Testicular dysgenesis syndrome and the estrogen hypothesis: a quantitative meta-analysis*

A síndrome da disgenesia testicular e a hipótese do estrogênio: uma meta-análise quantitativa

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Abstract ***Male reproductive tract abnormalities such as hypospadias and cryptorchidism, and testicular cancer have been proposed to comprise a common syndrome together with impaired spermatogenesis with a common etiology resulting from the disruption of gonadal development during fetal life, the testicular dysgenesis syndrome (TDS). The only quantitative summary estimate of the link between prenatal exposure to estrogenic agents and testicular cancer was published over 10 years ago; other reviews of the link between estrogenic compounds, other than the potent pharmaceutical estrogen diethylstilbestrol (DES), and TDS end points have remained inconclusive. We conducted a quantitative meta-analysis of the association between the end points related to TDS and prenatal exposure to estrogenic agents. Inclusion in this analysis was based on mechanistic criteria, and the plausibility of an estrogen receptor (ER)-α–mediated mode of action was specifically explored. Eight studies were included, investigating the etiology of hypospadias and/or cryptorchidism that had not been identified in previous systematic reviews. Four additional studies of pharmaceutical estrogens yielded a statistically significant updated summary estimate for testicular cancer. Results of the subset analyses point to the existence of unidentified sources of heterogeneity between studies or within the study population.***

Key words ***Cryptorchidism, DES, Estrogen, Hypospadias, Oral contraceptives, Testicular cancer***

Resumo ***Sugeriu-se que anomalias do trato reprodutivo masculino como hipospádia e criptorquidismo, assim como o câncer de testículo, componham uma síndrome comum com diminuição da espermatogênese, e de etiologia comum, a interrupção do desenvolvimento gonadal na fase fetal, a síndrome de disgenesia testicular (SDT). O único levantamento quantitativo da relação entre exposição pré-natal a agentes estrogênicos e câncer de testículo data de mais de dez anos; outras revisões da relação entre compostos estrogênicos diferentes do potente estrogênio sintético dietilstilbestrol (DES) e SDT continuam inconclusivas. Foi feita uma meta-análise quantitativa da relação entre SDT e exposição pré-natal a agentes estrogênicos. A inclusão na análise baseou-se em critérios mecanísticos e foi explorada a plausibilidade de um modo de ação mediada pelo receptor estrogênico-α (REα). Incluíram-se oito estudos sobre a etiologia das hipospádias e/ou criptorquidismo não identificados em revisões sistemáticas anteriores. Mais quatro estudos sobre estrogênios sintéticos resultaram em uma estimativa estatisticamente significativa para câncer de testículo. Os resultados das análises dos subconjuntos apontam à existência de fontes não identificadas de heterogeneidade entre estudos ou populações estudadas.***

Palavras-chave ***Criptorquidismo, SDT, Estrogênio, Hipospádias, Contraceptivos orais, Câncer de testículo***

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Impaired spermatogenesis, male reproductive tract abnormalities such as hypospadias and cryptorchidism, and testicular cancer have been proposed to comprise a common underlying syndrome with a common etiology resulting from the disruption of embryonic programming and gonadal development during fetal life, termed the testicular dysgenesis syndrome (TDS)[1,2]. A hormonal etiology most likely underlies this syndrome, although it is believed to have more than one cause, possibly including other than endocrine disruption. Some common causes of endocrine disruption include infection, diet and body weight, lifestyle, genetics, and environmental exposure, but endocrine-disrupting chemicals (EDCs), particularly those with estrogenlike properties, have received the most scientific attention.

The synthetic estrogenic drug diethylstilbestrol (DES) was prescribed to more than 5 million pregnant women from the late 1940s to the early 1970s to prevent abortions and pregnancy-related complications[3]. Evidence later showed that maternal ingestion of DES during early pregnancy increased the risk of vaginal clear cell adenocarcinoma in female offspring[4] and resulted in an increased incidence of malformations of the testes, the development of epididymal cysts, and impaired sperm quality in male offspring[5]. During pregnancy, maternal estrogen levels are significantly elevated. However, more than 90% of maternal endogenous estrogens are effectively sequestered via binding to sex hormone binding globulin (SHBG), and thus the fetus is relatively protected[6, 7]. On the other hand, DES and ethinylestradiol do not bind well to SHBG, having a higher biopotency if ingested[1,7]. Additionally, transgenerational exposure is also possible when lipophilic xenoestrogens are mobilized during pregnancy and lactation[8].

Previous systematic reviews of studies in which pregnant women were exposed to estrogens other than DES have failed to find evidence of an increased risk o f urogenital abnormalities in the male offspring[7, 9, 10, 11], and have raised the possibility that nonestrogenic or atypical estrogenic effects of DES exposure ***in utero*** induce male reproductive abnormalities. However, none of the effects of DES exposure on either male or female offspring of exposed wild-type pregnant mice were induced when administered to ERKO (ER-α knockout) mice[12], strongly suggesting an ER-α –mediated mechanism. There is, however, a body of experimental data that is consistent with an effect of antiandrogenic industrial chemicals on male sexual differentiation[13,14]. Moreover, mechanisms other than endocrine disruption may be involved in testicular toxicity; for example, the nematocide dibromochloropropane, an alkylating agent, is one of the most potent known testicular toxins in adults[6]. In this review we focus on the estrogen hypothesis of TDS.

Although several systematic reviews of the literature on the association between estrogenic agents and the disorders thought to belong to the TDS have been published, they are predominantly qualitative and the only quantitative summary estimate of the association between prenatal exposure to estrogenic agents and testicular cancer was published over 10 years ago[11]. The primary objective of a quantitative metaanalysis is to combine the results of previous studies examining a specific research question to arrive at a summary conclusion about a body of research. It has been found particularly useful when individual studies are too small to yield a valid conclusion, but it cannot, however, correct for bias and confounding. When applied to observational studies, subset analysis can be a useful tool to explore the reasons for discrepancies among the results of different studies.

