Long-term health effects after DES exposure in utero

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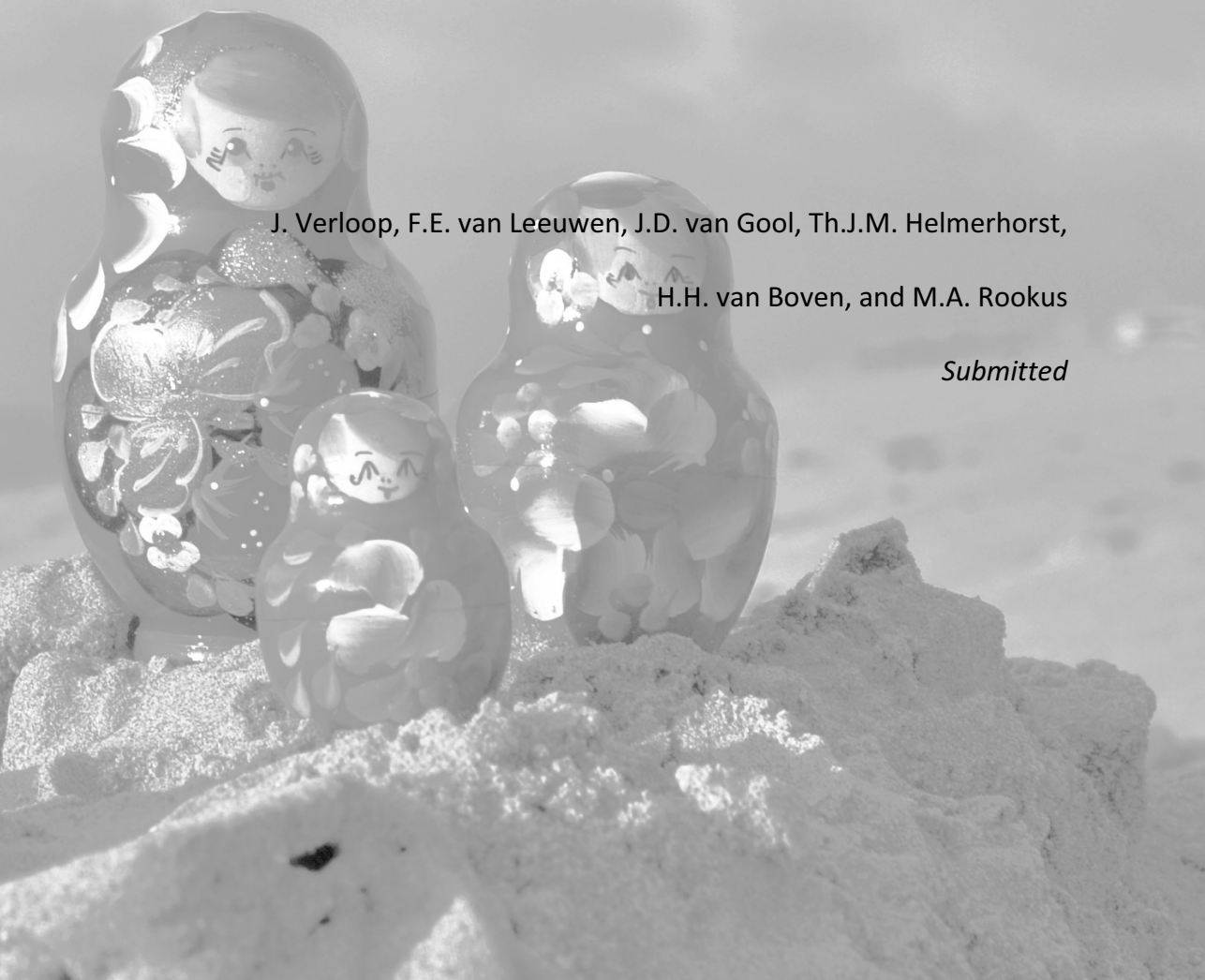
Chapter 5.2

Risk of hypospadias among DES grandchildren



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Submitted

5.2.1 Abstract

Purpose

There has been increasing interest in potential transgenerational adverse health effects of diethylstilbestrol (DES).

Methods

We studied prevalence of hypospadias and congenital urinary anomalies in 7,899 children born to DES daughters compared to 3,099 children born to unexposed sisters, and compared to reference rates of the European Registration of Congenital Anomalies (EUROCAT) and rates of a study examining prevalence of hypospadias among male newborns in Rotterdam. We calculated prevalence ratios (PRs) and 95% confidence intervals (95%CIs) based on a binomial distribution.

Results

Thirty-two cases of medically verified hypospadias were observed among sons of DES daughters, compared to three cases among sons of unexposed sisters (PR=4.2, 95%CI=1.3-13.7). The PR of mild hypospadias (coronary/glanular) was increased compared with EUROCAT (PR=3.9, 95%CI=2.4-6.4), but not compared with the internal control group (PR=2.2, 95%CI=0.7-7.6) or the Rotterdam study (PR=1.1, 95%CI=0.6-2.1). The PR of penoscrotal hypospadias was increased versus all reference groups (PR=7.9, 95%CI=3.3-18.8 and PR=5.3, 95%CI=1.1-26.5 for EUROCAT and the Rotterdam study, respectively). Penoscrotal hypospadias was not reported in the sons of unexposed sisters of DES daughters. Three of six cases of penoscrotal hypospadias were born to DES daughters with uterine cavum malformations. No increased risk of urinary malformations among children of DES daughters was observed.

