DDE AND PCBs: INTRA-INDIVIDUAL CHANGES, CORRELATIONS, PREDICTORS AND ROLE IN TIMING OF MENOPAUSE

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology

Chapel Hill
2007

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ABSTRACT

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(Under the direction of David Richardson)

Dichlorodiphenyldichloroethane (DDE) and polychlorinated biphenyls (PCBs) have been suggested to affect the timing of menopause. However, results from studies on DDE/PCBs and age at menopause have been inconsistent and may be limited by exposure assessment at widely divergent ages, remote from the time of etiologic relevance. Attempts to estimate measures at a common and etiologically appropriate age have been limited by the paucity of data on the predictors of long-term changes in organochlorines. Understanding the dynamics of individual body burdens of these organochlorines and the factors that predict them would improve exposure assessment, and determining whether DDE/PCBs affect age at menopause would contribute to the knowledge of their public health significance.

Data were collected from 512 participants from a 1978-1982 baseline study and a 2003-2004 follow-up study. Baseline and follow-up organochlorine measures from 123 participants were used to characterize the intra-individual changes and predictors of change in DDE/PCB levels, in order to provide a model for exposure estimation. Interview and ovarian hormone data at follow-up were used to classify menopausal status and define age at menopause, and the effect on timing of menopause from DDE/PCB exposures, estimated at age 40, was evaluated.

Serum DDE and PCBs dramatically declined (median drop of 84% and 55%, respectively) over the follow-up period. Baseline levels were strongly correlated with and predictive of follow-up levels (Spearman correlations (r_s): 0.72 for DDE; 0.43 for PCBs). Prediction of follow-up PCBs substantially improved with data on initial concentration, lactation duration, baseline body mass index, and percent change in body fat (r_s= 0.75 between predicted and actual follow-up levels), whereas DDE prediction improved slightly (r_s= 0.83). Effect estimates for age at menopause were inconsistent across organchlorine categories (compared to the lowest exposure category, menopause occurred -0.02, 0.4, 0.7, -0.3 years later across ascending categories of DDE, and -0.4, 0.4, 0.8, and 0.6 years later across PCB categories).

Results suggest a single organochlorine measure provides considerable information on relative ranking at distant times and prediction is improved with data on a few easily collected variables. However, the data provide no evidence for an effect of DDE/PCBs on age at menopause.

DEDICATION

I dedicate this dissertation to my parents

and brothers for their love, support and faith in me.

ACKNOWLEDGEMENTS

This dissertation could not have been achieved without the significant contributions of a number of people. First, I would like to thank my committee, beginning with my dissertation chair, David Richardson, for "adopting" me as his student and for making my dissertation process a most pleasant and positive experience, and for sharing his pragmatism in epidemiology.

I am deeply indebted to Beth Gladen for her selflessness in coming out of retirement to devote a tremendous amount of time, dedication, and expertise to this project and my education and growth—more than what any student can ever hope for. My academic experience has unquestionably been enriched through our interactions and for that, I will always be grateful.

I am grateful to Glinda Cooper for her vision and for giving me the opportunity to work on this fantastic project. Despite the distance, she has readily available and I have benefited from her wonderful advice, both academically and beyond.

I am extremely privileged to work with Donna Baird, who has provided critical contributions and supported my funding on this project. She offered excellent insight on the biology and technical aspects of the project.

I am fortunate to have worked with Marilie Gammon, who has continuously encouraged me from day one and has considered my best interest throughout this dissertation process. It was an honor to tap into her treasure trove of knowledge on menopause and organochlorines and speak with her on a more personal level.

I am lucky to have Julie Daniels as an advisor, as she has provided valuable insights and highly constructive comments on my manuscripts. Her expertise on the dynamics of organochlorines was extremely helpful.

I would also like to thank the people in the Epidemiology Branch of the National Institute of Environmental Health Sciences for their financial and research support.

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Ahr	Aryl hydrocarbon receptor
BMI	Body mass index
CI	Confidence interval
CYP	Cytochrome p450 enzyme
DDE	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichloroethane
E_2	Estradiol
FMP	Final menstrual period
FSH	Follicular stimulating hormone
GC-MS	Gas chromatography-mass spectrometry
HR	Hazard ratio
HRT	Hormone replacement therapy
IU/L	International units per liter
kg/m^2	Kilogram per meter squared
LH	Leutenizing hormone
LMP	Last menstrual period
LOD	Limit of detection
OC	Oral contraceptives
pbb	Parts per billion
PBB	Polybrominated biphenyls
PCBs	Polychlorinated biphenyls

ng/g	Nanogram per gram
ng/mL	Nanogram per millilter
pg/mL	Picogram per milliliter
r_s	Spearman correlation
r_p	Pearson correlation
S.E.	Standard error
TCCD	2,3,7,8-tetrachlorobibenzodioxin
TEQ	Toxic equivalence factor
ug/L	Microgram per liter

1. BACKGROUND AND SIGNIFICANCE

This chapter is organized into three sections pertaining to organochlorines, natural menopause, and the significance and implications of the proposed research. Section 1.1 begins with an overview of DDT/DDE and PCBs and discusses their use, biologic properties, reported health effects, human exposures and changes to those exposures. The goals of the research on the dynamics of these organochlorines were to evaluate the 1) intra-individual changes and 2) correlations in serial measures of p,p'-DDE and PCBs, as well as 3) develop a model for the prediction/estimation of organochlorine levels based on literature on the predictors of DDE/PCBs. Sub-sections 1.1.3 and 1.1.4 summarizes the epidemiologic studies pertaining to intra-individual changes and correlations in DDE and PCB measures, respectively. The last two subsections (1.1.5 and 1.1.6) begins with an overview of the literature on the predictors of a single measure of DDE and PCBs and leads into studies of the predictors of organochlorine change—the latter of primary relevance to predictive modeling.

Section 1.2 focuses on natural menopause and begins with an introduction into its epidemiology and definition. The goal of the research on menopause is to determine whether p,p'-DDE and PCBs affect the timing of menopause. Subsection 1.2.2 discusses the factors that have been associated with the timing of menopause and subsection 1.2.3 focuses in on the role of DDE and PCBs on age at menopause.

Section 1.3 provides an overview of the findings and limitations of previous studies on the temporal changes, predictors, and possible role of DDE and PCBs in the timing of menopause, as well as the goals and implications of the proposed research to address some of the limitations.

1.1 ORGANOCHLORINES: DDE AND PCBS

1.1.1 DDT and DDE: Changes in use and exposures

Dichlorodiphenyltrichloroethane (DDT) was widely used as a pesticide in the United States starting in the 1940s (FIGURE 1.1). In 1972, DDT was banned from agricultural applications in the United States, and thereafter, in many other parts of the world, as a result of observed toxic effects in wildlife [1]. Today, DDT is still used for emergency or public health purposes in many countries for the prevention of vector-borne diseases, such as malaria.

Technical grade DDT is a mix of primarily isomers p,p'(para-para)-DDT (85%), and to a lesser extent, o,p' (ortho-para)-DDT and o,o' (ortho-ortho)-DDT [2]. For the remainder of this document, DDT will refer to the sum of all DDT isomers and metabolites, whereas DDE will refer to p,p'-DDE, unless otherwise noted.

o,p'-DDT is considered weakly estrogenic and exposure occurs primarily in occupational settings, whereas exposure of the general population to p,p'-DDT and its metabolite, p,p'-DDE, occurs primarily via dietary intake [3]. p,p'-DDT is more readily degraded and excreted than DDE, which has a biologic half-life in humans of over 7 to more than 10 years [2, 4-6].

p,p'-DDT is reported to have neurotoxic [2] and carcinogenic [7] properties, while p,p'-DDE has anti-androgenic effects [8]. These properties prompted studies of their potential effects on cancer [9-11], neurodevelopment [12], and reproductive outcomes, such as spontaneous abortion [13, 14], and age at menopause [15, 16]. However, clear associations have not been established.

Upon introduction into the environment, DDT and DDE become immobilized in the soil and can be transported long distances via water or volatilization into the atmosphere—hence, their ubiquity in the environment and exposure to humans worldwide [1, 17, 18]. DDT use increased significantly until 1959, steadily declined until 1972, and then markedly dropped with its agricultural ban. A concomitant change in environmental concentrations and exposure to DDT and DDE ensued, with the predominant route of exposure via diet. In the Food and Drug Administration Total Diet Studies estimated average adult intakes of DDT from consumption of meat, fish, poultry, and dairy products declined from 240 ug/person/day in 1970 to 0.97 ug/person/day in 1986-91, corresponding to decreasing levels in foodstuffs during the same time period [1]. The sources of exposure and the corresponding human tissue levels varies with age, with infants having higher levels due primarily to ingestion of pesticide-rich foods (breast milk) and their having lower body fat content, and adults acquiring DDE primarily through fish and meat consumption [3].

Data from an annual tissue repository of surgical patients and autopsied cadavers, gathered by the Environmental Protection Agency in the National Human Adipose Tissue Survey (NHATS), reveal a steady decline in lipid-adjusted adipose tissue DDT from a geometric mean of 8 ppm in 1970 to 2 ppm in 1983 [19]. A similar decline in mean total DDT in human breastmilk can be seen worldwide, as evidenced by numerous cross-sectional

population studies [17, 20]. In the United States and Canada, the gradual decrease in the 1960s became progressively sharper in 1975, following their bans [18]. Despite the drop in environmental and human tissue levels, exposure, particularly to DDE, is ongoing due to their accumulation and continued presence in the environment.

1.1.2 PCBs: Changes in use and exposures

In the United States, commercial use of PCBs, marketed under the name Arochlor, was widespread from 1929 until 1977, when production was banned, except in totally closed systems. This ban was enacted because of concerns about their persistence and accumulation in the environment, and potential for causing toxic effects. Due to their chemical stability, they were used as plasticizers, coolants and lubricants in transformers and capacitors, surface coating and adhesives and other applications [21]. PCBs are produced by chlorinating the biphenyl structure at one or more of the 10 positions labeled 2 to 6 and 2' to 6' (FIGURE 1.2). The various number and structural arrangements (i.e. ortho-, para-, meta-) of chlorine atoms on the biphenyl ring confer 209 different PCB compounds, referred to as congeners [22]. Isomers are PCBs that have the same number of chlorine atoms but differing arrangement of the chlorines.

PCB congeners most often detected in human tissues have high molecular weights [2, 3, 23], including PCB-118, 138, 153 and 180 [24-27]. Higher molecular weight congeners are generally resistant to degradation (having half lives of ~3-24 years) [28-31] and are fairly correlated with one another [3, 32]. PCB-153, being the predominant congener, has been used as a marker of exposure for the other PCBs.

Fish and meat consumption are the major sources of adult PCB exposure, with secondary exposure via inhalation of contaminated air [23]. Children acquire additional exposure via mother's milk. Exposure to lower molecular weight PCBs occurs primarily in occupational settings.

PCBs congeners possess broad biologic properties, including both estrogenic and anti-estrogenic effects, activation of the ryanodine-sensitive calcium channels as well as toxicity similar to 2,3,7,8-tetrachlorobibenzodioxin (2,3,7,8-TCDD), which is the most toxic of the non-pesticide organochlorines [2, 33, 34]. Much literature has been published on the latter, with TCDD exerting its effect by interacting with the aryl hydrocarbon receptor (Ah) receptor, thereby inducing the gene expression of metabolic enzymes, such as cytochrome P450, CYP1A1 and CYP1A2 [35, 36]. As determined by their toxic equivalence (TEQ), a measure of PCBs toxicity relative to TCDD, coplanar PCBs, chlorinated at both the para (4,4') positions and two or more meta positions (3,5,3' and 5'), have the most potent dioxin-like activity, whereas mono-ortho-substituted PCBs are less potent [25, 36].

Dioxins cause chloracne and liver abnormalities in humans and are linked to other adverse health effects [37, 38]. Occupational exposure studies have consistently associated PCBs with abnormal liver function tests and chloracne [39-41]. Prenatal PCB exposure may also have effects on neurologic development and thyroid function [42-44]. Most epidemiologic data on health and PCBs address exposure to total PCBs, rather than specific congeners. There are fairly high correlations between the various PCB congeners [3, 45], making it difficult to draw conclusions about causal relationships between body burden of individual compounds and disease.

Environmental levels of PCBs have declined most precipitously in the late 1970s to mid-1980s, corresponding to the timeframe when regulatory controls were imposed on PCB use [46]. The Food and Drug Adminstration Total Diet Study showed that PCB levels in the diet in 1993 were significantly less than $1/1000^{th}$ the levels detected in the mid 1970s [46]. The estimated dietary intake of PCBs for an average adult was approximately 0.03 ug/kg/day in 1978 and declined to <0.001 ug/kg/day by 1991 [23].

Repeated cross-sectional studies of PCB show an overall decline in levels over time across populations [17, 47], reflecting the impact of the ban on PCB usage [46], and hence, environmental exposure levels [17, 46, 47]. However, interpreting and assessing trend from these data can be challenging given the variable component congeners of total PCBs across study populations and the non-standardization of measurement techniques across studies. In a Swedish study of breastfeeding women, where survey data have been collected using a fairly consistent method over time, there is evidence of an upswing in breastmilk PCBs levels in the 1960s followed by a downturn, with total PCB levels in 1997 30% of that in 1972 [47]. Analysis of the data from the National Human Adipose Tissue Survey in the United States shows a steady downward trend in the population percentage of individuals with total adipose tissue PCB levels >3ppm, from a high of nearly 10% in 1977 to 0% in 1983 [19]. Nevertheless, despite the reduction in PCB production and usage, exposure is ongoing due to bioaccumulation.

1.1.3 Intra-individual changes in DDE/PCBs over time

Given the ubiquity and potential health effects of these organochlorines, understanding the dynamics of individual body burdens of DDE and PCBs would have important health implications. Studies with serial organochlorine measures among

occupational cohorts [29, 30, 48, 49] and individuals poisoned by very high acute levels of PCBs [50, 51] have shown dramatic declines in levels over a period of decades. However, long term (>2 year) studies of intra-individual changes in DDE and PCBs among the general population, who are mostly exposed to relatively low, ongoing environmental levels of these pollutants have been limited [52-60] (TABLE 1.1). These studies range in size from 35 to approximately 1500 participants and were conducted in various populations at different time periods (from the 1970s to 2002). Eight of the 10 outlined studies had a follow-up interval of approximately 2 to 10 years, with the longest study about 20 years in duration.

Baseline organochlorine levels and changes in those levels varied widely across studies, depending on the region, study population, study time period, and type of organochlorine and method of assessment. However, there was an overall decline in intra-individual DDE and PCB levels across all studies. In a series of three studies, conducted from 1973 to 1995, in different subsets of the same cohort of Great Lakes fisheaters ($\geq$24 pounds of fish in past year) and their low or non-eating (<6 pounds fish in past year) counterparts, declining levels of serum PCBs were partly attributed to a reduction in fish consumption [58] as well as declining environmental levels in contaminated fish [54]. In studies in which both organochlorines were assessed, the decline was more striking for DDE than for PCBs, particularly when the exposure measures were taken 10 years [53] or 7 years [54] apart. These differences, however, were less noticeable in studies with follow-up measures taken over a span of 2 to 5 years [55, 60].

These prospective studies confirm the trend seen in serial cross-sectional surveys that show human tissue levels of DDE and PCBs have dropped across various study populations and time periods. This reduction may partly be explained by the declining environmental

levels resulting from their respective bans. However, as changes to an individual's body burden is related to a balance of intake and excretion, other host and environmental factors, including changes in metabolism, diet, weight, lactation, and age, may also play a role in shifting these levels over time.

1.1.4 Correlations between serial DDE/PCB measures

Correlation studies are important with respect to exposure assessment and determination of how well a measure at one time point reflects that at another time. A number of short-term (<2 years) studies report high Spearman correlations (r_s>0.8), a measure that evaluates the relationship between rank ordered measures, for serum DDE and/or PCB measures taken on average 2 months apart [61], before and after an overnight fast [62, 63], and across trimesters of pregnancy and shortly afterwards [64]. Similar correlations were found between breast milk DDE samples taken at birth to 6 weeks (r_s=0.89), birth to 3 months (0.87) and birth to 6 months (0.86) in an approximately 2 year study of 807 lactating mothers [65]. These respective correlations were lower for total PCBs (0.74, 0.68, and 0.59). It should be noted that in the latter study, lactation is a unique route of elimination that is generally not found at other times. Overall, these short term studies reveal higher correlations among measures taken closer in time to one another than those temporally remote.

Of the 10 aforementioned longitudinal studies of 2 or more years, four evaluated correlations between past and present organochlorine values [52, 53, 55, 60] (TABLE 1.2). In general, correlations between measures were sizeable (>0.5) for both DDE and PCBs, particularly highly chlorinated PCBs. In a 2006 study of Faroes Island children who had

various PCB congeners measured at ages 7 and 14, the Pearson correlations evaluating the linear relationship between the two measures, were mostly greater than 0.5 for highly chlorinated PCBs (PCB-118, 153, 156, 157 and 180) and less than 0.2 for less chlorinated PCBs (PCB-18, 28, and 44) [52]. Spearman correlations were higher for DDE than for total PCBs in two studies of breast cancer cases and controls with approximately 2 years of follow-up (0.9 for DDE and 0.8 for PCBs) [60] and 7 years duration (0.79 for DDE and 0.64 for PCBs) [55]. However, these differences were less noticeable when the predominant PCB-153 congener was evaluated; DDE and PCB-153 correlations were respectively 0.92 and 0.90 in a 10-year study of 39 males by Hagmar et al. [53] and 0.79 and 0.68 in the 7-year study by Hoyer et al. [55]. The latter study further showed that correlations between measures varied by PCB congener.

Overall, these short-term studies (<2 years) reveal relatively high correlations, which weakened the further apart organochlorine measures were taken in time from one another. Studies of 2- to 10-year duration show moderate to high (0.5 to 0.9) correlations for DDE and PCBs—the latter, depending on the specific congener analyzed. These correlations demonstrate that a measure at one time point may be reflective of those at another time point up to 10 years apart.

1.1.5 Predictors of DDE and PCBs levels

An important goal in studies of etiology is to optimize exposure assessment by capturing or predicting the appropriate exposure levels at the time of etiologic relevance, thereby, giving a more accurate picture of the possible associations between the exposure and disease risk. Numerous studies have evaluated a host of constitutive, demographic, and

behavioral factors that may predict a single measure of DDE and PCBs, including age, year of birth, gender, ethnicity, dietary factors (e.g. fish consumption), smoking, alcohol use, parity, lactation, measures of weight and adiposity, and relative weight change.

The majority of studies show a positive association between age and concentrations of both DDT/DDE and PCBs [57, 66-71]. The few studies that do not show a relationship, generally, had small sample sizes, a limited age range to allow detection of an age difference, or a low exposure population [61, 72-74]. The positive association may be a function of an age-dependent accumulation. Age may also be a marker for cohort-related changes in exposure levels, with older individuals being exposed to higher levels in the past [69, 75, 76], as well as a marker for age-related shifts in weight and metabolism.

In the large (n~6000) National Health and Nutrition Examination Survey (NHANES) from 2001-2002, no significant gender differences in levels of p,p'-DDE was reported. However, males had higher mean levels of PCB-153 and PCB-180 than females [25]. In the same study, Mexican Americans had higher levels of p,p'-DDE and PCB-153 than Non-Hispanic Blacks and non-Hispanic Whites. Non-Hispanic Blacks have higher levels of DDE, but similar levels of PCB-153 than non-Hispanic Whites. It is unknown whether these gender and racial differences are associated with pharmacokinetics or due to differences in fat content. Furthermore, differences with respect to occupation or residence (i.e. near agricultural areas that formerly used DDT) may contribute to the observed ethnic differences in DDE levels [76].

As previously mentioned, diet is a major source of exposure to DDE and PCBs, with fish consumption the primary source for adults [68, 77-79]. Dairy products have generally not been associated with serum or milk levels of DDT/DDE or total PCBs [69, 80-82].

Numerous studies report no association between serum DDE and total PCBs and smoking (or smoking intensity) [67, 71, 72, 77], or likewise, no to minimal positive associations with alcohol consumption [67, 77, 82-84].

Lactational transfer is a major route of elimination of organochlorines, with many studies showing an inverse association between lactation duration and DDE and PCB levels [65, 66, 81, 85-89]. Unifying this idea of lactational transfer is the positive associations found between breastfeeding duration and serum organochlorines in breastfed children [83, 90-92]. Studies that found no effect of lactation generally had few women who breastfed or breastfed for long periods (>6 months), or had predominantly older women (>40 years) [71, 80, 93-96]. The effect of lactation may have diminished by this older age.

Placental transfer of organochlorines from mother to fetus has also been documented by the detection and correlation of PCBs or pesticides in both maternal and umbilical cord blood, suggesting that organochlorine levels would decrease with parity [97-99]. However, the effect of parity is mostly secondary to the effect of lactation. Positive associations with parity are found in studies in which a majority of participants lactated [65, 82, 100, 101], whereas no association are generally found in studies with few women breastfeeding [72, 75, 80, 93, 95]. Overall, lactational transfer to the child is considered the major route of organochlorine excretion in women, as evidenced by the much lower organochlorine concentrations in cord blood compared to maternal blood and breastmilk, despite the high correlations between all three samples [91, 102, 103].

Since fatty tissue is the site of organochlorine sequestration, weight loss or a loss of body fat is expected to release organochlorines from fat, thereby elevating serum concentrations [104, 105]. Conversely, weight gain will lead to dilution of organochlorines

into fat tissue, thereby decreasing serum concentrations. Cross-sectional analyses of the effect of weight change, mostly weight change in the recent past (<2 years), on a single measure of organochlorine have shown either an inverse association [71, 82, 106-108] or no effect [69, 70, 84, 109, 110].

Body mass index (BMI) as a measure of adiposity is not related to DDE [65, 66, 69, 80, 96, 109, 111] or total PCBs [58, 65, 70, 72, 77, 80, 93, 109, 110, 112, 113] in numerous studies, some of which accounted for the effect of lactation or were conducted in young lactating women [65, 69, 80, 96, 109, 110, 112]. Other studies have found positive associations with DDE [68, 70, 71, 93, 106, 113-117] and negative associations with total PCBs [60, 66, 67, 69, 75, 84, 108, 117, 118], although these relationships have not always held [68, 75, 96, 106]. Furthermore, BMI associations with individual PCB congeners do not follow any obvious pattern [71, 94, 114].

These inconsistent associations with body mass index across studies and across organochlorine compounds have been postulated to be an outgrowth of multiple factors, including differential metabolism between heavier and leaner individuals, passive dilution of organochlorines in adipose tissue, and timing of sampling relative to the temporal changes in environmental exposure levels [106, 119]. Alcock et al. further suggested that the exposure patterns over time are a result of these complex interactions with other cohort-related and age-dependent changes in breastfeeding patterns, and dietary intake and composition [120]—relationships which are difficult to evaluate in cross-sectional analyses using a single measure of organochlorine. Nevertheless, these studies provide us with some clues on potential predictors of DDE and PCBs.

1.1.6 Predictors of intra-individual change in DDE and PCB levels

To date, six prospective studies [53-55, 57, 59, 60], with a follow-up period of 2 or more years, have assessed the effects of some of the aforementioned factors on intra-individual change in DDE and PCB levels (TABLE 1.2). Two studies found that a single measure of body mass index at study enrollment was positively correlated with DDE, but not PCB, half-life [59, 60]. Half-life of DDE in the lowest quartile of body mass index was 4 years compared to 7 years for the highest quartile [59]. Another study showed body mass index to be minimally associated (Odds ratio (OR)=1.07, 95% confidence interval (CI)=1.01-1.13) with an increase in total PCBs [57]. Body mass index may be associated with reduced metabolism by xenobiotic-metabolizing enzymes such as cytochrome p450 (CYP). CYPs of the 1A, 2B, and 3A family are most likely involved in the metabolism of various PCB congeners [23, 33, 121], and obesity has been associated with decreased activity of CYP 3A4 in several studies [122].

An inverse association between relative changes in body mass index or weight and changes in DDE/PCBs has been demonstrated in two studies [53, 55]. Since serum organochlorines are expected to be in passive pharmacodynamic equilibrium with adipose reservoir, an increase in weight may lead to the dilution of serum organochlorine concentrations, thereby giving a negative association [123].

Two studies which evaluated age at study entry, controlling for the effects of weight change or a single measure of body mass index, found no significant associations with changes in DDE or PCB levels [53, 57], which contrasts with previous studies showing a positive association between age and a single measure of organochlorine [67-69, 124]. It is

possible that age, per se, may not be related to metabolism and excretion of these organochlorines, independent of body mass index.

A number of short-term (<2 year) studies provide convincing evidence for the elimination of organochlorines via lactational transfer [65, 85, 86, 88, 89, 91, 125]. These studies show a decline in organochlorine levels with progressive lactations. Wolff et al., however, found no effect of lactation duration on change in DDE or PCBs over a median of 25.4 months of observation [60]. This study had only 35.2% of participants with a history of breastfeeding, and of those, 88% breastfed for 6 months or less, which may have limited the investigators' ability to see an effect.

Only two studies assessed the change in fish consumption on change in organochlorine levels over the periods of 1991-2001 [53] and 1982-1989 [54]. Although both studies found an overall reduction in serum DDE and PCB levels, these declines were not associated with altered fish consumption. The authors suggest that the declines in serum levels are more likely to be explained by the reduction in environment levels, following their respective bans, rather than fish consumption.

In addition to the factors mentioned above, initial concentration has also been evaluated as a potential predictor of change in organchlorine levels. Previous studies of occupational cohorts with measures taken prior to and years after their exposure had ceased, demonstrate faster elimination of PCBs with higher initial concentrations [29-31]. Similarly, in a study of 701 women environmentally exposed to PCBs (median 31 months of follow-up), those whose PCBs had decreased were over 3 times more likely to have higher initial concentrations than those who had maintained their levels [57]. Several possible explanations have been proposed for this phenomenon [30], including "continuing exposure,

preferential retention of more slowly metabolized congeners, recent non-equilibrated exposure and greater enzyme induction at higher doses" [28].

In summary, there are few long-term studies that have prospectively assessed the intra-individual changes, correlations and/or predictors of change in DDE and PCBs in populations exposed to relatively low levels. These studies show an overall decline in DDE and PCB levels across various populations and time periods. And these intra-individual changes may be a function of a multitude of factors, including lactation duration [56, 65], body mass index at study enrollment [59, 60], weight change [53, 55], and initial concentration [57]. Correlations between temporal measures were generally moderate to large (0.5-0.9) for both DDE and total PCBs, particularly higher molecular weight PCBs.

It is unknown whether the changes, correlations, and predictors of change seen in these studies would continue with longer durations between measurements. Understanding the long-term intra-individual changes in these organochlorines and the factors that influence organochlorine body burden would help to improve DDE/PCB exposure assessment in etiologic studies, particularly studies in which a single organochlorine measure is used. A single measure may provide an imprecise picture of the etiologically relevant exposure levels that initiate the onset of disease or health outcome, such as menopause.

1.2 NATURAL MENOPAUSE

1.2.1 Definition and Epidemiology

Natural menopause is the permanent cessation of menstruation resulting from loss of ovarian follicular activity. In the United States, the median age at menopause ranges from 48 to 52 years, based mostly on samples of Caucasian women [126-129]. The Study of Women's Health Across the Nation (SWAN) has estimated median age at menopause for multiethnic samples of US women to be 51.4 years [130]. The distribution of age at menopause includes a Gaussian portion between ages 47-55 years, with an extended lower tail [131-133].

Ovarian failure before age 40 is considered premature and occurs with a frequency of 0.3% to 0.9% [134, 135], whereas by age 45, about 5 to 10% of women have experienced menopause [129, 136]. Possible etiologies for premature ovarian failure include genetic [137, 138] or immune system defects [139-143], as well as ovarian insults, such as radiation [144] or chemotherapy [145].

Aside from the very early age at onset of ovarian senescence, diagnosis of premature ovarian failure is based on a minimum of two elevated measures of follicle stimulating hormone (FSH) taken one or more months apart. Various cutpoints have been used to define elevated FSH, including >20 and >40 international units per liter (IU/L) [146, 147]. In contrast, natural menopause is conventionally defined as the cessation of menstrual periods for at least 12 consecutive months [148, 149], in the absence of known causes, including pregnancy or lactation, and medical interventions [144, 145, 150, 151].

Menopause is characterized by the depletion of the pool of ovarian follicles with a shift in ovarian steroid and gonadotropin hormones to a relative low estrogen and high

gonadotropin state. Early age at natural menopause may be a marker for damage to the follicular pool or disruption of the hormonal feedback during the menopausal transition period (i.e. peri-menopause), which is typified by irregular menstrual cycle lengths and flow pattern up to five years prior to menopause [152]. Early age at menopause may influence the risk for heart disease [153] and osteoporosis [154], whereas late age at menopause may increase the risk for breast [155] and endometrial cancers [156]. Therefore, determining the factors that affect the timing of natural menopause is of substantial interest.

Approximately 6 million oocytes per ovary are present in-utero. At birth, about 2 million oocytes remain [157] and these steadily deplete as a result of atresia or entry into the growth pool [158]. At any given time, most follicles are "resting" or arrested at prophase I of meiosis. Beginning before birth and continuing throughout reproductive life, some of these resting primordial follicles, under the influence of unknown factors, are recruited into an initial growth phase whereby they transition through morphologically distinct stages: the primordial, primary, secondary (preantral) and antral follicles, the latter of which contain a developing fluid-filled antral cavity [159]. At the preantral and antral stage, most follicles undergo atresia until puberty and the reproductive years are reached, whereupon a few are "rescued" or recruited into a gonadotropin-dependent growth phase defined by the ovarian cycle, and the corresponding, menstrual cycle (See Figure 1.3).

The ovarian cycle is characterized by alternating follicular and luteal phases. The follicular phase is dominated by the presence of a maturing follicle ready for ovulation and fertilization. A complex of hormonal changes occurs with a gradual rise in estrogen (i.e. estradiol or E_2) and a surge in the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), resulting in ovulation (release of a mature follicle or ovum)

(FIGURE 1.3 and 1.4). This ovulation is followed by the luteal phase, which is characterized by the development of the corpus luteum in preparation for pregnancy. Should fertilization not occur, the endometrial lining of the uterus and its blood vessels degenerate, accompanied by a drop in LH, estrogen, and progesterone, thereby resulting in menstruation and the beginning of a new menstrual cycle.

During the reproductive years, about 400 oocytes will undergo ovulation, and the rest will undergo atresia. At menopause, there are virtually no remaining follicles [160], and the ovary stops producing estrogen, thereby disrupting the feedback mechanism that suppresses FSH and LH production. FSH levels increase 10-20 fold, and LH increase approximately 3 fold [161, 162] during menopause compared to the reproductive years (FIGURE 1.4 and 1.5). The characteristic changes in circulating hormonal levels during transition to menopause allow these ovarian hormones to be used in classifying menopausal status. As previously mentioned, serum FSH can be used in classification, with cutpoints at 20 IU/L or 40 IU/L. Some studies show better prediction of menopause with the latter cutpoint [163, 164]. Ideally, FSH measurement should be timed to the menstrual cycle, as a rise in FSH may be indicative of either menopause or a spike during ovulation. A ratio of FSH to LH greater than 1 or 1.5 has also been used to define postmenopausal status [151, 165], and may be a better discriminator than FSH alone, even among women using oral contraceptives [165-167]. FSH levels rise faster and higher than LH during menopause (FIGURE 1.5), whereas during the reproductive years, LH levels overtake those of FSH (FIGURE 1.4). In addition, estradiol (E_2) (<60 picogram per millliter for premenopause and >100 pg/mL for postmenopause), in combination with FSH, has also been used to discriminate menopause status among pre-menopausal and oophorectomized women [168].

Ovarian failure is typified by the depletion of the prenatally-determined primordial pool of ovarian follicles [159] and changes to ovarian steroid (estrogen) and gonadotropin (FSH and LH) levels [169]. Theoretical models based on biologic data suggest that the rate of atresia in the resting pool of primordial follicles during reproductive life, rather than the number of oocytes one is born with, has a greater influence on hastening the age at menopause [170]. It is possible that harmful exposures during menopausal transition can increase the rate of atresia [171] and disturb follicular growth and the ovarian hormonal milieu [172, 173] to a point at which the relatively small remaining pool of follicles cannot sustain recovery. Numerous studies showing a more consistent effect of current smoking, compared to former smoking, on the age at menopause [114, 172, 174] support the notion that menopausal transition may be particularly vulnerable to the effects of potential ovarian toxicants.

1.2.2 Factors associated with the timing of menopause

Smoking is the best documented factor consistently associated with early age at menopause. Numerous studies show an earlier age at menopause (generally, 1-2 years) for smokers, particularly current smoker, compared to non-smokers [129, 130, 134, 172, 175]. Former smokers have about the same risk as non-smokers [175-177], independent of duration of smoking [172]. The number of cigarettes currently smoked appears to be influential in some studies [172, 177] but not in others [129, 176, 178]. The inconstencies may reflect errors in estimates of quantity smoked, which have generally been based on self-reports of current rate of cigarette consumption [179].

There is evidence for a strong genetic component to the timing of menopause from twin studies [180, 181]. The effect of family history of early menopause was evaluated in a case-control study of 344 women with early menopause (< age 46) who were age-matched to women who were still menstruating or who had menopause after age 46. Women with early menopause were 6 times more likely to report a family history of menopause before age 46 compared to control women [182]. In another study of 1081 randomly sampled women, ages 45-54, those with premature (<age 40) and early (<age 46) menopause (defined as >6 months of amenorrhea) were also more likely to report a family history of early menopause (OR=6.0, 95%CI=3.4-10.7) as compared to those still menstruating or those who had menopause after age 46, after adjusting for age and smoking status [183].

Other studies have examined the effect of a hysterectomy or unilateral oophorectomy on menopause using hormonal measures of menopausal status [150, 151]. In a longitudinal study of 275 women who had an ovary conserving hysterectomy and 259 who did not have a hysterectomy, menopause (defined as FSH$\geq$40 IU/L) occurred 3.7 years earlier (95%CI=1.5-6) in the hysterectomy group [150]. Among those with a hysterectomy, the women (n=28) who had a unilateral oophorectomy reached menopause 4.4 years earlier (95%CI=0.6-7.6) than those who retained both ovaries. Similarly, a cross-sectional study of 1716 women, ages 35-49, found an increased prevalence of FSH>20 IU/L among those having had a hysterectomy with removal of one ovary compared to those who had not had hysterectomies or oophorectomies (OR=2.4, 95%CI=1.3-4.6) [151].

Parity, later age at menarche, irregular cycle length and oral contraceptive use have been postulated to delay menopause, based on the premise that they are associated with reduced ovulatory cycles, and hence, preservation of oocytes [175]. Nulliparous women

have been reported to have earlier menopause as compared to parous women [130, 175, 184], and increasing parity has been associated with later age at menopause in some studies [185-188]. These observed effects may be explained by the notion that women with an early menopause are likely to have a shorter fertile period compared with women who have a later menopause. Therefore, the relationship between age at menopause and parity may not be causal. On the other hand, studies evaluating oral contraceptive use, irregular menstrual cycles and age at menarche have yielded contradictory findings [127, 130, 175, 185, 189-191].

The association between body mass index and age at menopause has also been inconsistent, with cross-sectional studies reporting no effect [192], earlier [193] as well as later [177, 190, 193] menopause in relation to higher body mass index [177, 191, 194]. However, the association between higher body mass index and later age at menopause may partly be confounded by smoking status, with smokers generally being thinner. A 3-year prospective study enrolling 2014 menstruating women, ages 46-56 years, found no effect of body mass index on timing of menopause (defined as 12 months of amenorrhea), after stratifying on smoking status [178]. Another prospective study of over 66,000 nurses over a 2 year period found a weak relationship with age at menopause across quintiles of relative weight that was confined only to smokers [134].

The relationship between alcohol use and age at menopause has been equivocal. A few studies [189, 190] show a higher prevalence of early menopause among drinkers versus non-drinkers—one of which found a dose-response relationship [190]. Other studies report no effect [175, 195].

More recently, organochlorine pollutants, such as DDE and PCBs have been speculated to affect the timing of menopause. Few studies have investigated the role of these compounds on age at menopause, and their results have been inconsistent. Given the importance of menopausal age on the risk of late stage diseases and the environmental ubiquity of these organochlorines, further investigations are warranted to clarify their relationship.

1.2.3 DDE/PCBs and age at menopause

Many organochlorines are considered to be endocrine disruptors, in that they interfere with the synthesis or action of hormones in organs of the endocrine system, including the ovary. Little is known about the effect of PCBs and DDE on the timing of menopause. However, there are a number of postulated biological mechanisms for the influence of these chemicals. DDE has been detected in human ovarian follicular fluid [196] and repeated exposure to p.p'-DDE has been shown to decrease estrogen levels in porcine granulosa cells [197]. Whether this might influence age at menopause is unknown. p,p'-DDE also has anti-androgenic properties [8]. The reported effects of androgens on ovarian follicles can vary, and according to recent studies, may depend on the developmental stage of the follicles [198-200]. Androgens have been reported to enhance follicular atresia in immature rats [201, 202] and reduce the number of large follicles [203]. However, recent data in animals, including rhesus monkeys, show that androgens, such as testosterone, directly stimulates the proliferation and growth of preantral and small antral ovarian follicles during the early stages of folliculogenesis [200, 204, 205] in a dose-dependent fashion [198, 199, 206], and this stimulatory effect is abrogated by flutamide, an anti-androgen [199]. Menopause is linked to

the exhaustion of early primordial follicles [159], so if androgens increase the rate of their development, early menopause might ensue. The anti-androgenic effects of p,p'-DDE could be hypothesized to delay menopause.

PCBs may lead to premature menopause via the binding and activation of aryl hydrocarbon receptor (AhR), the same mechanism through which smoke-related toxicants [207] and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may exert their effects [208]. Ahr binding induces the estrogen-metabolizing enzyme, cytochrome P4501A1 [209], thereby increasing 2-hydroxyestrogen concentrations [210]. Increased concentrations of 2-hydroxyestrogen have been correlated with decreased serum estrogen levels in women who smoke [211]

Some PCBs (e.g. PCB-28, 105, 118, and 156) have AhR binding capabilities and hence dioxin-like toxicity, though to a lesser extent than dioxin [25, 212, 213]. Chronic low dose exposure to TCDD has been reported to hasten reproductive senescence in female rats via the induction of a dose-dependent loss of normal cyclicity and estradiol levels, without a depletion to follicular reserve [208, 214]. Evidence in humans is not clear; one epidemiologic study of TCDD showed an inverted U-shape dose response relationship between TCDD and risk of early menopause among women accidentally exposed to high levels of TCDD [215].

There are currently only four studies that have investigated the role of DDE and/or PCBs on the timing of menopause (see TABLE 1.3). A study of 219 naturally menopausal Hispanic women, ages 42-74, reported a mean age at menopause 5.6 years earlier ($p<0.01$) for women in the highest category of p,p'-DDT ($\geq$6 ppb) versus those below the limit of detection (referent category), but variable effect for the middle three categories [16]. These

results were based on sparse data in the exposure categories, as 67% of the samples were below the limit of detection. Similarly, for p,p'-DDE, the authors found an earlier mean age at menopause (1.5 years) for those in the upper quintile of exposure compared to the lowest quintile. However, there is no trend in the mean ages at menopause across the middle three categories of exposures and information on the use of hormone replacement therapy (HRT) is lacking. Because there was no data on HRT use, women who had undergone natural menopause but were still menstruating because of HRT use, may have been misclassified as being pre-menopausal.

Blanck et al. conducted a prospective study of 874 women, ages 24-60, who were orally exposed to PCBs and polybrominated biphenyls (PBB), and found no effect of Arochlor 1254 on the timing of menopause [216]. This study had a high proportion (55%) of women with levels below the limit of detection, possibly making it difficult to observe an association.

In a population-based study of breast cancer cases (n=748) and controls (n= 659), ages 21-74, Cooper et al. found little evidence for an association between PCBs and age at menopause [15]. However, women with higher compared to the lowest category of plasma DDE had monotonically higher rates of natural menopause, with those in the highest 10^{th} percentile of DDE having a hazard ratio=1.4, 95% CI=0.9-2.1. In this study, a sizeable proportion (25%) of women who were post-menopausal had their organochlorines measured 20 years after the self-reported age at menopause, and current measures of serum organochlorines may not reflect past exposures. In addition, 25 women with unknown menopausal status or who were taking hormone replacement therapy were excluded from analysis. These excluded women may be experiencing menopause, and if their exposure

potential differs from those of the study participants, then biased estimates can result. Lastly, those who reported natural menopause within a year of the interview were classified as pre-menopausal, because it was uncertain whether these women truly were undergoing menopause. If the last menstrual period (LMP) was actually the onset of menopause and censoring occurred at the interview (instead of at the LMP), this could lead to misclassification of menopausal status and mismeasurement of age at menopause.

Analysis of a cohort of Taiwanese (Yucheng) women, ages 30-59, accidentally poisoned by high levels of PCBs revealed no difference in the menopausal status or mean age at menopause between women who were exposed and their neighborhood controls [217]. This study was descriptive in nature and did not account for other covariates, and was furthermore, unique in terms of its study population being exposed to very high, acute levels.

The evidence for an effect of DDE and PCBs remains unclear due to the inconsistencies in results, which may have stemmed from differences in study population and analytical strategies, including classification of menopause, criteria for exclusion/inclusion, and control for potential confounders. One common thread between all of these studies is that organochlorines were measured in women at various ages (from 21-74 years), and for some studies in a large proportion of women who had already undergone menopause. Given that women at different ages have different amounts of time to bioaccumulate organochlorines, the measures at different ages are not comparable between persons. This incomparability is further complicated by the temporal changes in exposure levels over time as a result of cohort- and age-related shifts in environmental contamination, diet, metabolism, lactation, and weight. The authors have attempted to account for the differences in age by controlling for age at enrollment in the model. Nevertheless, exposures after the onset of

disease (or health outcome) are not the exposures of etiologic relevance. Studies on smoking and age at menopause provide us with clues to possible reference periods. Because current smoking has been consistently associated with the timing of menopause, the menopausal transition period is a likely time period that is sensitive to the effects of ovarian toxicants. Therefore, it is important to standardize organochlorine levels to a common age that is etiologically relevant.

1.3 DISCUSSION AND SIGNIFICANCE OF PROPOSED RESEARCH

DDE and PCBs are ubiquitous environmental pollutants that have been implicated in various health outcomes; more recently, the timing of menopause. The age at menopause can influence the risk for heart disease, osteoporosis, breast and endometrial cancers, and infertility. Though there is biological plausibility for their effects, the few epidemiologic studies that have investigated the role of these organochlorines on age at menopause have found inconsistent results. While differences and inadequacies in analytical methodologies across studies may partly account for these results, exposure assessment using a single measure at widely divergent ages, temporally remote from the period of etiologic relevance, was a shortcoming in all studies.

Efforts to estimate and standardize exposure measures to an age purported to be of etiologic significance have been limited by the lack of data on the changes and predictors of change in intra-individual body burdens of DDE and PCBs over the course of decades. The few long-term studies that have prospectively assessed the intra-individual changes and predictors of change in DDE and PCBs, in populations exposed to relatively low levels, were 2 to 10 years in duration and had variable or limited data on predictors and covariates.

Two previous studies have attempted to estimate past DDE and PCB levels using kinetic models that took into account current organochlorine values, the effect of lactation, and decreasing concentrations in the environment (i.e. fatty fish) [6, 218]. Rylander et al. modeled PCB-153 levels in the past (1973-1991) based on current (1995) values and found a higher agreement between predicted and actual past levels as compared to current and actual past levels (kappa statistic (κ) of 0.52 versus 0.30, respectively) [218]. In a follow-up study with similar back-calculations for PCB-153 and DDE, Axmon et al. found a relatively small median difference (40 ng/g lipid) between estimated and actual past (1991) levels for both PCB-153 and DDE. Lactation was found to have a major impact on estimation of past exposure [6].

To address some of the limitations in previous studies, the following research was conducted, based on a prospective analysis in a relatively large cohort over a long period of time using questionnaire data and serum measures taken at baseline enrollment (1978-1982) and at follow-up (2003-2004) among female participants in the North Carolina Infant Feeding Study. Serum organochlorines measures at baseline and follow-up will be used to characterize the intra-individual changes in DDE and PCBs over the ~25 year time period, in order to provide a model for exposure estimation. In addition, serum gonadotropin and ovarian hormones data were used to augment classification of menopausal status and define the age at menopause. A goal of this research is to enhance our understanding of the long-term intra-individual dynamics of these organochlorine compounds, so as to improve exposure assessment in etiologic studies. Another goal is to determine the effect on age at menopause of DDE and PCBs, estimated at an age posited to be of etiologic significance;

thereby contributing to our current knowledge of the public health significance of these organochlorines.

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FIGURE 1.1 Chemical structures of DDT and DDE [2]

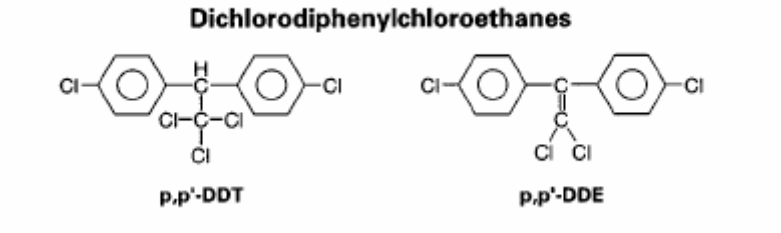

FIGURE 1.2 Chemical structures of PCBs and a specific coplanar PCB [2]

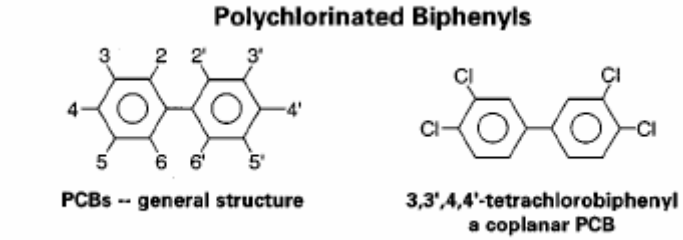

Table 1.1 Prospective studies with serial DDE/PCB measures: Intra-individual changes

Author, Yr, Location	Population	Study purpose to assess	DDE/PCBs measured	Sample period / follow-up time	Levels at baseline	Intra individual changes / Temporal trend
Barr, 2006, Faroes Island [52]	1022 children	PCB concentrations in a birth cohort	Total and specific PCBs (ng/g lipid)	At delivery, age 7 and age 14 for PCBs (sample sizes differ at age 7 and 14)	Median total PCB levels at age 7 • 799 ng/g	• At age 14, median total PCBs were 83% of their levels at age 7
Hagmar, 2006, Sweden [53]	39 males	intra-individual changes in DDE and PCBs	serum DDE and PCB-153 (ng/g lipid)	1991 and 2001	Median levels in 1991 • PCB = 340 ng/g • DDE = 650	Median 1991 levels of • DDE were 31% of baseline • PCB-153 were 50% of baseline
Hovinga, 1992, Michigan [54]	191 fisheaters and non-eaters (as designated in 1982 survey)	historical changes in PCBs and DDT (comprised mostly of DDE)	total serum DDT and PCBs (ppb)	1982 and 1989	In fisheaters, mean 1982 levels • DDT = 25.8 ppb • PCBs = 20.5 In controls, mean 1982 levels • DDT = 9.6 ppb • PCBs = 6.6	In fisheaters, median 1989 levels of • DDT were 60% of baseline • PCB were 43% of baseline In controls, median 1989 levels of • DDT were 71% of baseline • PCB were 103% of baseline
Hoyer, 2000, Denmark [55]	429 breast cancer cases and controls	risk of breast cancer and organochlorines	blood DDE, total and congener specific PCB-118, 138, 153, and 180 (ng/g lipid)	1976-78 and 1981-83	Median levels in 1976-8 • p,p'-DDE = 1197 ng/g • total PCBs = 1102 • PCB-118 = 64 • PCB-138 = 176 • PCB-153 = 223 • PCB-180 = 86	Median 1981-3 levels of • DDE were 97% of baseline • Total PCBs were 89% of baseline • PCB-118 were 67% of baseline • PCB-138 were non-detectable • PCB-153 were 91% of baseline • PCB-180 were 95% of baseline

Table 1.1 (continue)

Author, Yr, Location	Population	Study purpose to assess	DDE/PCBs measured	Sample period / follow-up time	Levels at baseline	Intra individual changes / Temporal trend
Sasamoto, 2006, Japan [56]	35 women	concentration of dioxin analogues in breast milk samples over time	breastmilk dioxin-like PCBs (TEQ pg/g fat)	1999-2000 and 2001-02	Average TEQ pg/g fat in 1999/00 • PCBs = 11 pg/g	• Average TEQ pg/g fat in 2001/2 were 60% of their TEQ in 1999/00
Sweeney, 2001, Michigan [57]	701 accidentally exposed to PBB and PCB sealant	predictors of change in PBB and PCB levels	serum total PCBs (ppb)	from 1975 to 1994 (median time between sampling is 31 months, range 1-146 months)	Median range of PCBs at enrollment, across age categories • 5-6 ppb	• 37.5% of participants maintained their levels • 12.2% increased their levels by a median of 2ppb • 50.3% decreased their levels by a median of 3.0ppb
Tee, 2003, Michigan [58]	179 fisheaters and noneaters (as defined in 1980/2 survey)	factors related to change in levels	serum total PCBs (ppb)	1980-82 and 1989-91 and 1993-95 (participants donated blood to at least 2 of 3 surveys)	Mean levels in survey periods • (1980-2) = 23 ppb • (1989-91) = 19 • (1993-5) = 14	• Median PCBs at 1989-91 survey were 82% of baseline • Median PCBs at 1993-5 survey were 61% of baseline • Authors suggest decline is due to a decrease in fish consumption
Wolff, 1999, Michigan [59]	302 farm residents accidentally exposed to PBB and followed up	sources & disposition of PBB with secondary analysis of DDE	serum and adipose DDE (ppb)	1976 and 1980	Geometric mean DDE in 1976 • (males) = 3900 ppb • (females) = 3200	Geometric mean 1980 levels were • 62% of baseline in males • 83% of baseline in females • Average half-life for adipose DDE ~6 years

Table 1.1 (continue)

Author, Yr, Location	Population	Study purpose to assess	DDE/PCBs measured	Sample period / follow-up time	Levels at baseline	Intra individual changes / Temporal trend
Wolff, 2000, New York [60]	280 breast cancer cases and controls	risk of breast cancer and organochlorines	blood DDE and PCBs (ng/mL)	1985-86 to 1987-90 (≥2 blood donations; median (range) of 25.4 (5.8-70.6) months between 1st and last blood	Geometric mean at 1st visit • DDE = 5 ng/mL • PCBs = 7.2	Geometric mean at last visit for • DDE was 89% of baseline • PCBs was 94% of baseline • Median half life among controls for DDE (8.6 years) and PCB (11.2 years)

PBB=polybrominated biphenyls, TEQ = toxic equivalence factor

TABLE 1.2 Prospective studies with serial DDE/PCB measures: Correlations and predictors of change

Author, Yr, Location	Population	DDE/PCBs measured	Sample period / follow-up time	Correlation between measures at	Predictors/control variables	Potential predictors of change in DDE/PCBs
Barr 2006 (Faroes Island) [52]	1022 children	Total and specific PCBs (ng/g lipid)	Age 7 and 14	Ages 7 and 14 • High chlorinated PCBs (mostly r_p>0.5) • Low chlorinates PCBs (mostly r_p <0.2)		
Hagmar 2006 (Sweden) [53]	39 males	serum DDE and PCB-153 (ng/g lipid)	1991 and 2001	Year 2001 and 2001 • DDE (r_s =0.92) • PCB-153 (r_s =0.90)	• Age • Relative BMI change • 1991 fish consumption • Relative change in fish consumption from 1991-2000 • fatty acid composition	• Age was not related to a change in DDE or PCB-153 • Relative increase in BMI weakly associated with decrease in DDE (β=-1.0, 95% CI=-2.3, 0.2) • Relative increase in BMI associated with decrease in PCB-153 (β=-1.0, 95%CI=-1.8, -0.2) • Change in DDE and PCB-153 not related to age, fish consumption in 1991, relative change in fish consumption, or fatty acid composition
Hovinga 1992 (Michigan) [54]	191 fisheaters and non-eaters (as designated in 1982 survey)	total serum DDT and PCBs (ppb)	1982 and 1989		• Age (-matched) • Fish consumption • Gender • Baseline 1982 serum levels	• Fish consumption not associated with change in DDT and PCB

TABLE 1.2 (continue)

Author, Yr, Location	Population	DDE/PCBs measured	Sample period / follow-up time	Correlation between measures at	Predictors/control variables	Potential predictors of change in DDE/PCBs
Hoyer 2000 (Denmark) [55]	429 breast cancer cases and controls	blood DDE, total and congener specific PCB-118, 138, 153, and 180 (ng/g lipid)	1976-78 and 1981-83	Year 1976-78 and 1981-83 • DDE (r_s =0.79) • PCBs (r_s =0.64) • PCB-118 (r_s =0.32) • PCB-138 (r_s =0.57) • PCB-153 (r_s =0.68) • PCB-180 (r_s =0.54)	• Weight loss/gain	• Those who lost weight had a drop in their DDE and total PCB levels significantly less than expected • Those who gained weight had a significantly greater than expected drop in their PCB levels only.
Sweeney 2001 (Michigan) [57]	701 accidentally exposed to PCB sealant	serum total PCBs (ppb)	from 1975 to 1994 (median time between sampling of 31 months, range 1-146 months)		• Age at enrollment • BMI at enrollment • Initial concentration • Having pregnancy between tests • Interval between 1st and last blood draw	• Age at exposure (i.e. age at study entry) not significantly associated change in PCBs • High BMI associated with a minimal increase in PCBs (1.07, 95%CI=1.01-1.13), but borderline associated with a decrease in PCBs (0.94, 95%CI=0.88-1.0) • High initial PCB minimally associated with an increase in PCB (1.34, 95%CI=1.17-1.53) but more strongly associated with a decrease (>-1 ppb) in PCBs (3.26, 95%CI=2.58-4.12) • Having a pregnancy and the interval between blood draws not associated with PCB change

TABLE 1.2 (continue)

Author, Yr, Location	Population	DDE/PCBs measured	Sample period / follow-up time	Correlation between measures at	Predictors/control variables	Potential predictors of change in DDE/PCBs
Wolff 1999 (Michigan) [59]	302 farm residents accidentally exposed to PBB and followed up later	serum and adipose DDE (ppb)	1976 and 1980		• BMI	• A single BMI measure in 1976 associated with DDE half-life • Half-life of adipose DDE in lowest quartile of BMI was 4 yr compared with 7 years for highest quartile of BMI
Wolff 2000 (New York) [60]	280 breast cancer cases and controls	blood DDE and PCBs (ng/mL)	1985-86 to 1987-90 (≥2 blood donations; median (range) of 25.4 (5.8-70.6) months between 1st and last blood	Visit 1 and 2: • DDE (r_s =0.93) • PCB (r_s =0.81) Visit 1 and 3: • DDE (r_s =0.95) • PCB (r_s =0.83)	• BMI • Ever lactated	• BMI positively correlated with DDE (r_s=0.26, p<0.02), but not PCB, half-life • No effect of lactation duration on half-lives

PBB=polybrominated biphenyls, r_p = Pearson correlation , r_s = Spearman correlation

FIGURE 1.3 Correlation between hormonal levels and cyclical ovarian and uterine changes [219]

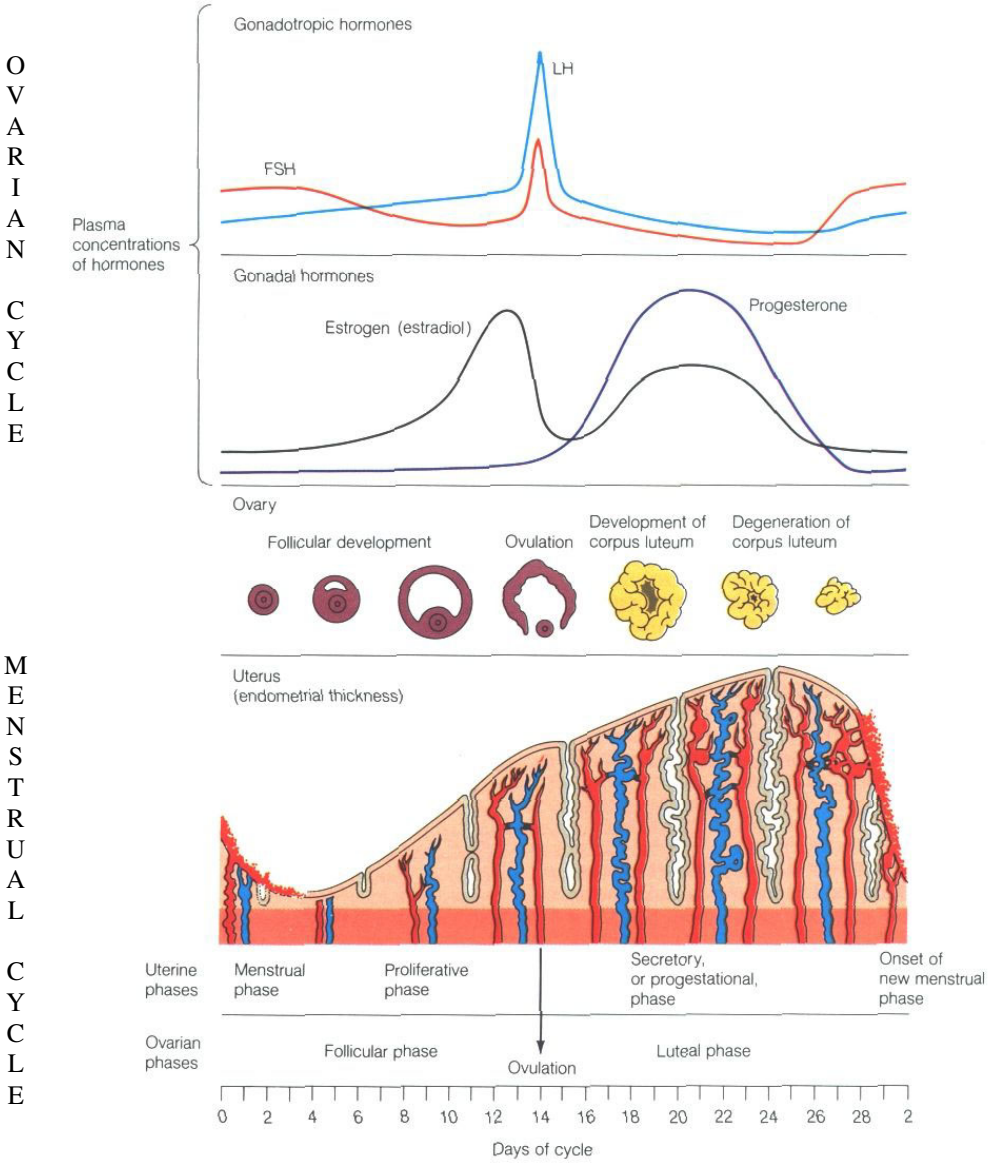

FIGURE 1.4 Hormone levels during the menstrual cycle [220]

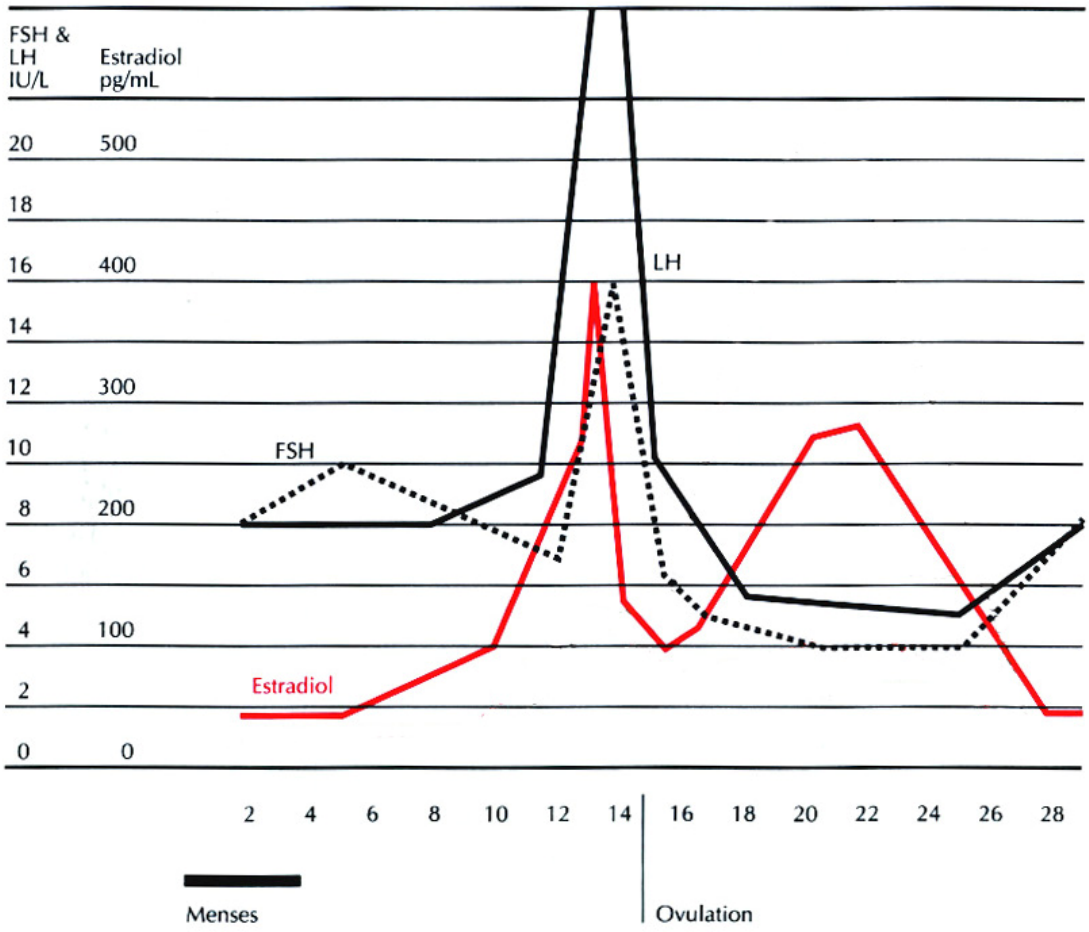

FIGURE 1.5 Hormone levels during menopausal transition and menopause [169]

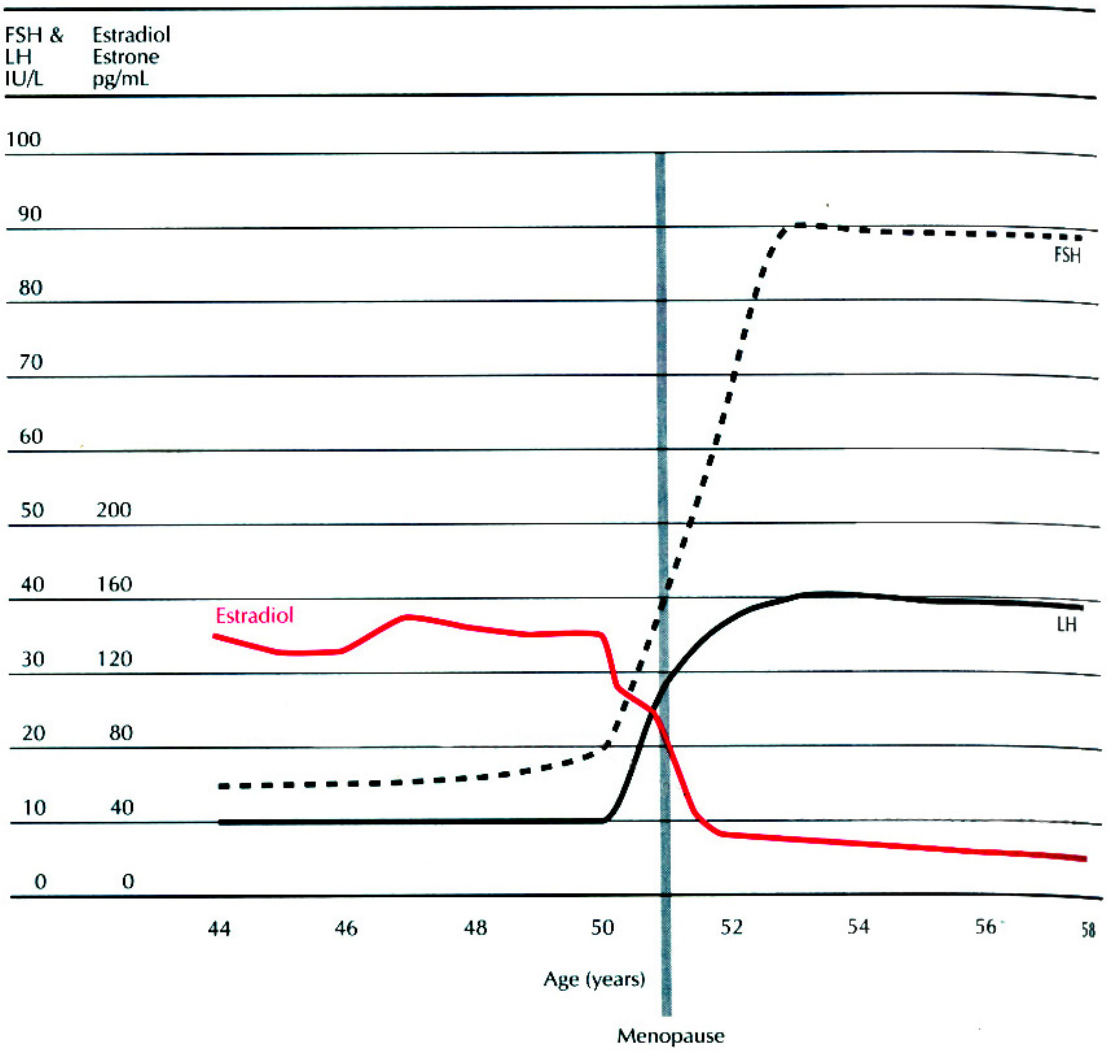

TABLE 1.3 Studies of DDE and/or PCBs and age at menopause

Author, Yr, Location	Population	Organochlorine exposure (median, range)	Definition of natural menopause / Censoring / Exclusion	Controlled for	Results
Akkina, 2004, Colorado [16]	219 Hispanic women from HHANES, ages 42-74	• p,p'-DDT (3.5 ppb) Categorized as <2, 2-2.9, 3-3.9, 4-5.9, ≥6) (67% below LOD=2 ppb) • p,p'-DDE (28.6 ppb) Categorized as ≤5.4, 5.5-10.3, 10.4-16.0, 16.1-23.5, ≥24.6	• Excluded women with menopause of less than 35 years • No info on HRT use	• Age at exam • Hispanic group • Marital status • Family income • Education • Ever smoked • Alcohol • Residence • Born in US • Farm work • OC use • Age at menarche	• Mean age at menopause across categories of p,p'-DDT: 48.8, 47.7, 48.5, 46.7, 43.2 (p-value for difference b/w 1st and 3rd category was <0.01) • Mean age at menopause across quintiles of p,p-DDE: 48.2, 48.8, 49.4, 48.0, 46.5 (all with p-values >0.1)
Blanck, 2004, Michigan [216]	990 women accidentally exposed to PBB, ages 24-60	• Arochlor 1254 Categorized as <5pbb, >5-11ppb, >11ppb) (55% below LOD=5ppb)	• 12 months amenorrhea (37%) • Censor at LMP is post-menopause, start of HRT if use HRT (pre- and post-; sens anal), age at surgery if surgical menopause, current age if otherwise pre-menopausal	• Age at time of PBB exposure • Smoking (time-varying) • BMI • Full-term pregnancies • Age at menarche • Ever use of OC • Household income • Education	• No effect of Arochlor 1254

TABLE 1.3 (continue)

Author, Yr, Location	Population	Organochlorine exposure (median, range)	Definition of natural menopause / Censoring / Exclusion	Controlled for	Results
Cooper, 2002, North Carolina [15]	1435 breast cancer cases and controls, ages 21-74	•p,p'-DDE (3.1, 0.04-93.8 ng/mL; 0.6 ug/g lipid) •Total PCBs (1.8, 0.3-26.1 ng/mL; 0.4 ug/g lipid)	• 12 months amenorrhea • Censored at LMP if postmenopause, age at surgery if surgical menopause, age at last menses if other medical reasons, at interview if otherwise pre-menopause, • Those who reported natural menopause within a 1 yr of interview classified as pre-menopause • Excluded women with unknown hormone status or missing data (n=59) • Samples collected after menopause (in 25% post-menopausal, this was >20 years after reported age at menopause)	• Age at interview • Smoking (ages 18-34 and 35-44) • