M. Sharpe. In *Fetal Programming: Influences on Development and Disease in Later Life,* P. M. S. O'Brien, T. Wheeler, D. J. P. Barker (Eds.), pp. 187–194, Royal College of Obstetricians & Gynaecologists Press, London (1999).
19. N. Atanassova, C. McKinnell, M. Walker, K. J. Turner, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome, R. M. Sharpe. *Endocrinology* **140**, 5364–5373 (1999).
20. K. Williams, C. McKinnell, P. T. K. Saunders, M. Walker, J. S. Fisher, K. J. Turner, N. Atanassova, R. M. Sharpe. *Hum. Reprod. Update* **7**, 236–247 (2001).
21. R. M. Sharpe, M. Walker, M. R. Millar, K. Morris, C. McKinnell, P. T. K. Saunders, H. M. Fraser. *Biol. Reprod.* **62**, 1685–1693 (2000).
22. J. Toppari, J. C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L. J. Guillette, Jr., B. Jégou, T. K. Jensen, P. Jouannet, N. Keiding, H. Leffers, J. A. McLachlan, O. Meyer, J. Müller, E. Rajpert-De Meyts, T. Scheike, R. M. Sharpe, J. S. Sumpter, N. E. Skakkebaek. *Environ. Health Perspect.* **104** (Suppl. 4), 741–803 (1996).
23. A. J. Wilcox, D. D. Baird, C. R. Weinberg, P. P. Hornsby, A. L. Herbst. *New Engl. J. Med.* **332**, 1411–1416 (1995).
24. H. Adami, R. Bergstrom, M. Mohner, W. Zatonski et al. *Int. J. Cancer* **59**, 33–38 (1994).
25. I. Dos Santos Silva, A. J. Swerdlow, C. A. Stiller, A. Reid. *Int. J. Cancer* **83**, 630–634 (1999).
26. H. Moller. *Hum. Reprod.* **16**, 1007–1011 (2001).
27. SEER (Surveillance, Epidemiology and End Results; National Cancer Institute, USA). Incidence and age-adjusted rates of testis cancer 1973–1999. Web site address: <http://seer.cancer.gov> (2002).
28. R. Bergstrom, H. Adami, M. Mohner, W. Zatonski et al. *J. Natl. Cancer Inst.* **88**, 727–733 (1996).
29. T. Zheng. T. R. Holford, Z. Ma, B. A. Ward, J. Flannery, P. Boyle. *Int. J. Cancer* **65**, 723–729 (1996).
30. H. Moller and N. E. Skakkebaek. *Br. Med. J.* **318**, 559–562 (1999).
31. R. Jacobsen, E. Bostofte, G. Engholm, J. Hansen, J. H. Olsen, N. E. Skakkebaek, H. Moller. *Br. Med. J.* **321**, 789–792 (2000).
32. M. O. Savage and D. G. Lowe. *Clin. Endocrinol.* **32**, 519–533 (1990).
33. B. E. Henderson, R. Ross, L. Bernstein. *Cancer Res.* **48**, 246–253 (1988).
34. K. P. Dieckmann, T. Endsin, U. Pichlmeier. *Eur. Urol.* **40**, 677–683 (2001).
35. H. K. Weir, L. D. Marrett, N. Kreiger, G. A. Darlington, L. Sugar. *Int. J. Cancer* **87**, 438–443 (2000).
36. W. C. Strohsnitter, K. L. Noller, R. N. Hoover, S. J. Robboy, J. R. Palmer, L. Titus-Ernstoff, R. H. Kaufman, E. Adam, A. L. Herbst, E. E. Hatch. *J. Natl. Cancer Inst.* **93**, 545–551 (2001).
37. T. Westergaard, J. H. Olsen, M. Frisch, N. Kroman, J. W. Nielsen, M. Melba. *Int. J. Cancer* **66**, 627–635 (1996).

38. A. J. Swerdlow, B. L. De Stavola, M. A. Swanwick, N. E. S. Maconochie. *Lancet* **350**, 1723–1728 (1997).
39. M. M. Braun, A. Ahlbom, B. Floderus, L. A. Brinton, R. N. Hoover. *Cancer Causes Control* **6**, 519–524 (1995).
40. N. E. Skakkebaek, J. G. Berthelsen, A. Giwercman, J. Muller. *Int. J. Androl.* **10**, 19–28 (1987).
41. K. P. Dieckmann and N. E. Skakkebaek. *Int. J. Cancer* **83**, 815–822 (1999).
42. M. Rorth, E. Rajpert-de Meyts, L. Andersson, K. P. Dieckmann, S. D. Fossa, K. M. Grigor, W. F. Hendry, H. W. Herr, L. H. Looijenga, J. W. Osterhuis, N. E. Skakkebaek. *Scand. J. Urol. Nephrol.* **205**, 166–186 (2000).
43. E. Rajpert-de Meyts, N. Jorgensen, K. B. Nielsen, J. Muller, N. E. Skakkebaek. *APMIS* **106**, 198–206 (1998).
44. J. Slowikowska-Hilczer, R. Walczak-Jedrzejowska, K. Kula. *Folia Histochem. Cytobiol.* **39**, 67–72 (2001).
45. J. Toppari, M. Kaleva, H. E. Virtainen. *Hum. Reprod. Update* **7**, 282–286 (2001).
46. L. Raman-Wilms, A. L. Tseng, S. Wighardt, T. R. Einarson, G. Koren. *Obstet. Gynecol.* **85**, 141–149 (1995).
47. L. J. Paulozzi, J. D. Erickson, R. J. Jackson. *Pediatrics* **100**, 831–834 (1997).
48. L. J. Paulozzi. *Environ. Health Perspect.* **107**, 297–302 (1999).
49. F. H. Pierik, A. Burdoff, J. M. Rien Nijman, S. M. de Muinck Keizer-Schrama, R. E. Juttmann, R. F. A. Weber. *Hum. Reprod.* **17**, 1112–1115 (2002).
50. N. Hussain, A. Chaghtai, C. D. Herndon, V. C. Herson, T. S. Rosenkrantz, P. H. McKenna. *Pediatrics* **109**, 473–478 (2002).
51. M. Joffe. *Hum. Reprod.* **17**, 520–523 (2002).
52. B. E. Henderson, B. Benton, J. Jing, M. C. Yu, M. C. Pike. *Int. J. Cancer* **23**, 598–602 (1979).
53. H. Klip, J. Verloop, J. D. van Gool, M. E. Koster, C. W. Burger, F. E. van Leeuwen. *Lancet* **359**, 1102–1107 (2002).
54. J. F. Couse, D. Dixon, M. Yates, A. B. Moore, L. Ma, R. Maas, K. S. Korach. *Devel. Biol.* **15**, 224–238 (2001).
55. G. S. Prins, L. Birch, J. F. Couse, I. Choi, B. Katzenellenbogen, K. S. Korach. *Cancer Res.* **61**, 6089–6097 (2001).
56. X. Li, E. Nokkala, W. Yan, T. Streng, N. Saarinen, A. Warri, I. P. Huhtaniemi, R. Santti, S. Makela, M. Poutanen. *Endocrinology* **142**, 2435–2442 (2001).
57. C. McKinnell, N. Atanassova, K. Williams, J. S. Fisher, M. Walker, K. J. Turner, P. T. K. Saunders, R. M. Sharpe. *J. Androl.* **22**, 323–338 (2001).
58. T. Haavisto, K. Numela, R. Pohjanvirta, H. Huuskonen, F. El-Gehani, J. Paranko. *Mol. Cell. Endocrinol.* **178**, 169–179 (2001).
59. G. Majdic, M. Sharpe, M. R. Millar, P. T. K. Saunders. *Mol. Cell. Endocrinol.* **127**, 91–98 (1997).
60. G. Majdic, R. M. Sharpe, P. J. O'Shaughnessy, P. T. K. Saunders. *Endocrinology* **137**, 1063–1070 (1996).
61. K. Williams, J. S. Fisher, K. J. Turner, C. McKinnell, P. T. K. Saunders, R. M. Sharpe. *Environ. Health Perspect.* **109**, 1227–1235 (2001).
62. A. Rivas, J. S. Fisher, C. McKinnell, N. Atanassova, R. M. Sharpe. *Endocrinology* (2002). In press.
63. J. M. Emmen, A. McLuskey, I. M. Adham, W. Engel, J. M. Verhoef-Post, A. P. Themmen, J. A. Grootegoed, A. O. Brinkmann. *Endocrinology* **141**, 846–849 (2000).
64. S. Nef, T. Shipman, L. F. Parada. *Devel. Biol.* **224**, 354–361 (2000).
65. S. Nef and L. F. Parada. *Nat. Genet*. **22**, 295–299 (1999).
66. S. Zimmermann, G. Steding, J. M. Emmen, A. O. Brinkmann, K. Nayernia, A. F. Holstein, W. Engel, I. M. Adham. *Mol. Endocrinol.* **13**, 681–691 (1999).

67. I. M. Adham, G. Steding, T. Thamm, E. E. Bullesbach, C. Schwabe, I. Paprotta, W. Engel. *Mol. Endocrinol.* **16**, 244–252 (2002).
68. T. M. Crisp, E. D. Clegg, R. L. Cooper, W. P. Wood, D. G. Anderson, K. P. Baetcke, J. L. Hoffmann, M. S. Morrow, D. J. Rodier, J. E. Schaeffer, L. W. Touart, M .G. Zeeman, Y. M. Patel. *Environ. Health Perspect.* **106** (Suppl. 1), 11–56 (1998).