Conclusion

In conclusion, the risk of penoscrotal hypospadias among sons of DES daughters appears to be increased compared to unexposed sons. Biological mechanisms are still unclear, although congenital uterine cavum constrictions in DES daughters might play a role.

5.2.2 Introduction

Diethylstilbestrol (DES) is a synthetic estrogen that was prescribed during the late 1940s - early 1970s to more than 5 million pregnant women worldwide, in order to prevent miscarriage. Several adverse health effects, including vaginal cancer and reproductive abnormalities, have been observed in prenatally exposed women[1-3]. In men, elevated risks of cryptorchidism, hypoplastic testes, and cysts of the epididymis have been reported[4]. There are no indications that the risk of hypospadias in DES sons (second generation) is increased[5;6], and it is unclear whether the risk of or testicular cancer in DES sons is elevated[7]. In the last decade, there has been increasing interest in the possible transgenerational adverse health effects after DES exposure *in utero*. Experimental studies have shown elevated risks of tumors of the reproductive tract in male and female descendants (third generation) of mice exposed to DES[8-11]. The mouse model, which replicates many DES outcomes observed in humans, suggests that DES causes epigenetic alterations and/or altered gene expression of specific estrogen-responsive genes, which are transmissible to male and female offspring of prenatally exposed female animals[10]. To date, only few studies have examined transgenerational adverse effects of DES in humans. A higher risk of hypospadias among DES grandsons has been suggested[12-15], but one study with the largest number of verified DES-exposed grandsons found no effect (4/1000 observed, 2.2/1000 expected; OR=1.7, 95%CI=0.4-6.8)[16]. Hypospadias is a congenital disorder characterized by an abnormal location of the urethral opening along the ventral surface of the penis. Risk factors for hypospadias are largely unknown, but its etiology is believed to be multifactorial[17].

In this study we evaluate the risk of medically verified urogenital anomalies and congenital heart disease in children in a large cohort of DES daughters (DES-net), as compared with children born to their non-exposed sisters, as well as with two external registries of congenital anomalies.

