Long-term health effects after DES exposure in utero

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Chapter 6

Summary and general discussion

6 Summary and general discussion

The studies described in this thesis "Long-term health effects after DES exposure *in utero*" have provided insight into the transplacental teratogenic effects of exposure to the hormone diethylstilbestrol, implicating health effects affecting both the second generation (DES daughters) and the third generation (DES grandchildren). In this chapter, firstly the main results of this thesis are summarized (chapter 6.1) and placed in a wider context of the literature (chapter 6.2). Subsequently, several methodological issues that have arisen from the different studies will be discussed (chapter 6.3). Next, biological mechanisms underlying DES effects are discussed (chapter 6.4). Finally, conclusions are drawn and implications for clinical practice (chapter 6.5) and recommendations for future research are given (chapter 6.6).

6.1 Summary of results

6.1.1 Cancer risk in DES daughters

Chapters 2.1 and 2.2 report on the long-term risk of cancer in women exposed to diethylstilbestrol (DES) *in utero*. In these two studies the same source population was used (namely women registered at the DES Center). The studies differed with respect to the design (cross-sectional with prevalent cases and prospective with incident cases, respectively), the year of data collection (the prevalence study was conducted in 1994 and the cancer incidence study in 2000), type of questionnaire (short and long, respectively) and response to the questionnaire (5,147 and 7,925 responders, respectively), see also introduction (chapter 1). Both studies make an important contribution to the literature, since worldwide there is only one other cohort of DES daughters (the NCI DES Combined Cohort Study) of the same size that enables research on long-term risks after intrauterine DES exposure.

The **first study** examined the risk of **prevalent cancer** in women who responded to the 1994 questionnaire, sent to all DES daughters registered at the DES Center. Cancers were reported in the questionnaire and verified by medical files. Clear cell adenocarcinomas (CCA) (n=7) were excluded from these analyses, since our focus was on non-CCA cancers. The agreement between self-reported information and the information in the medical file was poor

for cervical cancer (21%). A total of 33 prevalent cases of cancer was observed. An increased prevalence ratio (PR) of cervical cancer (ten patients with squamous cell cancer and two with adenocarcinomas) among DES daughters was observed (PR=5.4, 95%CI=2.8-9.5) compared to the general population (based on data from the Eindhoven Cancer Registry). No statistically significant increased risks were found for other tumors (vulva, breast, ovary, melanoma and colon). Six cases from the prevalence study were also included in the cancer incidence study (two patients with melanoma, three with breast cancer and one with an ovarian tumor). There was no overlap with patients examined in the study on effectiveness of screening (chapter 4).

In the **second study** on cancer risk we estimated **cancer incidence** (period 1992 to 2008) among the 12,091 subjects participating in the DES-net project. The DES-net project is a retrospective cohort study with prospective follow-up. Follow-up started at the date of registration at the DES Center (1992 for the majority of participants) and follow-up ended at November 2008, date of diagnosis or date of death, whichever came first. A 16-page self-administered questionnaire on risk factors for hormone-related cancers and medical history was sent to all women registered at the DES Center in 2000 (n=13,113) and the final response to the questionnaire was 60%. Cancer incidence was assessed through linkage with PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands, and the Netherlands Cancer Registry (NCR). The surveillance committees of PALGA and NCR granted us permission to link both responders and non-responders to the questionnaire under strict privacy procedures. Women who actively refused to participate in the study (5%) were excluded from analyses. The median age at end of the follow-up was 44 years compared to a median age of 30 years in the cancer prevalence study. Period and age-specific cancer incidence rates from the general population were used as a reference. A total of 348 incident cases of cancer was observed. Overall, no increased risk of cancer was found (standardized incidence ratio (SIR)=1.01; 95% confidence interval (CI)=0.91-1.13). The risk of clear cell adenocarcinoma of the vagina and cervix (CCA) was statistically significantly increased (SIR=24.23, 95%CI=8.89-52.74) and the increased risk seemed to continue past young adulthood (above 40 years of age). The incidence of squamous cell cancer of the cervix/vagina (nine located in the cervix and one in the vagina) was non-significantly decreased (SIR=0.64, 95%CI=0.31-1.17). This finding was in contrast with the elevated risk of cervical cancer (non-CCA) found in the prevalence study. The risk of melanoma diagnosed before age 40 was increased (SIR=1.59, 95%CI=1.08-2.26), which was in line with the slightly increased (but not

statistically significant) prevalence ratio of melanoma (PR=1.7, 95%CI 0.6-3.7) in the cancer prevalence study (results not shown). No excess risks were found for other sites, including breast cancer.

In summary, CCA incidence remained increased at older ages, no increased incidence of breast cancer was found, and the risk of invasive squamous cell cancer of the cervix/vagina (mainly located in the cervix) was (not statistically significantly) reduced.

6.1.2 Cervical dysplasia and cancer in DES daughters

In the next study we examined whether the risk decrease of cervical cancer as found in the prospective study was due to enhanced detection and treatment of precancerous cervical lesions among DES daughters. We compared the risk of cervical intra-epithelial neoplasia (CIN) and squamous cell cervical cancer in 11,895 DES daughters (particating in the DES-net study) with the screened general population. For these analyses follow-up was restricted to the period January 2000 till November 2008. Incidence of CIN and cervical cancer was obtained through linkage with PALGA. General population data were obtained from the Department of Public Health of the Erasmus Medical Center in Rotterdam that evaluates the national cervical screening program (these data were originally derived from PALGA as well).

The analyses were based on six women with invasive cervical cancer (diagnosed in the period 2000-2008), compared to 15 cases (including these six cases) in the cancer incidence study (diagnosed in the period 1992-2008). With respect to invasive cervical cancer (all morphologies) we again observed a decreased risk (not statistically significant) compared to the general population (SIR=0.4, 95%CI=0.09-1.3), similar to the risk found in the cancer incidence analyses. However, when precancerous lesions (CIN2 and CIN3) and cancer were combined into one estimate, the overall risk of CIN2+ (CIN2,CIN3 or cancer) was no longer decreased compared to the screened general population (SIR=0.96, 95%CI=0.69-1.3). Another remarkable finding was the borderline significantly increased risk for low-grade CIN lesions (CIN1) (SIR=1.5, 95%CI=0.99-2.3) compared to the screened general population with similar screening frequency. When we restricted the analysis to women with DES-related malformations who had had 1 to 2 screening episodes during follow-up (8.9 years), a strongly increased risks for CIN1 was observed compared to the general population with similar

screening frequency (once per 5 years) (SIR=4.6, 95%CI=2.6-7.5), whereas the risk of CIN2+ was not increased (SIR=0.87, 95%CI=0.38-1.7). Furthermore, the detection of CIN (all grades) appeared to be strongly determined by the frequency of screening.

In conclusion, an increased risk of CIN1 was observed, with the risk increase being most pronounced in women with DES-related malformations. The risk of CIN2+ (including cancer) was not increased, suggesting that DES daughters in general do not have an increased risk of squamous cell cervical cancer.

6.1.3 Effectiveness of screening in DES daughters

Because of their increased risk of clear cell adenocarcinoma of the vagina and cervix (CCA), women exposed to diethylstilbestrol (DES) *in utero* have been recommended to have frequent gynecological examinations in order to early detect and treat (pre)cancerous lesions. In chapter 4 we evaluated the effectiveness of cytological screening in DES daughters in a nested case-control study of 39 DES daughters with vaginal or cervical cancer and 186 DES-exposed controls, diagnosed in the period 1989-2007 and matched on age and date of birth. Women who had had a Pap smear during the period 5.5 to 0.5 years preceding (pseudo)diagnosis did not have a reduced risk of invasive vaginal and cervical cancer (OR=1.48, 95%CI=0.56-3.9) compared to women who were not screened during this period. A slightly favorable stage distribution was observed in DES daughters with cervical cancer compared to the patients in the general population, although not statistically significant (stage I in 90% and 77% of the cases, respectively, p=0.15). The majority of vaginal cancers (all screened) was diagnosed with stage I (57%). Women with moderate of severe dyskaryotic smears (MSD) detected during screening had a strongly increased risk of cervical and squamous cell cancer compared to women with a normal smear (OR=29.3, 95%CI=7.5-115). Women with DES-related malformations such as vaginal adenosis and squamous cell metaplasia more often presented with abnormal smears in their screening history. At higher ages (above age 40), screening might be protective against cervical cancer, although the result was not statistically significant and numbers were small (OR=0.35, 95%CI=0.06-1.9). Thus, cytological screening did not appear to be effective in preventing cervical and vaginal cancer in DES daughters aged 30-50 years,

although stage at diagnosis might be more favorable. Women with DES-related malformations more often presented with abnormal smears during screening.

In contrast, the decreased risk of invasive cervical cancer found in the DES-net cancer incidence analysis and the cervical cancer/CIN analysis (see previous sections) (SIR 0.74 and 0.43, respectively) was suggestive of a protective effect of screening. The older age in the latter two studies (mean age: 38.4 years and 42.0 years, respectively, compared with 35.6 years in the effectiveness study) might be part of the explanation.

6.1.4 Congenital malformations in offspring of DES daughters

Alerted by two case reports of hypospadias in sons of DES daughters, the risk of hypospadias was examined in a large nationwide cohort of subfertile women (n=19,840) of whom 75% had received in vitro fertilization treatment (the **OMEGA cohort**). When we were alerted to these two cases of hypospadias (2002), the data collection of the DES-net cohort had just started and analysis in this cohort were not yet possible. Therefore, we decided to analyze data of the 463 DES daughters participating in the OMEGA cohort, and planned to later use DES-net data for this purpose. The risk of hypospadias was examined among male offspring of 6,651 subfertile women (of whom 157 were exposed to DES *in utero*). A total of 8,934 boys were reported of whom 205 boys were born to DES daughters. Four of these boys had medically verified hypospadias. Among all other 8,729 children, only eight cases of hypospadias were observed, resulting in a strongly increased prevalence ratio of 21.3 (95%CI=6.5-70.1). The increased risk could not be explained by differences in subfertility treatment (natural conception, fertility drugs, insemination or in vitro fertilization), maternal age, birth weight and duration of gestation. Neither did the reason of subfertility (female factor, male factor, unexplained, both) differ between the DES-exposed and the non-exposed children, although DES daughters reported more subfertility problems related to cervical and uterine problems than unexposed women. Since the analysis in the OMEGA cohort was based on only a few cases confirmation in a larger DES-exposed cohort was needed.

