IN UTERO ENVIRONMENTAL EXPOSURES AND REPRODUCTIVE ENDPOINTS

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ABSTRACT

MAUREEN COONEY: *In utero* environmental exposures and reproductive endpoints
(Under the direction of Anna Maria Siega-Riz)

The etiology of age at menarche, infertility, and endometriosis, three common reproductive outcomes is largely unknown. Environmental chemicals exhibiting endocrine disrupting behavior have recently been implicated in a number of reproductive disorders, however little research has been done to examine the potential effects of these chemicals on adult reproductive health when the woman is exposed *in utero*. This research focuses on the effects of exposure to cigarette smoke and diethylstilbesterol (DES) experienced *in utero* and three reproductive outcomes: age at menarche, infertility, and endometriosis.

Using data from over 5,000 women enrolled in the National Cooperative DES and Adenosis Study (DESAD) and a subsequent follow-up we were able to ascertain *in utero* exposures from the mother and subsequent health outcomes from the daughters later in life. Overall, we found no suggestion for a delay or advance in age at menarche for women who were exposed *in utero* to tobacco smoke. Furthermore, we found null results for the associations between *in utero* tobacco smoke and self-reported infertility and endometriosis. This is in contrast to a few earlier studies which have found effects. We determined that women exposed *in utero* to DES had a 70% increase in the odds for developing endometriosis compared to women who were unexposed after controlling for age. We also used these data to determine whether self-reported age at menarche later in life is a reliable measure. Women were asked around the age of puberty to report their age at menarche, and

they were queried on this same information approximately twenty years later. We found that self-report of age at menarche later in life is not reliable when exact age at menarche is required, but the reliability is good within a year of the first reported age at menarche. The only covariates slightly associated with discordant responses were young age (<30 years) at the time of the follow-up survey and originally reported late age at menarche (>14 years). These three papers add to a small body of literature and address critical data gaps regarding the potential effects of the intrauterine environment on later reproductive health. These findings stress the importance of limiting potentially harmful exposures to pregnant women, as health effects may not only be seen at birth, but also years into adulthood. Furthermore our finding with respect to DES highlights the possibility that other endocrine disrupting chemicals, both pharmaceutical and those found in our environment may have similar adverse reproductive effects.

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ABBREVIATIONS

BMI	Body Mass Index
CI	Confidence Interval
CPP	Collaborative Perinatal Project
DAG	Directed Acyclic Graph
DDT	Dichlorodipehnyltrichloroethane
DES	Diethylstilbesterol
DESAD	National Cooperative Diethylstilbesterol and Adenosis Study
EDC	Endocrine Disrupting Chemical
GEE	Genearlized Estimating Equations
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamic-Pituitary-Gonadal
IRB	Institutional Review Board
LBW	Low Birth Weight
NCI	National Cancer Institute
NGS	Newton Girls Study
NHANES	National Health and Nutrition Examination Study
NHS	Nurses' Health Study
NSFG	National Survey of Family Growth
OR	Odds Ratio
PCB	Polycholorinated Biphenyl
PBB	Polybrominated Bipenyl
SD	Standard Deviation

SES	Socioeconomic Status
SGA	Small-for-Gestational-Age
TTP	Time-to-Pregnancy

CHAPTER I

INTRODUCTION

Overview

Age at menarche, infertility, and endometriosis are reproductive outcomes which affect millions of women and can cause physiological, psychological, and financial burden for women in this country and worldwide. Early age at menarche is known to be associated with eating disorders, early initiation of sexual intercourse, higher sexually transmitted disease and teenage pregnancy rates (Deardorff et al. 2005;Stice et al. 2001). Infertility affects approximately 10-20% of couples trying to conceive and can cause psychological as well as financial problems (Redshaw et al. 2007;Cousineau and Domar 2007). Endometriosis is a disease which can cause severe pelvic pain, infertility, and can account for many days of lost work due to debilitating symptoms (Missmer and Cramer 2003;Eskenazi and Warner 1997).

The etiology of these three outcomes is as yet unknown and while genetics play a role, evidence is beginning to accumulate that indicates there may be an environmental influence on these outcomes as well. Endocrine disrupting chemicals (EDCs) are anthropogenic compounds that block or mimic the action of endogenous hormones. There is a long list of chemicals that are considered EDCs; however, this proposal will concentrate on only two of these many: Diethystilbesterol (DES) and cigarette smoke. DES is a synthetic non-steroidal estrogen that was synthesized in 1938 and was prescribed to millions of

pregnant women over the course of three decades (1938-1971) with the mistaken belief it would prevent miscarriage and even enhance pregnancies by lengthening gestation and increasing birth weight. Cigarette smoke contains a mixture of over 4,000 chemicals including several which are considered to be EDCs (specifically those belonging to the polycyclic aromatic hydrocarbon family). Adult exposure to cigarette smoke is perhaps one of the most well studied environmental toxins, but the literature regarding *in utero* exposure to cigarette smoke is sparse.

This study uses data collected from women (both mothers and daughters) participating in two studies: National Cooperative Diethylstilbesterol and Adenosis Study (DESAD) cohort and the National Cancer Institute (NCI) DES Follow-up Study. Currently there is a dearth of knowledge regarding the intergenerational effects of environmental exposures and reproductive endpoints. This study will contribute to a sparse and sometimes absent literature regarding the *in utero* effects of these chemicals on reproductive outcomes. Furthermore, it will improve upon previous studies' exposure assessments because the exposure is captured across generations by mothers and daughters. This differs from the few previously published studies that use proxy exposure information (daughters reported their mothers' habits while they were pregnant).

Specific Aims

The dissertation has four specific aims which are contained in three manuscripts. The first aim is contained in paper one, the second, third, and fourth aims are addressed in paper two, and the fifth aim is contained in paper three.

Aim 1. To determine the reliability of self-reported age at menarche later in life using measures taken at puberty and approximately 20 years later.

Aim 2. To assess the association between *in utero* exposure to cigarette smoke and age at menarche among women not exposed to DES.

Aim 3. To examine the association between *in utero* exposure to cigarette smoke and infertility among women not exposed to DES and explore any interaction between this association and adult cigarette smoke exposure.

Aim 4. To examine the association between *in utero* exposure to cigarette smoke and endometriosis among women not exposed to DES and explore any possible interaction with adult cigarette smoke exposure.

Aim 5. To determine the association between *in utero* exposure to DES and endometriosis and explore any interactions with *in utero* cigarette smoke exposure and adult cigarette smoke exposure.

Background and Significance

The intrauterine environment may play an important role in determining future reproductive health for women. There is an evolving body of literature that suggests environmental exposures experienced *in utero* may result in higher risk status for adult onset diseases. This type of research is referred to as the early origins of disease hypothesis (Barker 1992). The time spent *in utero* may represent a critical window of exposure during which environmental insults may permanently and irreversibly alter germ cells and the development of the reproductive system in women.

Age at menarche is the age in years when a girl experiences the onset of her first menstrual period. Age at menarche is thought to be important not only as a sentinel event but also as a risk factor for adult disease and possibly as a marker of increased exposure to environmental agents.

A secular decline in the age at onset of puberty has been reported for some but not all developed countries (Castellino et al. 2005;Lindgren 1996;Parent et al. 2003). A recent paper reviewed age at menarche data from National Health and Nutrition Examination Study (NHANES) from 1988-1994 and compared this to data collected by NHANES in 1999-2002 (Anderson and Must 2005). The authors found that the average age at menarche decreased from 12.53 (95% Confidence Interval (CI) = 12.43, 12.63) years in 1988-1994 as compared to 12.34 (95%CI = 12.24, 12.45) in 1999-2002. These differences were even more pronounced when the results were stratified by race. Mexican American girls showed the greatest change in average age at menarche during this time with a difference of .15 years younger in 1999-2002 as compared to 1988-1994. Whether this difference can be attributed to exposures such as DES and cigarette smoke is speculative since DES exposure during this time period was rare and pregnancy smoking rates were highest in Non-Hispanic white women (Mathews 1998).

Earlier onset of puberty has been associated with earlier initiation of intercourse, substance abuse, higher incidence of sexually transmitted diseases, and teen pregnancy (Deardorff et al. 2005). Numerous psychological issues have also been attributed to girls experiencing an early age at menarche. A recent study indicated both depression and eating disorders were more prevalent among girls who had an earlier age at menarche compared to their peers (Stice et al. 2001).

Early age at menarche has also been linked to outcomes later in life including cardiovascular disease risk and reproductive outcomes including cancers. A recent study found that girls with an earlier age at menarche had an increased risk for cardiovascular disease later in life due to deleterious changes in adulthood including changes in insulin, glucose, and blood pressure (Remsberg et al. 2005). Two studies also found that early age at menarche conferred a higher risk for spontaneous abortions and poor fertility outcomes later in life (Martin et al. 1983;Sandler et al. 1984). Early age at menarche has also been associated with reproductive cancers. In particular, girls with an early onset of menses are at higher risk for breast and endometrial cancers (MacMahon et al. 1982;McPherson et al. 1996). It is unclear whether age at menarche itself is the impetus for this increased risk due to exposure to more menstrual cycles across the lifespan or if there is some shared effect earlier in development that programs the girl for both early age at menarche and high cancer risk in her adulthood.

The consequences of early age at menarche are critical for the reasons outlined above. Despite the importance of this commonly collected variable both as an endpoint and a risk factor for other outcomes, the reliability of age at menarche has not been well studied in populations. In lieu of a biomarker for puberty, self-reported age at menarche is widely used in many epidemiologic studies. It will be important to determine whether this measure is accurate.

Recent estimates from the 2002 National Survey of Family Growth (NSFG) show that 12% (7.3 million) women in the U.S. aged 15-44 experience impaired fecundity, while 15% of married women suffer from impaired fecundity (Martinez GM et al. 2006). The definition of impaired fecundity in this study included women who reported that a) it was physically

impossible for them or their partner to have a baby for any other reason than a sterilizing operation b) it was physically difficult or dangerous to carry a baby to term c) they had been continuously cohabitating or married, had not used contraception, and had not had a pregnancy for three years or longer. In 2002, 7.4% of married women were infertile (infertile was defined in this survey as not having used contraception and also not becoming pregnant within 12 months). Impaired fecundity and infertility as so defined are the cause of enormous emotional and financial burden for the couple trying to become pregnant (Cousineau and Domar 2007;Farley Ordovensky and Webb 2007).

The secular pattern of fertility has declined. This has made it difficult to compare fecundity and fertility across populations and investigators have found that rates differ across countries (Juul et al. 1999). The decline in fertility is in part due to pregnancy intentions and the desire and ability to limit family size. Research has called for the monitoring of fecundity on a population level as changes in fertility may be a sentinel of environmental exposures (Joffe 2003;Olsen and Rachootin 2003), however others have disputed this proposal (Sallmen et al. 2005).

Endometriosis is a complex disease that occurs when endometrial tissue grows outside of the uterus where it is normally found. This ectopic tissue responds to hormonal signals, does not grow normally, and can cause severe debilitating pain and infertility. The most accurate way to diagnose endometriosis is by visualization of the pelvis and some argue histopathology of explants as well through surgery (a laparoscopy or laporotomy). Most women who present with suspected disease are not taken to surgery right away as alternative therapies are available and can ameliorate symptoms of the disease for a large proportion of women. Availability and type of health insurance coverage often dictates treatment options.

For example, many women will be treated with hormonal therapies such as birth control or other drugs which suppress normal ovulatory function.

Because the gold standard of diagnosis requires surgery, not all women are symptomatic, and the severity of symptoms does not correlate with severity of disease, it is very difficult to estimate the prevalence of this disease in the population. Some women present with no symptoms but upon laparoscopy they have very advanced disease, while others have many symptoms and minimal or no disease detected with surgery.

Despite all the challenges in identifying disease in a population, a few estimates of the burden of disease on the population level have been offered. The only population-based incidence figure that persists in the literature comes from a study in Olmsted County, Minnesota where 8,229 women participated in a cohort study (Leibson et al. 2004). In this study, the incidence of endometriosis was 1.9 per 1,000 person-years. Prevalence figures vary considerably. In the general population, among women of reproductive age, the prevalence of disease has been estimated between 10-15% (Houston 1984;Olive and Schwartz 1993). Among women seeking treatment for pelvic pain, the prevalence of endometriosis increases to 20-65% (Strathy et al. 1982;Moen and Muus 1991;Mahmood and Templeton 1991;Wardle and Hull 1993;Carter 1994).

The disease can manifest itself in a number of ways already described. The most common complaint among women with endometriosis is pelvic pain and infertility. When the endometriosis has spread to areas such as the bowel or lung even more serious complications may arise resulting in necessary removal of the colon and even death. A study of women in the U.S. Army found the prevalence of disease to be at 6.2% among the forces accounting for 21,746 days of missed duty in five years of record review (Boling et al. 1988).

Recent studies have linked the presence of endometriosis to autoimmune diseases (Sinaii et al. 2002) as well as cancers of the breast and endometrium (Brinton et al. 1997).

Age at menarche

Genetic causes for early age at menarche have been established as girls whose mothers experienced early onset of menses have a higher risk for early onset themselves (Graber et al. 1995). These interesting results should be interpreted carefully as the study was conducted in a small sample (75 girls) that was homogenous with respect to race and socioeconomic status (SES) which may have influenced the results. Other factors that influence the timing of puberty and the onset of menses have been suggested including stressful life events and physical activity level (Goran et al. 1998;Graber et al. 1995). Both physical activity and stress are difficult exposures to measure. Since these studies were published, improvements have been made in refining measurement of physical activity and stress which warrant further study of this research question with the improved methodology. Perhaps the most commonly cited factor associated with an early age at menarche however is obesity. The declining age of menarche in the U.S. has been attributed to the increasing number of overweight and obese children in this country (Baker 1985;Slyper 2006). One theory supporting this evidence is that early menarche is preceded and perhaps influenced by pre-pubertal hyperinsulinaemia and insulin resistance. Accurate measurements of both body characteristics as well as age at menarche are critical in assessing this association. This is particularly important because it has also been shown that girls who mature early are heavier after the onset of puberty and are often at higher risk for adrenal hyperandrogenism (Ibanez et al. 1993). Finally with respect to EDCs, two studies have shown that girls with higher

blood lead levels reach puberty as defined by early age at menarche or attainment of Tanner stage 2 pubic hair at later ages than girls with lower blood lead levels (Modern Epidemiology 1998;Selevan et al. 2003;Wu et al. 2003).

Less research has focused on the potential effect for prenatal exposures on age at menarche; however, the few studies which have addressed this topic indicate that the early fetal environment may be important. An early study found that girls who were exposed *in utero* to alcohol had a later onset of menses than girls who were unexposed (Robe et al. 1979). This study relied on the daughter's self report of their mother's drinking habits and also on retrospectively ascertained age at menarche. A more recent study examined the association between postnatal exposure to polybrominated biphenyls (PBBs) which are EDCs and age at menarche (Blanck et al. 2000). The authors found that girls exposed to higher levels of PBBs through breast milk had an earlier onset of menses (11.6 years) than girls who were exposed to low levels through breast milk (12.6 years) or those who were not breastfed (12.7 years). This study was limited because there was no actual measure of the chemicals *in utero*. Instead PBBs transmitted via breast milk were a proxy for this exposure. This may have lead to some exposure misclassification. While suggestive, this study also relied on self-reported age at menarche that was asked up to 15 years after the actual event occurred. This presents problems for misclassification of the outcome.

Animal studies support the hypothesis that exposures experienced *in utero* affect future age at onset of menses (Colborn et al. 1993). EDCs in particular have been shown to be hormonally active and induce changes in age at menarche in various animal species. A study of female alligators exposed to dichlorodiphenyltrichloroethane (DDT) contaminated water found large differences in the plasma hormone concentrations and age at which these

levels peaked when compared to alligators in the control lake (Guillette, Jr. et al. 1994). When rats were exposed to DDT a constant estrus syndrome was produced (Heinrichs et al. 1971) and rats exposed neonatally to bisphenol A showed an advance in the age at puberty (Howdeshell et al. 1999). Studies in hamsters have also shown the reproductive toxicity of cigarette smoke, particularly on the ovary and uterus (Magers et al. 1995). These studies in animals provide evidence that EDCs, including those found in cigarette smoke could have human health effects as well, particularly effects related to reproduction and, specifically, the age at onset of puberty.

The timing of onset of menses is under control of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal systems and, thus, exposures experienced during the development of this system may also alter the timing of onset of menses. The onset and progression of puberty itself is actually the final stage of reproductive development and maturation (Forest MG 1990). The synthesis and secretion of lutenizing hormone, follicle stimulating hormone, human chorionic gonadotropin, and prolactin by the pituitary are initiated during fetal life. These hormones are critical in the onset of menses. It has also been shown that fetal pituitaries are capable of gonadotropin synthesis and release *in vivo* (Forest MG 1990).