69. H. M. Bolt, P. Janning, H. Michna, G. H. Degen. *Arch. Toxicol.* **74**, 649–662 (2001).
70. R. Melnick, G. Lucier, M. Wolfe, R. Hall, G. Stancel, G. Prins, M. Gallo, K. Reuhl, S. M. Ho, T. Brown, J. Moore, J. Leakey, J. Haseman, M. Kohn. *Environ. Health Perspect.* **110**, 427–431 (2002).
71. H. Leffers, M. Naesaby, B. Vendelbo, N. E. Skakkebaek, M. Jorgensen. *Hum. Reprod.* **16**, 1037–1045 (2001).
72. D. M. Henricks, S. L. Gray, J. J. Owenby, B. R. Lackey. *APMIS* **109**, 273–283 (2001).
73. I. G. Lange, A. Daxenberger, H. H. D. Meyer. *APMIS* **109**, 53–65 (2001).
74. J. S. Fisher, K. J. Turner, D. Brown, R. M. Sharpe. *Environ. Health Perspect.* **107**, 397–405 (1999).
75. E. P. Murono, R. C. Derk, J. H. de Leon. *Reprod. Toxicol.* **13**, 451–462 (1999).
76. H. Nikula, T. Talonpoika, M. Kaleva, J. Toppari. *Toxicol. Appl. Pharmacol.* **157**, 166–173 (1999).
77. S. S. Raychoudhury, C. A. Blake, C. F. Millette. *Toxicol. Appl. Pharmacol.* **157**, 192–202 (1999).
78. E. Bendsen, S. Laursen, C. Olesen, L. Westergaard, C. Andersen, A. Byskov. *Hum. Reprod.* **16**, 236–243 (2001).
79. F. S. Vom Saal, P. S. Cooke, D. L. Buchanan, P. Palanza, K. A. Thayer, S. C. Nagel, S. Parmigiani, W. V. Welschons. *Toxicol. Ind. Health* **14**, 239–260 (1998).
80. K. A. Thayer, R. L. Ruhlen, K. L. Howdeshell, D. L. Buchanan, P. S. Cooke, D. Preziosi, W. V. Welshons, J. Haseman, F. S. vom Saal. *Hum. Reprod.* **16**, 988–996 (2001).
81. M. Ema, S. Fujii, M. Furukawa, M. Kiguchi, T. Ikka, A. Harazano. *Reprod. Toxicol.* **15**, 505–523 (2001).
82. T. Nagao, Y. Saito, K. Usumi, S. Yoshimura, H. Ohno. *Reprod. Toxicol.* **16**, 123–130 (2002).
83. E. Mylchreest, M. Sar, R. C. Cattley, P. M. D. Foster. *Toxicol. Appl. Pharmacol.* **156**, 81–95 (1999).
84. E. Mylchreest, D. G. Wallace, R. C. Cattley, P. M. D. Foster. *Toxicol. Sci.* **55**, 143–151 (2000).
85. R. M. Sharpe. *Toxicol. Lett.* **120**, 221–232 (2001).
86. L. G. Parks, J. S. Ostby, C. R. Lambright, B. D. Abbott, G. D. Klinefelter, N. J. Barlow, L. E. Gray, Jr. *Toxicol. Sci.* **58**, 339–349 (2000).
87. O. P. Heinonen, D. Slone, S. Shapiro. *Birth Defects and Drugs in Pregnancy,* p. 176, John Wright, Littleton, MA, USA (1977).
88. B. E. Henderson, L. Bernstein, R. K. Ross, R. H. Depue, H. L. Judd. *Br. J. Cancer* **57**, 216–218 (1988).
89. T. J. Key, D. Bull, P. Ansell, A. R. Brett, G. M. Clark, J. W. Moore, C. E. Chilvers, M. C. Pike. *Br. J. Cancer* **73**, 698–701 (1996).
90. B. C. Blount, M. J. Silva, S. P. Caudhill, L. L. Needham, J. L. Pirkle, E. J. Sampson, G. W. Lucier, J. Jackson, J. W. Brock. *Environ. Health Perspect.* **108**, 979–982 (2000).
91. M. C. Kohn, F. Parham, S. A. Masten, C. J. Portier, M. D. Shelby, J. W. Brock, L. L. Needham. *Environ. Health Perspect.* **108**, A440–A442 (2000).
92. S. M. Haffner. *Hormone Res.* **45**, 233–237 (1996).
93. P. Preziosi, E. Barrett-Connor, L. Papoz, M. Roger, M. Saint-Paul, K. Nahoul, D. Simon. *J. Clin. Endocrinol. Metabol.* **76**, 283–287 (1993).
94. W. C. Song. *Ann. NY Acad. Sci.* **948**, 43–50 (2001).
95. M. H. A. Kester, S. Bulduck, D. Tibboel, W. Meinl, H. Glatt, C. N. Falany et al. *Endocrinology* **141**, 1897–1900 (2000).
96. M. H. A. Kester, S. Bulduck, H. van Toor, D. Tibboel, W. Meinl et al. *J. Clin. Endocrinol. Metabol.* **87**, 1142–1150 (2002).