A soon as the data collection of the **DES-net cohort** was completed the first preliminary analysis on the prevalence of hypospadias was conducted. A couple of years later the analyses was extended to other congenital anomalies (among which were congenital

urinary anomalies and congenital heart disease). In the final analyses we compared the prevalence of hypospadias, urinary anomalies and heart disease in 7,899 children born to DES daughters compared to 3,099 children born to unexposed sisters, and we also made a comparison with external reference rates from two sources; European Registration of Congenital Anomalies (EUROCAT) and data of a study examining the prevalence of hypospadias among male newborns in Rotterdam[1]. Thirty-two cases of medically verified hypospadias were observed, compared to three cases among the unexposed children (PR=4.2, 95%CI=1.3-13.7). No overall increased risk of hypospadias (all types) was found when we compared with the data of the Rotterdam study, which was the most conservative estimate (PR=1.1, 95%CI=0.6-2.1). However, the risk of penoscrotal hypospadias was increased compared to both EUROCAT and the Rotterdam study (PR=7.9, 95%CI=3.3-18.8 and PR=5.3, 95%CI=1.1-26.5, respectively). In the internal comparison group of children born to unexposed sisters no cases with severe hypospadias were reported. A remarkable finding was that half of the mothers of the cases with penoscrotal hypospadias reported uterine cavum malformations as cause of subfertility, compared to 10% of the overall DES daughters. No increased risk of urinary malformations among children of DES daughters was observed. In conclusion, the risk of penoscrotal hypospadias among sons of DES daughters appears to be increased compared to sons of unexposed women. Biological mechanisms are still unclear, although our study suggested that congenital uterine cavum malformations in DES daughters might play a role.

6.2 Results in perspective

In this paragraph, we place the overall findings of the studies described in this thesis in the wider context of the literature and we also try to explain some discrepancies between the results of the chapters of this thesis.

6.2.1 NCI DES Combined Cohort Study (NCI-DES study)

Besides the DES-net cohort, there is only one other cohort of DES daughters of the same size in the world that has examined long-term health risk in DES daughters: the NCI-DES study. This combined cohort is a combination of four different cohorts of DES daughters that were each

identified in the mid-1970s. The main cohort is the DESAD cohort currently including 4,015 DES daughters and 1,034 unexposed controls. In this cohort DES daughters were identified through prenatal record review (47%), or were referred to the DES clinic by their physicians (32%) or walked-in (self-referral) (21%). Unexposed women were identified through the same prenatal records as the exposed women (76%) or were sisters of the exposed women (24%)[2]. The second cohort is composed of the offspring of mothers who participated in the Dieckmann trial, a clinical trial to investigate the effectiveness of DES (n=296 DES daughters and n=246 unexposed women)[3]. The third cohort consists of offspring of DES-mothers and unexposed women participating in the Women's Health study (n=327 DES daughters and n=716 unexposed women)[4]. To be complete, we also mention the fourth cohort consisting of intrauterine DES-exposed and unexposed women born to mothers who were treated for fertility problems in a private obstetric clinic (the Horne cohort). Yet, this last cohort is not included in the cancer and CIN analyses done within the NCI-DES study. In total, the combined cohort exists of 4,653 DES daughters and 1,927 unexposed women. DES exposure of all DES daughters was validated by the mother's medical record.

In the first years after recruitment all women (both exposed and unexposed) yearly underwent gynecological examinations (DESAD cohort only). From 1984 onwards, information on disease was obtained through mailed questionnaires and for all self-reported cancers and CINs confirmation by medical files was sought. In some analyses only pathologically confirmed cases were included (CIN analysis)[5], but other analyses also included self-reported unverified cases[6]. For comparison, both internal (unexposed controls) and external reference data (cancer incidence rates for white women from the Surveillance, Epidemiology, and End Results Program SEER) were used (www.seer.cancer.gov). The completeness of follow-up has not been clearly described. This may be a problem since the U.S. is not covered by nationwide population cancer registries.

6.2.2 Cancer

Overall, we found no increased risk of cancer, which was in agreement with the overall cancer risk among DES daughters compared to the general population (SEER) in the NCI-DES study[6]. Both studies also found a highly increased risk of **CCA** compared to the general population

(SIR=39.0, 95%CI=15-104 and SIR=24.2, 95%CI=8.9-52.7) in the NCI-DES study and DES-net, respectively). In the NCI-DES cohort estimated relative risks of cancer in DES daughters were systematically lower for nearly all tumours when DES daughters were compared to the external reference group (SEER data) instead of when they were compared to the internal reference group. The researchers mention that, for instance, breast cancer risk profiles seem to be different if both DES-exposed and unexposed women are compared with the general population (with the most remarkably finding being a lower prevalence of overweight and obesity)[7]. Thus, this might be an indication that the comparison with the external reference group, like we did, suffers from uncontrolled confounding. Compared to the unexposed internal reference group, the risk for **breast cancer** (all ages) in the NCI-DES study was not increased[7]. However, at attained ages ≥40 and ≥50 years risks were significantly increased (incidence rate ratio (IRR)=1.91, 95%CI=1.09-3.33 and IRR=3.00, 95%CI=1.01-8.98, respectively). The risks attenuated when they were estimated compared to the general population data and only the risk in the 50+ age group remained significantly increased (IRR=1.77, 95%CI=1.05-3.00). The most recent analyses of the breast cancer risk above age 40 in the NCI-DES study revealed a HR of 1.82 (95%CI=1.04-3.18) compared to the unexposed group[2]. In the DES-net study, we used the general population as reference and found no increased breast cancer risk neither overall, nor above age 40 (SIR=1.05, 95%CI=0.90-1.23 and SIR=1.09, 95%CI=0.91-1.31, respectively). Although uncontrolled confounding cannot be fully excluded we do not expect this bias to be as important as in the US, as the Dutch general population is much less diverse. Another possible explanation for the inconsistent findings between our study and the NCI-DES study is that the DES daughters in the NCI-DES study were slightly older at the end of follow-up compared to the women in the DES-net study (median age 49 years and 44.0 years, respectively). However, chance might also play a role, because the number of cases was relatively small in the older age categories in both studies. Also, misclassification of DES exposure in our study might have attenuated the risks. Furthermore, risks for **squamous cell cervical cancer** and **melanoma** could not be evaluated in the NCI-DES study, because agreement between self-reported cancers and medical verification was poor and medical verification data were incomplete. The discrepancy between the increased risk of cervical cancer found in the cancer prevalence study and the decreased risk of cervical cancer observed in the cancer incidence study is probably be due to residual non-response bias in the first study, which will be discussed in more detail in section 6.3.5.

6.2.3 Cervical intraepithelial neoplasia

An important finding of our study is that we found no increased risk for CIN2+ (a combined estimate for CIN2, CIN3 and invasive cervical cancer), neither in the entire cohort of DES daughters nor in the subgroup of women with DES-related malformations. In these analyses we carefully adjusted for the frequency of screening. We defined an episode as starting with a primary smear, if necessary followed by secondary smears in case of an abnormal smear or a smear of inadequate quality. An episode ended when follow-up was complete. By definition, the extra smears taken because of a detected abnormal lesion were attributed to the same episode as the abnormal lesion itself. Thus, the extra smears did not result in more episodes.

Our findings do not agree with the findings from the DESAD study/ NCI-DES study[2;5;8]. The most recent paper on this study[2] extended the follow-up to approximately 26 years after study entry (1975) and included 208 DES-exposed women with biopsy-confirmed high grade CIN, compared to 232 cases with CIN2 and CIN3 in the DES-net cohort. The authors found a two-fold increased risk for high grade CIN lesions (invasive cancer not included), compared to non-exposed controls (HR=2.09, 95%CI=1.45-3.00, adjusted for frequency of Pap Smears)[2]. Quite similar to our study, an increased risk of CIN2+ was shown for women with VCEC (HR=2.40, 95%CI=1.60-3.61 and HR=1.7, 95%CI=1.2-2.3 in the NCI-DES study and our study, respectively). However, in our study the risk increase of CIN2+ among women with VCEC largely disappeared when we conducted an analysis stratified for screening episodes. In this stratified analysis we compared the risk of CIN2+ among women with VCEC and who had had 1-2 screening episodes during the 5-year interval with the screened general population (with similar frequency based on normal general population screening, i.e. once per 5 years). In the NCI-DES study the stratified analyses on the presence of VCEC were not adjusted for screening. Another important difference between the two studies was that information on CIN in the NCI-DES study was mainly based on **self-report** followed by verification by pathology records. Therefore, this information might be incomplete and reporting bias cannot be excluded. While overreporting may be solved by verification, underreporting by the unexposed group may stay present, potentially biasing the results away from zero. The authors indicate that the observed increased risks might be due to the incomplete adjustment for screening[5]. On the other hand, our study might have been biased toward zero because we may have included some women

without DES exposure, whereas 100% of the women in the NCI-DES study had verified DES exposure.

Thus, the NCI-DES study and the DES-net study differ with respect to some important issues, which might have biased the results of either study. In the NCI-DES study incomplete adjustment for screening and bias in the assessment of the outcome (reporting bias) might have biased the results away from zero. In the DES-net study, the incomplete confirmation of DES exposure may have lead to bias towards zero. In combination both studies show the lower and upper bounds of the real estimate, suggesting that DES daughters in general do not have an increased risk of squamous cervical cancer, or only a slightly increased risk.

6.2.4 Effectiveness of screening

The study on effectiveness of cytological screening among DES daughters described in this thesis shows that screening in DES daughters is not effective in preventing invasive vaginal and cervical cancer, while screening was effective in shifting diagnoses to slightly more favorable stages. The **screening of CCA** (of which 9 tumors were located in vagina and 5 in the cervix) appeared to be ineffective in all age groups (range age 30-54 years), whereas the **screening of squamous cell cervical cancer** seemed to be slightly effective at older ages (>40).

There is sparse evidence from the literature on the effectiveness of screening among DES daughters, and the studies concerned only examined effectiveness of screening on CCA, not squamous cell cancer. Furthermore, these studies were only focused on the detection of *invasive* cancer and not on the detection (and treatment) of *pre-invasive* lesions.

It may be questioned whether invasive **CCA** may be expected to be prevented by screening. Screening on cervical adenocarcinoma in general is assumed to be less effective than screening on squamous cell cervical cancer. Furthermore, precancerous stages of adenocarcinoma are less clearly understood and it is unclear whether glandular precursor lesions develop in the same manner as CIN lesions develop into squamous cell carcinoma [9;10]. With respect to CCA (which are located predominantly in the vagina), it has been suggested that the sensitivity of the cytological screening on CCA might be lowered by 1) inadequate sampling (cervix instead of vagina), 2) the location of the CCA tumor (submucosa) and 3) difficulties in recognizing CCA tumor cells which sometimes are highly differentiated or

obscured by e.g. inflammatory cells[11]. Although cytological examinations appeared to be ineffective in preventing invasive CCA in our study, a shift toward a lower stage distribution for CCA (and squamous cell cancer) cancers was observed. Interestingly, similar to our findings Hanselaar et al observed that women with a history of a cytological examination (in the period two years prior to the diagnosis) had a more favorable stage distribution (58% stage I) compared to women without cytogical examinations (20% stage I)[12].