Smoking's effect on the endocrine system has been well studied. It has been documented that some of the 4,000 constituents in cigarette smoke are reproductive toxins with endocrine reactive properties. Particularly, cigarette smoke has been investigated for its anit-estrogenic activity (Baron et al. 1990;MacMahon et al. 1982;Michnovicz et al. 1986). Smoking during pregnancy has also been shown to be associated with changes in the central nervous system of children, which further suggests the potential for an *in utero* effect on

onset of menses (Eskenazi and Trupin 1995;Naeye and Peters 1984). If the hypothalamic pituitary gonadal (HPG) system takes a hit from an environmental exposure early in development this may re-program the functions later in life; hence, fetal reprogramming.

Two previous studies have examined the association between *in utero* exposure to cigarette smoke and age at onset of menses in human populations. Windham and colleagues (Windham et al. 2004) used data from the Child Health and Development Study, a longitudinal pregnancy study of families in the San Francisco Bay Area of California who were members of the Kaiser Permanente Health Plan. The women were recruited while pregnant during the early 1960s and the children were followed through an adolescent study. Data regarding exposure was captured during the prospective pregnancy study using self reported smoking habits. Age at menarche was ascertained from a total of 994 girls from in person interviews done at a clinic when the girls were between 16 and 17 years old.

The authors categorized age at menarche into early (<12 years) and late (>13 years) with 12-13 years as the referent group. The mean age at menarche of the subjects was 12.96 years with 16% of the girls experiencing onset early and 24% experiencing it late. More than half of the girls were exposed prenatally to cigarette smoke. An earlier age at menarche was observed for those girls experiencing the highest exposures (>20 cigarettes per day) as compared to girls whose mothers did not smoke at all during pregnancy. After adjusting for potential confounders the mean age at menarche was a few months (Difference=-.31, 95% CI (-.65, 0.03)) earlier among daughters whose mothers smoked a pack or more of cigarettes per day compared to girls whose mothers did not smoke.

The study, however, also had some limitations. There was a fair amount of missing data (200 parents (20%) did not report smoking status) which the authors handled in the

analysis by substituting the other parent's value in for the non-reporting parent. Exposure and outcome misclassification were possible as both were self reported, however, bias is unlikely as the exposure was collected before the outcome was assessed. This was the first study to examine the effect of *in utero* exposure to cigarette smoke and age at menarche.

Recently, a second study by the same authors was published (Windham et al. 2008). In this study data from the Collaborative Perinatal Project (CPP) and a subsequent follow-up study were used to examine the association between *in utero* exposure to cigarette smoke and age at menarche in a different population. In this study age at menarche was ascertained when the daughters were young adults and smoking data was collected from the mother as part of the original study which was a prospective pregnancy study. In contrast to their earlier findings, the authors found that exposure to heavy smoking (>20 cigarettes/day) *in utero* was associated with an almost four month delay in onset of menses (OR=0.34 years (95%CI (-0.02, 0.66)) compared to women who were not exposed to cigarette smoke *in utero*. These contradictory findings serve as impetus for our study.

Age at menarche is used in many studies as both an outcome and a risk factor. In lieu of a convenient and inexpensive biomarker for puberty, most studies rely on self-reported age at menarche. Despite the widespread use of self-reported age at menarche as an indicator of age at onset of puberty in epidemiologic studies, the accuracy of recall of this variable has not been well studied. Self reported age at menarche is easy to capture in a questionnaire or interview and is often done years after the woman has experienced puberty and kept as part of her reproductive history. Two studies to date have addressed the long term recall of age at menarche with sufficient sample size and follow-up of participants. These studies show conflicting results which merit further study on this issue.

A retrospective follow-up of participants in the Newton Girls Study (NGS) (Must et al. 2002) assessed the accuracy of women's recall of age at menarche. The original study (1965-1975) was a prospective study of physical growth and sexual maturation which included 793 girls in Newton, Massachusetts. The girls were followed through their first menstrual period and for approximately two years afterwards. In 1998, up to 33 years after the original NGS, the participants were re-contacted and asked to recall information about their early menstrual characteristics. Approximately 57% of the original participants agreed to participate in the follow-up survey (n=448). Overall, the authors found that a woman's recall of menarcheal age was good. Original mean age at menarche was 12.93 (95%CI: 12.81, 13.06) and recalled mean age at menarche was 12.85 (95%CI: 12.69, 13.00). On average, women recalled their age at menarche as being 0.08 (95%CI: -0.18, 0.01) years earlier than their original age. Recalled and original age at menarche were highly correlated ($r=0.79$, $p<0.001$). Fifty five percent of women recalled their age at menarche to within a half year of the original age reported. Seventy-nine percent of women were accurate within one year of the original age.

This study has several strengths. One of the potential problems in the study, however, is that only a little more than half of the original participants also participated in the follow-up study. There is no reason to believe that the lack of participation was related to self reported age at menarche. However, if women who would report a younger or older age of menarche did not participate systematically this could be a potential source of bias. This point could have been further explored by characterizing the participating women in relation to data from the original study.

A recent British study found that the validity of age at menarche when self reported in middle age was actually quite poor (Cooper et al. 2006). This study re-contacted women who participated in the original Medical Research Council National Survey of Health and Development, initiated in 1946. Information on age at menarche during an interview administered when the girls came in for medical examinations between the ages of 14 and 15. A total of 1050 women participated in the follow up study by answering questions on age at menarche as part of a mailed survey in 1994. Of the 946 women with a valid age at menarche at both measurement points, 412 (43.6%) had recalled exactly the same age at menarche (in years) at age 48. Overall, 21% of the women had recalled their age at menarche as one year older than that recorded in adolescence and 21% remembered their age as one year younger than that which was recorded in adolescence.

This study had a much larger sample size and, thus, power to look at validity and predictors of accurate recall, albeit a 41% response rate raising the possibility of non-differential bias assuming no systematic differences. The differing conclusions from these two studies motivated our reliability study.

Infertility

Fecundity refers to the biologic capacity of a couple for reproduction while fertility denotes demonstrated fecundity as measured with live birth. While often the two terms are used interchangeably incorrectly the two terms have precise meanings.

Many potential risk factors for subfecundity have been examined in the literature. One of the most well studied factors is age and it has been well established that as a woman ages her fertility declines. Women with a high Body Mass Index (BMI) or who are obese are

also more likely to experience longer waiting times to pregnancy (Gesink et al. 2007;Ramlau-Hansen et al. 2007). Gesink and colleagues examined self reported time to pregnancy and BMI within the Collaborative Perinatal Project (CPP) dataset. The Ramlau-Hansen paper did a more thorough investigation of BMI and fecundity by taking into consideration both male and female BMI during pregnancy in the Danish Birth Cohort. This couple based approach to fertility is ideal when information from a male partner can be ascertained.

Environmental factors have also recently been investigated. Several studies have examined the effect of exposure to EDCs during adulthood and time to pregnancy (the number of months it takes a couple who is trying to become pregnant), conception delay (a time to pregnancy greater than six months), and infertility (a waiting time for pregnancy of 12 months or greater). Curtis and colleagues have shown negative effects of pesticides on fertility (Curtis et al. 1999;Thonneau et al. 1999) and others have shown similar effects for women working in agricultural settings exposed to pesticides (Bretveld et al. 2006). These three studies used self-reported exposure as the measure of pesticide use. Since the collection was retrospective there was the potential for recall bias as well as overall misclassification of exposure. Other authors have shown deleterious effects of cigarette smoke (Baird and Wilcox 1985;Hassan and Killick 2004;Munafo et al. 2002;Hughes and Brennan 1996;Bolumar et al. 1996;Alderete et al. 1995) on time to pregnancy. All of these studies used self-reported smoking as the exposure. While this measure is probably more accurate and reliable during a time period when cigarette smoking did not carry a social stigma (particularly during pregnancy), these studies were all conducted in a modern era where some biomarker of exposure such as cotinine levels would be preferable. Many of

these studies also used retrospective report of the exposure which may be subject to recall bias if the woman had difficulty conceiving. Polycholrinated biphenyls (PCBs) have also been implicated in research for their negative effects on TTP (Axmon et al. 2000;Buck et al. 2000;Law et al. 2005) while other authors have found no effect (Axmon et al. 2004;Yu et al. 2000). The studies differ in their exposure measurement as some of the authors measured PCB concentrations in the serum of the women studied and others used information gathered from a questionnaire to designate exposure. Furthermore, some studies assessing PCB levels in serum adjust for lipid values of the woman as these chemicals are lipophilic, whereas other studies do not.

Other lifestyle exposures have been examined as well. Results are mixed when it comes to the effects of self-reported alcohol and caffeine with some studies finding an effect while others do not (Juhl et al. 2001;Hassan and Killick 2004;Jensen et al. 1998;Jensen et al. 1998;Alderete et al. 1995;Zaadstra et al. 1994).

The anti-estrogenic activity of cigarette smoke has been described (Baron et al. 1990;MacMahon et al. 1982;Michnovicz et al. 1986) and it is through this activity that exposure *in utero* to the chemicals that make up cigarette smoke may be related to infertility, specifically by disturbing early egg genesis and development.

Ovarian development begins soon after conception (at approximately four weeks gestation). The embryonic germ cells migrate to the primitive gonadal folds (Witschi 1948). These primordial germ cells become oogonia dividing by mitosis. At eight to thirteen weeks gestation, the oogonia enter meiosis and then remain in a protracted state of meiotic arrest until just prior to ovulation as stimulated by the preovulatory gonadotropin surge (Gondos et

al. 1986). This arrested state lasts for several years until the onset of ovulation which leaves the oogonia in a vulnerable state susceptible to environmental toxicants.

Several animal studies have pointed to the potential for *in utero* exposure to EDCs affecting future fertility. Decreased adult fertility in mice has been shown following prenatal exposure to benzo (a) pyrene which is a component of cigarette smoke (MacKenzie and Angevine 1981). Another finding in mice demonstrates that oocytes were destroyed by prenatal exposure to polycyclic aromatic hydrocarbons (Dobson and Felton 1983). A review of the animal literature suggests that compounds in cigarette smoke may interfere with all events of reproduction from gametogenesis to early post-implantational development (Mattison 1982).

To date, there have been only three studies which have examined the relation between *in utero* exposure to cigarette smoke and time to pregnancy. All of these studies used proxy exposure information and two found an adverse effect between cigarette smoke exposure *in utero* and adult fecundability (Jensen et al. 1998;Weinberg et al. 1989) while the third study found no effect (Baird and Wilcox 1986).

The first study to address prenatal cigarette smoke exposure and adult fecundability was in a population of 600 women in Michigan who were trying to become pregnant (Baird and Wilcox 1986). Women who were pregnant were contacted by telephone and asked to report the number of months it took them to become pregnant. Subsequently, the authors sent out a questionnaire to the participants (93% of them responded) querying the couples about whether their parents smoked while pregnant with them to assess *in utero* exposure. No evidence was found in this study to support the hypothesis that exposure *in utero* to cigarette smoke caused a decreased time to pregnancy or reduction in fecundability. The

study was well powered, but the exposure information may be suspect as a proxy (the daughter) was used instead of the mother or father. The information on time to pregnancy and the exposure were both gathered retrospectively, which may lead to recall bias if the pregnancy took a long time to conceive.

Weinberg and colleagues tried to replicate the earlier findings from Michigan in a sample of women from North Carolina who participated in a prospective pregnancy study designed to examine the incidence of early losses. Two hundred twenty-one couples were recruited between 1983 and 1985. At the time of enrollment the women were asked whether their mother smoked while she was pregnant with them and also about their household exposure before age ten years. The women were then followed using daily urine collected at home, which was later analyzed for the presence of human chorionic gonadotropin. There was a strong negative association between fecundability and prenatal exposure even in the unadjusted data. Their final results (adjusted for age, frequency of intercourse, and the woman's own smoking status) showed that the estimated fecundablity ratio for women exposed *in utero* was 0.7 (95% CI 0.5, 0.9). This study had a prospective ascertainment of time to pregnancy. However, the measurement of the exposure was retrospectively ascertained from the woman as the authors asked women to report their mother's smoking history. This information on exposure was collected before the outcome so recall bias is not a concern, but there could be the possibility for misclassification because of the proxy used for information on exposure. Finally, this study was conducted in a homogenous population of highly educated white high SES women limiting the generalizability of the results.

Finally, Jensen and colleagues prospectively followed 430 Danish couples discontinuing contraception who began attempting pregnancy for up to six months (Jensen et

al. 1998). The participants in the study completed surveys regarding lifestyle behaviors in the menstrual cycles they were trying to conceive. The baseline questionnaire also included the following question regarding parental exposure to cigarette smoke: "Did your mother smoke when she was pregnant with you (Yes or No)?" The fecundability odds ratio for non-smoking women who were exposed *in utero* was 0.70 (95% CI 0.48, 1.03) compared to non-smoking women who were not exposed *in utero*. Women who were exposed *in utero* and smoked themselves had a fecundability odds ratio of 0.53 (95% CI 0.31, 0.91). The analyses were adjusted for BMI, alcohol intake, diseases of the female reproductive organs, semen quality, and duration of menstrual cycle. This study, again, had the benefit of prospectively captured exposure and outcome data. Like the Weinberg study though the authors asked daughters to report on their mother's smoking history while pregnant. While each of the three studies provided interesting information on fecundability none addressed the endpoint of infertility.

Endometriosis

Perhaps, the most commonly cited non-environmental etiology of endometriosis is the theory of retrograde menstruation. This was first described by Sampson in the early 20th century (Sampson JA 1927). The mechanism that underlies this theory is regurgitated menstrual effulge through the fallopian tubes which implants and requires neovascularization. However, recently it has been noted that retrograde menstruation is common among women of reproductive age (76% of women undergoing laparoscopy during menstruation), implicating that this may not be the only factor playing into the development of disease (Liu and Hitchcock 1986).

The environmental origin of endometriosis has recently been a topic of interest to epidemiologists. There are approximately seven studies to date which present data regarding environmental chemicals and risk for endometriosis. Four studies reported a positive association between dioxin (Mayani et al. 1997) or PCBs (Louis et al. 2005;Porpora et al. 2006;Gerhard and Runnebaum 1992) and endometriosis. The Mayani study was one of the first to use serum concentration levels of the chemicals albeit a small sample size (44 case and 35 controls). Louis and Porpora both assessed serum concentrations of PCBs in relation to endometriosis, but only the former studied laparoscopically confirmed disease. The small size of these studies (79 and 80 women respectively) was a limitation in both studies. . Two studies found two- to four-fold increases in risk for endometriosis with dioxin (Eskenazi et al. 2002) or PCB exposure (Pauwels et al. 2001) respectively. Eskenazi and colleagues also looked at a population in Seveso, Italy that suffered from an acute accidental chemical exposure which may have conferred different levels of risk than that seen in populations chronically exposed. Pauwels and colleagues conducted their study on a small sample size of 69 infertile, women which may have affected their ability to detect small differences.

It has been suggested that lifestyle factors may also play a role in risk for endometriosis. Women with lower body mass index (BMI) have recently been shown to be at higher risk for the disease when compared to women with larger BMIs (Hediger et al. 2005). The authors also found that women who tracked lean throughout their childhood and adolescence were at higher risk for the endometriosis suggesting a possible *in utero* origin for the disease. Through the hypothesized alteration of estrogen levels several studies have found smoking and exercise to be protective factors of risk for endometriosis (Cramer et al. 1986;Cramer and Missmer 2002;Eskenazi et al. 2002;Baron 1996;Matorras et al. 1995) while

others have found no such effect (Moen and Schei 1997). Self-reported smoking during the time periods in which it was collected for these studies may be inaccurate leading to the potential for exposures misclassification. Furthermore, exercise was self reported in all these studies by using simple questions regarding ever/never exercising. Even more detailed questions regarding how vigorously the exercise was may have improved the exposure measurement and helped to distinguish any real effects of the exposure from noise in the data.

Similar to the mechanisms discussed for the previous outcomes, the biologic basis for the hypothesis of *in utero* exposure to EDCs and their subsequent effect on risk for disease stems from the ability of these chemicals when present periconceptionally or pre-natally to damage vulnerable organ systems which are under development.

One theory underlying the biologic mechanism by which DES, cigarette smoke, or any other EDC might program the body for endometriosis is the possibility that the chemicals may cause embryonic müllerianosis rest (Batt et al. 1990). In this state, residual pieces of the müllerian ducts (an early embryonic structure) proliferate. It is hypothesized that these pieces later become endometrial explants found throughout the body.

There has only been one study that has explored the association between *in utero* exposure to DES and endometriosis. Similarly, there is only one study published that has examined the association between *in utero* exposure to cigarette smoke and endometriosis.

The recent study by Missmer and colleagues is the only one published that has examined the association between *in utero* exposure to DES and endometriosis. The authors used data from the Nurses' Health Study to examine this question (Missmer et al. 2004). During 566,250 woman-years of follow-up 1,226 cases of laparoscopically confirmed

endometriosis were reported among women with no past infertility. Nurses were followed with a questionnaire where they reported the presence or absence of endometriosis and the method of diagnosis. Additionally women were asked to recall their mother's exposure to DES while they were pregnant with them. A validation study was conducted on a subset of the women who reported laparoscopically confirmed endometriosis and the reporting was accurate 86.6% of the time when the diagnosis was checked against medical records. The authors used a model adjusting for age, calendar time, parity, race, and body mass index at age 18. They observed an 80% greater incidence of endometriosis among women who reported being exposed to DES *in utero* (RR=1.8, 95%CI =1.2, 2.8) compared to unexposed women.

