

Hypospadias: a transgenerational effect of diethylstilbestrol?

M.M.Brouwers^{1,3}, W.F.J.Feitz², L.A.J.Roelofs², L.A.L.M.Kiemeny^{1,2},
R.P.E.de Gier² and N.Roeleveld¹

¹Department of Epidemiology and Biostatistics (133) and ²Department of Urology (426), Radboud University Nijmegen Medical Centre, P.O.Box 9101, 6500 HB Nijmegen, The Netherlands

³To whom correspondence should be addressed. E-mail: M.Brouwers@epib.umcn.nl

BACKGROUND: In 2002, an increased risk of hypospadias was reported for sons of women exposed to diethylstilbestrol (DES) *in utero*, suggesting transgenerational effects of DES. The aim of this study was to further assess the association between parental DES exposure and hypospadias in a case–referent study. **METHODS:** Cases with hypospadias were retrieved from the hospital information system. Referents were recruited via the parents of cases. Both parents completed postal questionnaires. Associations were estimated by odds ratios (OR) with 95% confidence intervals (CI). Additionally, conditional logistic regression analyses were performed for a matched subset of parents. **RESULTS:** The final database included 583 cases and 251 referents. In the initial analyses, an indication was found for an increased risk of hypospadias when mothers were exposed to DES *in utero*: OR = 2.3 (95% CI 0.7–7.9). Conditional logistic regression resulted in a stronger risk estimate: OR = 4.9 (95% CI 1.1–22.3). Paternal exposure to DES did not increase the risk. **CONCLUSIONS:** The results confirm an increased risk of hypospadias when mothers were exposed to DES *in utero*. However, the excess risk appears to be of much smaller magnitude than in the 2002 study. Further research on the potential health risks for the third generation is of great importance.

Key words: diethylstilbestrol/hypospadias/offspring/transgenerational effects

Introduction

Klip *et al.* (2002) reported an increased risk of hypospadias for sons of women who were exposed to diethylstilbestrol (DES) *in utero*. Hypospadias is a common congenital defect in boys, which involves a dysplasia of the ventral penis and urethra and often requires several operative procedures (Sorber *et al.*, 1997). The disorder affects ~30–40 children out of 10 000 live births, which implies 1 in 150 live-born males (Pierik *et al.*, 2002). The increased prevalence found in offspring of DES daughters was the first suggestion of a transgenerational effect of DES in humans (Klip *et al.*, 2002).

In the years 1938 through 1975, ~220 000 women in The Netherlands and a few million women around the world were prescribed DES during pregnancy in order to prevent a threatening miscarriage or premature birth. Unfortunately, this synthetic estrogen was not only found to be ineffective, but also to have harmful effects. Daughters born out of DES-related pregnancies often show abnormalities of reproductive structures and have elevated risks of vaginal and cervical clear-cell adenocarcinoma, fertility problems, ectopic pregnancies, miscarriages, and premature births. There also appears to be an increased risk of breast cancer for DES mothers and of reproductive tract abnormalities for DES sons (Giusti *et al.*, 1995). Moreover, the question emerged whether the harmful effects of DES may be ‘transmitted’ to subsequent generations. Findings from animal studies suggest that DES causes certain genetic or

epigenetic changes, which may be transmitted to subsequent generations leading to increased susceptibility for tumours of the reproductive tract (Newbold *et al.*, 1998, 2000).

Hypospadias results from an incomplete fusion of the urethral folds between the eighth and 14th weeks of gestation. Male sexual differentiation in general depends on testosterone, dihydrotestosterone, and the expression of androgen receptors by target cells (Manson and Carr, 2003). Disturbances in the balance of this endocrine system by either endogenous or exogenous factors may lead to hypospadias. Well-established is the familial clustering of hypospadias and the association with low birth-weight (Weidner *et al.*, 1999; Hussain *et al.*, 2002; Fredell *et al.*, 2002a,b). Indications for some other risk factors have also been reported (Silver *et al.*, 1999; North and Golding, 2000; Wennerholm *et al.*, 2000; Fisch *et al.*, 2001; Pierik *et al.*, 2004). However, the aetiology of hypospadias remains largely unknown.

Klip *et al.* (2002) estimated the risk of hypospadias in sons of DES daughters in a cohort of women diagnosed with fertility problems. This cohort counted 205 sons of DES daughters, four of whom had hypospadias. In the remaining 8729 boys, only eight cases of hypospadias were reported, resulting in a prevalence ratio of 21.3 (95% CI 6.5–70.1). However, it is questionable whether these results can be extrapolated to the general population. The study exclusively involved women with fertility problems, who reflect a specific subset of DES daughters. A recently published study of registered data from

France confirmed the increased occurrence of hypospadias in sons of DES daughters, but presented a probably more realistic prevalence ratio of 5.0 (95% CI 1.2–16.8) (Pons *et al.*, 2005). In another recent investigation in the USA, an inconclusive prevalence ratio of 1.7 (95% CI 0.4–6.8) was observed (Palmer *et al.*, 2005). All three studies focused on maternal DES exposure only. In contrast, we conducted a population-based case–referent study in order to further evaluate the possible association between hypospadias and intrauterine DES exposure of both parents.

Materials and methods

Data of 937 patients diagnosed with hypospadias and born in The Netherlands between 1987 and 1997 were retrieved from the hospital information system of the Radboud University Nijmegen Medical Centre in The Netherlands. Mainly due to unknown address changes or misdiagnosis, 120 patients were non-eligible for the study. Both parents of the remaining 817 patients were asked to fill out a postal questionnaire addressing various potential risk factors for hypospadias, including DES exposure. In order to recruit the referent population, these parents were asked to give an extra set of questionnaires to the parents of a boy of about the same age as their son. Regarding paternal and maternal exposure to DES *in utero*, response options were ‘yes’, ‘probably’, ‘no’ and ‘unknown’. Parents who listed probable exposure to DES were asked to clarify this. All data were self-reported. Associations were estimated by odds ratios (OR) with 95% confidence intervals (95% CI) and were adjusted for confounding in logistic regression analyses. In addition, conditional logistic regression analyses were performed on the subset of case parents for whom referent parents were also available, to control for potential confounding by unmeasured factors as a result of the referent recruiting strategy. The study was approved by the Regional Committee on Research Involving Human Subjects.

Results

From the 817 eligible patients, the parents of 613 patients completed the questionnaires, resulting in a response rate of 75%. Additionally, completed questionnaires were received from the parents of 264 referents. For 30 cases and 13 referents information could only be obtained from one parent, so they were excluded. The final database included 583 cases and 251 referents. The mean age of cases and referents at time of data collection was 10.2 and 9.5 years respectively. Six mothers of cases reported DES-related pathology and fertility problems, of whom one achieved pregnancy with IVF. Table I presents the intrauterine exposure to DES for the parents of cases and referents. When mothers were probably or certainly exposed to DES *in utero*, their sons appeared to have an increased risk of hypospadias: OR=2.6 (95% CI 0.8–9.0). Educational level was considered to be a confounder, because it differed substantially between case and referent parents and might have been a proxy for other risk factors related to lifestyle or occupation. After adjustment for confounding by educational level, however, an indication for an increased risk of hypospadias remained: OR=2.3 (95% CI 0.7–7.9). Associations were not found between hypospadias and exposure of the father to DES *in utero*: OR=1.1 (95% CI 0.3–4.2). The variation of the potential for DES exposure over time was another possible source of confounding, but adjusting for the parent’s year of birth and additional analyses excluding parents born after 1976

Table I. Parental exposure to diethylstilbestrol (DES) *in utero* and the risk of hypospadias in offspring

	Cases (n = 583) yes/no ^a (%)	Referents (n = 251) yes/no ^a (%)	Odds ratio (95% CI)	Adjusted odds ratio ^b (95% CI)
Mothers probably or certainly exposed to DES <i>in utero</i>	18/565 (3.1)	3/248 (1.2)	2.6 (0.8–9.0)	2.3 (0.7–7.9)
Fathers probably or certainly exposed to DES <i>in utero</i>	8/575 (1.4)	3/248 (1.2)	1.2 (0.3–4.4)	1.1 (0.3–4.2)

^aParents with unknown exposure status (71 mothers and 136 fathers) were considered to be non-exposed.

^bAdjusted for educational level (three levels) of the mother and father respectively.

CI = confidence interval.

did not lead to different findings. Effect-measure modification or confounding by other factors could not be identified.

Because DES exposure was unknown for many parents (71 mothers and 136 fathers), these parents were considered non-exposed. Excluding cases and referents with unknown exposure status from the analyses did not change the effect estimates. The mothers of 11 cases and three referents were certain about their exposure to DES *in utero*. The remaining seven mothers of cases suspected DES exposure, most often because their mothers received an unknown medicine for preventing miscarriage or because they suffer from uterine abnormalities typical for intrauterine DES exposure. Exclusively focusing on mothers who were certain only about their DES exposure weakened the association with hypospadias substantially: OR=1.6 (95% CI 0.4–4.7). The fathers of four cases and two referents were certain about exposure to DES *in utero*, while the fathers of the other four cases and one referent suspected exposure.

On a subset of the parents of 232 cases for whom both referent parents were also available, conditional logistic regression analyses were performed in which the tie between cases and referents was considered a matching variable. As shown in Table II, the association between hypospadias and maternal DES exposure was much stronger in this analysis compared to

Table II. Parental exposure to DES *in utero* and the risk of hypospadias in offspring: conditional logistic regression analyses on a ‘matched’ subset of cases and referents^a

	Cases (n = 232) yes/no ^b (%)	Referents (n = 232) yes/no ^b (%)	Conditional odds ratio (95% CI)	Adjusted odd ratio ^c (95% CI)
Mothers probably or certainly exposed to DES <i>in utero</i>	12/220 (5.2)	3/229 (1.3)	5.5(1.2–24.8)	4.9(1.1–22.3)
Fathers probably or certainly exposed to DES <i>in utero</i>	2/230 (0.9)	2/230 (0.9)	1.0(0.1–7.1)	0.9(0.1–6.7)

^aAnalyses were performed on the subset of case parents for whom referent parents were also available.

^bParents with unknown exposure status (28 mothers and 63 fathers) were considered to be non-exposed.

^cAdjusted for educational level (three levels) of the mother and father respectively.

the initial analyses: OR=4.9 (95% CI 1.1–22.3). The same was observed when the analyses were restricted to mothers certainly exposed to DES: OR=3.0 (95% CI 0.6–14.9). Risk estimates concerning paternal DES exposure did not change.

Discussion

Our findings indicate an increased risk of hypospadias in offspring of women who were exposed to DES *in utero*. However, the excess risks estimated in our population appear to be of much smaller magnitude than in the previous findings in The Netherlands (Klip *et al.*, 2002). This may be explained by a different study design and study population, probably resulting in a more valid risk estimate, that is concordant with recent findings in France (Pons *et al.*, 2005). In the USA, Palmer *et al.* (2005) found no evidence for an increased risk of hypospadias in a combination of existing cohorts of DES daughters and their referents, but the broad confidence interval is compatible with our risk estimate. In both the US study and the previous Dutch study, the occurrence of hypospadias in offspring was self-reported and assessed in a manner that raises concern about underreporting. In contrast, Pons *et al.* (2005) studied the prevalence of hypospadias in children who were born in a hospital in France and their data collection showed many similarities with ours. Mothers who gave birth were systematically asked about their intrauterine DES exposure and diagnoses of hypospadias were confirmed by medical files. The fact that their risk estimate [prevalence ratio 5.0 (95% CI 1.2–16.8)] is concordant with ours adds support to the validity and reliability of the results in the current study.

However, the self-reported nature of DES exposure may have biased these results. Both case and referent parents may not have been aware of their mother's use of DES during pregnancy, possibly leading to non-differential misclassification of exposure status and underestimation of the effect of DES. Since the link between DES and hypospadias in grandchildren is not very obvious and certainly not common knowledge, it seems less likely that the effect estimates were overestimated due to recall bias, although this cannot be ruled out either. A strong argument against recall bias, however, is that the ORs for fathers centre around unity.

As mentioned before, Klip *et al.* assessed DES exposure in a cohort of women with fertility problems [prevalence ratio 21.3 (95% CI 6.5–70.1)]. Reduced fertility and infertility treatment may be intermediate factors in the causal pathway in which DES increases the risk of a son with hypospadias (Silver *et al.*, 1999; Wennerholm *et al.*, 2000). These women may therefore not reflect DES daughters in general, unlike the DES-exposed mothers in our study. Furthermore, the low prevalence of hypospadias in the control group is reason for concern that selective underreporting affected the risk estimated by Klip *et al.* (Hernandez-Diaz, 2002). The authors comment that mild cases were less well represented in both groups (Klip *et al.*, 2002), but this does not mean that mild cases were in fact underreported in both groups. Possibly, DES or DES-related causal factors lead to a more severe manifestation of hypospadias, so that mild cases are not to be expected in the exposed group.

Due to the referent recruitment strategy in our study, data were collected for a referent boy for less than half of the cases only. This resulted in a non-random sample of referents in which the referent parents on average seemed to be more highly educated than the parents in the case population. That is why we performed additional analyses focusing only on the cases for whom referent parents were also available. In this 'matched' subset, the association between maternal exposure to DES and hypospadias appeared to be stronger. We feel that this risk estimate (OR=4.9; 95% CI 1.1–22.3) is probably more valid than the estimate based on the total study population. Nevertheless, it remains uncertain whether the referents were an accurate reflection of the source population of the cases and to what extent this influenced the results.

The mechanism through which DES could cause adverse effects in the third generation is unclear. According to our findings, DES sons are unlikely to 'transmit' a predisposition to hypospadias to their sons. DES-related pathology of reproductive structures in DES daughters, however, may interfere with normal fetal development during pregnancy. Additionally, DES daughters could suffer from a disturbed hormonal balance during adult life. A third suggestion is that certain genetic or epigenetic defects are transmitted, which increases the risk of hypospadias for their sons (Klip *et al.*, 2002). The latter hypothesis is supported by findings from animal studies and implies that other health risks may also be expected in the third generation (Newbold *et al.*, 1998, 2000). Blatt *et al.* (2003) described a 15 year old girl with small cell ovarian carcinoma whose mother was exposed to DES *in utero* (Blatt *et al.*, 2003). Although it concerned only one case, this finding seems to be as alarming as the independent findings on hypospadias in this study and the previous studies. Further research, with a more thorough assessment of both exposure and outcome, should clarify the ability of DES to affect grandchildren, as it is of great importance to identify their potential health risks at present as well as in the future.

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