Remarkably, we found no protective effect of screening among 17 cases with **squamous cell cancer** (SCC) (of whom 2 were located in the vagina), whereas screening on cervical squamous cell cancer in the general population has been proven to be effective[13]. Another striking finding is the high number of moderate/severe dyskaryotic smears (SMD) that preceded the diagnosis of SSC. This percentage was higher than would be expected based on general population data[14]. The SMD lesions that preceded the cancer diagnosis occurred mainly in women with adenosis. An explanation might be that the occurrence of adenosis was misinterpreted as severe/moderate dyskaryosis, or, even more important, the presence of adenosis might have complicated the smear taking and may have obscured the detection of tumor cells in the cytological material. Also, the presence of adenosis may have lead to more colposcopic surveillance.

Although screening for cervical squamous cell cancer in DES daughters seems rather ineffective in our study due to the reasons just mentioned, at **older ages** the effectiveness seems to improve. As adenosis regresses when women are aging[15], cytological smears may gain sensitivity to detect tumor cells. This is also in line with our findings of a lower risk of invasive cervical cancer among DES daughters (chapters 2.2 and 3). In both analysis (cancer and CIN analysis) the average age at diagnosis was higher than in the efficacy study (38.4 years, 42.0 years and 35.6 years, respectively).

In conclusion, although the efficacy of preventing invasive cervical and vaginal cancer is low, cytological screening seems to result in a shift of the tumor stage distribution towards more favorable stages. However, this might not be the result of cytological screening itself but the overall effect of the more intensive gynecological surveillance (including clinical examinations and colposcopy). Unfortunately, we lacked detailed information to further explore this hypothesis.

6.2.5 Hypospadias

In the studies, described in this thesis we observed an increased risk of hypospadias, in agreement with other studies[16-19], but not confirmed by the NCI-DES study[20]. All studies used self-reported hypospadia, but the NCI-DES study was the only study that did not use medically verified diagnoses. On the other hand, the NCI-DES study was the only study with 100% confirmed DES exposure. As the severe types of hypospadias are less vulnerable to reporting bias, it is a pity that NCI-DES did not report on this subtype. When we analyzed risk of hypospadias according to severity, the increased risk was restricted to penoscrotal hypospadias (the most severe type of hypospadias). Only two other studies had information on type of hypospadias, but no estimates were given[17;19]. An interesting finding in our study was the relatively high number of uterine cavum malformations (50%) reported as cause of subfertility among mothers of sons with penoscrotal hypospadias, which was considerably higher than the number 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 (of which 5 were of the severe type)[19]. Whether these maternal uterine cavum malformations only occurred among the sons with severe hypospadias, however, was not reported. It seems a likely explanation that the increased risk of hypospadias among sons of DES daughters might be caused by the congenital (DES-related) malformation of the mother, since placental insufficiency and early-onset intrauterine growth retardation of the fetus are potential risk factors for (penoscrotal) hypospadias[21;22] and maternal uterine cavum constrictions limiting uterine space may result in growth retardation[23].

6.3 Methodological considerations

6.3.1 Study design

Several study designs were used in this thesis; a retrospective cohort study (DES-net, Chapters 2.2 and 3), a nested case-control study (Chapter 4) and three cross-sectional studies (Chapters 2.1, 5.1 and 5.2).

In a **retrospective cohort study**, the investigator defines a group of individuals (cohort) who are free of disease of interest at the start of follow-up and who experienced a specific exposure or condition. Preferably, also a reference cohort that is unexposed and also free of disease is defined[24]. Sometimes the general population is used as the reference cohort (as described in this thesis), but that does not fully meet the criteria of an unexposed cohort (see also paragraph on reference groups). A retrospective (or historical) cohort study means that the identification of subjects took place after the exposure has occurred. Some cohort members might be already diseased at recruitment, but new cases may arise during prospective follow-up. Mostly, this type of study is based on existing records or registries. With respect to the DES-net cohort, the identification of subjects was based on the registry of the DES Center (started in 1992), and the actual exposure to DES had occurred long before the registry was established and the recruitment into the study took place. A disadvantage of this type of study is that the investigators must rely on records from the past which may be problematic. First, detailed information about timing and dose of DES was generally not available and, worse, many medical records had been destroyed before the start of the study. Consequently, the DES registry was largely based on the daughters' report of maternal DES use. An advantage of a retrospective cohort design over a prospective cohort design is the relatively short time to complete the study. The initiation of and progression to cancer after exposure to a potential carcinogen takes many years, and prospectively, it would take decades to complete a study. Since DES exposure is a rather rare exposure (prevalence in the general female population is estimated between 1.2-3.2%), a case-control study, in which persons with a specific disease and persons without a disease are compared, would not be an efficient design. The number of DES-exposed individuals in such a study would be far too low. Another advantage of a cohort study above a case-control design is that various outcomes can be (exploratively) studied, whereas a case-control study is only focused on a single disease.

Prospective follow-up in a retrospective cohort design means that data are also collected after the recruitment of subjects. Completeness of follow-up is crucial in a cohort study, since incomplete follow-up may lead to selection bias.

Another type of study design used in this thesis was a **cross-sectional design** (chapter 2.1, 5.1 and 5.2). A cross-sectional design is a type of study that includes all living persons in the population (or a sample) at one point in time, including persons with the disease. With respect to the Cancer Prevalence study, data collection took place in 1994 when a questionnaire was sent to all women enrolled in the registry of the DES Center, by employees of the DES Center. The aim of the study was to quantify the prevalence of health problems in all DES daughters. One of the major disadvantages of this type of data collection is the problem of under-representation of deceased patients. The main aim of the DES registry was to get compensation of DES-related problems and thus, deceased persons generally stayed in the registry in order to provide their next of kin with the compensation (see 6.3.2.2).

Finally, a **nested case-control design** has been used in this thesis (study on effectiveness of screening, chapter 4). A nested case-control study is a case-control study conducted within a cohort study. Furthermore, we used a matched design, meaning that cases and controls were made comparable with respect to specific confounders. In the study described in this thesis, we matched on age and date of birth. A great advantage of this type of study is its efficiency with respect to supplementary data collection or processing. With regard to the DES-net cohort, the analyses on screening history needed very detailed coding of all information supplied by PALGA, which would have been very time consuming when this should have been conducted for the entire cohort (n=12,182), instead of the subsample of 39 cases and 186 matched controls.

6.3.2 Cohort identification (DES-net)

6.3.2.1 Source population of DES daughters

The precise number of DES daughters in the Netherlands is unknown. The first estimates of the number of DES-exposed pregnant women (the so-called DES-mothers) ranged between 190,000 and 380,000, based on US sales figures extrapolated to the Dutch situation, resulting in approximately 90,000-190,000 DES daughters[25]. In the eighties, the estimate was refined to 110,000 DES daughters, based on the absolute risk of clear cell adenocarcinoma among DES

daughters (1 per 1,000 through age 34)[26], and the number of CCA cases registered in the Nijmegen CCA Registry (n=55 cases in 1988)[27]. The most recent paper on the CCA registry in Nijmegen reported that the number of DES-related CCA cases had **increased to 76** in 2005, with a mean age at diagnosis of 24.2 (± 7.0) years[28]. In this study it was unclear how many cases arose after age 34, but it was reported that 95% of the CCA cases occurred before age 38.2. Based on this updated number of CCA cases and the absolute risk of CCA risk of 1 per 1000 DES daughters, the maximum number of DES daughters in the Netherlands can be re-estimated at 76,000.

We made an estimation of the number of DES daughters based on a pilot-study that we performed in the period 1999-2003 (not used for this thesis). In this study (our **archival cohort**) we identified DES daughters through the mothers' obstetrical records. We traced DES daughters (and controls) in the historical archives of six hospitals (Westeinde ziekenhuis Den Haag, UMCG Groningen, Atrium ziekenhuis Heerlen, Vroedvrouwenschool Kerkrade, Ziekenhuis Rivierenland Tiel, Gelre ziekenhuizen Apeldoorn), which had kept all historical obstetrical records. From these selected hospitals it was known that DES had been prescribed on a large scale. We collected data on timing and dose of DES exposure and other pregnancy characteristics. A total of 126,233 medical files were checked for the prescription of diethylstilbestrol, and 849 DES daughters were traced. When we assessed the overlap between the registry of the DES Center and this archival cohort, only 21% (range 13-33% for the different hospitals) was known in both registries. This implies that 79% of all DES daughters traced through the medical records was not known at the DES Center. Thus, if we extrapolate these percentages, the source population of DES daughters in the Netherlands is estimated to amount 60,000 (range 40,000-100,000).

6.3.2.2 DES Center registry as sampling frame

Our cohort members were identified through the registry of the DES Center. The number of registered DES daughters at the DES Center at time of enrollment (n=13,044) was much lower than the estimated 60,000 DES daughters in the source population. The DES registry was established in 1992 because of a change in civil law shortening the period of liability. This event urged the need for registration of all DES-involved individuals in order to guarantee future health claims for compensation by the pharmaceutical industry. A big media campaign in 1992 resulted in the registration of more than 17,000 DES-exposed individuals (among whom were

13,500 DES daughters). All individuals with suspected DES exposure were advised to register, diseased individuals as well as healthy individuals, in particular because of possible health problems in the future. To enter the registry documented DES exposure was asked but not required.

It is worth mentioning that there may be several reasons for a DES daughter *not* to be included in the DES registry. First, women might have been unaware of their intrauterine DES exposure. In the DESAD study (later referred to as NCI-DES study), 37% of DES mothers who were identified through medical record review, were unaware that they used DES (29% did not recall DES use and 8% denied the use of DES, while it was recorded in their prenatal record)[29]. Second, women might have felt no urge to register because they experienced no health problems. Third, women may have been uncertain about their *in utero* exposure to DES. Fourth, some women did not want to be confronted with DES matters any longer. Fifth, women who were deceased because of DES-related disease before 1992 may not have been registered by their relatives, although they were advised to do so in order to retain the possibility to pose a health claim for compensation. Furthermore, we assume that only a small fraction of women died before 1992 since DES daughters had an average age of 31 at start of the follow-up (date of registry). We included 59 patients with CCA in our DES-net cohort, of whom 53 patients were diagnosed before start follow-up (1992). Currently, 81 patients with CCA are known at the DES Fund of whom 11 were deceased (personal communication 2012), which implies that we missed 22 cases (=81 -59).