The objectives of this research were therefore to carry out a quantitative meta-analysis of the association between three of the end points related to TDS and prenatal exposure to estrogenic agents that would account for both the size and quality of the studies included and yield updated summary estimates in light of the body of research carried out since the formulation of the estrogen hypothesis. Inclusion in this analysis was based on mechanistic criteria, and the plausibility of an ER-α–mediated mode of action was specifically explored. Moreover, subset analysis has been applied to categories of compounds with estrogenic potencies differing by several orders of magnitude in an attempt to detect the existence of any potency–response trend. Most of the studies of sperm quantity or quality have been concerned with time trends rather than etiology, and this end point was not considered further here.

Material and methods

Identification and selection of literature

A computerized search was conducted using the databases PubMed[15] and Web of Science[16] for the period 1970 to April 2007. The general search keywords were “estrogen,” “risk,” “dose,” and ei-

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ther "hypospadias," "cryptorchidism," or "testicular cancer." A preliminary identification was performed by screening the titles and, if relevant, the abstracts of retrieved literature. The next stage was to check the citations and references of selected studies. This was an iterative process, repeated until no new study could be identified. A set of both inclusion and exclusion criteria was defined, and all relevant literature was then checked for eligibility. The inclusion criteria considered were ***a***) study design, namely, either a case–control, cohort, or clinical trial; ***b***) written in English; ***c***) exposure to one or a mixture of known estrogenic compounds; and ***d***) sufficient data reported to be used in meta-analysis.

The following exclusion criteria were used:

. Exposure to a group of compounds (suspected endocrine disruptors) for which mode of action was unspecified, for example, pesticides.

. Studies of exposure to phytoestrogens. Some phytoestrogens have been found to have a greater binding affinity for ER-β than for ER-α and can result in agonistic or antagonistic effects[17].

. Studies of maternal endogenous hormones.

. Studies of the same cohort as this would bias the results towards the particular studies.

. Incomplete data.

Data extraction and quality rating

In addition to the number of exposed and nonexposed cases and controls, and risk ratios (RRs) with their confidence intervals (CIs), information regarding the study design, estrogenic agent, geographic location of the study, and year of publication were extracted from the selected literature to allow subset analysis to be carried out. When more than one RR was reported, the following priorities were set for choice:

. Adjusted RRs were used, except when the study provided only unadjusted estimates.

. When multiple estimates were given, the RR estimator on which the authors had relied for their assessment of causal association was used.

. Overall RRs were chosen instead of those derived from further stratifications. If an overall estimate was not provided, the RRs of the maximum duration of exposure or the maximum exposure concentration were chosen.

Several aspects of the quality of each study were also recorded according to a rating scheme adapted from those previously described[18, 19]. Every criterion was assessed on a scale of 0 to 2, 0 suggesting that it was not present, 1 when it was unclear, and 2 when that criterion was satisfied. A maximum score of 50 and 52 could be assigned for retrospective (case–control) and prospective (cohort and clinical trials) studies, respectively. This enabled a quality sensitivity analysis to be performed to check the influence of studies with low quality on the pooled estimate.

Data analysis

Graphical representation

The RRs and CIs were plotted against the year of publication to determine whether any positive or negative trends in reporting RRs had occurred over time. Similarly, quality scores were plotted against the year of publication to investigate whether the quality of studies improved over time. To assess publication bias, a funnel plot (SE vs. RR) was produced based on the assumption that smaller studies are less precise in their RRs and thus have less weight and larger SE and should scatter more widely at the lower end of the graph, whereas larger studies will tend to be closer together[20]. Forest plots present the RRs against the reference of the study and help check homogeneity visually.

Statistical pooling

Pooled estimates and 95% CIs were calculated using both a fixedeffects model (Mantel–Haenszel method) and a random-effects model (DerSimonian–Laird method), allowing evaluation of the dependence of the conclusions of the analysis on the model assumptions. A summary estimate is considered statistically significant at the 0.05 level if its CI does not include unity.

The Mantel–Haenszel pooled effect estimate was used in a chi-square statistical test of homogeneity to assess the between-study variance. The magnitude of the test statistics depends on the weight of each study. When the number of studies is low or the studies themselves are small, the test statistic ***Q*** tends to be small. Tests of heterogeneity in metaanalyses are generally low in their power to reject the null hypothesis of homogeneity. For this reason, the chi-square statistical test of homogeneity was carried out at both 0.05 and 0.1 significance levels. Additionally, pooled estimates calculated using fixed effect and random effect models differ only if there is lack of homogeneity between studies. The estimates obtained by both methods were therefore compared to better assess potential heterogeneity between studies, in which case a single summary estimate of effect may be considered inappropriate.

Subset and sensitivity analyses

To investigate potential sources of heterogeneity between studies, we performed subset analyses for the study design, estrogenic agent, and geographic location.

Some studies exploring the influence of hormonal treatment during pregnancy did not specify the type of hormone. From what is known of the hormonal treatment of common conditions occurring during pregnancy, it was deemed reasonable to assume that they would have been likely to include estrogens, and these studies were included in the analysis. The validity of this assumption was tested by applying stricter criteria and calculating a summary estimate of effect excluding any study in which the hormone used had not been specified. Further sensitivity analysis was performed by excluding low-quality studies and extremes (exclusion of the studies with the largest and smallest RR estimators and exclusion of the studies with the largest and smallest weights) to verify that either the quality of the studies or one particular study did not have an excessive influence on the pooled estimate.

Results

A total of 50 studies were identified for the association between ***in utero*** exposure to estrogenic agents and hypospadias and/or cryptorchidism, including 16 that had not been included in previous systematic reviews. Sixteen studies, of which 8 were new studies, were included in the calculation of a summary estimate of effect for either or both end points (Table 1). Studies predating the formulation of the TDS hypothesis often were designed to explore the association of ***in utero*** exposure to a range of pharmaceuticals with birth malformations. Other than 2 recent studies for which pesticide exposure was determined by chemical analysis of specific compounds, assessment of exposure to pesticides is generally derived from the occupation of the mother and specific agents are not identified.

Of the 12 studies identified for the association with testicular cancer, only 3 were excluded from the calculation of a summary estimate of effect (Table 2).

Table 1. Studies identified for the association between in utero exposure to estrogenic agent and hypospadias and cryptorchidism.

Reference	End point	Comment	Previous reviews[a]
Aarskog[21]	Hypospadias	Data on progestins treatment only	
Beard ***et al.***[22]	Cryptorchidism	Included	R-W, T, S
Beral and Colwell[23]	Cryptorchidism	Study too small to calculate risk ratio	Sx
Berkowitz and Lapinski[24]	Cryptorchidism	Use of clomiphene before pregnancy recognized	
Bernstein ***et al.***[25]	Cryptorchidism	Maternal endogenous hormones	S
Bhatia ***et al.***[26]	Cryptorchidism	Included	
	Hypospadias	Included	
Bianca ***et al.*** [27]	Hypospadias	Occupational exposure of fathers to pesticides	
Burton ***et al.*** [28]	Cryptorchidism	Maternal endogenous hormone levels	S
Calzolari et al.[29]	Hypospadias	Oral contraceptive use before pregnancy recognized	R-Wx, S
Cosgrove ***et al.***[30]	Cryptorchidism Hypospadias	No control data for documented abnormalities	R-Wx, Sx
Czeizel ***et al.***[31]	Hypospadias	Progesterone treatment	R-Wx
Czeizel ***et al***.[32]	Cryptorchidism Hypospadias	Ecological study design	
Davies ***et al.***[33]	Cryptorchidism	Oral contraceptive use before pregnancy recognized	S
Depue[34]	Cryptorchidism	Same cohort as Depue (1988)	R-Wx, S
Depue [35]	Cryptorchidism	Included	

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Table 1. continuation

Reference	End point	Comment	Previous reviews[a]
Flores-Luevano ***et al.*** [36]	Hypospadias	Included	
Garcia-Rodriguez ***et al.***[37]	Cryptorchidism	Ecological study design	V, S
Gill ***et al.***[38]	Cryptorchidism	No genitourinary abnormalities in exposed infants	T
	Hypospadias		
Gill ***et al.***[39]	Cryptorchidism	Cryptorchidism in men with testicular hypoplasia	T,S
Harlap ***et al.***[40]	Cryptorchidism	All cases exposed to progesterone	R-W
	Hypospadias		
Harlap and Eldor[41]	Cryptorchidism	No cases after oral contraceptive use during pregnancy	R-W
	Hypospadias		
Harlap ***et al.***[42]	Cryptorchidism	Included	R-W
	Hypospadias	Included	
Heinonen ***et al.***[43]	Hypospadias	Included	
Hemminki ***et al.***[44]	Hypospadias	No exposed controls	
Henderson ***et al.***[45]	Cryptorchidism	No unexposed cases	T, V
	Hypospadias	No unexposed cases	
Janerich ***et al***.[46]	Hypospadias	Data for hypospadias not reported	R-Wx
Källén[47]	Hypospadias	Included	R-W
Källén and Winberg[48]	Hypospadias	No exposed controls	R-Wx, S
Källén ***et al.***[49]	Hypospadias	Included	R-W, S
Key ***et al.***[50]	Cryptorchidism	No exogenous hormone use	S
Klip ***et al.***[51]	Hypospadias	Included	
Kristensen ***et al.***[52]	Cryptorchidism	Exposure to unspecified pesticides	V, S
Longnecker ***et al.***[53]	Cryptorchidism	DDE is antiandrogenic	V, S
	Hypospadias		
McBride ***et al.*** [54]	Cryptorchidism	Included	R-W, S
Monteleone-Neto ***et al.***[55]	Hypospadias	Included	R-W, V, S
North and Golding[56]	Hypospadias	Phytoestrogens	S
Palmer ***et al.***[57]	Hypospadias	Included	
Pierik ***et al.***[58]	Cryptorchidism	Phytoestrogens, unspecified pesticides, or EDCs	
	Hypospadias		
Polednak and Janerich[59]	Hypospadias	Included	R-W, S
Pons ***et al.***[60]	Hypospadias	Included	
Restrepo ***et al.***[61]	Cryptorchidism	Unspecified pesticides	V
Rothman and Louik[62]	Hypospadias	Oral contraceptive use before pregnancy recognized	S
Sorensen ***et al.***[63]	Hypospadias	Clomiphene is estrogenic but does not act via ER	
Stoll ***et al.***[64]	Hypospadias	Oral contraceptive use before pregnancy recognized	R-Wx, S
Sweet ***et al.***[65]	Hypospadias	No exogenous estrogens during pregnancy	R-W
Torfs ***et al.***[66]	Cryptorchidism	Same cohort as Bhatia et al. (2005)	R-Wx
	Hypospadias		
Vessey ***et al.***[67]	Cryptorchidism	Included	S
	Hypospadias	No unexposed cases	S
Vrijheid ***et al.***[68]	Hypospadias	Included	
Weidner ***et al.***[69]	Cryptorchidism	Exposure to unspecified pesticides	V, S
	Hypospadias		
Whitehead and Leiter[70]	Cryptorchidism	No controls	T

[a] The letters R-W, T, V, and S refer to Raman-Wilms ***et al.***[9], Toppari ***et al.***[11], Vidaeff and Sever[7], and Storgaard et al.[10], respectively, where the suffix "x" indicates study was excluded from that review.

Table 2. Studies identified for the association between in utero exposure to estrogenic agent and testicular cancer.

Reference	Comment	Previous reviews [a]
Brown ***et al.***[72]	Included	T, S
Depue ***et al.***[73]	Included	T, S
Dieckmann ***et al.***[74]	Maternal endogenous hormone levels	
Gershman and Stolley[75]	Included	S
Hardell ***et al***.[76]	Included	
Hemminki ***et al.***[44]	No cases	
Henderson ***et al.***[77]	Included	T, S
Moss ***et al.***[78]	Included	T, S
Schottenfeld ***et al.***[79]	Included	T, S
Strohsnitter ***et al.***[80]	Included	S
Walcott et al.[81]	Phytoestrogens	
Weir ***et al.***[82]	Included	S

[a] The letters T and S stand for Toppari ***et al***.[11] and Storgaard ***et al.***[10], respectively.

Hypospadias

The data from studies included in the meta-analysis for hypospadias are summarized in Table 3. Three extreme values, two greater than and one lower than unity, can be identified visually from the forest plot of the RRs and their CIs (Figure 1). These extremes correspond to studies with larger SEs, and the shape given to the funnel plot (Figure 2) by those smaller positive studies would be consistent with publication bias. These two extreme positive risk ratios were, however, reported after what is commonly referred to as "third-generation exposure" to DES, when the mother herself had been exposed to DES prenatally. It was recognized that the inclusion of such studies in the metaanalysis could have introduced heterogeneity, and the influence of this choice was investigated in the subset analysis. Plots of the quality score and RRs versus year of publication did not suggest any significant trends in quality of the studies or estimates of effect over time (not shown).

The pooled estimates of effect by both the Mantel–Haenszel and DerSimonian–Laird methods are very close to unity, and no relationship between ***in utero*** exposure to estrogenic agents and hypospadias could be detected (Table 4). None of the chi-square tests allowed the rejection of the null hypothesis of homogeneity between the studies at the 0.05 or 0.1 level of statistical significance. The subsets of studies in which exposure to DES and pharmaceutical estrogens were investigated yielded statistically significant risk ratios with both models, although the modest discrepancy between the fixed-effects and randomeffects estimates suggests heterogeneity. Summary estimates for the latter subset were no longer significant when studies that included DES exposure were excluded. Although these results were based on four studies that all addressed ***in utero*** exposure to oral contraceptives, some heterogeneity between studies remained. Excluding the studies of third-generation exposure to DES, values for the summary estimate of effect were found to be 1.33 (95% CI, 0.63–2.83) by the Mantel–Haenszel method and 1.31 (95% CI, 0.52–3.26) by the DerSimonian–Laird method, a very modest and nonsignificant increase in risk. Excluding third-generation exposure from the DES subset yielded estimates of 2.02 (95% CI, 1.12–3.65) by the Mantel–Haenszel method and 2.00 (95% CI, 0.97–4.15) by the DerSimonian–Laird method, on the basis of two studies investigating exposure to any estrogenic drug during the first trimester of pregnancy. The difference between the results obtained by the two models for studies of third-generation exposure to DES was reduced only slightly by excluding the study by Klip ***et al.***[51]; the Mantel–Haenszel method yielded an estimate of 2.46 (95% CI, 0.91–6.67) and the DerSimonian– Laird method of 2.18 (95% CI, 0.64–7.46). The latter study's cohort had been recruited in a fertility clinic, and whether results obtained with subfertile women are generalizable to all women exposed to DES ***in utero*** has been questioned[71].

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Table 3. Summary of data used for the meta-analysis of the association between prenatal estrogenic agents and hypospadias.

Reference	Design	Agent	Location
Bhatia ***et al***.[26]	Case–control	DDT	California, USA
Flores-Luevano ***et al.***[36]	Case–control	DDT	Mexico City
Harlap ***et al.***[42]	Cohort	Oral contraceptives	North Carolina, USA
Heinonen ***et al.***[43]	Cohort	Estrogenic drugs	United States
Källén ***et al.***[47]	Case–control	Oral contraceptives	Sweden
Källén ***et al.***[49]	Case–control	Oral contraceptives	8 countries
Klip ***et al***.[51]	Cohort	DES (mother exposed prenatally)	Netherlands
Monteleone-Neto ***et al***.[55]	Case–control	Sex hormones	Latin America
Palmer ***et al.***[57]	Cohort	DES (mother exposed prenatally)	United States
Polednak and Janerich[59]	Case–control	Oral contraceptives	New York, USA
Pons ***et al.***[60]	Case–control	DES (mother exposed prenatally)	Paris, France
Vrijheid ***et al***.[68]	Case–control	Phthalates (occupational)	United Kingdom

Cases		Controls		RR (95% CI)	SE	Weight	Quality score
E	NE	E	NE				
9	34	42	117	0.79 (0.33–1.89)	0.38	7.07	41
8	33	5	23	1.13 (0.24–5.29)	0.65	2.39	37
3	98	847	32,597	1.10 (0.10–3.90)	0.64	2.47	36
4	184	295	25,069	1.60 (0.44–4.04)	0.69	2.12	45
5	43	6	109	2.11	0.79	1.58	23
16	830	11	835	1.36 (0.64–2.92)	0.43	5.40	30
4	8	205	8,729	21.30 (6.50–70.10)	2.34	0.18	27
21	252	12	307	2.20 (1.04–4.91)	0.44	5.11	24
10	3	2,522	1,336	1.70 (0.40–6.80)	0.72	1.95	36
1	98	3	96	0.33	0.82	1.48	27
3	44	237	17,349	4.99 (1.20–16.80)	1.30	0.59	17
147	3,324	1,399	31,092	0.90 (0.74–1.10)	0.09	129.31	31

Abbreviations: E, exposed; NE, nonexposed.

Although the equality of the results obtained by both methods for the environmental estrogens subset suggests those results are robust, the influence of the weight of the study by Vrijheid ***et al***.[68] cannot be underestimated, as shown by the sensitivity analysis. Exclusion of this study from the analysis yielded a statistically significant Mantel–Haenszel estimate but a lower and not statistically significant DerSimonian–Laird estimate, revealing heterogeneity. A statistically significant estimate was obtained for prospective studies by the Mantel–Haenszel method, but the wide difference with the estimate using the random effect model was suggestive of heterogeneity. Geographic subsets point to a higher risk in Latin America, although the pooled estimates for this location were based on only two studies and did not reach statistical significance.

In addition to the results of the sensitivity analysis presented in Table 4, a pooled estimate of effect was calculated when a stricter inclusion criterion was applied, namely, excluding results from the study by Monteleone-Neto ***et al***.[55]. This had little influence on the overall result, generating summary estimates of 0.97 (95% CI, 0.83–1.13) for the fixed effect model or 0.93 (95% CI, 0.80–1.09) for the random effect model.

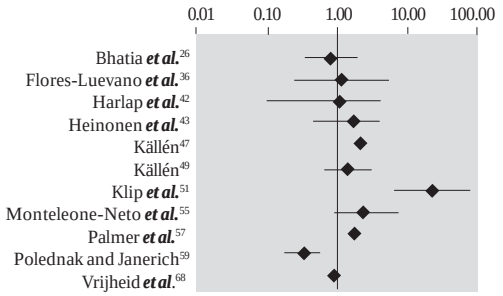

Figure 1. Forest plot of the risk estimates and their 95% CIs from the studies included in the metaanalysis of the association between prenatal exposure to estrogenic agents and hypospadias.

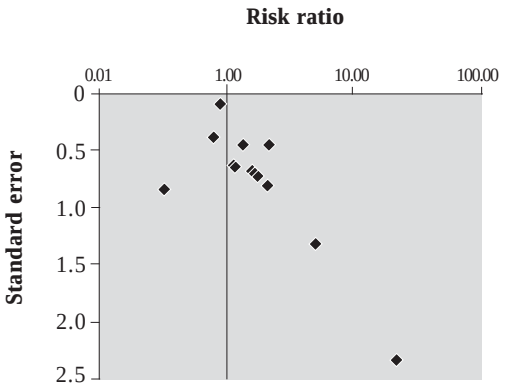

Figure 2. Funnel plot of the risk estimate studies included in the meta-analysis of the association between prenatal exposure to estrogenic agents and hypospadias and their SEs.

Cryptorchidism

Data for the six studies included in the meta-analysis for cryptorchidism can be found in Table 5. The results of only two studies significantly differ from unity, as illustrated by the forest plot (Figure 3). The small number of eligible studies renders analysis of the funnel plot and potential for publication bias difficult (Figure 4). The SEs do, however, illustrate well that the studies were all relatively small. No time trends for the estimate of effect or the quality of studies could be detected (not shown).

As presented in Table 6, the pooled estimates of effect by both the Mantel–Haenszel and DerSimonian–Laird methods are marginally superior to unity, and their relative divergence implies there may be sources of heterogeneity. Chi-square tests did not, however, detect that any of the subsets were significantly heterogeneous. Excluding studies in which DES exposure was examined, either exclusively or along with hormonal therapeutics, yielded summary estimates consistent with no relationship. Statistical pooling of the studies including DES exposure generated a statistically significant estimate by the Mantel–Haenszel method, suggesting a doubling of the risk of cryptorchidism after ***in utero*** exposure to DES. The same estimate by the DerSimonian–Laird method did not, however, reach statistical significance and the difference relative to the fixed effect model is indicative of heterogeneity. The heterogeneity introduced by the DES subset of studies can again be observed by comparing the results obtained for all pharmaceutical estrogens with those obtained by pooling the two studies of accidental use of oral contraceptives during pregnancy. Study design also appeared to be a source of heterogeneity. If case–control studies are prone to recall bias, this subset also included the study with the highest estimate, itself a source of heterogeneity, as shown by the sensitivity analysis. Excluding the study by Depue[35] reduced the difference between estimates by both models, the Mantel– Haenszel estimate then calculated as 1.29 (95% CI, 0.87–1.91) and that by the DerSimonian–Laird method as 1.23 (95% CI, 0.81–1.86). This was also observed for the American subset of studies. When the Depue[35] study is omitted, the Mantel–Haenszel method yielded a no longer statistically significant estimate of 1.34 (95% CI, 0.84–2.14) and the DerSimonian–Laird method an estimate of 1.27 (95% CI, 0.72–2.23).

Applying a stricter exclusion criterion to studies examining hormonal treatment did not affect which studies were included in the meta-analysis of cryptorchidism. The study with the highest weight appears to lower the overall estimates, whereas increasing quality seems to reduce heterogeneity and lower the estimate of effect toward unity. These variations did not, however, influence the overall conclusion that aside from the DES studies subset, summary estimates did not detect any association between ***in utero*** exposure to estrogenic substances and cryptorchidism.

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Table 4. RRs (95% CIs) of the summary estimate of effect, subsets, and sensitivity analyses for the association between hypospadias and prenatal exposure to estrogenic agents.

Subset of studies	No. of studies included	Mantel–Haenszel method (fixed effects)	$\chi 2$ p-Value	DerSimonian–Laird method (random effects)
All studies	12	1.02 (0.88–1.19)	0.30	1.16 (0.83–1.62)
Excluding DES exposure	7	0.93 (0.79–1.09)	0.69	0.91 (0.78–1.07)
Studies including DES exposure	5	2.49 (1.54–4.02)	0.75	2.14 (1.15–3.98)
Mothers exposed to DES prenatally	3	3.73 (1.58–8.80)	0.40	2.54 (0.78–8.33)
Pharmaceutical estrogens only	9	1.85 (1.30–2.64)	0.45	1.54 (1.00–2.36)
Pharmaceutical estrogens excluding DES	4	1.27 (0.74–2.19)	0.36	1.13 (0.61–2.10)
Environmental estrogens only	3	0.90 (0.76–1.06)	0.89	0.90 (0.76–1.06)
European studies	4	0.96 (0.81–1.14)	0.18	0.96 (0.72–1.27)
North American studies	5	1.03 (0.63–1.68)	0.50	0.93 (0.56–1.55)
Latin American studies	2	1.86 (0.99–3.48)	0.39	1.78 (0.87–3.64)
Excluding highest risk ratio	11	1.00 (0.86–1.16)	0.37	0.99 (0.82–1.20)
Excluding lowest risk ratio	11	1.03 (0.87–1.20)	0.35	1.02 (0.84–1.25)
Excluding highest weight	11	1.55 (1.13–2.11)	0.42	1.29 (0.90–1.85)
Excluding lowest weight	11	1.00 (0.86–1.16)	0.37	0.99 (0.82–1.20)
Case–control studies only	8	0.98 (0.84–1.15)	0.22	1.00 (0.78–1.28)
Cohort studies only	4	2.10 (1.14–3.85)	0.54	1.46 (0.59–3.57)
Excluding studies with quality score < 30	7	0.94 (0.80–1.10)	0.85	0.93 (0.79–1.09)
Excluding studies with quality score < 35	5	1.11 (0.69–1.77)	0.83	1.06 (0.65–1.73)

Table 5. Summary of data used for the meta-analysis of the association between prenatal estrogenic agents and cryptorchidism.

References	Design	Agent	Location
Beard ***et al.***[22]	Case–control	Estrogenic drugs	Minnesota, USA
Bhatia ***et al.***[26]	Case–control	DDT	California, USA
Depue [35]	Case–control	Estrogenic drugs	United States
Harlap ***et al.***[42]	Cohort	Oral contraceptives	North Carolina, USA
McBride ***et al.***[54]	Case–control	Oral contraceptives	British Columbia, Canada
Vessey ***et al.***[67]	Clinical trial	DES	United Kingdom

Cases		Controls		RR (95% CI)	SE	Weight	Quality score
E	NE	E	NE				
9	104	15	211	2.20 (0.70–7.20)	0.47	4.60	34
11	32	42	117	0.95 (0.43–2.07)	0.39	6.65	41
5	380	3	765	5.15	1.01	0.99	29
6	196	844	27,595	1.10 (0.10–3.90)	0.42	5.78	36
18	226	34	454	1.10	0.31	10.50	38
6	6	138	126	0.91	0.58	3.00	18

Abbreviations: E, exposed; NE, nonexposed.

Testicular cancer

Nine studies were included in the meta-analysis of testicular cancer and the data used are summarized in Table 7. Of these, 4 had not been included in the summary estimate previously calculated by Toppari ***et al.***[11]. The lack of homogeneity between studies is evident from the forest plot (Figure 5). Further, the funnel plot (Figure 6) also illustrates the relatively small size of the

included studies. Although a positive trend over time was found for the quality of the included studies (Figure 7), no significant time trend could be detected for the effect size (not shown).

Both the fixed and random effect models yield a statistically significant estimate; however, the discrepancy between the two results is suggestive of heterogeneity despite the result from the chi-square test (Table 8). Conversely, the subset analysis was limited by the similarity of the question addressed by the studies included. Eight of the nine studies were interested in hormonal exposure and were conducted in the United States. Despite this, statistically significant heterogeneity between the studies was detected at the 0.1 level. Pooling the two studies examining DES exposure specifically produced a raised but statistically nonsignificant result. Despite the unexplained heterogeneity, all estimates that were calculated point to a doubling of the risk of developing testicular cancer after exposure to estrogenic agents ***in utero***. The work on chlorinated biphenyls (PCBs) by Hardell ***et al.***[76] was the only study examining environmental estrogens. Its size was relatively small, and it did not detect such an effect.

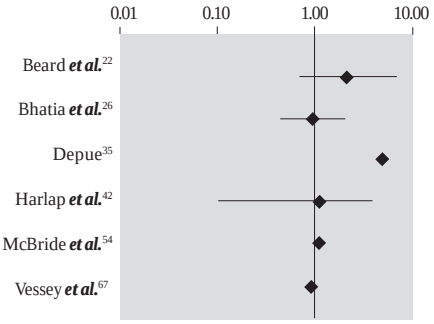

Figure 3. Forest plot risk estimates and their 95% CIs from the studies included in the metaanalysis of the association between prenatal exposure to estrogenic agents and cryptorchidism.

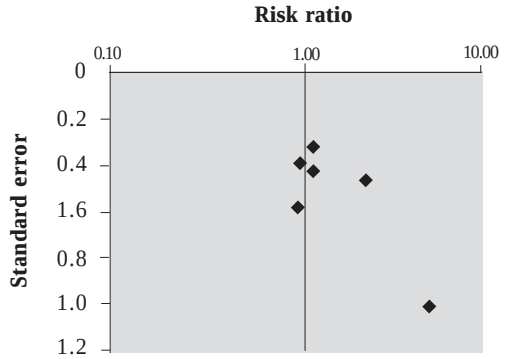

Figure 4. Funnel plot of the risk estimate studies included in the meta-analysis of the association between prenatal exposure to estrogenic agents and cryptorchidism and their SEs.

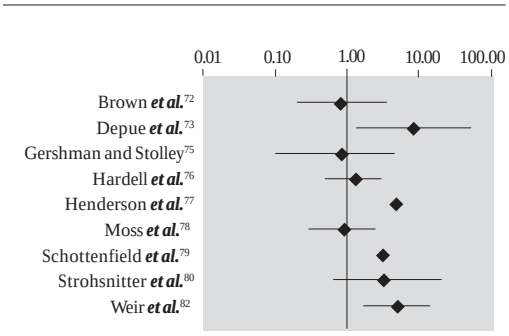

Figure 5. Forest plot risk estimates and their 95% CIs from the studies included in the meta-analysis of the association between prenatal exposure to estrogenic agents and testicular cancer.

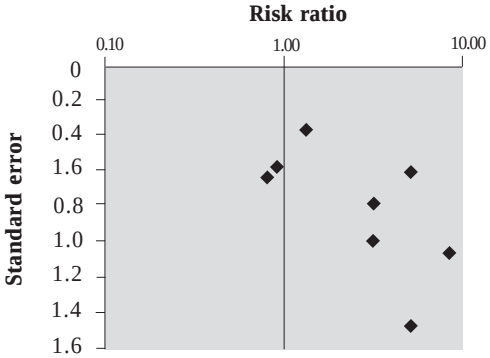

Figure 6. Funnel plot of the risk estimate studies included in the meta-analysis of the association between prenatal exposure to estrogenic agents and testicular cancer and their SEs.

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Applying a stricter exclusion criterion to studies examining hormonal treatment excluded four studies from the meta-analysis; namely, Brown ***et al.***[72], Gershman and Stolley[75], Henderson ***et al.***[77], and Weir ***et al.***[82]. This resulted in a slightly lower Mantel–Haenszel estimate of 1.98 (95% CI, 1.23–3.18) and if the DerSimonian–Laird estimate remained equal to 1.59, because of the wider confidence interval (95% CI, 0.93–2.69), statistical significance was no longer achieved. The sensitivity analysis is consistent with some heterogeneity between the studies, the estimates obtained being relatively sensitive to the exclusion of particular studies varying above and below a risk estimate of 2. The quality of the studies seemed to explain at least some of this heterogeneity.

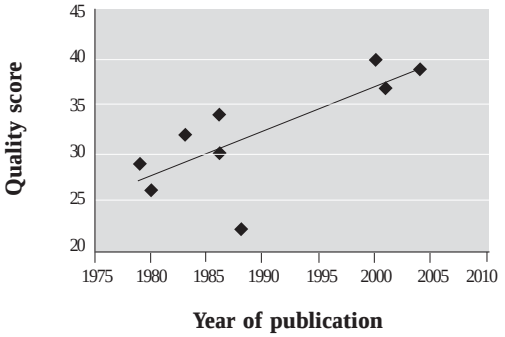

Figure 7. Time trend for quality showing quality score attributed to studies included in the metaanalysis of the association between prenatal exposure to estrogenic agents and testicular cancer by year of publication. R2 = 0.5711.

Discussion

While it is clear that hypospadias, cryptorchidism, and testicular cancer are all positively associated with prenatal exposure to DES, this meta-analysis was unable to produce evidence that such effects were associated with environmental estrogens or even accidental use of oral contraceptives during pregnancy. This is consistent with the results obtained in earlier meta-analyses[9,11].

The main limitations of meta-analysis are ***a***) the susceptibility of its summary results to publication bias, ***b***) the influence of the quality of studies, ***c***) the possibility of including multiple results from the same study, and finally, ***d***) heterogeneity between studies that could lead to invalid conclusions. The methodology employed in this present review attempts to address these issues. Additionally, the importance of carrying out and reporting a sensitivity analysis was illustrated by the case of hypospadias where the weight attrib-

Table 6. RRs and 95% CIs of the summary estimate, subsets and sensitivity analyses for the association between cryptorchidism and prenatal exposure to estrogenic agents.

Subset of studies	No. of studies included	Mantel–Haenszel method (fixed effects)	$\chi 2$ p-Value	DerSimonian–Laird method (random effects)
All studies	6	1.34 (0.96–1.87)	0.44	1.22 (0.86–1.73)
Excluding DES exposure	3	1.06 (0.70–1.59)	0.95	1.05 (0.70–1.59)
Studies including DES exposure	3	2.09 (1.13–3.86)	0.24	1.80 (0.83–3.93)
Pharmaceutical estrogens	5	1.44 (0.99–2.10)	0.37	1.31 (0.87–1.96)
Pharmaceutical estrogens excluding DES	2	1.10 (0.49–2.49)	1	1.10 (0.49–2.49)
Case–control studies only	4	1.45 (0.98–2.15)	0.24	1.38 (0.81–2.34)
Cohort studies	2	1.04 (0.53–2.02)	0.79	1.03 (0.53–2.00)
American studies	4	1.55 (1.00–2.39)	0.24	1.40 (0.82–2.41)
Excluding highest risk ratio	5	1.21 (0.86–1.72)	0.66	1.16 (0.81–1.66)
Excluding lowest risk ratio	5	1.38 (0.97–1.97)	0.34	1.27 (0.86–1.87)
Excluding highest weight	5	1.46 (0.97–2.19)	0.32	1.30 (0.82–2.06)
Excluding lowest weight	5	1.21 (0.86–1.72)	0.66	1.16 (0.81–1.66)
Excluding studies with quality score < 30	4	1.25 (0.86–1.80)	0.53	1.19 (0.82–1.73)
Excluding studies with quality score < 35	3	1.06 (0.70–1.59)	0.95	1.05 (0.70–1.59)

uted to one particularly large study had a non-negligible influence on the results. In this particular case, the study by Vrijheid ***et al.***[68] inferred exposure to phthalates from registry data about occupation, and although such an approach can allow the analysis of a great number of cases, assessment of exposure is much more likely to be prone to confounding. The number of studies included in meta-analyses lies typically between 5 and 15, and the results presented here also fall within this range. The size of the homogeneity test statistic depends on both the number and size of individual studies. The funnel plots offer a good visual representation of the precision and size of individual studies, and it is clear that most studies published on the association between estrogenic agents and the probable end points of a TDS were found to be relatively small. The chi-square tests had, therefore, a relatively low power to detect heterogeneity. However, in the absence of statistical heterogeneity, the results of the fixed effect and random effect models should be virtually identical, and the comparison of results obtained by applying both the Mantel–Haenszel and DerSimonian–Laird models enabled the exploration of sources of heterogeneity despite this low statistical power.

If the quality of the studies was found to explain some of the heterogeneity observed, particularly in the case of testicular cancer, the remaining heterogeneity could not be explained solely by the fact that environmental, and therefore generally much weaker, estrogens were included in the analysis. The systematic review of published literature yielded relatively few studies examining the association of male urogenital abnormalities or testicular cancer with environmental estrogens specifically; a number of studies concerned with an association with broad categories of putative endocrine disruptor, most often pesticides, were excluded from the meta-analyses. This illustrates the difficulties associated with as-

Table 7. Summary of data used for the meta-analysis of the association between prenatal estrogenic agents and testicular cancer.

Reference	Design	Agent	Location
Brown ***et al.*** 1986	Case–control	Sex hormones	Washington, DC, USA
Depue ***et al.*** 1983	Case–control	Estrogenic drugs	Los Angeles, USA
Gershman and Stolley 1988	Case–control	DES	Connecticut, USA
Hardell ***et al.*** 2004	Case–control	Estrogenic PCBs	Sweden
Henderson ***et al.*** 1979	Case–control	Hormone treatment	Los Angeles, USA
Moss ***et al.*** 1986	Case–control	DES or other hormones	California and Nevada, USA
Schottenfeld ***et al.*** 1980	Case–control	DES or other hormones	United States
Strohsnitter ***et al.*** 2001	Cohort	DES	United States
Weir ***et al.*** 2000	Case control	Hormone treatment	Ontario, Canada

Cases		Controls		RR (95% CI)	SE	Weight	Quality score
E	NE	E	NE				
4	198	5	201	0.80 (0.20–3.50)	0.64	2.43	30
8	88	2	103	8.00 (1.30–49)	1.07	0.88	32
4	75	5	74	0.80 (0.10–4.50)	0.65	2.37	22
29	29	30	31	1.30 (0.50–3.00)	0.37	7.31	39
6	72	1	77	5.00	1.47	0.46	29
7	202	6	204	0.90 (0.30–2.60)	0.59	2.89	34
11	170	3	133	3.05	0.79	1.61	26
6	2	1,359	1,392	3.05 (0.65–21.96)	1.01	0.99	37
15	310	7	483	4.90 (1.70–13.90)	0.61	2.66	40

Abbreviations: E, exposed; NE, nonexposed.

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Table 8. RRs and 95% CIs of the summary estimates, subsets and sensitivity analyses for the association between testicular cancer and prenatal exposure to estrogenic agents.

Subset of studies	No. of studies included	Mantel–Haenszel method (fixed effects)	$\chi 2$ p-Value	DerSimonian–Laird method (random effects)
All studies	9	2.14 (1.48-3.10)	0.12	1.59 (1.04–2.43)
DES exposure exclusively	2	2.53 (0.79–8.09)	0.77	2.47 (0.61–10.00)
Pharmaceutical estrogens	8	2.57 (1.66–3.99)	0.09	1.94 (0.98–3.87)
Case–control studies only	8	2.10 (1.43–3.07)	0.09	1.71 (0.92–3.17)
North American studies	8	2.57 (1.66–3.99)	0.09	1.94 (0.98–3.87)
Excluding highest risk ratio	8	1.89 (1.29–2.78)	0.21	1.56 (0.93–2.61)
Excluding lowest risk ratio	8	2.31 (1.56–3.40)	0.14	1.94 (1.08–3.48)
Excluding highest weight	8	2.57 (1.66–3.99)	0.09	1.94 (0.98–3.87)
Excluding lowest weight	8	2.08 (1.42–3.03)	0.10	1.68 (0.95–2.97)
Excluding studies with quality score < 30	6	2.16 (1.42–3.29)	0.08	1.79 (0.91–3.52)
Excluding studies with quality score < 35	3	2.33 (1.39–3.91)	0.13	2.23 (0.98–5.05)

sessment of exposure, pesticide exposure often being inferred from parental occupation rather than direct measurement. Furthermore, there is increasing evidence that, in accordance with pharmacokinetic theory, the effects of xenobiotics acting via the same mechanism can be predicted fairly accurately by concentration addition[83]. Accurately accounting for combined exposure or adjusting for the confounding introduced by environmental exposures will probably require the development of mechanism-specific biomarkers of exposure.

When DES is excluded, there is no conclusive evidence of an effect of pharmaceutical estrogens. Exposure to such estrogens is related mainly to the accidental use of oral contraceptives during pregnancy or hormonal pregnancy tests. Such estrogenic pharmaceuticals often are given in combination with progestagens, and it is legitimate to question whether unopposed estrogens would have the same effects as opposed estrogens. This also highlights another difficulty associated with exposure assessment, that of critically sensitive periods of development and the ascertainment of whether exposure took place during a "window" of susceptibility to hormone disruption. Nonetheless, studies in which maternal levels of hormones were measured in the first and third trimester of pregnancy do not support an association with elevated estrogen levels but rather indicate that a lower estrogen/androgen ratio and/or higher levels of α-fetoproteins may be beneficial[84,85]. If in animals both estrogenic and antiandrogenic compounds have been associated with end points consistent with those of human TDS[86,87], epidemiologic evidence remains elusive. Alternatively, the doubling of the risk estimates of all three effects associated with DES exposure would be consistent with a shared etiology and the TDS hypothesis. It does not constitute conclusive evidence of an estrogenic mode of action, however, as common etiologic factors could be related to the underlying condition for which DES was prescribed. Furthermore hypospadias, cryptorchidism, and testicular cancer have all been found to be associated with low birth weight, suggesting a potential association with an underlying placental defect.

The understanding of the importance of endogenous estrogens in normal adult testicular function is becoming clearer. Their roles during fetal life, however, remain relatively unclear, but those mediated by the ER-α or ER-β have been shown to differ[88]. Interestingly, DES has been found to have similar affinity for both receptors, whereas estradiol has only a slightly stronger affinity for ER-α compared with ER-β [17]. ER-α has been detected in undifferentiated gonads as early as 10 days postconception in the mouse and found to be localized in the Leydig cells of fetal testis in rodents[88]. Studies of the expression of ER-α and ER-β in human and nonhuman primates have so far yielded inconsistent results. Gaskell ***et al.***[89] reported that ER-α could not be

detected in human fetal testes between weeks 12–19 of gestation, whereas Shapiro ***et al.***[90] found that ER-α was apparent by week 12, its levels peaked at 16 weeks before diminishing, and it was localized in Leydig cells. Current research focus has shifted to the role played by testosterone, anti-Müllerian hormone and insulin-like factor 3 produced by the fetal testes during masculinization. In the male rat, exposure to high levels of estrogens has been shown not only to suppress testosterone production but also to downregulate the expression of the androgen receptor protein in reproductive target tissues including the testes, Wolffian duct, and prostate[91]. Further research in this area may help shed light on possible mechanisms of injury or relevance of the rodent model. The subset analyses did not generate many clues to explain the heterogeneity of the collected data. This is, however, consistent with the wide geographic variability in the incidence of the conditions of interest[92,93]. Interactions between genetic susceptibility and the environment have been the focus of research in this area[94], and advances in genomics have allowed the identification of polymorphisms associated with hypospadias, cryptorchidism, and testicular cancer[95-98]. Such discoveries may, however, give rise to as many questions as they offer to answer. This is well illustrated by the recent identification of the association of a variant of the gene for the ER-α with hypospadias and cryptorchidism in Japanese cohorts[98,99] that has now been found to be associated with a decreased incidence of hypospadias in a European cohort[100].

Conclusion

The modest increase in risk for all three end points associated with DES exposure is consistent with a shared etiology and the TDS hypothesis, whereas the results of the subset analyses suggest the existence of yet unidentified sources of heterogeneity between studies or within the study populations. Although 10 years of further research on the potential effects of endocrine disruptors on male reproductive health have provided some clues regarding the etiology and mechanism of conditions such as hypospadias, cryptorchidism, and testicular cancer, there is still no conclusive evidence of the role played by environmental estrogens.

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