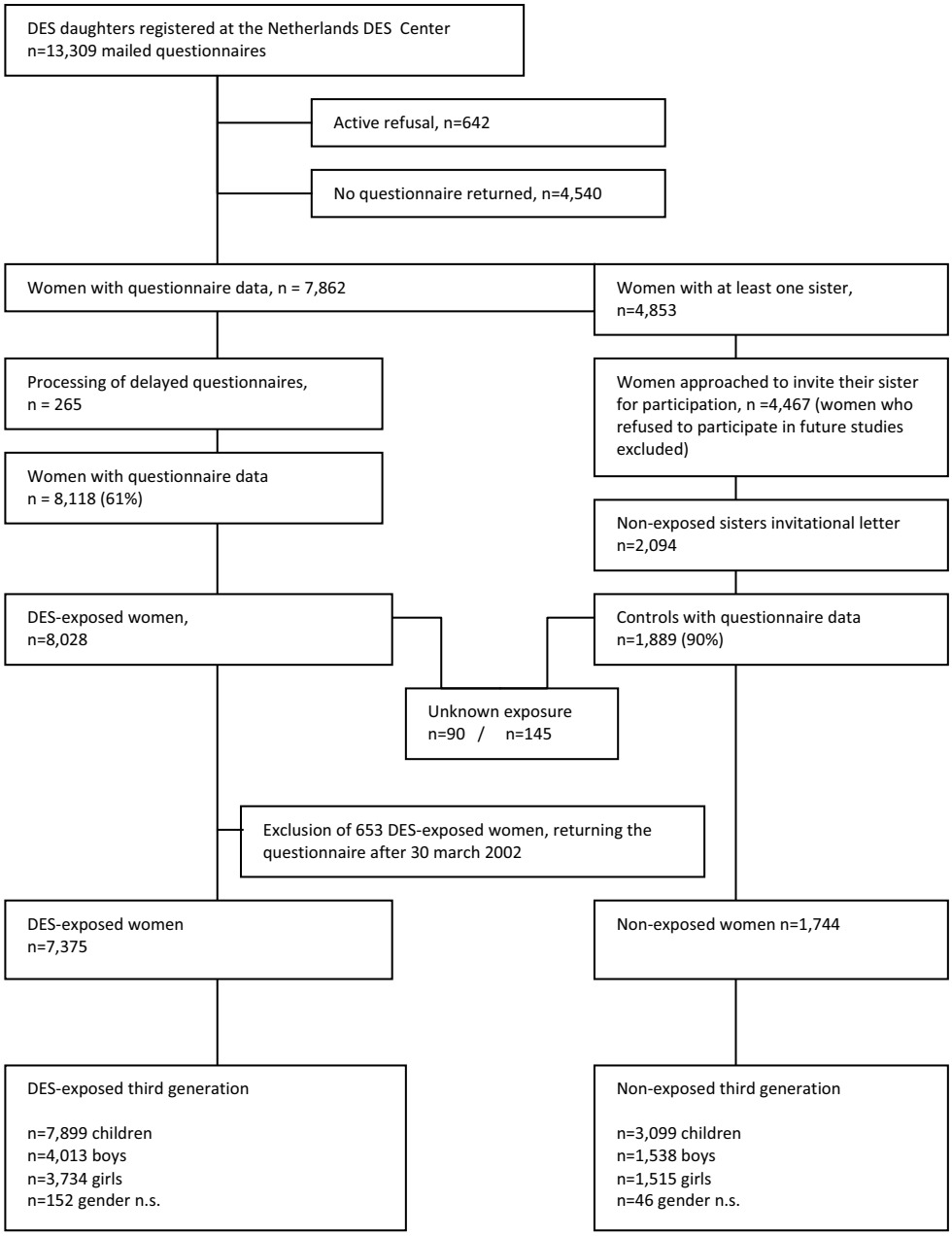

Figure 1 Third generation cohort of DES-net study

5.2.3 Methods

Study population

The DES-net project is a nationwide retrospective cohort study with prospective follow-up of 13,309 DES daughters in the Netherlands and their 1,889 non-exposed sisters (Figure 1). The study was designed to investigate adverse health effects in women exposed to DES *in utero*. All DES daughters were recruited through the registry of the Netherlands DES Center. The study design has been described in more detail elsewhere [18]. In short, the DES Center registry was established in 1992, in order to deal with future health claims. Between 2000 and 2005, 13,309 registered women were sent a 16-page questionnaire to collect data on DES daughters' own medical and reproductive history. Information was also obtained on the number of sisters and brothers and their exposure to DES. Furthermore, information was collected on children's birth weight, duration of gestation, the presence of congenital diseases and whether surgery for such diseases had taken place. In addition to the questionnaire, women were asked to provide a copy of the medical file of their mothers in which DES exposure *in utero* was confirmed. After two reminders the final response rate to the questionnaire among DES-exposed women was 61% (Figure 1). A study among non-responders showed that uncertainty about DES exposure was the main reason for non-response [18]. In November 2004, we asked 4,467 DES daughters who reported one or more sisters in the questionnaire and who consented to participate in future studies to invite their non-exposed sisters to participate in our study. Based on self-reported information in the questionnaire, we estimated that the number of eligible non-exposed sisters was between 4,199 and 5,262 (the maximum estimate included 264 non-exposed relatives with unknown gender, 678 sisters with unknown exposure and 121 relatives with unknown gender and exposure). A total of 1,905 DES daughters sent back a reply form with the addresses of one or more sisters (in total 2,094 non-exposed sisters). Subsequently, we sent an invitational letter to these sisters accompanied by a questionnaire and a leaflet, of whom 1,889 (90% of those invited, ~40% of all sisters) responded by returning the questionnaire.

Because of possible selection bias, we excluded 653 DES daughters who returned the questionnaire after March 2002, because around that date the first paper on a positive association between DES exposure and hypospadias in grandsons was published [12]. These 653

daughters reported 4 sons with glandular and 4 sons with penile hypospadias. Furthermore, 145 sisters with doubts about DES exposure were excluded from the analyses (Figure 1).

For the purpose of the present research question, the study population consisted of all liveborn offspring of DES daughters and the offspring of unexposed sisters of DES daughters. A total of 10,998 liveborn children were included of whom 7,899 were born to DES daughters and 3,099 to unexposed sisters. The study was approved by the Institutional Review Board of the Netherlands Cancer Institute.

Verification of DES exposure

Since the tracing of mothers' medical records was extremely difficult, documented DES exposure was available for only 10% of the subjects. In a validation study in a subgroup of participants (n=115), self-reported DES exposure was verified by medical records from four hospitals which had kept all medical records in their archives. DES exposure was confirmed for 76% of the women. In 3%, a medication other than DES was recorded in mothers' hospital medical file and in 21% no proof of DES prescription or another medication was found, which still left open the possibility that DES mothers had received DES through their general practitioner, which often occurred in the Netherlands. Since the agreement between self-report and verified DES exposure was good we included all women, irrespective of DES exposure validation, in the analyses.

Table 1 Main characteristics the external reference data on congenital malformations used in this study

Reference group	EUROCAT (1)	Rotterdam study (2)
Coverage	Approximately 80% (parents have to give informed consent for inclusion of the child in the registry)	95% of all newborns in Rotterdam
Region	Northern Netherlands (Groningen, Drente, Friesland), accounting for 10% of all births in the Netherlands	Rotterdam
Case ascertainment	Notification by midwives, general practitioners, well baby clinic doctors and other treating physicians on voluntary basis. Active search for cases by registry personnel (obstetric records, hospital administration data, pathology records) and case lists by the regional pediatric cardiology center (University Medical Center Groningen)	Child Health Care Centres (CHC) received notification of all newborns registered in the Municipal Birth register of Rotterdam. Parents were invited to visit the CHC for examination of their child
Inclusion	Live births	Live births
Time window	No lower limit –age 16	Birth – 6 months after birth
Detailed information	**Hypospadias**: subtypes of hypospadias available since 1992 (incomplete coverage for glandular hypospadias). Complete coverage glandular hypospadias since 2005	**Hypospadias**: subtypes available **Heart anomalies**: not available **Urinary anomalies**: not available
Period in analysis	1981-2004	1998-2000

(1) Greenlees R *et a*l. Birth Defects Res A Clin Mol Teratol 2011 March;91 Suppl 1:S51-S100.
(2) Pierik FH *et al*. Hum Reprod 2002 April;17(4):1112-5.

Assessment and medical verification of congenital disorders

Mothers of liveborn children with congenital heart malformations or malformations of the urogenital system (cryptorchidism excluded) were asked by the researchers for permission to retrieve their child's medical file, in order to verify the reported malformation. In case a child had already reached the age of 18, informed consent from the child itself was asked. After 4 weeks, all women who did not return the informed consent form were sent a reminder; non-responders to the reminder were approached by telephone. Subsequently, pediatricians were asked to confirm the diagnosis, and to provide us with information on the severity of the disorder and applied surgical procedures, if any.