Thus, of the estimated 60,000 DES daughters in the Netherlands, approximately a quarter (i.e. 13.500/60,000) entered the DES registry. As described above, there may have been many different reasons not to register, most of which may not affect the external validity of the study. However, it cannot be excluded that women may have differentially enrolled based on signs and symptoms of disease and therefore might have a different predisposition for other diseases of interest. For instance, if the presence of DES-related adenosis would increase the risk of CCA of the vagina/cervix, and women with adenosis were more inclined to enter the registry, the risk of CCA may be overestimated if adenosis was not taken into account in the analysis. If handled adequately in the analyses no bias arises in prospective cohort analyses, although the external validity stays dependent on the (unknown) prevalence of adenosis in the entire group of DES daughters. In cohort studies this type of bias can be viewed as confounding rather than selection bias[24]. Interestingly, when we compared the percentage

of women with DES-related malformations (which might be indicators of selective enrollment into the registry as well as into the group with questionnaire data; see 6.3.5) between our cohort and the NCI-DES study, 36% of the subjects in our cohort reported to have had DES-related malformations (chapter 3) compared to 45% in the NCI-DES study[2]. The percentages might not be entirely comparable since the occurrence of DES-related malformations is also correlated with early timing of DES exposure[30] and DES treatment protocols might differ between the two cohorts. However, our data suggest that women with health problems are not overrepresented in our cohort.

In conclusion, although our DES-net study population might not be entirely representative for all Dutch DES daughters, this does not appear to be a major problem. However, for the subgroup analyses in women with questionnaire data in combination with an outcome that preceded the questionnaire and in the studies that used prevalence as major outcome, selection bias might occur. The potential impact of these types of selection bias on these studies will be discussed section 6.3.5.

6.3.2.3 Choice of reference groups

An important step in the design of a cohort study is the choice of the reference group, in order to compare the disease experience of exposed individuals with that of non-exposed individuals. The unexposed group is preferably similar to the exposed group with respect to the factors determining the disease of interest. Other factors unrelated to the disease may differ and this might even be true for factors related to the disease, provided that the latter can be measured and be controlled for[24]. Two kinds of reference groups are possible. In the sections below we discuss the use of internal reference groups and external comparisons groups, which are both used in this thesis.

Internal reference groups

In the two cross-sectional studies examining the prevalence of hypospadias described in this thesis, internal reference groups were used. Regarding the **OMEGA** analyses on hypospadias, both DES-exposed and unexposed subjects originated from the same cohort of subfertile women. Compared to the unexposed group, the sons of DES daughters had a shorter duration of gestation, and a higher proportion had an extremely low birth weight (lower than 1500 gram). Adjustment for these confounders did not materially alter the risk of hypospadias.

In the analyses of hypospadias among **DES-net** participants both an internal reference group and two external reference groups were used. The internal reference group consisted of sons unexposed sisters of DES daughters. An advantage of a sister control group is that these women share part of the genetic, *in utero* factors and life style background with their exposed sister. The recruitment of the unexposed sisters of DES daughters into the DES-net study was a rather complex process. DES daughters who already participated in the study were asked to invite their unexposed sister(s) for participation and were asked to sent back a reply form with names and addresses. Subsequently, we sent an invitational letter together with a questionnaire. Since the invitational procedure was a multi-step process, non-response could occur in several ways; 1) DES daughters may not have approached their sister(s); 2) sisters may have refused to participate or may not have responded after they were invited by their DES-exposed sister (these two categories 1) and 2) could not be distinguished), and 3) actual non-response after we sent the questionnaire to the sister concerned (205/2094=10%). Use of the sister control group in our study was hampered by the rather high non-response. Unfortunately, we lacked detailed data on the total number of eligible non-exposed sisters, but we estimated that between 4,199 and 5,262 sisters were eligible for inclusion into the study and thus the potential non-response ranged between 55% and 64%. Given the uncertainty of potential bias through this non-response, we also used two external reference groups, which will be discussed in the next section. As the results were similar for the internal and external reference groups, non-response among the sisters did not seem to be related to the prevalence of hypospadias in sons.

External reference data

For four studies described in this thesis (cancer incidence and prevalence, cervical dysplasia incidence, hypospadias) the general Dutch population was used as the external comparison group. For the different outcomes we used different sources:

- Cancer prevalence (chapter 2.1): Eindhoven Cancer Registry
- Cancer incidence (chapter 2.2): Netherlands Cancer Registry and PALGA
- CIN (chapter 3): PALGA
- Hypospadias (chapter 4): EUROCAT and the Rotterdam study (i.e. study data, not based on general population)[1].

The use of the general population as the "unexposed" cohort in the cohort studies described in this thesis has several drawbacks[24]. First, the unexposed cohort is not entirely unexposed, since a small proportion has been exposed to DES *in utero*. However, since this proportion is estimated to be very small in case of *in utero* exposure to DES (the estimated prevalence in the Dutch female population is between 1.2 and 3.2%), bias towards null (no effect) will be minimal. Second, information for the general population might differ from the information collected in the cohort regarding the quality and extent of detail. When using external reference rates it is important that assessment of diagnosis has been done in a similar way as in the exposed populations. Third, the general population may differ from DES-exposed individuals with respect to risk factors for the disease of interest and adjustment for confounding on an individual basis is not possible. In the next sections the characteristics of the different registries will be described in more detail.

Netherlands Cancer Registry/Eindhoven Cancer Registry

Since 1989, the Netherlands has a population-based nationwide Cancer Registry (NCR). Newly diagnosed cancer patients are identified through PALGA (patients with a microscopically confirmed diagnosis) and through the Dutch National Hospital Discharge Registry (clinically diagnosed patients). The completeness of the registry is estimated to be at least 95%[31]. For each tumor additional data on tumor characteristics (topography, morphology, stage) and treatment are abstracted from the medical records by trained registration clerks. Since 2005, additional information on prevalence is available in the NCR (by addition of vital status to the core dataset through linkage with data from the municipal population registries (de Gemeentelijke Basis Administratie, GBA). For the analyses for the cancer prevalence study, where the data collection already took place in 1994, we used data from the Eindhoven Cancer Registry (ECR), because this registry already had data on cancer prevalence available from 1970 onwards. A comparison of mortality data between the ECR region and the Netherlands as a whole has shown that cancer risk is rather comparable between the ECR region and the Netherlands. For our comparisons, we used sex-, age- and calendar period-specific data on invasive tumors from both the ECR (chapter 2.1 cancer prevalence) and the NCR (chapter 2.2 cancer incidence).

PALGA/PALEBA database (reference data for CIN incidence)

The PALEBA project is a collaboration between PALGA and researchers of the Department of Public Health, (Erasmus Medical Center Rotterdam), assigned to monitor and evaluate the National Cervical Screening program. PALEBA is the Dutch acronym for: "PALGA, input for the national cervical cancer screening program evaluation" ('Landelijk Bevolkingsonderzoek Evaluatie Baarmoederhalskanker'). All cervical cytological and histological examinations were retrieved from PALGA and processed to form the PALEBA database. We used the PALEBA database as our reference population in the analyses on incidence of CIN (Chapter 3). An important step in the processing of the data of PALGA was to combine the separate pathology records into individual screening histories and to define a screening episode. The identification of an individual was based on the first four letters of the maiden name in combination with date of birth. To avoid false clustering of women with common surnames (e.g. Jansen), in which case screening histories of two different women might be combined into one, 0.5% of the most common surnames were excluded[32]. By definition, a screening episode started with a primary smear if necessary followed by secondary smears in case of an abnormal smear or a smear of inadequate quality. An episode ended when follow-up was complete (i.e. three consecutive negative smears after a primary smear with a high grade squamous cell intra-epithelial lesion (HSIL, severe moderate dykaryosis), two consecutive negative smears after a primary smear with a low grade squamous cell intra-epithelial lesion (LSIL, borderline or mild dyskaryosis), one consecutive adequate smear after a primary smear of inadequate quality within 6 months, or when no (adequate) follow-up smears were done within 4 years from the primary smear. In the DES-net study evaluating CIN incidence we defined the episodes conform this (PALEBA) definition.

As reference rates we used information on the number of histologically verified cervical lesions per five year age category per calendar year (2000-2008) for the Dutch female population in the age range 30-64 years. We also used number of cervical lesions with exclusion of lesions detected during the first episode of each woman, in order to adjust for the occurrence of prevalent lesions, because it is known that first screens have generally higher detecting rates than following tests.

For the processing of the DES-net data, a problem of the exclusion of the 0.5% common surnames in the reference dataset was that we had to use the same approach for our cohort. However, we lacked information on which surnames exactly should be excluded.

Therefore, we used an approximation by multiplying the person-years in each age category by a fraction calculated as number of lesions after exclusion of 0.5% common surnames divided by the number of lesions without exclusion of the common surnames, like was done by Rebolj *et al* [33].

In order to calculate incidence, one needs not only information about outcome (PALEBA) but also about the population at risk. The latter data (per 5-year age group) were obtained from Statistics Netherlands (www.cbs.nl). To adjust for women without an uterus information from the Dutch Hospital Discharge database (LMR) was used[34].

EUROCAT (reference data for congenital malformations)

EUROCAT is a registry of congenital malformations which covers the northern part of the Netherlands. The registry is dependent on voluntary reporting by midwives, general practitioners, well-baby clinic doctors and other treating physicians. Furthermore, informed consent from the parents is needed for admission to the registry. There is no lower age bound for gestational age and no upper age limit for reporting of congenital malformations to EUROCAT. There are some limitations of the use of the EUROCAT as a reference group. First, based on the parental response, the completeness of the EUROCAT registry is estimated at 80%, which results in a underestimation of the prevalence. Second, only the northern regions in the Netherlands are covered by the EUROCAT registry, which might not be representative for the total Dutch population. And last but not least, glanular hypospadias were not registered until 1995 (unless they were part of a syndrome) resulting in underestimation of the prevalence of mild hypospadias and hypospadias in total. In conclusion, our prevalence ratios compared to EUROCAT tend to be slightly overestimated, especially with respect to hypospadias overall (all types) and glanular hypospadias.

Cohort study in Rotterdam (reference data for hypospadias)

As a second reference group we used the results of a Dutch cohort study among 7,292 newborn boys in Rotterdam who were neonatally screened for the presence of hypospadias within 6 months after birth[1]. Child Health Care Centres (CHC) received notification of all newborns registered in the Municipal Birth register of Rotterdam. Parents were subsequently invited to visit the CHC for examination of their child (response was 95%). Because all children were actively screened on hypospadias, the prevalence in this cohort was higher than in the

other birth anomalies registries. Therefore, since the children in the DES-net cohort were not actively screened on hypospadias, the prevalence ratios using the Rotterdam cohort as a reference were probably underestimated.