This large study provides initial evidence that women exposed *in utero* to DES may be at higher risk for developing endometriosis later in life. One of the major flaws of the study was the ascertainment of DES exposure. Women were asked to recall their mother's exposure. The nurses who participated in this study were asked this during their adulthood when their mother may or may not have been living. Furthermore, this study did not take into account the potential effect of smoking and the possible interaction between smoking and DES which may also be an important predictor of disease risk. Finally, the authors adjusted for several variables, including parity and BMI which may be on the casual pathway between exposure and disease. We sought to improve upon these limitations in our study of the same research question.

Only one study has examined the association between *in utero* cigarette smoke and endometriosis. Louis and colleagues found that *in utero* exposure was associated with a lower odds of being diagnosed with endometriosis (Buck Louis et al. 2007). This study had

laparascopically confirmed disease status on women, however, they relied upon proxy exposure information (daughter's were asked to recall their mother's smoking behavior while pregnant). These authors found the odds for endometriosis were lower among women exposed to cigarette smoke *in utero* (OR=0.22, 95%CI (0.06, 0.82)) who never smoked themselves compared to women who were unexposed. Furthermore, they found the association to be even stronger amongst women who were exposed *in utero* and had smoked themselves (OR=0.05 95%CI (0.01, 0.42)) compared to women who were unexposed. This study served as impetus for our work in a different population.

Summary of the literature

Overall, the data are sparse regarding the research questions. There are only a few published papers that examine *in utero* exposures and adult reproductive outcomes. Except for two studies examining *in utero* exposure to cigarette smoke and age at menarche, all previous studies use proxies to determine the main exposure measurement. Our study improved upon these exposure measurements with data collected from the mother. Therefore, we expect our measurement of exposure is more accurate and reflective of the actual *in utero* exposure. For DES exposure, physician confirmation was obtained for all women classified as exposed and unexposed in our study, which has not been done in previous studies looking at *in utero* DES exposure and endometriosis.

For age at menarche, two existing studies have examined the association between *in utero* exposure to cigarette smoke and age at onset of menses, for infertility, no studies have investigated the effects of *in utero* exposure to cigarette smoke and infertility, one published study has assessed the association between *in utero* exposure to cigarette smoke and

endometriosis, and only one study has been published looking at DES exposure and endometriosis. Because many of these studies have not yet been replicated or tested formally, we had a unique opportunity to improve upon previous research methodologies and address these gaps in the literature.

It is hypothesized that women who experience menarche at a later age are also at higher risk for endometriosis. The DESAD dataset offered the possibility of examining a continuum of disease by investigating the overlap of outcomes within a woman in the study. Specifically, we focused on whether the women who experienced altered age at menarche are the same women who developed endometriosis later in life, and ultimately also had problems with infertility. This type of continuum operates under the premise that the woman with all of these outcomes may have taken a hit *in utero* from an environmental chemical that altered her reproductive profile throughout the course of her reproductive development.

The following tables summarize the research to date on environmental factors and the three reproductive outcomes discussed in this dissertation: age at menarche, infertility or subfecundity, and endometriosis. Table 1.1 highlights a sampling of the general environmental risk factors that have been studied while Table 1.2 details comprehensively the studies which have looked at *in utero* environmental exposures and the proposed outcomes.

Table 1.1 Selected environmental risk factors and various reproductive outcomes

Author/Date of Study	Exposure	Outcome	Effect
Goran et al. 1998	Physical activity	Age at menarche	Decreased physical activity associated with earlier age at menarche
Graber et al. 1995	Stressful events	Age at menarche	No effect
Baker 1985	Obesity	Age at menarche	Obesity associated with

			earlier age at menarche
Slyper 2006	Obesity	Age at menarche	Overweight and obesity associated with earlier age at menarche
Robe 1976	*In utero* alcohol	Age at menarche	Alcohol exposure *in utero* associated with later age at menarche
Blanck et al. 2000	Postnatal PBB exposure	Age at menarche	PBB exposure through breast milk associated with earlier age at menarche
Gesink et al. 2007	Pre-Pregnancy BMI	TTP	Higher BMI associated with longer TTPs
Ramlau-Hansen et al. 2007	Male and Female BMI in early pregnancy	TTP	Higher BMIs of both partners associated with longer TTPs
Curtis et al 1999	Self reported pesticide use	TTP	Pesticide use associated with longer TTPs
Thonneau et al. 1999	Self reported pesticide use	TTP	Pesticide use associated with longer TTPs
Bretveld et al. 2006	Self reported agricultural pesticide use	TTP	Pesticide use associated with longer TTPs
Baird and Wilcox 1985	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Hassan and Killick 2004	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Munafo et al. 2002	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Hughes and Brennan 1996	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Bolumar et al. 1996	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Aldrete et al. 1995	Self reported smoking	TTP	Cigarette smoking associated with longer TTPs
Juhl et al. 2001	Alcohol	TTP	No effect
Hassan and Kllick 2004	Alcohol and Tea	TTP	Alcohol and tea consumption associated with longer TTPs
Jensen et al. 1998b	Alcohol	TTP	Alcohol was associated with longer TTPs

Jensen et al. 1998c.	Caffeine	TTP	Caffeine was associated with longer TTPs
Aldrette et al. 1995	Coffee	TTP	No effect
Zaadstra et al. 1994	Alcohol	TTP	No effect
Axmon et al. 2000	Fish Consumption	TTP	No effect
Buck et al. 2000	Serum PCB levels	TTP	PCB levels associated with longer TTP
Law et al. 2005	Serum PCB levels	TTP	PCB levels associated with longer TTP
Axmon et al. 2005	Serum POP levels	TTP	POP levels associated with longer TTP
Yu et al. 2000	PCB levels as identified from accidental exposure	TTP	No effect
Mayani	Serum dioxin levels	Endometriosis	Dioxin associated with endometriosis
Eskenazi et al. 2002	Serum dioxin levels	Endometriosis	Dioxin associated with endometriosis
Louis et al. 2005	Serum PCB levels	Endometriosis	PCB levels associated with endometriosis
Popora et al. 2006	Serum PCB levels	Endometriosis	PCB levels associated with endometriosis
Gerharad and Runnebaum 1992	Serum PCB levels	Endometriosis	PCB levels associated with endometriosis
Pauwels et al. 2001	Serum PCB levels	Endometriosis	PCB levels associated with endometriosis
Hediger et al. 2005	BMI	Endometriosis	Lower BMI associated with endometriosis
Cramer et al. 1986	Smoking and exercise	Endometriosis	Smoking and exercise are negatively associated with endometriosis
Cramer and Missmer 2002	Smoking and exercise	Endometriosis	Smoking and exercise are negatively associated with endometriosis
Eskenazi et al. 2002	Smoking and exercise	Endometriosis	Smoking and exercise are negatively associated with endometriosis
Baron 1996	Cigarette Smoking	Endometriosis	Smoking is negatively associated with endometriosis
Matorras et al. 1995	Smoking	Endometriosis	Smoking is negatively associated with endometriosis
Moen and Schei	Smoking and	Endometriosis	No effect

1997	exercise		
Cramer and Missmer 2002	Caffeine and alcohol	Endometriosis	Caffeine and alcohol intake are associated with endometriosis

*TTP (time to pregnancy)
PCB (polychlorinated bi-pheynyls)
POP (persistent organopollutant)

Table 1.2 *In utero* environmental exposures and reproductive outcomes: the critical studies.

Author/Date of Study	Exposure	Outcome	Effect
Windham et al. 2004	*In utero* smoke	Age at menarche	*In utero* exposure to smoke associated with younger age at menarche
Windham et al. 2008	*In utero* smoke	Age at menarche	*In utero* exposure to smoke is associated with a later age at menarche
Jensent et al 1998a.	*In utero* smoke	TTP	*In utero* exposure to smoke associated with longer TTPs
Weinberg et al. 1989	*In utero* smoke	TTP	*In utero* exposure to smoke associated with longer TTPs
Baird and Wilcox 1986	*In utero* smoke	TTP	No effect
Louis et al. 2007	*In utero* smoke	Endometriosis	*In utero* exposure to smoke associated with a reduced odds of endometriosis
Missmer et al. 2004	*In utero* DES	Endometriosis	*In utero* exposure to DES associated with endometriosis

CHAPTER II

METHODS

Overview

To evaluate our research hypotheses, we used data from two linked studies which are coordinated by the National Cancer Institute (NCI). The first study initially followed the mothers and daughters exposed and not exposed to DES in the early 1970s. The second study was initiated by the NCI as a follow-up study to track the long term health effects of DES in these women. All data were de-identified and are the property of the NCI who gave us access for these purposes after approval from the University of North Carolina Institutional Review Board. This was a secondary analysis of already de-identified data and an exemption to IRB review was ascertained.

The study was divided into three separate papers as designated by the five specific aims of the dissertation. The study sample from which the analytic plans were established is outlined in detail in the following sections. The study ultimately assessed the questions of whether *in utero* exposure to cigarette smoke and/or DES were associated with three reproductive outcomes: age at menarche, infertility, and endometriosis and an additional methodologic question of whether age at menarche as self-reported later in life is reliable.

Study Sample

Numerous studies of women exposed to DES began in the early 1970s, however systematic follow up of the women and their offspring ceased in the 1980s due to lack of funding. In 1992, the National Cancer Institute (NCI) began a large collaborative study with DES researchers at five field centers to reassemble and consolidate cohorts of mothers, daughters, and sons who had previously been studied. Seven thousand four hundred thirty-nine DES exposed and unexposed mothers and offspring were included in this study sample.

The study population that comprises the NCI DES Combined Cohort Follow-Up Study is complex. The NCI DES Combined Cohort Follow-Up Study re-contacted daughters from these studies in the nineties to ascertain additional information on health outcomes. There are five studies that constitute the NCI DES Combined Cohort Follow-Up Study, however for the purposes of this dissertation only the National Cooperative Diethylstilbestrol Adenosis (DESAD) study was used.

The largest cohort of DES exposed and unexposed daughters in the NCI DES Combined Cohort Follow-Up study is the DESAD cohort. The objective of the DESAD cohort was to follow daughters exposed *in utero* to DES yearly with clinical exams to monitor for abnormalities of the vagina and cervix. In 1975, over 4,000 exposed and 1,000 unexposed daughters were enrolled into the DESAD study at one of five sites across the country. The participating sites included: Baylor College of Medicine, Gundersen Clinic, Massachusetts General Hospital, Mayo Clinic, and University of Southern California. The mothers were asked to complete a pregnancy questionnaire at the baseline visit. The daughters completed detailed health history questionnaire and baseline clinical exam at enrollment which was completed for most participants by 1978. The daughters were offered free yearly exams until 1983 and then mailed annual questionnaires from 1984-1989.

Three types of exposed women were followed during the DESAD study: 1) women whose exposure to DES was ascertained by review of prenatal records (47%), 2) women referred to the study by outside physicians (32%), and 3) women who "walked-in" to the local DESAD clinics seeking free evaluation (21%). Exposed daughters who were referred by outside physicians or who “walked-in” to the clinics were required to have written evidence of exposure from prenatal records or a letter from a physician who had provided prenatal care during the pregnancy. Two types of unexposed women were selected: sisters of exposed participants (24%) and non-relatives identified from the same record sources as the exposed (76%), most of whom were matched to the exposed on year of birth and mother's age at delivery.

Standardized questionnaires were sent to all of the daughters included in the NCI DES Follow-up Study. The questions included: demographic factors, cancer risk factors, hormone replacement therapy and use of other hormones, history of sexually-transmitted diseases, autoimmune diseases, cancers, infertility and other reproductive problems, and major mental illness. The sex and birthdates of all live-born children were obtained to enable possible future study of third-generation offspring.

Most of the cohorts followed in the past had extensive information available for tracing, including social security numbers, last known address, and names and addresses of contact persons. If subjects had moved since the last contact and could not be found through listed contact persons, additional methods of tracing were used these included credit bureau searches, telephone and alumni directories, town books of addresses, Health Care Financing Administration tapes, and a National Death Index Search. Some subjects had during a previous follow-up requested no further contact, and no attempt was made to re-contact

them.

Questionnaire mailings began in January of 1993 and continued through July of 1996 as subjects were traced. Seventy-five percent of the questionnaires were completed by April 1, 1995. Subjects who did not return the first questionnaire within one month were sent a second one, and subsequently, interviewers at each field center attempted to conduct the interview by telephone. Study personnel at the field centers inspected the questionnaires for completeness and accuracy and telephoned respondents when there were discrepancies or large amounts of missing information.

Exposure Measurement

As previously outlined, *in utero* exposure to cigarette smoke was the primary exposure of interest for several of the aims and a potential effect measure modifier in another one of the aims. The mothers were asked if they smoked during the pregnancy which culminated in the birth of the index daughter who was enrolled into the study. If they answered yes, the mothers were then asked to report how many cigarettes they smoked per day while pregnant.

Recent studies discount the value of self-reported smoking habits, particularly during pregnancy, as there is a strong stigma attached to this behavior. However, the dangers of smoking during pregnancy were unknown in the late fifties and early sixties when most of the mothers in this study were pregnant. It was not until years later that the Surgeon General's report outlined the consequence of smoking while pregnant and even later (1981) when Congress mandated the Surgeon General's warning be printed on all packages of cigarettes. Furthermore, the reporting of smoking in this era has been found to be highly

accurate. A study of 448 pregnant women during the 1960s who participated in the Collaborative Perinatal Project revealed self-reported cigarette smoking was highly correlated with cotinine levels measured in stored blood samples obtained during pregnancy (Klebanoff et al. 1998). Based on the assumption that a serum cotinine concentration of >10 ng/ml represented active smoking, 94.9% of women who denied smoking and 87.0% of women who stated that they smoked (kappa=0.83) reported their status accurately.

Smoking was coded in three ways. A simple yes/no dichotomous variable was created indicating if the mother ever smoked during her pregnancy or never smoked during her pregnancy and was coded numerically as one for yes and zero for no. A second variable showed the amount of cigarettes that the mother smoked per day during her pregnancy and was examined in a continuous fashion (0-60). The third variable showed the number of packs of cigarettes a woman smoked per day during her pregnancy (≤1 or >1).

In utero exposure to DES was the primary exposure of interest for aim five. This study used information from the mothers themselves which had been verified against medical records or physician report for all women enrolled. Exposure to DES was a dichotomous variable coded as either yes (1) or no (0).

Outcome Measurement

This study was unique in that the daughters were examined yearly around the time of onset of puberty and were asked to report their age at menarche. Therefore, in terms of accuracy, this study should have a close measure of age at menarche. Furthermore, the women were queried about age at menarche approximately 20 years later. These data were

compared to the earlier data to determine the reliability of self-reported age at menarche later in life.

Age at menarche was measured in years and was coded as both a continuous (8-20 years) variable and as a categorical variable (<12, 12-13, and ≥14). Early, normal, and late were defined according to previously established standard cut-points (Lee 1980). Fractions of years obtained upon analysis were converted to months.

Information on infertility was collected in the follow-up questionnaire. Women were asked several questions related to infertility, which were used in the analysis. Specifically, they were asked if they had ever tried to become pregnant without success for 12 months or more and if they sought treatment for infertility. A small validation study was conducted on a subset of the women reporting infertility. In this study 63 participants who had reported infertility were selected so that all the centers and diagnoses were represented equally. Thirty-six women gave permission for review of their medical records. The treating physician was asked to complete a one-page medical record abstract form. Medical abstracts were obtained for 29 of the women. The reason for infertility reported by 26 of the 29 participants (90%) in the study was confirmed with medical records (Palmer et al. 2001).

Infertility was used primarily as a dichotomous variable and coded as yes (1) or no (0). In order to be classified as infertile a woman had to report that she had tried to become pregnant for 12 months or more without success and that she had sought medical care for infertility.

Endometriosis was the primary outcome for aims four and five of the dissertation. In this study, the presence of endometriosis was assessed during the NCI follow-up questionnaire through the use of several questions. The questions included were: Have you

ever been diagnosed with endometriosis? If yes, what year were you diagnosed? How were you diagnosed?

As described earlier, defining endometriosis is difficult on a population level because laparoscopy is not always indicated for the symptoms of endometriosis and, in fact, women with disease may present with no symptoms at all. The perfect study of this outcome would require all women to undergo laparoscopy so that disease or lack of disease could be documented for each woman; however, this is unethical and prohibitively expensive. This study was able to restrict the definition of disease to include only women who were diagnosed by laparsocpy, biopsy, hysterectomy, or other gynecologic surgery. Endometriosis was simply used in the analysis as a dichotomous yes (1) /no (0) variable.

To evaluate the extent that women in this study had more than one of these three outcomes a sub-analysis was conducted to determine how many women who had altered menarche and also had endometriosis and/or infertility. We modeled the chemical exposures in similar logistic regression models taking into consideration that altered menarche and endometriosis may be on the casual pathway from exposure to infertility.

Statistical analysis

Each exposure, outcome and covariate was inspected first using univariate analyses. Each continuous variable was assessed to determine the mean, median, mode, standard deviation, kurtosis and skewness values. Continuous variables were examined for appropriate cut points for categorizations based on biologic evidence in the bivariate analyses and modeling. Frequencies were assessed for categorical variables. Missingness was addressed and documented in this initial step. Women who had missing values for the

exposure and/our outcome were dropped from the analysis, but missing values on covariate data were treated as missing at random. Descriptive statistics on variables with missing values were documented.

Each variable was assessed in a bivariate manner. Each covariate including the exposure was crossed with the outcome of interest. Odds Ratios (ORs) and 95% Confidence Intervals (CIs) were calculated using a referent group for each of the covariates. After bivariate analyses were run, the presence of potential confounding and effect measure modification was reassessed using a Directed Acyclic Graph (DAG)s produced for each aim as the *a priori* information.

Effect measure modification was assessed formally by stratifying the results in all models according to the variables suggested as effect measure modifiers. A combination of statistical testing along with evaluation of the magnitude of difference between the estimates when stratified will be used as the approach to determine final effect measure modifiers. The likelihood ratio test for homogeneity was also used as a statistical test of the presence of modification (Modern Epidemiology 1998).

Confounding was based on both information from the DAG (representing *a priori* knowledge in the matter) and an assessment of the change in estimate criteria (ten percent) when factors were subtracted from the full model.

Multivariable models were built based on information from the bivariate analyses. Logistic regression models were used and the exposure was classified both as a dichotomous variable (yes/no) and when the exposure of interest was *in utero* exposure to cigarette smoke, a continuous measure using the number of cigarettes smoked per day and a categorical measure of packs of cigarettes per day during pregnancy were used as well. We created an

indicator variable to distinguish the sisters in the sample from those who were not sister and then used Generalized estimating equations (GEE) with logistic regression models (Liang and Zeger 1986;Zeger and Liang 1986) to account for the dependency between sisters.

Aim one was different from the other aims because it did not involve a traditional exposure-disease research question. Instead, aim one was a reliability study. The univarite analyses were conducted in the same manner described above. Bivariate analyses were conducted with the covariates and stratified by whether or not the woman reported the same age on both questionnaires or not. Frequencies were run on the number of women who accurately reported their age at menarche in the follow-up questionnaire and these data were be analyzed for the amount of disagreement between the two measures if they were not the same. A formal weighted kappa statistic was calculated based on the two measures. Models were built to determine which covariates may influence correct reporting.

Further details regarding the methods particular to each of the specific aims are contained in the following chapters.

CHAPTER III

PAPER 1: RELIABILITY OF AGE AT MENARCHE

Introduction

Menarche, defined as the age in years when a young woman experiences the onset of her first menstrual period, is an important milestone in a woman's reproductive life. It is thought to be important not only as a sentinel event, but also as a risk factor for adult disease and, possibly, as a marker of increased exposure to lifestyle factors and environmental agents both *in utero* and postnatally.

Age at menarche is often assessed based on recall many years later. Many studies, particularly those employing the popular life-course approach, rely on the accuracy of this variable as reported decades after the initial event took place. Recalled age at menarche is also an integral part of breast cancer risk assessment tools. Despite the widespread use of self-reported age at menarche, the accuracy of recall of this variable has not been well studied.

The National Cancer Institute (NCI) Diethylstilbesterol (DES) Combined Cohort Follow-up Study offers a unique opportunity to investigate the reliability of self-reported age at menarche from a large dataset with information on age at menarche ascertained at two time points: An initial observation of age at menarche was recorded during the DESAD Study, when the participants were teenagers or young adults. The second observation of age at menarche was obtained approximately twenty years later. This study also provides an

opportunity to determine factors that may influence the reliability of reporting of age at menarche later in life. This study will provide the data needed to respond to the recent conflicting findings (Cooper et al. 2006;Must et al. 2002) on the reliability of age at menarche as reported later in life.

Methods

Study Population

The largest group of DES exposed and unexposed daughters studied in the Combined Cohort Follow-Up Study is the DESAD Cohort. The objective of the DESAD Cohort was annual follow up of daughters exposed *in utero* to DES yearly with clinical exams to detail incident abnormalities of the vagina and cervix. DES use in pregnancy began in 1940 and stopped in 1971, with the height of its use in the 1950s. More than 4,000 exposed and 1,000 unexposed daughters were enrolled into the DESAD Study at one of five clinical sites across the country: Baylor College of Medicine, Gundersen Clinic, Massachusetts General Hospital, Mayo Clinic, and University of Southern California.

At the baseline visit in 1976, mothers of exposed daughters provided information for the index pregnancy. Subsequently, the daughters completed detailed health history questionnaires and underwent a baseline clinical exam at enrollment which was completed for most participants by 1978. Correspondingly, the average age at filling out the baseline questionnaire in 1976 was 22 (±5), with over two-thirds of the participants between the ages of 16 and 25. The daughters were offered yearly exams until 1983 and mailed self administered questionnaires from 1984-1989. Daughters were asked to report the age (in years) they first experienced menstruation or they were referred to a gynecologist if menses

was not yet initiated. These women were asked when menarche began or a year later (whichever occurred first).

Three types of exposed daughters were followed during the DESAD Study: 1) daughters whose exposure to DES was ascertained by systematic review of prenatal records (47%); 2) daughters referred to the study by outside physicians (32%); and 3) daughters who "walked-in" to the local DESAD clinics seeking evaluation (21%). Exposed daughters referred by outside physicians or who were “walk-ins” to the clinics were required to have written evidence of exposure from prenatal records or a letter from a physician who had provided prenatal care during the pregnancy. Two types of unexposed women were selected: sisters of exposed participants (24%) and non-relatives identified from the same record sources as the exposed (76%), most of whom were matched to the exposed on year of birth and mother's age at delivery.

From 1993-1995, women were re-contacted from various studies for a follow-up study regarding women’s adult health. All daughters from the DESAD Cohort were included in the NCI DES Follow-up Study if they had documented exposure status (either as exposed or unexposed from medical records) and had responded to at least one mailed questionnaire between 1984 and 1989. Standardized questionnaires were sent to the daughters included in the National Cancer Institute DES Combined Cohort Follow Up Study. The questions included: demographic factors, use of oral contraceptives, cancer risk factors, hormone replacement therapy and other hormones, history of sexually-transmitted diseases, autoimmune diseases, cancers, infertility and other reproductive problems, and major mental illness. Women were asked in this follow-up questionnaire to report the age when they started their first period. Women who did not return the first questionnaire within one month

were sent a second one, and subsequently, interviewers at each field center attempted to conduct the interview by telephone.

Data analysis

The original reported age at menarche (in years) queried of the initial visit in 1976-1978 was assumed to be the most accurate report. In both surveys, the question was phrased as "How old were you when you had your first menstrual period?" The participant recorded their answer in years on both surveys.

A weighted Kappa statistic and the Pearson correlation coefficient were used to assess concordance for age at menarche. Additionally, odds ratios and 95% confidence intervals were calculated for selected covariates to determine if any demographic characteristics or prenatal exposures were associated with concordant responses.

Additionally, a sensitivity analysis was conducted to assess the magnitude of effect change when different definitions of age at menarche were used in a typical exposure-outcome study where age at menarche was the outcome. Specifically, we tested the association of *in utero* exposure to smoke and age at menarche using the original report of age at menarche as the outcome and then using only the age given at the follow-up to examine any differences. The same analytic techniques were used for both analyses (logistic regression).

Results

Of the 5,049 women in the DESAD Follow-Up Study 79% (n=3998) had values for both reports of age at menarche. Women who were missing either the original age at menarche value or the age from the follow-up were excluded from the analysis. However,

these women did not differ statistically from the women included on important covariates including education, race, DES exposure status, maternal smoking status, gravidity, or attained age when menarche age information was provided (data not shown).

Overall, 55% of the women in the study reported the same age at menarche approximately 20 years later, whereas 45% of the women reported a different age (Table 3.1). However, the mean values for age at menarche from the two surveys were comparable at 12.8 (± ~1.4). When comparing the distributions of age at menarche from the two surveys, no difference in the two reports is observed (Figure 4.1). When the definition of concordance was extended to be within a year on either side of the true value, 90% (N=3,614) of the women reported the same age at menarche. Of those who misreported their age, 842 underestimated the age and 938 overestimated the age. Among women who misreported their age by three or more years, women whose first reported age at menarche was older than normal (>14 years) tended to under-report their age compared to women whose first reported age at menarche was normal or early. Approximately 80% of the women over- or under- reported age by only one year. The weighted kappa statistic reflecting agreement among the original and follow-up reports of exact year of menarche was 0.62 95%CI (0.60, 0.63) and the Pearson correlation coefficient was 0.71($p<0.05$).

Multiple characteristics including prenatal exposures were examined to assess their association with a concordant report later in life. Of all the characteristics examined, only first reported age at menarche and attained age at the follow up survey were meaningfully associated with the accuracy of report later in life (Table 3.2). Notably, DES exposure status was not associated with concordance despite the theory that exposed daughters might

remember events in their reproductive history more accurately. Even the time lapse between the two surveys did not affect accuracy of recall. Women who were less than thirty years old at the time of the second survey were less likely to accurately report their age at menarche than women who were 40-49 years old at the time of the survey (OR=0.7, 95%CI (0.6, 0.9)). Finally, women whose first reported age at menarche was late (>14 years) (OR=0.7, 95%CI (0.6, 0.8)) were less likely to report accurately later in life compared to women whose age at menarche was normal (12-14 years).

In the sensitivity analysis, we found that when age at menarche was categorized using the data collected later in life did alter the effect estimates, however, when age at menarche was examined continuously the estimates did not change. Specifically, it shifted them towards the null value. In the original analysis with the first reported age at menarche the odds ratio for the association between *in utero* smoke exposure and early age at menarche was 1.3 (95%Ci 0.8, 1.8) whereas with the age reported at the follow-up survey (OR=1.0, 95%CI (0.7, 1.5)). Similarly, using the original report, *in utero* smoke exposure was associated with late age at menarche (OR= 1.1, 95%CI (0.8, 1.5)) and this result was again attenuated when the follow-up age at menarche was used (OR=0.9 95%CI (0.6, 1.2)).

Discussion

Exact recall of age at menarche was found to be limited with only 55% of the participants correctly remembering their age at menarche. Overall, however, there was no difference in the mean age at menarche reported from the two surveys.
Furthermore, when the definition of accurately reporting was extended to encompass a year on either side of the original age at menarche, 90% of women were classified as correctly remembering their age at menarche. It is possible that the narrow age span over which

menarche actually occurs might allow for comprehensive coverage by small errors or that the mean actual age at menarche may be equal to the mean recalled age and still have considerable misclassification if the under and over reporting balance each other out.

The only covariates that influenced the concordance of the two reports of age at menarche were the age of the woman at the follow-up survey and the first reported age at menarche (Table 3.2). Girls who experienced onset of menses late (>14 years) were less likely to report the same age at menarche years later as compared with girls who had a normal age at menarche (12-14 years) (OR=0.7, 95%CI (0.6, .08)). When very late menarche (>16 years) was examined this association was no longer present.

The age at the time women filled out the survey also proved to be an interesting factor affecting the probability of remembering age at menarche correctly. One might assume women who were younger (≤ 30 years) at the follow-up survey would be more accurate in their reporting as they were closer in time to when the event actually occurred; however, we found the opposite to be true. This was not explained by confounding due to the high proportion (21%) of women in this group whose age at menarche was on the later side of normal >14 years.

A few reports on the reliability of age at menarche have previously been published. Much of the early research was done on historical cohorts with differing intervals between actual menarche and recall (Bean et al. 1979;Bergsten-Brucefors 1976;Casey et al. 1991;Damon and Bajema 1974;LIVSON and McNEILL 1962). The correlation coefficients in these studies ranged from (0.60-0.80) despite the interval of recall being between 1 and 39 years. However, two of these studies had limited sample sizes (n=160) and (n=43), respectively (LIVSON and McNEILL 1962;Bean et al. 1979;Bergsten-Brucefors 1976) and

one was only able to successfully follow up one third of the surveyed population(Casey et al. 1991).

Two more recent studies have come to differing conclusions, serving as the impetus for this study(Cooper et al. 2006;Must et al. 2002). A retrospective follow-up of participants in the Newton Girls Study (NGS) investigated the reliability of various menarchal characteristics (Must et al. 2002). Girls were recruited in third and fourth grade to participate in the study and their mothers completed monthly questionnaires about their daughter's growth, development, and onset of their first menstrual period (in years and months). In 1998, approximately 57% of the daughters were re-contacted 33 years later and provided recalled menarche in years and months. Overall, the authors found that a woman's recall of menarchal age reported by her mother was good. Original mean age at menarche was 12.93 95% CI (12.81, 13.06) and recalled mean age at menarche was 12.85 95%CI (12.69, 13.00). On average, women recalled their age at menarche as being 0.08 95%CI (0.01, 0.18) years earlier than their original age. While our results are similar, our conclusions differ from Must and colleagues who find that recall of age at menarche was generally quite good. The Pearson correlation coefficient from this study (r=0.79) is similar to that found in our study (r=0.71). These authors found that women with an earlier age at menarche remembered their age better than those whose menarchal age was closer to the mean. Also, women with older ages at menarche (>16 years) remembered better than women with the mean menarchal age. We did not make these same observations when we looked at women with older age at menarche (>16 years) in our data. Finally, they did not have information on early life exposures, birth weight, or adult covariates such as gravidity which could be associated with reporting correctly.

In contrast, our conclusions are more similar to Cooper and colleagues (Cooper et al. 2006) who found that at best there was only moderate agreement between the two measures of age at menarche. This study re-contacted women who participated in the original Medical Research Council National Survey of Health and Development, that was initiated in 1946. Nine hundred forty-six women (37.1% response) from the original study participated and answered questions on age at menarche as part of a mailed survey in 1994. While these authors found a slightly lower Pearson correlation coefficient (r=0.66) between the two measures compared with ours, they also found that only 43.6% (compared with our 45%) of the women queried recalled exactly the same age at menarche and concluded that the 'validity' of age at menarche when self-reported in middle age was actually quite poor (Cooper et al. 2006). Overall, they found 21% of the women had recalled their age at menarche as one year older than that recorded in adolescence and 21% remembered their age as one year younger than that which was recorded in adolescence. This study was larger than Must and colleagues with 946 women participating, but had some weaknesses because women who were originally interviewed at age 14-15 years and had not yet experienced menses were not followed further to ascertain their age at menarche. Cooper and colleagues also found greater agreement among women with higher education levels, which we did not observe.

In summary, we found that self-reported age at menarche as reported 20 years after it was initially collected is prone to recall errors. Although there was a great amount of misreporting of the exact age at menarche (44%), the degree of misreporting was actually quite small, with most women misreporting by only one year. To the best of our knowledge, our study is the largest to date on this issue, and has extensive information on potential

covariates. We are limited by the participation rate in our study, which while higher than previous studies still leaves 20% of the women for whom we do not have information. However, this should not introduce bias as characteristics of the women who were not included are similar to those who were included. We are also limited because age at menarche was measured in whole years and therefore subtle differences may not be seen with a unit of measure this large. It is possible that when women were asked to recall their age at menarche that they interpreted the question as asking when they regularly started menstruating, which is often times different from the onset of first menses. Finally, we cannot be confident of the accuracy of the daughter's first report as it was not captured prospectively in time.

Results from our sensitivity analysis suggest that when age at menarche is categorized and examined as an outcome in a study, the effect estimates that are generated can be affected by the misclassification of age at menarche as reported later in life. In our example the estimates were shifted towards the null by approximately thirty percent in one instance, which would arguably change the conclusions of the analyses.

These results translate to a dual conclusion. If the exact year a girl reaches menarche is an integral part of calculations for risk (such as in some calculators and tools for estimating cancer risk), then self-reported age at menarche reported later in life may be unreliable. Furthermore, studies which depend on remotely recalled age at menarche to categorize the outcome of menarche by early, normal, and late, should also be wary of this variable. A year error in either direction may put women into the incorrect category. For instance in our study 596 women originally reported having an early age at menarche (<12 years), yet at the follow-up survey only 74 percent of these women reported an early age at

menarche. Therefore, 152 women would have been misclassified. For early age at menarche this resulted in a sensitivity of 74% and a specificity of 94%. If early age at menarche was associated with a health outcome and those who misreported did not differ on their outcome status, this misclassification would push the effect estimate towards the null. As age at menarche is a risk factor for various diseases, including breast cancer, the impacts of this type of misclassification on different outcomes should be explored in detail by researchers in such fields. Finally, for studies examining age at menarche as the outcome, small effects (such as those observed in environmental and hormonal exposures) would also be missed or distorted with slight misreporting as was observed in our study. However, for studies interested solely in the mean age at menarche, remotely recalled age at menarche is reliable.

Table 3.1 Errors in age at menarche for women reporting their age in the follow up survey, DESAD study, 1975-1994 (N=3998).

	N	%
Concordant reports	2218	55.5
Discordant reports	1780	44.5
Number of years under-reported		
1	671	79.7
2	135	16.0
3	24	2.9
4	8	1.0
5+	4	0.5
Total # under-reporting	842	
Number of years over-reported		
1	725	77.3
2	166	17.7
3	31	3.3
4	10	1.1
5+	6	0.6
Total # over-reporting	938	

Table 3.2 Characteristics of women in DESAD study, 1975-1994 with values for age at menarche from both the initial visit and the follow up survey by concordance of age at menarche values (N=3998).

	Concordance of Age at Menarche					
	Agree (n=2218)		Disagree (n=1780)		OR[a]	95%CI[b]
	n	%	n	%		
Highest grade completed						
High School or Less	269	12.1	281	15.8	0.9	(0.8, 1.0)
Some College	510	23.0	429	24.1	1.0	---
Graduated College	1432	64.6	1067	59.9	1.1	(1.0, 1.1)
Missing	7	0.3	3	0.2		
Ethnic background						
White	2185	98.5	1754	98.5	1.1	(0.8, 1.4)
Non White	28	1.3	26	1.5	1.0	---
Missing	5	0	1	0		
Ever smoked						
Yes	884	39.9	777	43.7	0.9	(0.9, 1.0)
No	1332	60.1	1002	56.3	1.0	---
Missing	2	0	1	0		
Exposed to DES						
Yes	1765	79.6	1409	79.0	1.0	(0.9, 1.1)
No	453	20.4	371	20.8	1.0	---
Missing	0	0	0	0		
Ever pregnant						
Yes	1725	77.8	1431	80.4	0.9	(0.9, 1.0)
No	493	22.2	346	19.4	1.0	---
Missing	0	0	3	0.1		
Age at menarche (as reported from original survey)						
< 12	336	15.1	260	14.6	1.0	(0.9, 1.1)
12-14	1744	78.6	1324	74.3	1.0	---
> 14	138	6.2	196	11.0	0.7	(0.6, 0.8)
Mean age (SD)[c]	12.7	(1.3)	12..9	(1.4)		

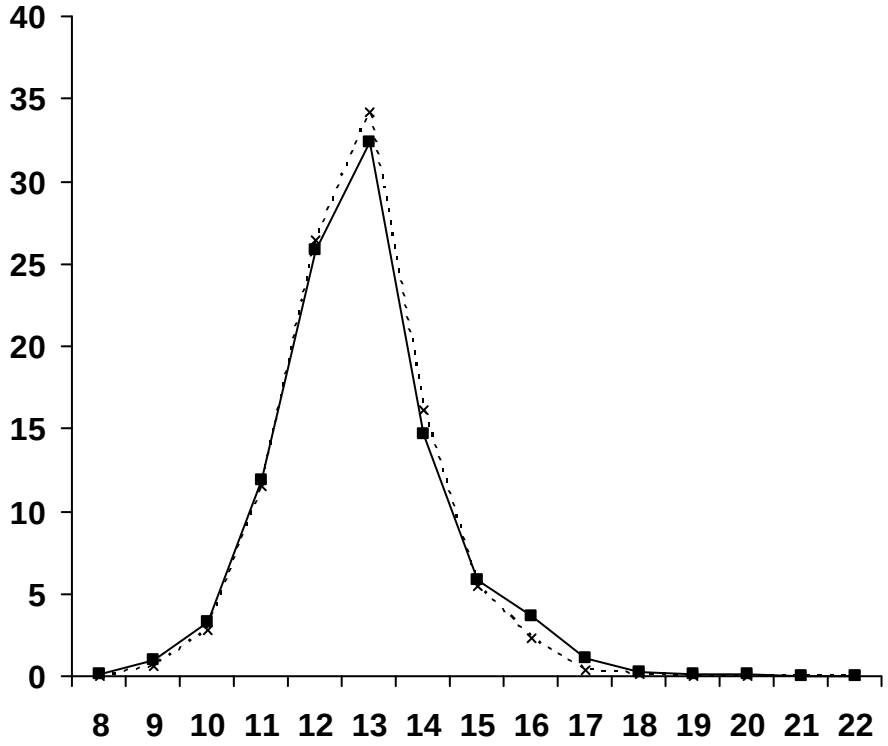
Age at 1994 survey						
≤ 30	42	1.9	57	3.2	0.7	(0.6, 0.9)
31-39	797	35.9	725	40.7	0.9	(0.9, 1.0)
40-49	1342	60.5	972	54.6	1.0	---
≥ 50	37	1.7	26	1.5	1.0	(0.8, 1.2)
Mean (SD)	40.6	(4.6)	40.0	(4.8)		
Missing	0	0	0	0		
Time lapse between surveys (years)						
≤14	162	7.3	119	6.7	1.0	---
15-17	700	31.6	558	31.3	1.0	(0.9, 1.1)
18-20	1356	61.1	1103	62.0	1.0	(0.9, 1.1)
Low birth weight (grams)						
< 2500	245	11.0	202	11.3	1.0	(0.9, 1.1)
≥ 2500	1933	87.2	1546	86.9	1.0	---
Missing	40	1.8	32	1.8		
Mean (SD)	3139.4	(535.4)	3136.2	(564.7)		
BMI at age 20 (kg/m^2)						
< 18.5	292	13.2	251	14.1	1.0	(0.9, 1.0)
18.5-24.9	1666	75.1	1317	74.0	1.0	---
25-29.9	136	6.1	124	7.0	0.9	(0.8, 1.1)
30+	34	1.5	29	1.6	1.0	(0.8, 1.2)
Missing	90	4.1	59	3.3		
Mean (SD)	20.9	(3.0)	21.0	(3.1)		
BMI at 1994 (kg/m^2)						
< 18.5	82	3.7	72	4.0	1.0	(0.8, 1.1)
18.5-24.9	1399	63.1	1140	64.0	1.0	---
25-29.9	439	19.8	322	18.1	1.0	(1.0, 1.1)
30+	253	11.4	209	11.7	1.0	(0.9, 1.1)
Missing	45	2.0	37	2.1		
Mean (SD)	24.1	5.0	24.1	5.1		

Mom's age at birth						
< 20	43	1.9	37	2.1	1.0	(0.8, 1.2)
20-24	428	19.3	360	20.2	1.0	(0.9, 1.1)
25-29	789	35.6	630	35.4	1.0	---
30-34	582	26.2	438	24.6	1.0	(0.9, 1.1)
35-40	247	11.1	181	10.2	1.0	(0.9, 1.1)
≥ 40	55	2.5	42	2.4	1.0	(0.9, 1.2)
Missing	74	3.3	92	5.2		
Mean (SD)	29.2	(5.1)	29.0	(5.1)		
Mom smoked in pregnancy						
Yes	741	33.4	651	36.6	0.9	(0.9, 1.0)
No	1363	61.5	1027	57.7	1.0	---
Missing	114	5.1	102	5.7		
Packs per day (among smoking moms)						
≤ 1	319	43.0	275	42.2	1.0	---
> 1	386	52.1	343	52.7	1.0	(0.9, 1.1)
Missing	36	4.9	33	5.1		

[a] Crude Odds Ratio
[b] 95% Confidence Interval
[c] Standard Deviation

Figure 3.1 Frequency of age at menarche as reported from original study and follow up survey.

---- original study
___ follow up survey



CHAPTER IV

PAPER 2: *IN UTERO* EXPOSURE TO CIGARETTE SMOKE AND REPRODUCTIVE OUTCOMES

Introduction

Age at menarche, infertility, and endometriosis are three important reproductive outcomes impacting millions of women and each can be associated with substantial physical, psychological, and financial burden for affected individuals. The epidemiology of altered age at menarche, infertility, and endometriosis is largely unknown. A common origin has been hypothesized, but as yet, the ordering of events is imprecise. For example is early or late onset puberty in the pathway to endometriosis and/or infertility? An evolving body of evidence indicates the intrauterine environment may play a role in the development of these and other female reproductive outcomes (Buck Louis et al. 2007;Missmer et al. 2004;Baird and Newbold 2005;Hatch et al. 2006;Palmer et al. 2001;Windham et al. 2004;Colbert et al. 2008;Baird and Wilcox 1986;Jensen et al. 1998;Weinberg et al. 1989;Windham et al. 2008). A recent review of endocrine disrupting chemicals and their effects on female reproductive disorders highlights the importance for more research focusing on the intrauterine environment as a potential risk factor (Crain et al. 2008).

In utero exposure to cigarette smoke has been reported to be associated with adverse female fecundity. Exposure *in utero* to cigarette smoke was associated with a 3 month earlier

age at menarche in a study by Windham and colleagues done in California (Windham et al. 2004), and a 3.7 month delay in another recent study by the authors (Windham et al. 2008). Similarly, early work by Baird et al. found no association between *in utero* cigarette smoke exposure and fecundability (Baird and Wilcox 1986). However, subsequent authors later reported that women exposed *in utero* required a longer time-to-pregnancy in comparison to unexposed women (Weinberg et al. 1989;Jensen et al. 1998). Only one study to date has examined *in utero* exposure to cigarette smoke and endometriosis. Louis and colleagues found that *in utero* exposure was associated with a lower odds of being diagnosed with endometriosis (Buck Louis et al. 2007).

Mechanisms underlying these observations are not fully understood despite recognition that cigarette smoke contains 4,000 constituents purportedly to be reproductive toxins with endocrine reactive properties (Baron et al. 1990;MacMahon et al. 1982;Michnovicz et al. 1986). Studies examining the effect of *in utero* exposure to cigarette smoke on reproductive outcomes are sparse; however, animal evidence suggests a plausible biologic mechanism for such an association. The timing and onset of menses are under control of the hypothalamic-pituitary-gonadal (HPG) and the hypothalamic-pituitary-adrenal (HPA) axes. Exposures experienced during the development of this system which occurs *in utero* may be important for the activation of other mechanisms directing the onset of puberty later in life. A recent review of environmental chemicals and their effect on timing of puberty stresses the possibility that these exposures could occur early on *in utero* not manifesting their effects until puberty or even later (Buck Louis et al. 2008). Studies on alligators, hamsters, and rats exposed to various endocrine disrupting chemicals such as dichlorodiphenyltrichloroethane (DDT), bisphenol A (BPA), and tobacco smoke have shown

a range of effects from differences in age of peak plasma hormone concentrations, an advance in the age of puberty, and effects on the ovary and uterus, respectively (Guillette, Jr. et al. 1994;Heinrichs et al. 1971;Howdeshell et al. 1999;Magers et al. 1995). Several animal studies have also shown the deleterious effects of cigarette smoke on mouse fecundity. Decreased adult fertility in mice has been shown after *in utero* exposure to benzo (a) pyrne, a constituent of tobacco smoke (MacKenzie and Angevine 1981). Another finding demonstrates that oocytes were destroyed after prenatal exposure to polycyclic aromatic hydrocarbons (Dobson and Felton 1983). Finally, endometriosis is widely known as an estrogen-dependent disease. The reduced odds ratios for endometriosis among smokers that have been observed (Cramer et al. 1986;Missmer and Cramer 2003) may be due to a hypoestrogenic state that can be induced in some women by smoking and/or the interference of nicotine and cotinine (important components of cigarette smoke) with steroid synthesis converting androgens into estrogens (Barbieri et al. 1986). Also, *in utero* cigarette smoke may affect müllerianosis, the ability of embryonic tissue from the Müllerian Duct to proliferate, which leads some to hypothesize that endometriosis is essentially a disease that develops *in utero* and which is activated later in life when hormonal stimuli are present (Batt et al. 1990).

We sought to examine the association between *in utero* cigarette smoke and three reproductive outcomes - age at menarche, infertility and endometriosis - in a large cohort of women with intensive medical follow-up to examine the consistency of effects across outcomes and a possible shared *in utero* origin.

Methods

Study Population

We used data from the National Cooperative Diethylstilbesterol Adenosis (DESAD) study which followed daughters exposed and unexposed *in utero* to Diethylstilbestrol (DES) yearly with clinical exams to monitor for abnormalities of the vagina and cervix. In 1975, over 4,000 exposed and 1,000 unexposed daughters were enrolled into the DESAD study at one of five sites across the country. The participating sites included: Baylor College of Medicine, Gundersen Clinic, Massachusetts General Hospital, Mayo Clinic, and University of Southern California. In 1994, the National Cancer Institute (NCI) sent mailed questionnaires to the original participants querying them on subsequent health exposures and outcomes.

Only women who were not exposed to DES (n=1034) were chosen for this analysis to assess the specific effects of *in utero* cigarette smoke and the three fecundity outcomes in the absence of a known reproductive and developmental toxicant, i.e., DES. Information on menarche, infertility and endometriosis accompanied with *in utero* exposure to cigarette smoke was available for 950 (92%), 764 (74%), and 738 (71%) of the eligible women, respectively.

In the age at menarche analysis, there were 745 women who had no sisters, 86 women who had one sister in the study, and 11 women who had two sisters in the study. For the infertility analysis, 610 women had no sisters in the study, 68 had one sister and six had two sisters. Finally, for the endometriosis analysis 590 women had no sisters in the study, 65 had one sister and six women had two sisters in the study.

Mothers completed a pregnancy questionnaire, and daughters completed a detailed health history questionnaire followed by a clinical examination in the mid-1970s. Daughters

were followed with annual physical examinations through 1984 and then mailed self administered questionnaires from 1985 through 1989. A follow-up questionnaire querying the daughters about subsequent health outcomes and potential exposures was initiated by the NCI in January of 1993 and continued through July of 1996. Eighty one percent of daughters from the eligible unexposed to DES cohort completed this 1994 questionnaire.

Data Analysis

Information on *in utero* exposure to cigarette smoke was ascertained at the baseline questionnaire from the mother when she brought her daughter in for the first DESAD visit. Mothers were asked about cigarette smoking (yes/no) during the index pregnancy. If women reported smoking, they were asked to specify how many cigarettes they smoked per day while pregnant.

Daughters' age at menarche was ascertained from the baseline questionnaire which asked daughters to report their age (in whole years) when menstruation first occurred. Age was categorized into the widely accepted categories of early ($\leq$ 11 years), average (12-13 years), and late ($\geq$ 14 years) (Lee 1980). Infertility and endometriosis were self reported on the 1994 follow-up questionnaire. We assessed infertility with three separate definitions. The strictest definition we applied was when a woman was considered infertile only if she answered yes to both of the following questions:

> Have you ever tried to become pregnant for 12 months or more without success? (yes/no)
> Have you ever been seen by a physician for difficulty getting pregnant? (yes/no)

We relaxed this definition to answering yes to either of the above criteria and also to just answering yes to the question of trying for more than 12 months to account for the many women who may have difficulty conceiving but who do not seek care (Chandra A et al.

2005). Women were classified as having endometriosis if they reported ever having been diagnosed by any one of the following methods: laparoscopy, biopsy, hysterectomy, or any other gynecologic surgery.

All covariates were examined using frequencies (for categorical variables) and means (for continuous variables) by the three outcomes. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each covariate. Unconditional logistic regression was used to examine the association between *in utero* cigarette smoke exposure and the dichotomous outcomes: early age at menarche, late age at menarche, infertility, and endometriosis. Age at menarche was also examined in a continuous fashion to look for any subtle differences between groups. Each outcome was examined individually and also simultaneously with the other outcomes to account for the possibility that the exposure affected multiple outcomes on the same biologic pathway. Generalized estimating equations (GEE) were utilized to account for the dependency between observations of sisters included in the study (Zeger and Liang 1986;Liang and Zeger 1986).

Potential confounders were initially determined *a priori* based on an association with both *in utero* exposure to cigarette smoke and the outcome. In all three models where outcomes were considered individually education was considered a potential confounder (as a surrogate for socio-economic status). Daughter's race was a potential confounder in the age at menarche analysis. History of sexually transmitted diseases and daughter's smoking status were considered as potential confounders in the models for the outcomes of endometriosis and infertility. Finally, infertility was considered as a potential confounder in the model with the outcome of endometriosis. In the model considering any of the outcomes combined, only education was included as a potential confounder due to the possibility that

many of these covariates and outcomes could be on the pathway between exposures and disease. If inclusion of the confounders did not change the effect estimate >10%, the covariate was excluded from the final model.

We stratified the *in utero* exposure to cigarette smoke-outcome relationship by the daughter's smoking status as this was considered a potential effect modifier for all of the outcomes except for age at menarche (we did not have data on the daughter's smoking habits before she attained menarche). Additionally, a cutoff value of $p<.10$ for a pseudo likelihood ratio test was used for the interaction term in all models to confirm effect modification. All analyses were done using SAS® statistical software program version 9.2 (SAS Institute 2006).

Results

Of the 950 women in the age at menarche analysis, approximately 37 percent were exposed *in utero* to cigarette smoke. One hundred twenty-two women were classified as having an early age at menarche, 572 had an average age at menarche, and 256 had a late age at menarche. Age at menarche was not associated meaningfully with any of the covariates except for body mass index (BMI). Women with lower BMI had an increased odds for late age at menarche compared to women with a normal BMI (Table 4.1). The odds for having either an early or a late age at menarche were slightly increased (OR=1.3, 95%CI (0.8, 1.8)) and (OR=1.1, 95%CI (0.8, 1.5)), respectively, among women who were exposed *in utero* to cigarette smoke compared to unexposed women. However, these associations disappeared when packs/day and cigarettes/day were assessed (Table 4.2). Education and the daughter's race did not change the effect estimates more than 10%, and the unadjusted models were

appropriate for this outcome (Table 4.2). No association was observed for age at menarche when it was considered as a continuous variable (mean change in age= -0.07, 95%CI (-0.14, 0.01)).

Overall, 93 women were classified as infertile in this analysis according to the strictest definition requiring physician diagnosis. Forty-five of the women were exposed *in utero* to cigarette smoke irrespective of infertility status (Table 4.3). None of the other covariates examined were associated with infertility except for education. Women who graduated from college had an elevated odds for infertility compared to women with only some college education (OR=1.7, 95%CI (1.0, 2.9)). This association, however, may be more of a function of care seeking behavior rather than infertility. Regardless of what definition of infertility was used, estimates of the odds for infertility comparing women who were exposed *in utero* to cigarette smoke compared to women who were unexposed hovered around one (Table 4.4). These null results persisted when cigarettes/day and packs/day were examined, and when results were stratified by the daughter's smoking status as an adult. Adjustment for sexually transmitted diseases (STDs) and education as a surrogate for socioeconomic status did not change the estimates and, thus, unadjusted models are presented.

Of the 738 women in the endometriosis analysis, 58 (8%) women were classified as having disease according to our definition (Table 4.5). The only covariate associated with endometriosis risk was ever having been pregnant, which showed a reduced risk (OR=0.4, 95%CI (0.2, 0.6)). Logistic regression analyses showed a decreased odds of endometriosis for women exposed *in utero* to cigarette smoke (OR=0.7, 95%CI (0.4, 1.2)), even when results were stratified by whether the daughter smoked and when packs/day or cigarettes/day

were examined (Table 4.6). STDs, education, and infertility were included in the initial models as potential confounders, but did not change the estimates and, therefore, were not included in the final model.

All three outcomes were simultaneously assessed to address the shared etiology and the possibility that a woman might have multiple outcomes. The hypothesized pathway is that exposure *in utero* to cigarette smoke would be associated with an altered age at menarche followed by infertility and endometriosis. Only four women in our study had all three outcomes, while 16 had both infertility and endometriosis, 18 had late menarche and infertility, 15 had late menarche and endometriosis, 15 had early menarche and infertility, and 11 women had early menarche and endometriosis. These sample sizes were too small to produce stable estimates. When the combined outcomes were examined in the full cohort of DES exposed and non-exposed daughters, the result for the effect of *in utero* cigarette smoke exposure on all combinations of the outcomes was esentially null after adjusting for appropriate confounders (data not shown).

Discussion

Overall, we found no evidence to suggest that *in utero* exposure to cigarette smoke was associated with age at menarche or infertility, however there was a decreased odds for endometriosis among women exposed *in utero* to cigarette smoke compared to unexposed women participating in the DESAD study. The findings for age at menarche and infertility are in contrast to the few previous studies published in this area (Baird and Wilcox 1986;Buck Louis et al. 2007;Jensen et al. 1998;Weinberg et al. 1989;Windham et al. 2004;Windham et al. 2008), but those for endometriosis are in the same direction as the one previous study on this topic (Buck Louis et al. 2007).

Two previous studies have examined the association between *in utero* cigarette smoke exposure and age at menarche (Windham et al. 2004;Windham et al. 2008). In her first study, Windham and her colleagues used data from the Child Health and Development Study, a longitudinal pregnancy study of families in the San Francisco Bay Area of California who were members of the Kaiser Permanente Health Plan to examine whether exposure *in utero* to cigarette smoke affected age at menarche. Maternal smoking during pregnancy was assessed upon enrollment into the study and daughters were asked to recall their age at menarche in adolescence in years and months. The mean age at menarche was a few months earlier among daughters whose mothers smoked a pack or more of cigarettes per day compared to girls whose mothers did not smoke(-.31, 95% CI (0.65, 0.03)). The authors also found that the odds of late menarche were slightly reduced when comparing mothers who smoked heavily (OR=0.69, 95%CI (0.38, 1.23)) to mothers who did not smoke at all. In our study, we found no difference between the mean age of menarche for women exposed and unexposed *in utero* to cigarette smoke (mean=12.9 in each group). However, our study is limited by only having data on menarche recorded in years, whereas the Windham study had years and months for 55% of the girls. Additionally, Windham and colleagues had information on environmental tobacco smoke exposure in childhood which could have affected their results. These data were not available in our study. In a second study using data from two cohorts which were followed after participating in the Collaborative Perinatal Project in the 1960s, Windham and colleagues actually found results contradicting her earlier work. Girls born to mothers who were heavy smokers (20+ cigarettes/day) during pregnancy had a delay of 0.31 years (95%CI (0.008, 0.61)) when compared to girls born to mothers who were not smokers. This translates to an almost four month delay in age at

menarche. This second study had smoking measured across the pregnancy and, thus, the categorization of the exposure may be better than previous studies. However, the authors had to rely on recalled age at menarche (in whole years) later in life (around reproductive age) for the outcome. Again, in our study we did not find such a strong association between *in utero* exposures and age at menarche. We assessed menarche as a continuous variable and found the mean difference in age at menarche for daughters whose mothers smoked during pregnancy versus daughters who were not exposed *in utero* was -.07 (95%CI (-0.2, 0.1)). This difference is much smaller than that seen in previous studies by Windham and her colleagues.

To date, there have been only three studies which have examined the association between *in utero* exposure to cigarette smoke and time to pregnancy (TTP) a measure of fecundability. All of these studies used proxy exposure information, and two found an adverse effect between cigarette smoke exposure *in utero* and adult fecundability (Weinberg et al. 1989;Jensen et al. 1998), while the third study found no effect (Baird and Wilcox 1986). There are no published studies to our knowledge that look at the outcome of infertility, *per se,* with respect to *in utero* cigarette smoke exposure. We restricted our definition of infertility by first including only women who reported that they had experienced a waiting time of more than 12 months before becoming pregnant and who saw a physician for difficulty becoming pregnant. This is in keeping with a previous work examining the effect of DES on infertility in the same population (Palmer et al. 2001). However, it is possible that infertility was not accurately captured in our study as choices about family size and care seeking behaviors of women may have led to misclassification of a woman's infertility status. We also examined less stringent criteria for infertility to account for

possible misclassification of disease status and our results did not change. A small validation study of specific infertility diagnoses showed that women in this cohort can accurately report their infertility diagnosis (90% of the diagnoses were confirmed) (Palmer et al. 2001). However, only 50% of those women queried agreed to have their records searched. There is no reason to believe that any misclassification would have been differential on the exposure, as mothers and daughters were queried separately on respective exposure/outcome information.

We are only aware of one paper assessing the relation between *in utero* exposure to cigarette smoke and endometriosis (Buck Louis et al. 2007). This study of a laparoscopic cohort found a reduced odds ratio for endometriosis among women who were exposed *in utero* to cigarette smoke (OR=0.2, 95%CI (0.1, 0.8)) compared to unexposed women. Our study found a similar effect for endometriosis. Two important differences in these studies may have accounted for our estimate's lack of statistical significance. First, the Louis et al. study used proxy exposure information, as they relied on daughters reporting of maternal smoking status while pregnant while our study queried the mothers. Secondly, we relied on self-reported endometriosis in the DESAD cohort, while the Louis et al. study had laparoscopically-confirmed disease status. Self-reported endometriosis diagnosis can be unreliable, since disease manifestation is varied in different individuals. Previous studies have found that endometriosis based on symptoms or medical history has low concordance with laparoscopic diagnosis (Duleba 1997;Eskenazi et al. 2001). Therefore, we sought to improve this diagnosis by restricting the definition to women who had confirmed endometriosis by either laparoscopy, biopsy, hysterectomy, or other gynecologic surgery. Despite this effort, it is possible that cases of endometriosis in the population were missed,

particularly those cases which may not have presented with symptoms that may have led to the procedures listed above. In our study eight percent of the sample was diagnosed with endometriosis, perhaps somewhat lower than expected. To our knowledge, one population based estimate on incidence for endometriosis has been reported. Overall incidence of diagnosed endometriosis was 1.9 per 1,000 person years in a population of 8,229 women 15 years and older in Minnesota (Leibson et al. 2004). Prevalence figures on endometriosis depend on the study sample and range from 10-15% in the general reproductive aged population (Houston 1984;Olive and Schwartz 1993).

In conclusion, given the lack of published epidemiologic studies on the effects of *in utero* cigarette smoke and reproductive outcomes. The follow-up of DESAD allowed for initial assessment of the reproductive toxicity of *in utero* exposure to cigarette smoke and three outcomes among women undergoing intensive follow-up and medical evaluation. Furthermore, this cohort has information on maternal exposure, which sets it apart from the prior studies relying on proxy response by offspring. The data on exposure were collected in an era free from the stigma currently attached to women who smoke cigarettes during pregnancy. Therefore, our exposure data are likely to result in non-differential bias, if any. Despite the strengths and size of this study, we had to rely on self-reported outcomes, which for infertility and endometriosis can present misclassification problems. However, it is unlikely that any misclassification would be differential as the exposure information was ascertained from the mother, and occurred long before any of the reported outcomes.

Table 4.1 Characteristics of women by early, average, and late age at menarche, DESAD study, 1975-1994, (n=950)[a]

	Early ≤ 11 years (n=122)				Average 12-13 years (n=572)		Late ≥ 14 years (n=256)			
	n	%	OR[b]	95%CI	n	%	n	%	OR[b]	95%CI
Highest grade completed										
High School or Less	13	10.7	0.9	(0.5, 1.7)	91	15.9	44	17.2	1.1	(0.8, 1.5)
Some College	19	15.6	1.0	---	117	20.5	50	19.5	1.0	---
Graduated College	66	54.1	1.4	(0.9, 2.3)	260	45.5	121	47.3	1.1	(0.8, 1.4)
Missing	24	19.7			104	18.2	41	16.0		
Ethnic background										
White	120	98.4	1.1	(0.3, 3.8)	562	98.3	256	100	---	---
Non White	2	1.6	1.0	---	9	1.6	0	0	1.0	---
Missing	0	0			1	0.2	0	0		
Mom smoked in pregnancy										
Yes	50	41.0	1.2	(0.9, 1.8)	198	34.6	97	37.9	1.1	(0.9, 1.4)
No	72	59.0	1.0	---	374	65.4	159	62.1	1.0	---
Missing	0	0			0	0	0	0		
Packs per day in pregnancy (among smoking mothers)										
≤ 1	28	56.0	1.0	---	80	40.4	40	41.2	1.0	---
>1	19	38.0	0.6	(0.3, 1.0)	109	55.1	50	51.5	0.9	(0.7, 1.3)
Missing	3	6.0			9	4.5	7	7.2		

BMI at age 20 (kg/m^2)										
< 18.5	8	6.6	0.8	(0.4, 1.5)	51	8.9	38	14.8	1.4	(1.1, 1.8)
18.5-24.9	76	62.3	1.0	---	360	62.9	159	62.1	1.0	---
25-29.9	7	5.7	1.1	(0.6, 2.2)	29	5.1	14	5.5	1.1	(0.7, 1.7)
30+	2	1.6	1.0	(0.3, 3.7)	9	1.6	0	0	---	---
Missing	29	23.8			123	21.5	45	17.6		
Mean (SD)	21.4 (2.8)				21.1 (3.1)		20.5 (2.3)			
Low birth weight (grams)										
< 2500	4	3.3	0.8	(0.3, 2.0)	25	4.4	5	2.0	0.5	(0.2, 1.2)
≥ 2500	118	96.7	1.0	---	544	95.1	251	98.0	1.0	---
Missing	0	0			3	1.0	0	0		
Mean (SD)	3252.8 (470.7)				3332.9 (478.5)		3341.2 (423.6)			
Mother's age at birth										
< 20	2	1.6	0.4	(0.1, 1.6)	24	4.2	5	2.0	0.6	(0.3, 1.3)
20-24	26	21.3	1.0	(0.6, 1.5)	117	20.5	49	19.1	1.0	(0.7, 1.3)
25-29	49	40.2	1.0	---	217	37.9	95	37.1	1.0	---
30-34	32	26.2	1.0	(0.7, 1.5)	141	24.7	69	27.0	1.1	(0.8, 1.4)
35-40	11	9.0	0.9	(0.5, 1.5)	59	10.3	23	9.0	0.9	(0.6, 1.4)
≥ 40	2	1.6	0.8	(0.2, 2.9)	12	2.1	11	4.3	1.6	(1.0, 2.5)
Missing	0	0			2	0.3	4	1.6		
Mean (SD)	28.7 (5.1)				28.7 (5.2)		29.2 (5.2)			

Abbreviations: OR, Odds Ratio CI, confidence interval SD, standard deviation

[a] Analysis restricted to women not exposed to DES with information on age at menarche and *in utero* cigarette smoke exposure

[b] Odds Ratios comparing early or late age at menarche to average age at menarche

Table 4.2 Regression results examining *in utero* cigarette smoke exposure and age at menarche in the DESAD study, 1975-1994 [ab].

	Age at menarche (continuous)		Early age at menarche		Late age at menarche	
	Mean change	95%CI	OR	95%CI	OR	95%CI
Yes/No	-0.07	(-0.14, 0.01)	1.3	(0.8, 1.8)	1.1	(0.8, 1.5)
Cigarettes/Day	0	---	1.0	(0.9, 1.0)	1.0	(0.9, 1.0)
Packs/Day	-0.02	(-0.07, 0.03)	1.0	(0.8, 1.3)	1.1	(0.9, 1.3)

Abbreviations: OR, odds ratio CI, confidence interval

[a] Analysis restricted to women not exposed to DES with information on age at menarche and *in utero* cigarette smoke exposure

[b] Generalized estimating equations used to estimate mean changes and odds ratios in respective models

Table 4.3 Characteristics of women in DESAD study by infertility status, 1975-1994 (n=764) [a]

	Infertility Reported (n=93)		No Infertility Reported (n=671)		OR	95%CI
	n	%	n	%		
Highest grade completed						
High School or Less	15	16.1	129	19.2	1.2	0.6, 2.4
Some College	15	16.1	163	24.3	1.0	---
Graduated College	63	67.7	377	56.2	1.7	1.0, 2.9
Missing	0	0	2	0.3		
Ethnic background						
White	92	99.0	663	98.8	1.1	0.2, 7.0
Non-White	1	0.1	8	1.2	1.0	---
Missing	0	0	0	0		
Ever smoked						
Yes	39	41.9	307	45.8	0.9	0.6, 1.3
No	54	58.1	364	54.3	1.0	---
Missing	0	0	0	0		
Mom smoked in pregnancy						
Yes	28	30.1	255	38.0	0.7	0.5, 1.1
No	65	69.9	416	62.0	1.0	---
Missing	0	0	0	0		
Packs per day in pregnancy (among smoking mothers)						
≤ 1	14	50.0	111	43.5	1.0	---
>1	14	50.0	130	51.0	0.9	(0.4, 1.7)
Missing	0	0	14	5.5		

BMI at age 20 (kg/m^2)						
< 18.5	11	11.8	83	12.4	0.9	0.5, 1.7
18.5-24.9	72	77.4	508	75.7	1.0	---
25-29.9	6	6.5	44	6.6	1.0	0.4, 2.1
30+	1	1.1	9	1.3	0.8	0.1, 5.2
Missing	3	3.2	27	4.0		
Mean (SD)	20.8 (3.0)		21.0 (3.1)			
BMI at 1994 (kg/m^2)						
< 18.5	2	2.2	30	4.5	0.5	0.1, 2.1
18.5-24.9	55	59.1	413	61.6	1.0	---
25-29.9	18	19.4	141	21.0	1.0	0.6, 1.6
30+	15	16.1	69	10.3	1.5	0.9, 2.6
Missing	3	3.2	18	0		
Mean (SD)	24.2 (5.1)		24.1 (5.0)			
Low birth weight (grams)						
< 2500	1	1.1	26	3.9	0.3	(0.1, 2.1)
≥ 2500	91	97.9	644	96.0	1.0	---
Missing	1	1.1	1	0.1		
Mean (SD)	3132.3 (542.9)		3151.3 (556.7)			
Age at menarche						
≤ 11	15	16.1	81	12.1	1.2	0.7, 2.0
12-13	60	64.5	398	59.3	1.0	---
≥ 14	18	19.4	192	28.6	0.7	0.4, 1.1
Missing	0	0	0	0		
Mean age (SD)	12.6 (1.4)		12.9 (1.4)			

Mother's age at birth						
< 20	0	0	26	3.7	---	---
20-24	24	25.8	130	19.4	1.1	0.7, 1.8
25-29	41	44.1	250	37.3	1.0	---
30-34	16	17.2	182	27.1	0.6	0.3, 1.0
35-40	9	9.7	66	0.1	0.9	0.4, 1.7
≥ 40	3	3.2	11	1.6	1.5	0.5, 4.3
Missing	8	8.6	32	4.8		
Mean (SD)	28.8 (5.0)		29.2 (5.2)			

Abbreviations: OR, odds ratio CI, confidence interval SD, standard deviation

[a] Analysis restricted to women not exposed to DES with information on infertility (reported more than 12 months trying to become pregnant without success and saw a physician for difficulty getting pregnant) and *in utero* cigarette smoke exposure

Table 4.4 Regression results examining *in utero* cigarette smoke exposure and reported infertility in the DESAD study, stratified by woman's smoking status 1975-1994 [ab].

	Tried to get pregnant >12 months and sought care for infertility		Either tried to get pregnant >12 months or sought care for infertility		Tried to get pregnant >12 months	
	OR	95%CI	OR	95%CI	OR	95%CI
Yes/No	0.7	(0.4, 1.1)	0.9	(0.6, 1.3)	0.8	(0.5, 1.2)
Cigarettes/Day	1.0	(0.9, 1.0)	1.0	(0.9, 1.0)	1.0	(0.9, 1.0)
Packs/Day	0.8	(0.6, 1.1)	0.9	(0.7, 1.2)	0.9	(0.7, 1.1)
Among daughters who smoked						
Yes/No	0.7	(0.4, 1.4)	0.8	(0.4, 1.4)	0.7	(0.4, 1.4)
Cigarettes/Day	1.0	(0.9, 1.0)	1.0	(1.0, 1.1)	1.0	(0.9, 1.0)
Packs/Day	0.8	(0.5, 1.1)	0.9	(0.6, 1.2)	0.8	(0.6, 1.2)
Among daughters who did not smoke						
Yes/No	0.7	(0.4, 1.3)	0.9	(0.6, 1.6)	0.7	(0.4, 1.3)
Cigarettes/Day	1.0	(0.9, 1.1)	1.0	(0.9, 1.0)	1.0	(0.9, 1.0)
Packs/Day	0.9	(0.6, 1.3)	1.0	(0.7, 1.4)	0.9	(0.6, 1.3)

Abbreviations: OR, odds ratio CI, confidence interval

[a] Analysis restricted to women not exposed to DES with information on infertility and *in utero* cigarette smoke exposure

[b] Generalized estimating equations used to estimate odds ratios

Table 4.5 Characteristics of women in DESAD study, by endometriosis diagnosis 1975-1994 (n=738)[a]

	Endometriosis (n=58)		No Endometriosis (n=680)		OR	95%CI
	n	%	n	%		
Highest grade completed						
High School or Less	8	13.8	124	18.2	0.9	(0.4, 2.3)
Some College	11	19.0	160	23.5	1.0	---
Graduated College	38	65.5	379	55.7	1.4	(0.7, 2.7)
Missing	1	1.7	17	2.5		
Ethnic background						
White	57	98.3	673	99.0	0.6	(0.1, 4.0)
Non-White	1	1.7	7	1.0	1.0	---
Missing	0	0	0	0		
Ever pregnant						
Yes	38	65.5	578	85.0	0.4	(0.2, 0.6)
No	20	34.5	100	14.7	1.0	---
Missing	0	0	2	0.3		
Ever smoked						
Yes	21	36.2	309	45.4	0.7	(0.4, 1.1)
No	37	63.8	362	53.2	1.0	---
Missing	0	0	9	1.3		
Mom smoked in pregnancy						
Yes	17	29.3	253	37.2	0.7	(0.4, 1.2)
No	41	70.7	427	62.8	1.0	---
Missing	0	0	0	0		

Packs per day in pregnancy (among smoking mothers)						
≤ 1	9	52.9	113	44.7	1.0	---
>1	7	10.4	128	50.6	0.7	(0.3, 1.8)
Missing	1	1.5	12	4.7		
BMI at age 20 (kg/m^2)						
< 18.5	11	19.0	74	10.9	1.7	(0.9, 3.2)
18.5-24.9	41	70.7	510	75.0	1.0	---
25-29.9	4	6.9	43	6.3	1.1	(0.4, 3.1)
30+	0	0	10	1.5	---	---
Missing	2	3.4	43	6.3		
Mean (SD)	20.5 (2.9)		21.0 (2.9)			
BMI at 1994 (kg/m^2)						
< 18.5	3	5.2	26	3.8	1.4	(0.4, 4.2)
18.5-24.9	34	58.6	414	60.9	1.0	---
25-29.9	14	24.1	138	20.3	1.2	(0.7, 2.2)
30+	5	8.6	71	10.4	0.9	(0.4, 2.1)
Missing	2	3.4	31	4.6		
Mean (SD)	23.7 (4.2)		24.1 (5.0)			
Low birth weight (grams)						
< 2500	2	3.4	23	3.4	1.0	(0.3, 3.9)
≥ 2500	56	96.6	656	96.5	1.0	---
Missing	0	0	1	0.1		
Mean (SD)	3311.1 (461.6)		3309.9 (463.8)			

Age at menarche						
≤ 11	11	19.0	88	12.9	1.5	(0.8, 2.9)
12-13	32	55.2	404	59.4	1.0	---
≥ 14	15	25.9	188	27.6	1.0	(0.6, 1.8)
Missing	0	0	0	0		
Mean age (SD)	12.6 (1.4)		12.9 (1.4)			
Mother's age at birth						
< 20	2	3.4	20	2.9	1.4	(0.4, 5.8)
20-24	14	24.1	135	19.9	1.5	(0.8, 2.9)
25-29	18	31.0	266	39.1	1.0	---
30-34	15	25.9	178	26.2	1.2	(0.6, 2.4)
35-40	6	10.3	64	9.4	1.4	(0.6,3.3)
≥ 40	2	3.4	12	1.8	2.3	(0.6, 8.8)
Missing	1	1.7	5	0.7		
Mean (SD)	28.9 (5.7)		28.7 (4.9)			

Abbreviations: OR, odds ratio CI, confidence interval SD, standard deviation

[a] Analysis restricted to women not exposed to DES with information on endometriosis (only women who had diagnosis by laparoscopy, biopsy, hysterectomy, or other gynecologic surgery included) and *in utero* cigarette smoke exposure

Table 4.6 Regression results examining *in utero* cigarette exposure and endometriosis stratified by woman's smoking status in the DESAD study, 1975-1994 [ab].

	OR	95%CI
Yes/No	0.7	(0.4, 1.3)
Cigarettes/Day	1.0	(0.9, 1.0)
Packs/Day	0.6	(0.2, 1.7)
Among daughters who smoked		
Yes/No	0.6	(0.2, 1.6)
Cigarettes/Day	0.9	(0.8, 1.0)
Packs/Day	0.5	(0.1, 2.5)
Among daughters who did not smoke		
Yes/No	0.8	(0.4, 1.6)
Cigarettes/Day	1.0	(0.9, 1.1)
Packs/Day	0.9	(0.2, 3.3)

Abbreviations: OR, odds ratio CI, confidence interval

[a] Analysis restricted to women not exposed to DES with information on endometriosis (only women who had diagnosis by laparoscopy, biopsy, hysterectomy, or other gynecologic surgery included) and *in utero* cigarette smoke exposure

[b] Generalized estimating equations used to estimate odds ratios

CHAPTER V

PAPER 3: *IN UTERO* EXPOSURE TO DES AND ENDOMETRIOSIS

Introduction

Although endometriosis is the third leading cause of gynecologic hospitalization in the United States, its etiology is largely unknown (Eskenazi and Warner 1997). Endometriosis is a complex disease that occurs when endometrial glands and stroma grow outside the uterus and respond to hormonal signals, often growing in an aberrant manner. Endometriosis is a difficult disease to study at the population level, given that clinical diagnosis requires laparoscopic visualization and its asymptomatic presence in an uncertain percentage of fertile women (Leibson et al. 2004).

Of late, there is considerable speculation that many adult onset diseases originate *in utero,* though little research has focused on gynecologic conditions including endometriosis. In searching the literature, we were able to identify only a few papers focusing on possible intrauterine exposures (Buck Louis et al. 2007;Missmer et al. 2004) of which diethylstilbestrol (DES) was observed to be associated with increased risk of disease. Specifically, an 80% increased risk of endometriosis was observed for self reported DES exposure among women participating in the Nurses' Health Study (NHS) compared to unexposed women (Missmer et al. 2004). DES has been associated with a host of adverse health outcomes, including infertility in some (Palmer et al. 2001;Senekjian et al. 1988;Kaufman et al. 1986;Berger and Alper 1986) but not all studies

(Barnes et al. 1980;Cousins et al. 1980), and also is reported to be associated with a 50% increase in the odds of endometriosis among infertile women seeking diagnostic evaluation for infertility (Berger and Alper 1986). In addition, *in utero* DES exposure has been associated with cervical stenosis which results in a back flow of menstrual blood (Stillman and Miller 1984). This is one of the most commonly accepted etiologies of endometriosis. Thus, *in utero* exposure to a potent estrogenic compound and risk of endometriosis remains understudied serving as the impetus for this study.

Methods

Study Population

In 1975, over 4,000 exposed and 1,000 unexposed daughters were enrolled into the National Cooperative Diethylstilbesterol and Andenosis Study (DESAD) at one of five sites across the country: Baylor College of Medicine, Gundersen Clinic, Massachusetts General Hospital, Mayo Clinic, and University of Southern California. Three types of exposed women were followed during the DESAD study: 1) women whose exposure to DES was ascertained by review of prenatal records (47%); 2) women referred to the study by outside physicians (32%); and 3) women who "walked-in" to the local DESAD clinics seeking evaluation (21%). Exposed daughters who were referred by outside physicians or who "walked-in" to the clinics were required to have written evidence of exposure from prenatal records or a letter from a physician who had provided prenatal care during the pregnancy. Two types of unexposed women were selected: 1) sisters of exposed participants (24%) and 2) non-relatives identified from the same record sources as the exposed (76%), most of whom were matched to the exposed on year of birth and mother's age at delivery.

In 1976 mothers were asked to complete a pregnancy questionnaire and daughters filled out a detailed health history questionnaire and underwent a baseline clinical exam at enrollment. The daughters were offered yearly exams until 1983 and then mailed annual self-administered questionnaires from 1984-1989. In 1994, the National Cancer Institute (NCI) sought to re-contact participants to query them about subsequent health issues, including the development of cancers and reproductive outcomes experienced later in life. Women were included in our analysis if they responded to the 1994 self-administered questionnaire and had complete information on the question for endometriosis diagnosis. This included 3876 (77% eligible) women.

Data Analysis

Exposure to DES was coded as a dichotomous variable (yes/no) and was confirmed for all women in the study at the initial DESAD study visit. Medical records for some women included dose and timing information on DES, and when available these data were used. Gestational age (in completed weeks) at first exposure was categorized based on the distribution of data. Information on birth weight and gestational age were available for 80% of the women in the study from obstetrical charts. The remaining 20% of the data was ascertained from the mothers at their daughter's enrollment into the study. Body mass index (BMI) was calculated as weight in kilograms per height in meters squared and was divided into categories of underweight, normal, overweight, and obese based on established cut-points set by the Centers for Disease Control and Prevention.

Endometriosis was self-reported at the 1994 questionnaire. Women were asked if they had ever been diagnosed by a physician as having endometriosis (yes/no). If they

answered yes, they were asked how the endometriosis was diagnosed. Only women who reported that their endometriosis was diagnosed by laparoscopy, hysterectomy, biopsy, or other gynecologic surgery were included as endometriosis cases.

All covariates were examined using frequencies (for categorical variables) and means (for continuous variables) by endometriosis status. Odds ratios and 95% confidence intervals were calculated for each covariate. Unconditional logistic regression was used to examine the association between *in utero* DES exposure and endometriosis. Gestational age (in completed weeks) at first exposure was considered in models comparing women who were exposed at varying times during pregnancy to women who were unexposed. Gestational age at first exposure, birth weight, and gestational age at birth of the daughter were also examined as continuous variables in the logistic models. Generalized estimating equations were utilized to account for the dependency between observations of sisters included in the study (Zeger and Liang 1986;Liang and Zeger 1986). Confounders were determined based on an *a priori* association with both *in utero* exposure to DES and the outcome. Education (as a surrogate for socio-economic status), history of sexually transmitted diseases, daughter's smoking status (ever/never) and mother's smoking status during the index pregnancy, age, and gravidity were considered as potential confounders. If inclusion of a confounder did not change the effect estimate >10%, it was not included in the final model. Effect modification was assessed by stratifying the results. Reported infertility of the daughter, smoking status of the mother while pregnant, and if the daughter ever smoked were considered as potential effect modifiers. Additionally, a cutoff value of $p<.10$ for the pseudo likelihood ratio test was used for the interaction term in the logistic model to confirm effect modification.

We also examined the potential effect of birth weight, preterm delivery and small-for-gestational age (SGA) on risk of endometriosis as these have been hypothesized and shown in previous studies to be associated with onset of disease later in life. SGA was defined using the Brenner curves for distinguishing those infants born <10% percentile for that time period (Brenner et al. 1976). Briefly, this entailed using rounded measures for gestational age and the birth weight limits to determine the 10^{th} percentile for growth in each gestational age category (in completed weeks of gestation) . All analyses were performed using SAS statistical software version 9.1 (SAS Institute 2006).

Results

Data were available for 3,876 (77% eligible) women including 2,943 women who had no sisters in the study, 382 women with one sister, 45 women had two sisters, seven women with three sisters, and one woman with five sisters in the study. Overall, 472 women were classified as having endometriosis (12%). The majority of women were white and highly educated, consistent with the characteristics of who was given DES during pregnancy. Seventy-nine percent of the women had ever been pregnant at the 1994 survey and 41% had ever smoked in their lifetime. Seventy percent of the women were of normal BMI at age 20 years, while this number decreased to around 60% at the 1994 survey. Finally, over 80% of the women were exposed to DES in this sample (Table 5.1).

Higher body mass index (BMI) at age 20 was associated with a decreased odds for endometriosis (OR=0.6, 95% CI (0.2, 1.5)) as was mother's smoking status while pregnant (OR=0.9, 95%CI = (0.7, 1.1)). Conversely, DES exposure, the daughter's birth weight of

<2500 grams, and nulligravid status were associated with an increased odds of endometriosis in the categorical analyses (Table 5.1).

In the adjusted models, we accounted for age and gravidity of the daughter and found an increased odds for endometriosis among women who were exposed *in utero* to DES as compared to women who were not exposed (OR=1.6, 95%CI (1.2, 2.1)). When women with infertility were looked at separately, the increased odds among those exposed to DES was attenuated (OR=1.2, 95%CI (0.7, 2.0)). The same was true when women without infertility diagnoses were examined (Table 5.2).

Women who were exposed very early in gestation (≤ 7 weeks) had an 80% greater odds for endometriosis (OR=1.8, 95%CI (1.3, 2.4)) compared to unexposed women. Similar increases in risk were seen across all other categories of gestational age, including a 90% increase in odds of disease among women exposed between 11 and 14 weeks compared to unexposed women (Table 5.3). However, women exposed between 8 and 10 weeks gestation did not have an increased risk for disease (OR=1.2, 95% (0.8, 1.6)). Gestational age was also examined in a continuous fashion and only a slight increase in risk was observed (data not shown).

We also examined the potential effect of birth weight and preterm delivery on risk of endometriosis, given earlier reports of an association. We observed no association for low birth weight (<2500 grams) and preterm birth (<37 weeks) even after controlling for age, gravidity, and DES exposure respectively ((OR=1.0, 95%CI (0.7, 1.3)) and (OR=1.1, 95%CI (0.8, 1.5))). There was a slight increase in the odds for endometriosis (20%) associated with LBW status among the fertile population; however, this was not statistically significant (Table 5.4). Preterm delivery also conveyed a slightly higher risk for endometriosis after

adjustment in the total population and in the fertile population, but not in the infertile population (Table 5.4). Birth weight and gestational age were also examined in a continuous fashion and no increase in the odds for disease was seen in either model. Null results were obtained when we examined small-for-gestational-age (SGA) status (Table 5.4).

None of the interaction terms for the potential effect modifiers were statistically significant, but the results were stratified by infertility for comparability with previous publications. Furthermore, none of the potential confounders substantially changed the effect estimates except for daughter's age and gravidity which were retained in the final models.

Discussion

Overall, we found a 70% increased odds of self-reported endometriosis among women exposed *in utero* to DES compared to unexposed women supporting an earlier finding (Missmer et al. 2004). However, the effect was attenuated when stratifying or removing infertile women from the analysis suggesting women who underwent infertility evaluation may have been more likely to receive a diagnosis of endometriosis. This discrepancy could be a result of differing definitions of infertility, which has been shown to vary in prevalence by stringency of the definition and when asked lifetime versus current. The Nurses' Health Study was able to distinguish between concurrent infertility and past infertility, whereas our study did not have this information and relied on an ever/never diagnosis of infertility.

We limited our definition of endometriosis to only those women reporting physician diagnosed infertility by one of several operative approaches including hysterectomy, which was not recognized in NHS. While it may be hypothesized that women exposed to DES

would undergo more hysterectomies and gynecologic surgeries than women who were unexposed due to other concerns about gynecologic health, and/or their heightened awareness about gynecologic health given their exposure status, we found no evidence of this for this study cohort. In fact, unexposed women were slightly more likely to report hysterectomies than exposed women (18.8% and 17.2%), respectively. Our results remained unchanged when women diagnosed with hysterectomy (n=33) were removed from the analysis (data not shown).

These data concur with earlier work by Hediger and colleagues which suggests an *in utero* origin to endometriosis resulting from the higher risk for disease among women who have tracked lean (measured by BMI) historically and at the time of endometriosis diagnosis (Hediger et al. 2005). We decided not to adjust for adolescent BMI, given that it may in the pathway between the exposure and endometriosis. While not statistically significant, women in the leanest categories for BMI (<18.5 kg/m^2) at age 20 and again at the follow-up survey had a 20 and 30 percent (respectively) increased odds for endometriosis when compared to women in the normal range for BMI (18.5-24.9 kg/m^2).

A recent study in a laparoscopic cohort found an decreased risk for endometriosis among women whose mothers smoked when they were pregnant (Buck Louis et al. 2007). We did not find such an effect in our data; however, this could be due to the difference in the way exposure was ascertained in the two studies (proxy report by the daughter versus maternal report, respectively). We also did not see a protective effect for ever smoking, which has been seen in a few previous studies (Buck Louis et al. 2007;Cramer et al. 1986;Cramer and Missmer 2002).

In addition to examining overall odds for DES exposure and endometriosis in this cohort, we also investigated the potential effects of gestational age in completed weeks at DES exposure which was available for 62% of women and dose for 26% of women. Over seventy percent of women in both the case group and those without endometriosis were missing information on dose of DES which may have led to the lack of effect seen for dose on endometriosis risk. The categorical analysis of gestational age at first exposure showed higher risks among women who were exposed at varying gestational ages, however, when gestational age was examined in a continuous fashion only a slight increase in odds for disease were observed, suggesting the lack of an effect for gestational age at exposure.

No increase in the odds of endometriosis was observed for women's birth weight, gestational age or SGA status. The effect for birth weight was not consistent with results from NHS where a thirty percent increase in risk for endometriosis was found in women whose birth weight was <5.5 pounds as compared to women who were 7-8.4 pounds (Missmer et al. 2004). However, these authors failed to control for DES exposure in their models, which may have confounded the LBW-endometriosis association. The discrepancy may also be explained by the difference in methods for ascertaining birth weight (mostly via medical records in our study versus self-reported by the daughter in NHS). The Nurses' Health Study also did not see an increase in risk for women born preterm in their data. When the outcomes of birth weight and gestational age were examined in a continuous fashion, the null results persisted (data not shown).

A strength of this study is its exposure measurement, being the first study to examine this question with information on medically verified maternal DES exposure. However, important limitations impact the interpretation of findings most notably, the reliance on self

reported endometriosis and other relevant covariates. Despite this, the direction of recall bias is not in the expected direction of a possible exaggerated effect. Our prevalence is comparable or less than that reported for women of reproductive age (12%) suggesting that misclassification on disease status is minimal. We recognize that DES exposed women may be more vigilant about preventive screening and, hence, have more gynecologic visits and thereby be more likely to be diagnosed with endometriosis. To address this potential detection bias, we assessed the frequency of screening visits for cervical cancer (via PAP smear) and breast cancer (via mammography) and observed comparable percentage for exposed and unexposed women (10% and 9%, respectively).

Finally, it is possible given the long lapse in time between *in utero* exposure to DES and diagnosis of endometriosis, potential unknown or unmeasured variables could be associated with both the exposure and outcome and residual confounding may have distorted our results. Despite these potential limitations, the findings are from the largest known cohort with medically verified documentation on DES exposure and are suggestive of an association between DES exposure and diagnosis of endometriosis. While DES has been removed from the market, it is a model of the influence of endocrine disrupting chemicals. These results emphasize the need for studies to look at long term reproductive health effects of exposure to both pharmaceutical and environmental endocrine disrupting chemicals, starting with exposure in the womb.

Table 5.1 Characteristics of women in DESAD study by endometriosis diagnosis, 1975-1994 (N=3876)*

	Endometriosis (n=472)		No Endometriosis (n=3404)		Odds Ratio	95%CI
	n	%	n	%		
Highest grade completed						
High School or Less	57	12.1	429	12.6	1.0	---
Some College	94	19.9	783	23.0	0.9	(0.7, 1.2)
Graduated College	295	62.5	2019	59.3	1.1	(0.8, 1.4)
Missing	26	5.5	173	5.1		
Ethnic background						
White	467	98.9	3357	98.6	1.2	(0.5, 2.9)
Non-White	5	1.1	46	1.6	1.0	---
Missing	0	0	171	0		
Exposed to DES						
Yes	405	85.8	2679	78.7	1.6	(1.2, 2.0)
No	67	14.2	725	21.3	1.0	---
Missing	0	0	0	0		
Total dose of DES (grams)						
< 500	17		105		1.0	---
500-999	10		66		0.9	(0.5, 2.0)
1000-1999	15		89		1.1	(0.5, 1.1)
2000-2999	15		96		1.0	(0.5, 1.8)
3000-3999	15		55		1.3	(0.7, 2.5)
≥ 4000	78		462		1.0	(0.6, 1.7)
Missing	325	76.1	2531	74.4		
Mean (SD)	4966.7	8084.6	3706.3	6543.3		
Gestational Age at First Exposure						
≤ 7 weeks	104	22.0	587	17.2	1.6	(1.2, 2.2)
8-10 weeks	57	12.1	555	16.3	1.0	---
11-14 weeks	78	16.5	402	11.8	1.7	(1.3, 2.4)
≥ 15 weeks	70	14.8	549	16.1	1.2	(0.9, 1.7)
Missing	233	34.5	1860	38.5		
Mean (SD)	9.6	7.8	9.1	8.4		

Ever Pregnant						
Yes	337	71.4	2706	79.5	1.0	---
No	121	25.6	644	18.9	1.4	(1.2, 1.7)
Missing	14	3.0	54	1.6		
Ever Smoked						
Yes	197	41.7	1402	41.2	1.0	(0.9, 1.2)
No	262	55.5	1907	56.0	1.0	---
Missing	13	2.8	95	2.8		
Mom Smoked in Pregnancy						
Yes	151	32.0	1205	35.4	0.9	(0.7, 1.1)
No	287	60.8	2027	59.5	1.0	---
Missing	15	3.2	172	5.1		
Packs per day (among smoking mothers)						
≤ 1	69	45.7	516	42.8	1.0	---
>1	74	41.7	632	52.4	0.9	(0.7, 1.2)
Missing	18	5.3	57	5.0		
BMI at age 20 (kg/m^2)						
< 18.5	70	14.8	417	12.3	1.2	(0.9, 1.5)
18.5-24.9	331	70.1	2429	71.4	1.0	---
25-29.9	31	6.6	211	6.2	1.1	(0.8, 1.5)
30+	4	0.8	53	1.6	0.6	(0.2, 1.5)
Missing	36	7.6	294	8.6		
Mean (SD)	20.7	(2.9)	21.0	(3.0)		
BMI at 1994 (kg/m^2)						
< 18.5	21	4.4	113	3.3	1.3	(0.9, 2.0)
18.5-24.9	281	59.5	2067	60.7	1.0	---
25-29.9	90	19.1	623	18.3	1.1	(0.8, 1.3)
30+	50	10.6	370	10.9	1.0	(0.8, 1.3)
Missing	30	6.4	231	6.8		
Mean (SD)	23.9	(4.9)	24.2	(5.0)		

Table 5.2 Odds Ratios and 95% Confidence Intervals for association between DES and endometriosis by infertility status among women in the DESAD cohort, 1975-1994.*

	Total population		Among infertile women		Among fertile women	
	OR	95% CI	OR	95% CI	OR	95% CI
DES exposed†	1.7	(1.3, 2.2)	1.2	(0.7, 2.0)	1.1	(0.8, 1.6)
DES exposed††	1.6	(1.2 2.1)	1.2	(0.7, 2.0)	1.2	(0.8, 1.7)

*Only women who had diagnosis by laparoscopy, biopsy, hysterectomy, or other gynecologic surgery included.

†Adjusted for age
†† Adjusted for age and gravidity

Table 5.3 Odds Ratios and 95% Confidence Intervals for endometriosis by DES exposure at varying gestational ages among women in the DESAD cohort, 1975-1994*

	OR†	95% CI	OR††	95% CI
Unexposed	1.0	---	1.0	---
≤ 7 weeks	1.8	(1.3, 2.4)	1.7	(1.3, 2.4)
8-10 weeks	1.2	(0.8, 1.6)	1.1	(0.8, 1.6)
11-14 weeks	1.9	(1.4, 2.6)	1.9	(1.4, 2.7)
≥ 15 weeks	1.4	(1.0, 1.9)	1.3	(1.0, 1.9)

*Only women who had diagnosis by laparoscopy, biopsy, hysterectomy, or other gynecologic surgery included.

†Adjusted for age
†† Adjusted for age and gravidity

Table 5.4 Odds Ratios and 95% Confidence Intervals for endometriosis by various birth outcomes among women in the DESAD cohort, 1975-1994

	Total population		Among infertile women		Among fertile women	
	OR	95% CI	OR	95% CI	OR	95% CI
LBW ††	1.0	(0.7, 1.3)	0.8	(0.5, 1.3)	1.2	(0.7, 1.8)
Preterm delivey ††	1.1	(0.8, 1.5)	0.9	(0.5, 1.5)	1.4	(0.9, 2.1)
SGA ††	0.9	(0.6, 1.4)	0.8	(0.4, 1.6)	1.0	(0.5, 1.8)

†† Adjusted for age, gravidity, and DES exposure
*Only women who had diagnosis by laparoscopy, biopsy, hysterectomy, or other gynecologic surgery included

CHAPTER VI

CONCLUSIONS

Introduction

A growing body of evidence suggests the intrauterine environment may play an important role in the development of adult onset disease. Despite the accumulating literature regarding reproductive effects of exposure to environmental chemicals, little has been published regarding the potential effects of *in utero* exposure to environmental chemicals on human reproductive health.

We sought to expand upon two previous studies examining *in utero* cigarette smoke exposure and age at menarche (Windham et al. 2004;Windham et al. 2008), three papers examining *in utero* cigarette smoke exposure and fecundability (Baird and Wilcox 1986;Jensen et al. 1998;Weinberg et al. 1989), one study which found an association between *in utero* cigarette smoke exposure and endometriosis (Buck Louis et al. 2007) and one study that found an association between *in utero* DES exposure and endometriosis (Missmer et al. 2004). Many of these studies had not been replicated in different populations and only two of them (the age at menarche analyses) did not rely upon proxy exposure information. Additionally, we used reported age at menarche collected at two different time periods (one around the age at puberty and one twenty years later) to test the reliability of self-reported age at menarche later in life. Two recent studies came to differing conclusions on the

accuracy of recall later in life (Cooper et al. 2006;Must et al. 2002) serving as the impetus for this methodologic analysis.

Overall, we were unable to confirm previous findings of an effect (either a delay or an advance) in age at menarche after exposure *in utero* to cigarette smoke. We also did not find that exposure *in utero* to cigarette smoke increased the odds of developing infertility or decreased the odds for developing endometriosis, as other authors have suggested (Buck Louis et al. 2007;Jensen et al. 1998;Weinberg et al. 1989). We did however, find that exposure *in utero* to DES was associated with an increase of 70% in the odds for developing endometriosis compared to women who were unexposed, similar to the one previous published study (Missmer et al. 2004). Finally, we concluded that self-reported age at menarche later in life is not reliable when exact age at menarche in years is required, but improves when age within a year is considered, confirming a previous study's suggestion (Cooper et al. 2006).

Strengths and Limitations

This study was able to utilize a resource of over 5,000 women enrolled in 1976 and followed through the nineties to examine the potential effects of an *in utero* exposure and adult reproductive disease. The size and time period covered by this study are impressive and a strength for all the analyses. Papers two and three utilized information gathered about smoking exposure *in utero* ascertained from the mother several years after her pregnancy. This is an advantage over previous studies which have relied upon the daughters to report their mother's pregnancy smoking habits. Furthermore, the stigma currently attached to smoking during pregnancy was absent during the era when mothers were queried about this

information, so any reporting bias should be limited. The exposure measurement for DES was also a strength for paper three. DES exposure was reported by the mother and confirmed by medical records for all the participants in the study. This is in contrast to previous studies which rely upon the daughter to report the mother's exposure status. Furthermore, gestational age at first exposure was available for a considerable proportion of the women in the study which allowed us to examine whether timing of exposure played a role in the effect of DES on development of endometriosis.

There were several limitations in this study, which may have affected our conclusions. Firstly, we had to rely upon self-report for all three outcomes. Self-reported age at menarche is perhaps more widely used than self-reported infertility and endometriosis, however, more precise timing of puberty, using markers such as breast development and development of pubic hair in addition to age at first menses lend more information about the onset of puberty. Self-reported infertility is problematic because women who do not choose to have children, but who may be infertile are not captured by this definition. We restricted our definition further and only included women who sought care for infertility. This could be problematic because access to resources and knowledge and/or timing about when to seek care may have excluded some women from this category. Self-reported endometriosis was a limitation in our study because many women may not recognize endometriosis even if it is present in their bodies because of the varying degrees and symptoms associated with such a complex disease. While we restricted our definition to only women who had been diagnosed with surgery, this still may have excluded asymptomatic women or women who did not seek a diagnosis for any associated symptoms. Despite our reliance on self-reported outcomes, it is unlikely that any misclassification would be differential with respect to exposure to *in*

utero cigarette smoke. We also looked at indicators of detection bias in paper 3 to address the possibility that women who were exposed to DES might have been more likely to receive a diagnosis of endometriosis. We were also limited by the lack of ascertainment of childhood environmental exposure to cigarette smoke. This may have played a role in the onset of menses or may have interacted with the *in utero* cigarette smoke to affect any of the outcomes, but was not available in this dataset. Finally, we did not have specific data on adult smoking behaviors, beyond whether the woman smoked or not. Such information would have been helpful to tease out any interaction or confounding that may have occurred in conjunction with *in utero* exposure to cigarette smoke.

Public Health Importance

While our results from the smoking analyses were largely null, other health consequences associated with prenatal exposure to smoke are well known. While our study did not find any effects for age at menarche, previous studies have found both delays and advances in age at menarche among women whose mother's smoked when they were pregnant. While more studies are needed to clarify the potential risks, caution about exposure during pregnancy is always prudent. Our results from the DES analysis stress the ability for endocrine disrupting chemicals to cause reproductive health effects much later in life. While DES has been removed from the market these findings highlight the possible adverse effects of future pharmaceuticals as well as chemicals found in the environment which have similar properties. Finally, our results with respect to the reliability of self-reported age at menarche later in life may be un-nerving for practitioners and researchers who rely on this recalled marker to calculate risks for such things as breast cancer, where age

at menarche is considered to be a risk factor. When exact recall in years is necessitated it is better to collect these data prospectively, or at least, around the time of puberty, as we observed that report later in life did not concur with earlier report in 45% of the women sampled.

Directions for Future Research

Our findings add to a small existing literature on the effects of *in utero* exposures on adult reproductive outcomes. Further research is needed to clarify other potential exposures as well as to consider multiple outcomes which may be on the same biologic pathway. Our sample size in the unexposed to DES population was too small to draw any meaningful conclusions from the joint analyses, but future work should consider this possibility. Improved measures of exposure could also build upon our work. Biologic markers of exposure in the mothers and daughters would help clarify exposure. It would be ideal to also collect cord blood from daughters to tease out various exposures experienced *in utero.* Future studies should also focus on accurate classification of the outcome. Clinical evaluation of girls as they enter puberty or self-reported breast development and pubic hair development with tools such as Tanner staging (Tanner JM 1962) would improve the outcome measurement in future studies. Collection of a measure such as time-to-pregnancy would give a more accurate reflection of women who had problems trying to conceive, rather than having to rely upon self-reported infertility. Finally, although expensive and time consuming, proper evaluation via laparoscopy of women enrolled in studies of endometriosis is critical for identifying disease and could improve our understanding of this disease's etiology.

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