External reference data

For the external comparison we used two different reference populations (table 1). The first concerned the EUROCAT registry, which is part of the European network for the registration of congenital malformations. This registry covers the northern part of the Netherlands. Anomalies are reported on a voluntary basis by midwives, general practitioners, well-baby clinic physicians, and other treating physicians. Additionally, written consent of all involved parents is needed before the children's data can be entered into the registry. Based on the parental response, the completeness of the EUROCAT registry is estimated at 80%[19]. Prevalence was calculated as the number of liveborn malformed children per 10,000 live births, without a lower limit for gestational age or an upper age limit.

As a second source of external reference rates we used the data from the Rotterdam study, a Dutch cohort study among 7,292 newborn boys in Rotterdam, who were medically examined for the presence of hypospadias within 6 months after birth[20]. Prevalence was estimated as number of congenital anomalies per 10,000 live births.

Statistical analyses

We calculated prevalence ratios (PRs) with 95% confidence intervals (95%CIs) of the selected congenital anomalies, based on an exact binomial distribution.

We used logistic regression analyses to estimate odds ratios (OR) and 95% confidence intervals (CI) for the risk of hypospadias associated with DES use of the grandmother, adjusted for confounders (internal comparison only). Birth weight and duration of gestation were identified as confounders (more than 10% change of the ORs) and included in the multivariate model.

Table 2 Maternal and child characteristics of all DES-exposed and non-exposed study participants and their offspring

Characteristic	DES-exposed N	%	Unexposed N	%	Chi-square p-value
Women	7,375		1,744		
Women, no children	3,204	43%	372	21%	<0.001
Women, with children	4,171	57%	1,372	79%	
Age at date questionnaire	37.9	±5.9	44.0	± 8.0	
Number of children					0.33
1 child	1,393	33%	201	15%	
2 children	1,975	47%	734	53%	
>2 children	775	19%	431	31%	
Unknown	28	1%	6	0%	
Diagnosis of subfertility					<0.001
Yes	1,038	25%	140	10%	
No	3,072	74%	1,217	89%	
Unknown	61	1%	15	1%	
Cause of subfertility					
Tubal adhesion or occlusion	203	5%	17	1%	<0.001
Malformations uterus	421	10%	10	1%	<0.001
Cervical problems	155	4%	1	0%	<0.001
No or impaired ovulation	280	7%	39	3%	<0.001
Hormonal problems	136	3%	19	1%	<0.001
Endometriosis	158	4%	23	2%	<0.001
Male factor	141	3%	37	3%	<0.002
Other	313	8%	35	3%	<0.001
Unknown	175	4%	23	2%	<0.001
Adverse pregnancy outcome					<0.001
No	2,324	56%	940	69%	
Yes	1,745	42%	404	29%	
Unknown	102	2%	28	2%	
IVF treatment					<0.001
No	3,920	94%	1,330	97%	
Yes	203	5%	26	2%	
Unknown	48	1%	16	1%	
Confirmation of DES exposure			n.a.		
Medical file of the mother	434	10%			
DES-related malformation	877	21%			
Participated in DES surveillance	1,023	25%			
No confirmation	1,837	44%			
Self-reported certainty about DES exposure			n.a.		
Certain	3,095	74%			
Probable	1,076	26%			

Table 2 continued

Characteristic	DES-exposed N	%	Unexposed N	%	Chi-square p-value
Medically verified DES-related malformations					
No DES-related malformations	2,973	71%			
Epithelial changes (VEC)	349	8%			
Anatomical malformations	285	7%			
DES-malformations n.s.	112	3%			
Epithelial and anatomical malformations	364	9%			
Unknown	88	2%			
Number of unexposed sisters					
0	3,999	96%	767		
1	579	14%	409		
2 or more	193	5%	196		
Offspring	7,899		3,099		
Gender					
Male	4,013	51%	1538	50%	
Female	3,734	47%	1515	49%	
Unspecified	152	2%	46	1%	
Year of birth					<0.001
1961-1970	43	1%	119	4%	
1971-1980	652	8%	536	17%	
1981-1990	2,682	34%	960	31%	
1991-2000	4,405	56%	1,088	35%	
2001-2005	15	0%	372	12%	
unknown	102	1%	24	1%	
Maternal age (years)					
average	29.0	(±4.1)	29.1	(±4.4)	0.50
≤24	1,054	13%	481	16%	<0.001
25-29	3,256	41%	1,205	39%	
30-34	2,738	35%	1,034	33%	
≥35	734	9%	351	11%	
missing	117	1%	28	1%	
Number of siblings					
0	1,420	18%	206	7%	<0.001
1	3,934	50%	1,464	47%	
2	1,884	24%	954	31%	
3	520	7%	360	12%	
4	135	2%	115	4%	
5	6	0%	0	0%	

Table 2 continued

Characteristic	DES-exposed N	%	Unexposed N	%	Chi-square p-value
Duration of gestation (weeks)					
average	38.0	(±3.3)	39.4	(±2.0)	<0.001
24-32	567	7%	37	1%	<0.001
33-36	1,184	15%	170	5%	
37-38	1,864	24%	641	21%	
39-40	2,548	32%	1,360	44%	
≥41	1,373	17%	783	25%	
<24	46	1%	3	0%	
missing	317	4%	105	3%	
Birth weight (g)					
average	3,118	(±753)	3,422	(±585)	<0.001
1000-1499	192	2%	14	0%	<0.001
1500-2499	1,003	13%	122	4%	
2500-3499	3,432	43%	1,294	42%	
3500-3999	1,734	22%	985	32%	
≥4000	761	10%	480	15%	
<1000	136	2%	18	1%	
missing	641	8%	186	6%	
Medically verified DES-related malformations					
No DES-related malformations	5,711	72%			
Epithelial changes (VEC)	655	8%			
Anatomical malformations	506	6%			
DES-malformations n.s.	207	3%			
Epithelial and anatomical malformations	201	3%			
Unknown	619	8%			

The addition of other potential confounders such as maternal age, subfertility treatment with in vitro fertilization (IVF), treatment with Clomiphene, subfertility cause, and educational level, did not change the risk estimates and were therefore not included in the model. Furthermore we conducted a subgroup analysis among subfertile women, since our earlier results showing an increased risk of hypospadias among sons born to DES daughters were obtained in a cohort of subfertile women[12]. All analyses were conducted with Stata SE10.

5.2.4 Results

We included 10,998 offspring of 7,375 DES daughters and 1,744 non-exposed sisters in the study (Table 2, Figure 1). DES daughters were more often nulliparous and had fewer liveborn children compared to their sisters. Similar differences were found when we restricted the analysis to women above childbearing age (> age 45) at time of responding to the questionnaire (in this group 32% of the DES-exposed women was nulliparous compared to 14% among non-exposed women). In line with this finding, DES daughters also reported more adverse pregnancy outcomes, like miscarriages and stillbirths, than their non-exposed sisters. In addition, a subfertility diagnosis was more often reported by DES daughters compared to controls, with malformations of the uterus and ovulation disorders being the most frequently reported reasons of subfertility. Five percent of the DES-exposed women reported to have received IVF treatment, compared to one percent of the controls. Twenty-seven percent of our DES-exposed participants had DES-related malformations, like adenosis and structural abnormalities.

Offspring of DES daughters and their unexposed sisters included 5,551 boys, 5,249 girls and 198 children with unspecified gender. Children of unexposed women were more often born after 2000, due to the later recruitment of the unexposed sisters of DES daughters into the study. Maternal age did not differ between exposed and non-exposed DES grandchildren (mean maternal age 29.0 and 29.1 years for exposed and non-exposed children, respectively). Mean duration of gestation was slightly shorter for DES-exposed grandchildren than for non-exposed children (38.0 and 39.4 weeks respectively, results not shown). Birth weight was also lower for children born to DES daughters compared to children of their non-exposed sisters (mean birth weights of 3,118 grams and 3,422 grams, respectively).

Table 3 describes the number of reported and verified congenital malformations among children born to DES daughters, compared to non-exposed children. In total, 256 anomalies of the urogenital system and heart were reported. Information on reported surgery was used as an indication of the severity of the disease, assuming that reporting bias is less likely for more severe diseases. For 76 reported malformations (of which 33% were reported to have been treated with surgery) no informed consent was obtained to retrieve the medical file, among which were 5 cases of hypospadias (3 treated with surgery). Of the 43 reported children with congenital heart diseases without permission to retrieve the medical file, 30% were

Table 3 Number of reported and verfied congenital diseases in offspring DES-net cohort

Exposure	Congenital disease (ICD-10 code)	Self-report		No consent to retrieve medical file	No response physician	No medical file available	Medical file available			% verified
		n	% treated with surgery				Not verified	Verified	% of verified with surgery	
Non-exposed	Genital anomalies* Q54	5	60%	1	0	0	1	3	100%	60%
	Urinary anomalies† Q60-Q64	20	50%	8	1	1	3	7	57%	35%
	Heart anomalies‡ Q20-Q28	22	36%	6	2	4	0	10	40%	45%
DES-exposed	Genital anomalies* Q54	50	62%	4	0	2	12	32	72%	64%
	Urinary tract anomalies† Q60-Q64	46	33%	14	2	4	3	23	39%	50%
	Heart anomalies‡ Q20-Q28	110	34%	37	2	8	6	57	53%	52%

* Genital anomalies included hypospadias

† Urinary tract anomalies included: Renal agenesis, unilateral renal, cystic kidney (Potter type II/IV, simple renal cyst), hydronephrosis, stenosis ureter/uteropelvic junction, megaureter hydroureter, vesicoureteral reflux, atresia of urethra and bladder neck, malformation urethral valves, accessory kidney

‡ Heart anomalies included transposition great arteries,ventricular septum defect,atrium septum defect, ostium secundum, atrium septum defect, ostium primum, tetralogy fallot, pulmonary valve anomaly, tricuspid atresia/stenosis, aortic valve stenosis, bicuspidale aortic valve, patent ductus arteriosus, coarctation aortae, aorta stenosis

treated with surgery according to the mothers' report, whereas of the 22 reported urinary tract anomalies without consent for verification, nine cases (41%) were reported to have been treated with surgery. Regarding the unverified hypospadias cases, the true diagnosis appeared to be meatal stenosis (n=3), a short frenulum (n=2), a penile malformations (n=2), or no notification of any urogenital malformation in the medical file (n=5). The true diagnoses of the six medically unverified heart disease cases appeared to be heart murmurs. With respect to the five unverified urinary anomalies, these children appeared to suffer from urinary complaints, without obvious congenital malformations.

In table 4 the prevalence of medically-verified malformations among children of DES daughters is compared with the prevalence among the children of their non-exposed sisters and with the two external reference groups. The risk of hypospadias (all types) was statistically significantly increased compared to both the internal reference group of children born to unexposed sisters of DES daughters and EUROCAT, but not compared to the prevalence in the Rotterdam study[20]. The prevalence of penoscrotal hypospadias (the most severe type of hypospadias) was increased compared to all reference groups. Compared to EUROCAT and the Rotterdam study the PRs were 7.9, 95%CI=3.3-18.8 and PR=5.3, 95%CI=1.1-26.5, respectively, while the PR compared to the internal group was infinite due to zero observed cases in the control group. For urinary anomalies, no increased risk was found compared to the internal control group, and a slightly (borderline significant) increased risk was found compared to EUROCAT (PR=1.6, 95%=1.0-2.4). The prevalence of heart anomalies was borderline statistically significantly increased compared to the internal reference group (PR=2.0, 95%CI=1.0-4.0), but not increased compared to EUROCAT (PR=1.1, 95%CI=0.8-1.5). The prevalence of ventricular septum defect (VSD), one of the most frequently occurring clearly defined cardiac anomaly, was not increased among children born to DES daughters compared to the internal reference group and EUROCAT.

Stratified analyses on the presence or absence of maternal subfertility problems revealed that five out of six cases with penoscrotal hypospadias occurred in sons of DES daughters with subfertility problems (PR=19.0, 95%CI=3.7-98.1 and PR=1.2, 95%CI=0.1-13.1) for DES daughters without subfertility problems compared to the entire Rotterdam cohort, whereas the PRs for children with milder types of hypospadias were similar between women with and without subfertility problems (PR glanular hypospadias 0.8, 95%CI=0.3-2.8 and 1.2 95%CI=0.6-2.3 compared to the Rotterdam study, respectively).

Table 4 Prevalence ratios (PR) of **medically verified** congenital diseases among children born to DES daughters compared to children born to unexposed sisters (internal comparison group) and two external reference populations

Cohort		**DES-net, exposed**		**DES-net, unexposed**				**EUROCAT (1)**				**Rotterdam study (2)**			
Period		1960-2004		1960-2004				1981-2004				1998-2000			
Total live births		7,899		3,099				386,371				14,075			
ICD-10	**Congenital disease**	**N**	**Prev***	**N**	**Prev**	**PR***	**95%CI**	**N**	**Prev**	**PR**	**95%CI**	**N**	**Prev**	**PR**	**95%CI**
Q54	Hypospadias	32	40.5	3	9.7	4.2	1.3-13.7	482	12.5	3.2	2.3-4.6	53	37.7	1.1	0.7-1.7
Q54.0	Coronary and glanular	17	21.5	3	9.7	2.2	0.7-7.6	213	5.5	3.9	2.4-6.4	27	19.2	1.1	0.6-2.1
Q54.1	Penile	9	11.4	0	0.0			162	4.2	2.7	1.4-5.3	19	13.5	0.8	0.4-1.9
Q54.2	Penoscrotal	6	7.6	0	0.0			37	1.0	7.9	3.3-18.8	2	1.4	5.3	1.1-26.5
Q60-Q64	Urinary tract anomaly	23	29.1	7	22.6	1.3	0.6-3.0	724	18.7	1.6	1.0-2.4			n.a.†	
Q60.0.Q60.2	Renal agenesis	2	2.5	0	0.0			168	4.3	0.6	0.1-2.3			n.a.	
Q61. Q60.6	Cystic kidney	5	6.3	0	0.0			161	4.2	1.5	0.6-3.7			n.a.	
Q62.7	Vesicoureteral reflux	7	8.9	4	12.9	0.7	0.2-2.3	79	2.0	4.3	2.0-9.4			n.a.	
Q20-Q28	Heart anomaly	52	65.8	10	32.3	2.0	1.0-4.0	2,303	59.6	1.1	0.8-1.5			n.a.	
Q21.0	Ventricular septum defect	17	21.5	5	16.1	1.3	0.5-3.6	998	25.8	0.8	0.5-1.3			n.a.	

* Prev=prevalence, number per 10,000 live born children, PR=prevalence ratio, 95%CI=95% confidence interval based on a exact binomial distribution

† n.a.=not assessed

(1) Greenlees R *et a*l. Birth Defects Res A Clin Mol Teratol 2011 March;91 Suppl 1:S51-S100.

(2) Pierik FH *et al*. Hum Reprod 2002 April;17(4):1112-5.

Characteristics of all six cases with penoscrotal hypospadias are described in table 5. A striking finding was the high proportion of uterine cavum malformations reported as cause of subfertility in the mothers of these cases (n=3, 50%). In the overall group of sons of DES daughters this proportion was 10% (table 1).

5.2.5 Discussion

In the largest study examining the prevalence of hypospadias in DES grandchildren so far, we observed an increased risk of penoscrotal hypospadias in sons born to DES daughters based on comparisons with each of the three different comparison groups. Regarding the risk of milder types of hypospadias, an increased risk was found compared to EUROCAT, but not compared to the internal reference group and the Rotterdam study. No increased risk of urinary anomalies was observed among sons born to DES daughters, compared to the internal and external reference groups. A slightly increased risk of congenital heart anomalies was found among children born to DES daughters compared to the children born to their unexposed sisters, but there was no increased risk compared to EUROCAT.

We confirmed an overall increased risk for hypospadias among sons born to DES daughters as found in other studies [12-15], largely determined by the increased risk observed for penoscrotal hypospadias. Two out of five other studies reported on the severity of hypospadias [12;14], but did not use severity for risk estimation. In the NCI DES combined follow-up study in the United States, no significantly increased risk of hypospadias was observed (OR=1.7, 95%C 0.4-6.8) [16]. The published studies are quite different with respect to the number of sons born to DES daughters included, varying from 21 [15] to 2,522 [16]. In all studies, except the NCI DES follow-up study [16], DES exposure was based on self-report, and partially on DES daughters' own medical fertility record [12]. In the NCI DES follow-up study, documented DES exposure was available for all study subjects. Although in our study documented DES exposure was available for only 10% of the women, we demonstrated by a validation study (see methods) that self-reported DES exposure could be confirmed in 76% of the participants. Since the prevalence of women with DES-related malformations in our study was quite similar to the prevalence in the DESAD cohort (36%, of which was 28% were medically verified, and 45%[21], respectively) this

Table 5 Characteristics of six boys with penoscrotal hypospadias (all born to DES daughters)

Case	Diagnosis subfertility	DES-related malformations	SGA ‡	Ever IVF*	Ever KI*	Ever Clomiphene*	Klip et al†	Birth weight (grams)	Duration of gestation (weeks)	Toxemia	Maternal Age	Relatives with hypospadias	Medical indication description DES
1	No subfertility	No	No	No	No	No	No	3,620	41	No	29	No	Habitual abortion
2	Cervical malformation, tubal adhesion/occlusion, malformation uterus	Malformation uterine cavum	No	Yes	No	No	Yes	2,980	38	No	35	No	Threatened abortion/ habitual abortion
3	Subfertile with unknown cause	No	Yes	Yes	Yes	No	Yes	2,430	39	Yes	39	No	Threatened abortion/ habitual abortion
4	Subfertile with unknown cause	Squamous cell metaplasia/ cervical ridges/ malformation uterine cavum	Yes	No	Yes	No	No	1,042	33	No	36	No	Habitual abortion
5	Tubal adhesion/ occlusion, surgery ovaries, but no treatment	No	No	No	No	No	No	6,170	40	No	19	No	Threatened abortion
6	Malformation uterus	Uterine cavum malformation reported but no verification procedure	Yes	Yes	Yes	No	No	700	28	No	36	No	Threatened abortion/ habitual abortion

* IVF=in vitro fertilization, KI=insemination. Treatment not necessarily during this pregnancy

† Reference: Klip *et al*. Lancet 2002 March 30;359(9312):1102-7

‡ SGA=small for gestational age, defined as weighing less than the tenth percentile of birth weight data (reference: Alexander GR *et al*. Obstet Gynecol 1996 February;87(2):163-8)

further confirms the assumption that the majority of women in our study was truly exposed to DES *in utero*.

Many environmental and genetic factors have been examined in relation to hypospadias, but findings are inconclusive and the etiology of hypospadias remains largely unclear[22;23]. Hypospadias has been associated with maternal pregnancy-related problems, such as low birth weight, intrauterine growth retardation and placental abnormalities, maternal hypertension and pre-eclampsia[22-24]. In this regard, a remarkable finding in our study was the relatively high proportion of uterine cavum malformations (50%) reported as the cause of subfertility among mothers of sons with penoscrotal hypospadias, which, though based on small numbers, was considerably higher than the proportion of uterine malformations reported in the total group (9% and 1% in DES-exposed and unexposed grandchildren, respectively). Similarly, Kalfa *et al*, observed maternal uterine cavum malformations in two out of eight DES grandsons with hypospadias (five of which were of the severe type)[14]. Malformations of the uterine cavum might be important predictors for the receptivity of embryo implantation[25]. In addition, limited uterine space may result in placental insufficiency and early-onset intrauterine growth retardation of the fetus[26], while both conditions have also been associated with an increased risk of hypospadias[27;28]. Interestingly, the study by Yinon *et al* also found a correlation between the early onset and severity of placental dysfunction and severe hypospadias[27]. In another study by Lang *et al,* maternal intra-uterine exposure to DES appeared to be a risk factor for having a child that is small for gestational age (SGA) (OR=1.8, 95%CI=1.2-2.8)[29]. In the same study slightly (not statistically significantly) increased risks for SGA were found for women with an incompetent cervix (OR=1.3, 95%CI=0.8-2.3) or uterine anomaly (OR=1.6, 95%CI=0.6-4.2). Other factors that have been associated with hypospadias are maternal or paternal exposure to environmental chemicals (like pesticides, polychlorinated biphenyls and dichlorodiphenyltrichloroethane), dietary components (like phytoestrogens), exposure to exogenous gonadal hormones (as part of infertility treatment or pregnancy complications) and exposure to other medications (like valproic acid or corticosteroids)[22]. Also, inheritance might play a role and many genes might be involved. Candidate genes for hypospadias that have been examined in humans are homeobox genes (involved in the patterning of the embryo and of specific organ systems [30], genes coding for enzymes needed for biotransformation of sex hormones (like the gene coding for 5a-reductase type II which

converts testosterone to dehydroxytestosterone), or genes coding for hormone receptors (like the androgen receptor), reviewed in [22].

The biological mechanisms underlying a third generation effect of DES are still unclear. Several mechanisms have been postulated, especially changes to the germ cells or epi-genetic effects[10]. Another explanation might be a hormonal dysbalance or placental malfunction in DES daughters which might interfere with the development of the male urinary tract[14]. Interestingly, elevated risk of hypospadias has only been observed through maternal lineage, not through paternal lineage, although the numbers of children examined were very small[14]. An effect through maternal placental malfunction seems a likely explanation given our findings, but epigenetic effects, such as found in mice, cannot be excluded[10].

Additional to the risk of hypospadias, we examined whether the risk of *urinary anomalies* in sons born to DES daughters was increased. From mice studies it is known that intrauterine exposure to DES interferes with the normal differentiation of the urethra, testicles and epididymis from the Wolffian ducts and regression of the Müllerian duct[10]. If, for instance, epigenetic effects might result in similar developmental effects in the third generation, one might expect higher prevalence of urinary anomalies among offspring of DES-exposed women. To our knowledge, an increased risk of urinary tract anomalies in children born to DES daughters has not been reported. Titus-Ernstoff *et al*. examined birth defects in the third generation of the NCI-DES follow-up study, but urinary tract anomalies were not reported as a separate category[31]. We found no increased risk of urinary tract anomalies among children born to DES daughters in our study.

We aimed to use *heart anomalies* in our study as a marker to track possible reporting bias as it seems unlikely that hormonal risk factors play an important role in their origin (reviewed in [32]). In the NCI-DES follow-up study an increased risk of heart anomalies among daughters born to DES daughters was also attributed to underreporting of controls (OR=4.56. 95%CI=1.27-16.3)[31]. We found a borderline significantly increased risk in our study of congenital heart disease (CHD) among children (both genders) born to DES daughters compared to the offspring of the unexposed sisters (PR=2.0, 95%CI=1.0-4.0), but not compared to EUROCAT. Noteworthy, VSD, which was most clearly defined, was not increased. This supported our impression that reporting bias might occur for more general categories of diseases and may be to a lesser degree for more specific and/or severe types of disease. Equally, response bias that may originate from selective response of DES daughters with

children with health problems as compared with their unexposed sisters, may be less likely for severe types of diseases. Many risk factors for CHD have been identified, but it seems unlikely that hormonal risk factors play an important role (maternal oral contraceptive use was not associated with a higher risk of CHD and evidence with respect to maternal use of clomiphene was sparse and inconclusive (reviewed in [32]). Some recent studies, however, found an increased risk of septum heart defects after artificial reproductive techniques (ART)[33;34], but this might also be due to underlying causes of subfertility and not to the exposure to hormones (as part of the ART treatment) itself. Thus, the elevated risk found in our study might be a chance finding.

A limitation of our study is that women with children with congenital malformations may have responded more often to the questionnaire than women without health problems, resulting in potential selection bias. It is unclear whether such selection is differential or non-differential since higher response among mothers with diseased children may apply equally to both DES daughters as their unexposed sisters. Bias by indication in this study is not very likely for several reasons. The risk of hypospadias was not found to be increased in DES sons [5;6]. If uterine cavum malformations were the reason for DES use by the grandmother (and was inherited by the mother of the exposed sons), such an increased risk in DES sons might have been expected. Moreover, the unexposed group would have had the same 'exposure', i.e. chance of having a mother with the inherited uterine cavum malformation. In our study the increased risks were found for the internal as well as the external reference groups. Another limitation of our study is that the medical verification of congenital malformations in our study was incomplete. Thus, true prevalence ratios for all reported malformations are likely to be higher. Three cases of hypospadias in the present study (two penoscrotal and one midshaft hypospadias) proved to be in the overlap between offspring of the DES-net cohort and offspring of another Dutch national cohort of subfertile women on which we reported earlier (11). The prevalence ratio of hypospadias in 8,729 sons of 6,651 subfertile women (including 157 DES daughters) of whom the majority were treated with IVF was 21.3 (95%CI=6.5-70.1).

In conclusion, the risk of penoscrotal hypospadias among sons of DES daughters appears to be increased compared to sons of unexposed sisters of DES daughters. Biological mechanisms are still unclear, although our study suggests that congenital uterine cavum malformations in DES daughters may play a role.

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Supplement Table 1 Prevalence ratios (PR)* of **self-reported** congenital diseases among DES daughters, compared to different reference populations (non-confirmed cases excluded)

Cohort		DES-net, exposed		DES-net, non-exposed				EUROCAT(1)				Rotterdam study (2)			
Period		1960-2004		1960-2004				1981-2004				1998-2000			
total live births		7,899		3,099				386,371				14,075			
icd-10	Congenital disease	N	Prev*	N	Prev	PR*	95%CI	N	Prev	PR	95%CI	N	Prev	PR	95% CI
Q20-Q28	Heart anomaly	101	127.9	21	67.8	1.9	1.2-3.0	2303	59.6	2.1	1.8-2.6				
Q50-52	Female genital anomalies	1	1.3	0	0.0			72	1.9	0.7	0.1-4.9				
Q54	Hypospadias	38	48.1	4	12.9	3.7	1.3-10.4	482	12.5	3.9	2.8-5.4	53	37.7	1.3	0.8-1.9
Q60-Q64	Urinary tract anomaly	43	54.4	17	54.9	1.0	0.6-1.7	724	18.7	2.9	2.1-3.9				

* Prev=prevalence, number per 10,000 live born children, PR=prevalence ratio, 95%CI=95% confidence interval based on a exact binomial distribution

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