In conclusion, by using different comparison groups in our analyses on hypospadias we, in fact, conducted a sensitivity analysis, with the EUROCAT as the highest estimation and the Rotterdam comparison as the most conservative estimation of the prevalence ratios.

6.3.3 Assessment of DES exposure

A major limitation of the studies described in this thesis is the small number of women with documented DES exposure. In the DES-net study huge efforts were made to trace the mothers' medical files, however without much success. In this paragraph, we will discuss the difficulties that we came across.

Copies of mothers' medical files

In the DES-net study, women were asked to provide a copy of their documented DES exposure. In case no documentation was available, the mother was asked to give written informed consent to have her medical record traced by the researchers. A total of 1,117 copies of mothers' medical files were returned in addition to the questionnaire, in which DES exposure *in utero* was confirmed for 914 participants.

Table 6.1 Retrieval of mother's medical files for 183 DES daughters with cancer

Medical files inquired	Gynecologist	General practitioner
DES exposure confirmed	6 (6%)	3 (4%)
DES exposure not confirmed	3 (3%)	5 (7%)
No medical file found	76 (70%)	57 (76%)
Non-response	23 (21%)	10 (13%)
Total	108 (100%)	75 (100%)

Tracing of mothers' medical files

Our initial goal with respect to the DES-net cohort was to trace all mothers' records. For this purpose, we asked in March 2000 all mothers of participating DES daughters to fill in a written informed consent and to provide us with names and addresses of the treating physicians. Unfortunately, it appeared that the tracing the mothers' medical records was increasingly difficult. Many medical files of the period 1947-1975 appeared to have been destroyed. Moreover, the fact that DES has been prescribed not only by gynecologists but also on a large scale by general practitioners in the Netherlands (estimated between 33-37% of all prescriptions), made the tracing of the records even more difficult.

As a pilot we mailed 108 gynecologists and 75 general practitioners asking for the old medical files of the mothers, concerning 183 DES daughters with prevalent cancer (see

Table 6.1). No medical files were found for 70% and 76% of the requested medical files for gynecologists and physicians, respectively. In only four percent of all cases confirmation of DES exposure was obtained.

Verification of self-reported DES exposure

In another pilot study, we verified self-reported DES exposure in 115 participants of whom the mothers were treated in one of the four hospitals where the old archives were still kept (these hospitals also participated in the archival cohort, see paragraph on source population). For 76% of the women DES exposure was confirmed, in 3% a medicine/drug other than DES was recorded and in 21% no DES was mentioned in the hospital medical file (Table 6.2). For the latter group it was still possible, however, that the mothers concerned had received DES through their general practitioners.

Table 6.2. Validation of self-reported DES exposure in four hospitals with preserved historical medical archives

Mothers with self-reported DES exposure (n=115)		
No medical file available	45	31%
Medical file mother available	70	61%
DES exposure confirmed in medical file		
Yes	53	76%
No	15	21%
Other medicine reported	2	3%

In conclusion, even in hospitals with maintained historical archives, as was the case in the pilot study described above, the proportion of medical files that could be traced was low (70/115=61%) and the tracing of the files had been time consuming. The agreement between self-report and verified DES exposure was rather good (>76%). Therefore, we included all women in the analyses, irrespective whether DES exposure was medically verified. We also decided to discontinue the tracing of mothers' medical files.

DES daughters with **documented DES exposure** in our study might be a selective group, since women with health problems might have been extra motivated to retrieve their mothers' medical record at a moment that these records were still available. Indeed, the risk of CCA was higher among women with documented DES exposure than among women without verified exposure (although based on 2 cases only) (cancer incidence analysis, Chapter 2.2). Similarly, among women with medically verified DES-malformations (like adenosis and structural anomalies) 17.7% had documented DES exposure whereas among women without malformations only 9.3% had verified DES exposure (results not shown). However, another explanation might be that among women with CCA or DES-related malformations less misclassification occurred (that they were more likely to be truly DES-exposed), whereas among women who were not aware of any malformations unexposed women might be included as well. Thus, higher risks among the group with documented DES exposure cannot be interpreted as a more valid estimate of the real effect, because the initiative to retrieve the mother's medical record may be driven by the outcome.

DES-related malformations

An alternative approach to deal with missing information on documented DES exposure was to conduct subgroup analyses for women with (medically) DES-related malformations (like adenosis, coxcomb, vaginal ridges, T-shaped uterus, hypoplastic cervix). These malformations have a DES attributable risk higher than 90%[35]. Thus, DES-related malformations can be used in analyses as a proxy for true DES exposure. However, one should keep in mind that DES-related malformations are also associated with early DES use (first trimester) As a result, restriction of the study-population to women with evidence of DES-related malformations implies a shift to early timing of *in utero* DES exposure. Theoretically, this might mean a stratification on a different predisposition for other DES-related diseases.

Bias by indication

Bias by indication may occur if the reason that the DES-mother used DES is related with the health problems of the offspring, and not the DES usage itself[36]. For instance, if uterine malformations of the mother caused a threatened abortion during the pregnancy of the mother for which she was prescribed DES, DES usage may be falsely attributed to the uterine malformation of the daughter, which in fact is inherited. By the use of sister controls this type of bias by indication can be controlled for, at least partially. In addition, animal models showed uterine malformations in the neonatally DES-exposed mice, whereas in the unexposed mice no malformations were found.

Verification of DES exposure in OMEGA

In the OMEGA study on hypospadias no mothers' medical files were available, but DES exposure was based on self-report in the questionnaire and partly verified by DES daughters' subfertility records (64%). For the four cases with hypospadias additional information on *in utero* DES exposure was collected by telephone interview.

6.3.4 Assessment of outcome

In the studies described in this thesis we examined different outcomes (cancer prevalence, cancer incidence, CIN incidence, screening history, hypospadias) obtained by using different sources.

Medical verification of self-reported disease

Information on the prevalence of **hypospadias** (chapters 5.1 and 5.2) and **cancer** prevalence (chapter 2.1) was collected by means of a self-administered questionnaire. Informed consent was asked in order to verify reported disease with information from the medical file. A disadvantage of this approach was that some women did not respond to our request to give informed consent for verification, implying that a number of reported diseases could not be verified; 28% non-response overall and 10% for the congenital malformations in the DES-net hypospadias study and 20% non-response in the cancer prevalence study. Moreover, part of the medical records, especially records dating from more than ten years ago, were already

destroyed due to privacy regulations in the Netherlands. Altogether, this may have resulted in underestimation of prevalence in both studies. With respect to the congenital malformations, the prevalence of mild hypospadias and the other congenital malformations might be underestimated, although the sister comparison group may have suffered from the same bias. Thus, underestimation will be mainly a problem concerning estimated PRs compared to the external reference populations. Regarding the cancer prevalence study, fortunately we were capable of repeating these analyses in the DES-net cohort.

Reporting bias

Another aspect of using prevalence data from a questionnaire survey is the potential differential reporting by exposed and unexposed women (reporting bias). DES-exposed women may be more aware of their own health and that of their children than their unexposed sisters. We dealt with this problem of potential overreporting by the medical verification of all reported diseases. However, underreporting of disease, which might be especially the case in the unexposed group, could not be ruled out. In particular in the OMEGA study this was problematic since hypospadias was asked in a more general way ("Were there any *serious* health problems in one or more of your children"), whereas in the DES-net study the question was more specified ("Did one of your children have hypospadias"). The prevalence of all types of hypospadias among women in OMEGA not exposed to DES was 0.9/1,000, and in DES-net 1.9/1,000 liveborn boys, compared to the prevalence of 2.4 per 1,000 liveborn boys in the general population according to EUROCAT (1981-2004). The way the question was asked in OMEGA might have caused some underreporting of mild hypospadias (since it was an open-ended question and we asked for "serious" health problems). Thus, underreporting in the OMEGA study might have been a bigger problem than in the DES-net study. Whether this bias was differential or non-differential is not clear. With regard to the more severe types of hypospadias, like penoscrotal hypospadias, reporting bias is less likely.

Outcome retrieved by linkage

Various outcomes in the studies described in this thesis were obtained through linkage with population registries. Information on *cancer incidence* was obtained from the Netherlands Cancer Registry and PALGA (cancer incidence), information on screening history from PALGA, and information on death from the Central Bureau of Genealogy (CBG), a nationwide registry

of all deceased Dutch citizens. Before 1994 this registry was based on paper records "persoonskaarten"; since October 1994 this registry was digitalized. For a detailed description of NCR and PALGA, see chapter 6.3.2.3 on reference groups.

The Surveillance Committees of the NCR and PALGA allowed us to link all study subjects (both responders and non-responders) under strict privacy procedures, so that non-response bias was kept to a minimum. Only women who actively refused to participate in the study were excluded from linkage, which concerned only 5% of women. Another advantage of linkage to population registries was that all outcomes were medically verified. However, there were also some limitations: 1) incomplete data before 1989 and 2) the possibility of duplicate linkages.

Incomplete data before 1989

Since both PALGA and the NCR only reached national coverage in 1989[37], cancer and CIN outcomes that occurred before 1989 were incompletely registered (studies on effectiveness of screening and cancer prevalence). Therefore, all diagnosed prevalent cancers before 1989 were medically verified by mailing short questionnaires to treating physicians, but only after informed consent from the participant was obtained (80%). With respect to incomplete screening histories in PALGA, cases diagnosed before 1989 were excluded from analyses (chapter 4), which affects generalizability of the study. Evaluation of the effectiveness of screening among case diagnosed before age 30 (mainly diagnosed with CCA) was therefore not possible, which was unfortunate since screening might have had a different effect at younger ages.

Duplicate linkages

Since the linkage code used by us was not always unique, especially for common surnames (like "Jansen"), duplicate linkages were possible, linking the wrong records to a DES-net cohort member. In such case it is imperative to carry out additional checks to reduce false positive linkages. With respect to the PALGA linkage, we could only perform a few additional checks (first initial and place of birth), but these were available for responders only. As a consequence some linkage errors may have remained unnoticed. On the other hand we might have wrongly deleted too many linkage records when the initials did not match. Regarding the linkage with

the NCR, more checks were possible to determine correct linkages (like place of birth but also e.g. place of last residence).

6.3.5 Selection bias

Selection bias in cohort studies may arise if the chance to stay in the study is 1) not equally distributed among exposure categories and 2) dependent on disease status at the end of the observation period[38]. In other words, when loss to follow-up occurs that is associated with exposure of interest and also dependent on the disease outcome. Fortunately, loss to follow-up in the studies described in this thesis is minimal, since our main outcomes of interest (cancer and CIN incidence) are collected through linkage with nationwide registries, yielding very complete data and both responders and non-responders to the questionnaire were linked (see also paragraph on assessment of outcome). Still, selection bias might be present in some of the studies described in this thesis due to 1) diagnostic suspicion bias and 2) non-response to the questionnaire. Both will discussed in the next sections.

Diagnostic suspicion bias

Diagnostic suspicion bias means that knowledge about possible negative effects of an exposure influences intensity and the outcome of the diagnostic process[39]. In case of DES exposure, knowledge about adverse health effects might result in 1) a more intensive medical surveillance, not only of the women themselves, but also of their children and 2) a different interpretation of diagnostic outcomes. An example of the first potential bias might be the detection of milder types of hypospadias in sons of DES daughters, whereas in unexposed women minor malformations may remain unnoticed. Another example is the intensive cytological screening of DES daughters, resulting in an increased detection of transient CIN1 lesions that would be left undetected if screening was less frequent. An example of the second type of bias is that the presence of adenosis in a DES daughter might trigger the physician to be suspicious about a possible cancer risk, resulting in more colposcopy guided biopsies, and also in assigning the diagnosis of dysplasia (misclassification bias). Although diagnostic suspicion bias might be present in the studies described in this thesis, we could partially adjust for it (e.g. by adjustment for screening frequency). Also, our results on severe health outcomes (like

penoscrotal hypospadias or invasive cancer) can be expected to be much less influenced by diagnostic suspicion bias.

Non-response to the questionnaire

The overall analyses on incidence of cancer and CIN did not suffer from selection bias due to non-response, since these outcomes could be assessed for both responders and non-responders by linkage to the NCR and PALGA. Only refusers (5%) could not be linked with the NCR and PALGA. However, in the analyses that were restricted to women with questionnaire data (e.g. subgroup analyses), or the studies that used prevalence as major outcome or used medically verified diseases for which additional informed consent was needed (cancer prevalence, hypospadias, DES-related malformations), selection bias might be present.

In the next sections the impact of selection bias on the results of the different studies will be discussed.

Selection bias in the cancer prevalence study

The high **non-response** (59%) to the survey questionnaire mailed to all women registered at the DES Center in 1994 may have introduced selection bias. We tried to quantify the selective response by using two marker-tumors (colon cancer and melanoma), for which no association with DES was assumed. The analyses revealed that both tumors occurred more often among our DES-exposed study subjects compared to the general population, indicating that, indeed, selection bias appeared to be present. However, unexpectedly, we found an increased risk of melanoma in DES daughters a few years later in the cancer incidence analyses (chapter 2.2), so melanoma proved not to have been a good marker for selection bias after all. Nevertheless, we strongly doubt whether we succeeded in complete adjustment for selection bias in the cancer prevalence study. Moreover, the fact that we did not confirm the increased risk of cervical cancer in our subsequent analyses on cancer incidence and CIN (chapter 3) further underscores the potential for residual bias in this study.

Selection bias in the studies on hypospadias among DES grandsons

The response to the questionnaire in the OMEGA- and DES-net studies was quite comparable (67% and 61%, respectively). It is conceivable that DES daughters with children with congenital

abnormalities were more inclined to *respond* to the questionnaire or to *report* congenital malformations in the questionnaire. In this paragraph we will focus on selection bias. For the discussion of reporting bias, see 6.3.4. Because selection and reporting bias are strongly connected, arguments mentioned in this paragraph might be also applicable to reporting bias.

In the **OMEGA study**, the prevalence of hypospadias was compared between sons of (subfertile) DES daughters and unexposed (subfertile) women. The problem of selection bias was extensively addressed. Since all OMEGA participants were diagnosed with subfertility problems, it seems unlikely that response to the questionnaire (because of children's health status) differed between DES-exposed and unexposed women, which could have biased the results. Furthermore, in the OMEGA study the prevalence of other congenital malformations was also assessed (but not medically verified) and congenital heart defects did not appear to be increased in the DES-exposed group compared to the unexposed group (PR=1.5, 95%CI=0.2-11.1, based on 29 cases), suggesting that no major selection bias was present.

In the **DES-net study**, the prevalence of hypospadias was compared between sons born to DES daughters and sons of unexposed sisters. Similarly, no increased risk of congenital heart diseases was observed**,** although the mechanisms of selection bias in DES-net may differ from OMEGA since not all women had subfertility problems. Moreover**,** there was a substantial additional non-response in the process of medical verification of the reported diseases**.** We did a sensitivity analyses in which we included all non-responders (n=13,309 women, with an estimated number of 14,255 children). The risk (prevalence ratio, PR) of penoscrotal hypospadias remained increased, although no longer statistically significant when compared to the Rotterdam cohort, which was the most conservative estimation (PR=3.0, 95%CI=0.6-14.7); compared to EUROCAT the PR was 4.4 (95%CI=1.9-10.4). Thus, selection bias in the DES-net-study, although it could not be entirely excluded, does not seem to explain the increased risk of penoscrotal hypospadias.

Selection bias in the studies on incidence of cancer and cervical intra-epithelial neoplasia

Selection bias due to non-response to the questionnaire did not affect the results of the overall analyses on incidence of cancer and CIN, since we could both link responders and non-responders with the NCR and PALGA. However, selection bias might be present in subgroup analyses restricted to women with questionnaire data. Indeed, we found higher risks for cervical and vaginal cancer among women with questionnaire data, although risks for most

other cancers were quite similar to those of the total study population (chapter 2.2). With respect to women with DES-related malformations, we only had information on such malformations when questionnaire data were available. In some analyses we used self-reported malformations (cancer incidence, chapter 2.2) and in other analyses we used medically verified malformations (CIN, chapter 3). In the latter group additional non-response was present (because not all malformations were medically verified, since, for instance, the medical record was destroyed). In subgroup analyses stratified by DES-related malformations, selection bias may have occurred if the outcome took place before the date of questionnaire completion (cancer incidence, chapter 2.2). In the CIN analysis (chapter 3) the period of diagnoses followed the date of questionnaire (2000-2008), so selection bias is less likely. To investigate reasons for non-response to the questionnaire, we conducted a study among 948 non-responders. Of the women who did not want to participate, 31% reported to be certain after all not to have been exposed to DES *in utero* (but this information was generally **not** based on medical file information), 16% reported to have doubts regarding DES exposure, 21% reported emotional problems and 19% no longer wanted to be confronted with DES. The reasons for non-responding were quite different, thus selection bias might have affected the results of the analysis using questionnaire data in either direction (from or towards zero).

In conclusion, by conducting the cancer incidence study we solved the potential selection bias in the cancer prevalence study. Some selection bias may still be present in analyses restricted to the group with DES-related malformations where the outcome occurred before the date of completion of the questionnaire.

6.3.6 Confounding

A limitation of all of the studies discussed in this thesis is on the one hand the lack of information on potential confounders and on the other hand the inability to adjust for confounding when we used the general population as a reference. To be a confounder a risk factor for the outcome concerned should also be related to the DES use of the mother. Although certain familial or socio-economic status-related risk factors (like overweight) may be such potential confounders, it is not very likely that they will have strong effects. Behavioral changes because of awareness of being a DES daughter is another issue. Because of an enhanced health awareness DES daughters might reduce their smoking behavior, may express

more careful sexual behavior (lowering the risk of HPV infection), and most likely, will increase their screening behavior. Therefore, some residual confounding still might occur in some of our analyses.

Smoking

In the DES-net questionnaire we did not collect data on cigarette smoking. This turned out to be an issue in the DES-net cancer analyses when we found a decreased risk of **lung cancer** in DES daughters. We tried to estimate the level of confounding by smoking by estimating the proportion of smokers among 205 DES daughters in the OMEGA cohort (chapter hypospadias OMEGA); indeed, it appeared that DES daughters smoked a bit less than unexposed subfertile women (13.2 cigarettes and 15.9 cigarettes, respectively. Although women in this OMEGA cohort were not representative for the total population of DES daughters, we think that it is justified to conclude that the reduced risk of lung cancer might be largely, if not completely, due to the smaller percentage of smokers among DES daughters.

Parity

In our cancer incidence analyses, we found no increased risk of breast cancer in DES daughters compared to the general population. Differences by nulliparity might have occurred since we lacked data on this variable in our non-responders. It is known that nulliparity is associated with a reduced risk of **breast cancer** at young ages and an increased risk at older ages. At the other hand, differences in the proportion of nulliparous women in the cohort of DES daughters as compared with the general population, may also be considered the result of DES exposure (nulliparity as intermediate variable). Indeed, we found a higher percentage of nulliparity among DES daughters (33%) compared to 17% in the general population. To further explore the nulliparity effect we conducted a partially stratified analysis comparing strata of nulliparous and parous DES daughters with the general population. However, results in both strata were similar. Thus, nulliparity did not appear to explain the inconsistent findings on risk of breast cancer as compared with the NCI-DES study.

6.4 Biological mechanisms

In this paragraph the possible biological mechanisms underlying the teratogenic effects of exposure to DES *in utero* are described. Mechanisms will be addressed which might explain the elevated risk of CCA in the second generation and the risk of hypospadias in the third generation. Different mechanisms have been proposed, among which are direct (ERα-mediated) actions of DES exposure *in utero* on the expression of genes involved in the development of the urogenital tract and epigenetic effects.

Metabolism of DES

Diethylstilbestrol is a synthetic non-steroidal estrogen that is rapidly absorbed from the proximal gastro-intestinal after oral administration (reviewed in [40]). Plasma levels reach a peak after 20 to 40 minutes and then decrease and level off. The half-life for the second phase is 2-3 days. Studies in rhesus monkeys and mice (hamster, rat) have shown that DES is able to cross the placental barrier and that it accumulates in the fetal reproductive tract with levels three times higher than in the fetal plasma[41;42]. In mice, peak concentrations of radiolabeled DES were highest in fetal liver, followed by intestine, plasma and fetal reproductive tract. Moreover, oxidative and conjugative metabolism occurs in the fetus[42].

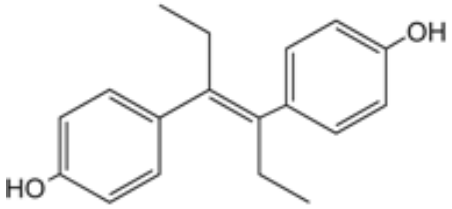
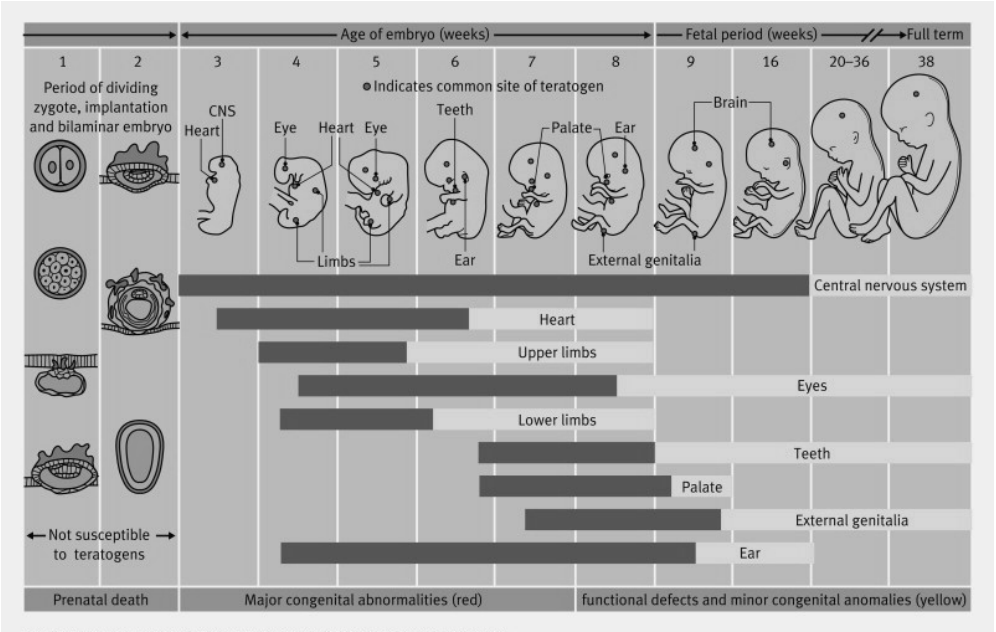


Figure 6.1 Chemical structure of DES

Normal fetal development

In normal fetal development, internal genitalia in humans are formed out of two embryonic structures: the Wolffian ducts (which form male genitals: the testicles, epididymus and urethra) and the Müllerian ducts (which form the female genitals: the ovary, the uterus and the upper part of the vagina[43]. Both structures, which each consist out of two symmetric ducts, are



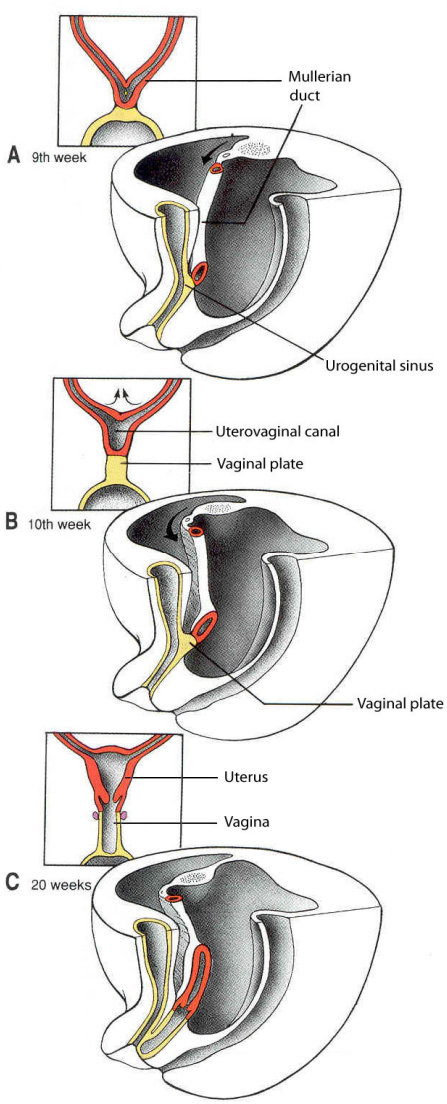
Dark grey indicates highly sensitive periods when teratogens may induce major anomalies
Source: Moore KL, Persaud TVN. *The Developing Human: Clinically Oriented Embryology*. 6th edn. Philadelphia: WB Saunders Company, 1998: 548.

Figure 6.2 Critical periods in human development

present in both male and female fetuses during the embryonic period. In absence of male hormones in females the Wolffian ducts regress (from week 10), and in males the Müllerian ducts regress. This process is under control of the hormones produced by the fetal testes (reviewed by McLachlan *et al*[44]). The configuration will be female unless the fetal testis intervenes by secreting Müllerian Inhibiting Substance (MIS) and testosterone to maintain the Wolffian duct (review in: [44;45]). During embryogenesis the human vagina develops from a fusion of the Müllerian ducts (which will form the uterovaginal canal) with the urogenital sinus at approximately 8 weeks. When the top of the uterovaginal canal have reached the urogenital sinus, the columnar cells of the uterovaginal canal are replaced by squamous epithelium that arises from the vaginal plate[46], see figure 6.3.

Malformation of the Müllerian ducts

The DES-induced reproductive malformations have been hypothesized to be caused by a disturbance of normal differentiation and regression of the Müllerian duct and the Wolffian duct in females and males, respectively. Incomplete regression of the Müllerian ducts **in females** leads to all kinds of DES-related malformations like vaginal ridges, T-shaped uterus, cervical collars and adenosis[30]. The teratogenic effect of DES is hypothesized to act through downregulation of **Wnt** and **Hox genes** that play a significant role in the normal development of the reproductive tract. In mice, DES downregulates Hoxa genes expression along the anterior to posterior axis of the developing Müllerian duct[47]. In each part of the Müllerian duct (from anterior to posterior) different Hoxa genes are expressed, responsible for the differentiation of the duct into the different female reproductive organs. Expression of Hoxa-9 occurs at the location where the Müllerian duct differentiates into the fallopian tubes, Hoxa-10 expression occurs at the location of the uterus, Hoxa-11 expression at the location of the uterus and uterine cervix and Hoxa-13 expression at the location of the upper vagina[48;49]. DES disrupts Hox gene expression by a posterior shift of the gene expression resulting in an anterior transformation. For example, Hoxa-10 and Hoxa-11 expression are decreased at the location where normally the uterus would develop; instead Hoxa-9 is expressed, resulting in a uterus developed like an oviduct (T-shaped uterus). Another example is the presence of



Source: Larsen WJ. *Human embryology*. 2nd edition ed. New York: Churchill Livingstone, 1997

Figure 6.3 Development of the human vagina

glandular tissue in the vagina that would normally occur in the uterus (resulting in adenosis). The malformations of the reproductive tract due to the decreased Hoxa-10 expression (as well as Hoxa-11 and Hoxa-13) in neonatal mice appeared to be similar to the malformations *in utero* DES-exposed women. In mice, DES was found to delay the onset of the development of the Müllerian ducts[45]. Like Hoxa knockout mice, Wnt7a knockout mice appear to have malformations that closely resemble the malformations of the reproductive tract of neonatally DES-exposed mice. Altered expression of Hoxa genes might be secondary to the disruption of the Wnt7a gene expression[50]. The downregulation of Hox- and Wnt genes is found to be estrogen receptor mediated (in mice)[51].

In **males**, DES might disrupt normal regression of the Müllerian duct, resulting in epidymal cysts (which are remnants of the Müllerian duct)[45].

Carcinogenic effects of DES

Although DES has not been shown to be mutagenic in the Ames test, studies have shown that DES might disrupt DNA replication in several ways[41;52;53]. When DES is used by pregnant women from the 7th week of pregnancy onwards, the estrogen passes the placenta in a period when breasts and gonadal organs go through critical phases in their embryonic development (see figure 6.2). The presence of hormone receptors may subsequently enable DES to reach the nucleus and to directly interfere with fetal mitotic processes by binding to tubulin, thus inducing genomic instability[53]. Simultaneously or alternatively, DES metabolites may bind to DNA. Binding on coding areas of mismatch repair genes may leave the replication errors unfixed, whereas binding on coding areas of tumor suppressor genes or oncogenes may result in a growth advantage of these cells. Neonatal DES exposure might induce decreased apoptosis in the vagina and uterus of adult mice, through downregulation of Fas (member of the tumor necrosis factor receptor family, signaling cells to go into apoptosis) and persistent expression of Bcl-2 (protects cell against DNA damage, but overexpression inhibits apoptosis)[54]. Also TNF-α, (tumor necrosis factor alfa) appears to be downregulated by DES[55]. Furthermore, neonatally exposure to DES might increase cell proliferation through induction of growth factor genes (like epidermal growth factor (EGF), transforming growth factor- α (TGF-alfa) and possibly (but not consistently found) Insuline-like Growth Factor-I (IGF-I)[56;57]. Also other estrogen responsive genes, like the lactoferrin gene, are induced by DES exposure (see review [58]).

Epigenetics

Epigenetics is a process by which heritable modifications in gene expression may occur while the genomic DNA sequence remains unchanged. These modifications are mediated through, for instance, changes in DNA methylation, modification of histones and changes of the chromatin[59;60]. The fact that *in utero* exposure to DES results in permanent changes in gene expression, long after the exposure has stopped, suggests that epigenetic mechanisms might play a role. Mice studies have demonstrated that neonatal DES exposure might induce hypomethylation of certain genes (c-fos (a gene that promotes uterine cancer)[61;62] or permanent hypermethylation of other genes (Hoxa-10 genes)[63], although not confirmed by Li *et al* [64]. Yet, although altered gene expression induced by DES has been demonstrated, it remains unclear by which molecular mechanism intrauterine DES exposure leads to tumorigenesis specifically in the reproductive tract.

Transgenerational effects (hypospadias)

In humans, so far, the first (and only) consistently transgenerational effect of DES that has been consistently described in the literature, is the increased risk of hypospadias among sons born to DES daughters[16-19]. The etiology of hypospadias is largely unclear[65;66]. Several environmental and genetic factors have been examined, among which were low birth weight, intrauterine growth retardation, placental malfunction[65-67], maternal or paternal exposure to environmental chemicals (like pesticides, PCB and DDT) and exposure to exogenous sex hormones. Interestingly, candidate genes for hypospadias that have been examined in humans are homeobox genes, genes coding for enzymes needed for biotransformation of sex hormones (like the gene coding for 5a-reductase type II which converts testosterone into dehydroxytestosterone), or genes coding for hormone receptors (like androgen receptor) (reviewed in: [65]). The **biological mechanism** of a third generation effect of DES on hypospadias through the maternal lineage is still unclear. Several mechanisms have been postulated, like (epi-)genetic changes to the primordial oocytes of the DES daughters, thus directly affecting the next generation. Another hypothesis is that DES exposure *in utero* has caused epi-genetic effects in somatic cells of the fetus (for instance in the Androgen Receptor gene). A remarkable finding in this respect is that the elevated risk of hypospadias was only observed through maternal lineage, not through paternal lineage[16;19], although paternal lineage has not been investigated intensively (also noted in[58]) rendering this hypothesis less likely.

In view of our findings of a higher risk of penoscrotal hypospadias among sons of women with uterine cavum malformations, also observed by Kalfa *et al*[19], a more likely explanation might be that placental malfunction interferes with the development of the male urinary tract in sons born to DES daughters. The underlying mechanism of the association between **placental malfunction** and hypospadias is unclear. The most severe (placental-mediated) intrauterine growth retardation starts during early gestation (week 7 and 8), when the male genitals are developed[21]. Fetal testosterone secretion is found to be under placental control, suggesting that a disturbance in fetal androgens levels by insufficient placental function might lead to hypospadias[21;68].

In general, transgenerational effects of DES exposure *in utero* (other than hypospadias) have been mainly studied in animal studies. Increased risks of uterine tumors have been found in both the F1 mice (DES daughters) and F2 mice (DES grand-daughters)[69;70], although the incidence was lower in the F2 generation. Furthermore, F1 mice differed from F2 mice, in the sense that they were subfertile whereas F2 mice were normally fertile[70].

In a recent review by Skinner *et al* [71], it has been questioned whether the endocrine disrupting effect of diethylstilbestrol is transgenerational, since the phenotypes found in the F1 en F2 generations of neonatally DES-exposed mice differed (and no major phenotype was found in the F3 generation in rats[58]), whereas a true transgenerational effect would produce similar phenotypes. Therefore, intrauterine DES might rather be seen as a multigenerational exposure, implying direct exposure of the individual, fetus or germline, without permanent alterations in the epigenetic programming. Future studies will be needed to further investigate possible transmission of DES-induced diseases to next generations.

6.5 Conclusions and clinical implications

CCA, cervical cancer and CIN

The studies described in this thesis show that the overall risks of cancer and cervical dysplasia in DES daughters are not increased. Nevertheless, two important findings urge the need for the continuation of the surveillance of DES daughters. First, the risk of CCA among DES daughters appears to remain increased at older ages. This seems to be in concordance with recent data from the US SEER cancer registry where a second peak of CCA after age 40 was observed among women born in the period that DES was prescribed (based on ecological data), compared to women of the same age born prior to the DES era[72]. However, based on the Dutch CCA registry, absolute numbers of DES-related cases declined in the most recent period (2001-2005)[28], although this might be partly due to the inability to verify DES exposure. Secondly, DES daughters with DES-related malformations more often had moderate/severe dyskaryotic smears and appeared to have a higher risk of developing squamous cell cancer of the cervix/vagina, despite treatment of the abnormal smears according the guidelines.

Surveillance and screening

These findings underscore the importance of keeping DES daughters under surveillance, especially when DES-related malformations are present, where the main purpose is not the prevention of invasive cancer, but the diagnosis of invasive cancer at an *early* stage. At the same time one needs to be cautious with respect to invasive diagnostic procedures and treatment since overdiagnosis seem to occur in a group of women which is already strongly medicalized. Our study on the incidence of CIN among DES daughters (chapter 3) showed an increased risk of CIN1, especially in DES daughters who were screened more frequently compared to the general screened population, Therefore, an important concern is how to organize the surveillance for DES daughters. Our results indicate that vaginal cancer (9 CCA and 2 squamous cell cancers) could not be prevented by cytological smears (even when smears were taken from the vagina). Furthermore, the detection of (clear cell) adenocarcinoma and precursors might be difficult and successful removal of precursor lesions hard to achieve. In women with squamous cell cervical cancer many abnormal (dyskaryotic) smears occurred preceding the diagnosis. Thus, management of severe/moderate dyskaryosis did not appear to be successful. However, screening on squamous cell cervical cancer seemed to become more

efficient at higher ages, probably due to the decrease of adenosis. Recently, the Dutch Health Council ("Gezondheidsraad") has made a proposal for reorganization of the general population screening on cervical cancer. In this proposal, cytological smears will be first tested on the presence of HPV and if positive be followed by a cytological test of the (same) smear for the detection of abnormal cells. With regard to the frequency of cervical screening, in women aged 30-40 a Pap smear is recommended every 5 years and from age 40 onwards every 10 years until age 60 (in total 5 Pap smears during a woman's life). Since it is unclear whether DES-related tumors are HPV-related and whether single testing on HPV will be adequate for DES daughters, it might be recommended that cytological evaluation of smears in DES daughters remains required and will not be restricted to the evaluation on the presence of HPV. Furthermore, the screening frequency of once per 5 years seems minimal for DES daughters especially when a woman has DES-related malformations like (former) adenosis or has a screening history of HSIL. Therefore, we would recommend that these DES daughters have at least one cervical smear per three years.

Breast cancer

Our findings do not indicate that DES daughters have an increased breast cancer risk. Therefore, recommendations for more intensive mammographic screening or lowering of the age at first screening do not seem to be justified. In the United States no specific recommendations for DES daughters are given, but women are (urgently) advised to follow the recommendations for the general population[73]. These recommendations include clinical breast examinations every 3 years (in women aged 20-39), annual clinical breast examinations in women aged 40 and older, annual or bi-annual mammograms (in women aged 40-49) and annual mammograms from age 50 onwards. Also monthly breast self-examinations are recommended. The recommendations for the general population in the Netherlands are restricted to biannual mammograms between age 50 and 75 and are, thus, less intensive. As we could not confirm an increased risk of breast cancer among DES daughters, so far we do not see a reason to advise a more frequent mammographic screening for DES daughters.

Hypospadias

The risk of penoscrotal hypospadias among sons of DES daughters appeared to be increased compared to unexposed sons. Biological mechanisms are still unclear, although our study

suggested that congenital uterine cavum restrictions in DES daughters might play a role. This would imply that the teratogenic effect of DES stops in the third generation and would not affect next generations. Screening for hypospadias in sons of DES daughters does not seem to be necessary as severe forms of hypospadias come to clinical attention anyway or become clinically apparent.

6.6 Recommendations for future research

Medical files

One of the major problems encountered is this study was the lack of medical confirmation of DES exposure, which was mainly due to the fact that medical files had been destroyed. To be able to evaluate long-term health risks of medication or treatment in general, we consider it important that medical files are kept much longer than 15 years, as the law currently prescribes, and preferably, as long as 100 years after date of birth.

Dutch social security number

Since 2007, a unique personal identifier for all inhabitants of the Netherlands has been implemented by all governmental organizations, the “Burger Service Nummer (BSN)”. Additionally, in 2008 all healthcare providers, defined as such by the law “BIG”, record the BSN for each patient. The possibility to use of the BSN number for data exchange with health registries in epidemiological studies, like the ones described in this thesis, would greatly simplify the procedure of linkage and would improve the quality of the follow-up data, by increasing the percentage of true positive matches and by decreasing the work involved in checking of potentially false positive matches. Therefore, we would like to stress the importance of the availability of the BSN number to researchers, obviously in agreement with the current privacy legislation.

Extended follow-up

Since DES daughters in our study were still relatively young at the end of follow-up (median age at the end of follow-up in November 2008 was 42.0 years), extended follow-up of the DES-net cohort is warranted and will increase power. Also, longer follow-up will give more insight into the question of whether long-term risk of specific cancers will increase as the cohort is aging,

with special attention for the risks of breast cancer, cervical cancer, melanoma and CCA. In future studies, the comparison group existing of non-exposed sisters can also be linked to the NCR and PALGA with sufficient power, enabling internal comparisons and adjustment for potential confounders.

Reproductive problems and infertility

Since most DES daughters are now at the end of their reproductive life span (> 45 years of age), it would be interesting to examine to which extent DES daughters differ from their unexposed sisters with respect to reproductive outcomes, infertility and age of menopause, in order to validate the findings by the NCI-DES study with more power[74-76].

Outcomes of infertility treatment

Limited data are available on the outcome of assisted reproductive techniques (ART) in women with a history of *in utero* exposure to DES[77-80]. It has been suggested that IVF treatment might be less successful in DES daughters with respect to pregnancy rates per embryo transfer, implantation rates and pregnancy outcomes. In the OMEGA study (described in chapter 5.1) detailed medical file information on IVF treatment has been collected and the study included 463 DES daughters. So the dataset is already available to conduct this analysis.

Risk factors for CCA

In the United States, risk factors for the development of DES-associated CCA have been studied by comparing DES-associated CCA cases from the CCA registry with DES daughters participating in the NCI-DES follow-up study[81]. Since we have a sufficient number of CCA cases in the DES-net study (n=59), pooling with the data of the Dutch CCA registry is not necessary to conduct a similar analysis on cofactors that might influence the development of CCA. Interesting factors to study are factors such as hormone use and family history of cancer. It would be interesting to study timing and dose of DES exposure, but regretfully we have only sparse and incomplete information on these variables.

Role of HPV in cervical cancers (excluding CCA)

It would be of much interest to investigate whether the gynecological cancers in DES daughters are HPV-related. In a recent study by Kocken *et al*, tumor specimens of 21 CCA patients

registered at the CCA registry in Nijmegen (among whom 10 were exposed to DES) were tested for hrHPV[82]. In none of the DES-related tumors a causal role of hrHPV could be identified. Unfortunately, for the remaining patients registered at the CCA registry no tumor material was left, and therefore the number of patients examined was small. To further improve our understanding of the role of HPV in DES-associated cervical and vaginal cancer, a new study may be conducted to test HPV, not only in (newly diagnosed) patients with CCA, but also in patients with other morphologies, such as squamous cell cancer.

Third generation

At the time the studies described in this thesis were conducted, the National Perinatal Database had just started[83]. Therefore, linkage with the National Perinatal Database was not yet possible. Future linkage might be a good option to complete our data on congenital disorders, so that the prevalence of all congenital disorders occurring in the children born to DES daughters can be examined with a larger sample size of DES grandchildren. Again, for the sister comparison group the same linkage might be conducted, so that internal comparisons can be made. It is also important to conduct research into potential biological mechanisms underlying the increased risk of severe hypospadias in sons of DES daughters. Furthermore, it would be interesting to collect additional questionnaire data on reproductive problems and DES-related malformations (such as a T-shaped uterus) in the DES granddaughters, as was done in the NCI-DES study[84]. Data on congenital malformations of the reproductive tract are not available in the National Perinatal Database[83], since these malformations only become apparent when women reach the fertile age and the registry is restricted to malformations observed by clinicians and midwives until 28 days after birth. In this respect, the EUROCAT database will probably also be incomplete. If we can confirm in future studies that DES granddaughters do not have an increased risk of reproductive problems, this would, together with the findings on hypospadias in DES grandsons, suggest that DES effects come to an end in the third generation.

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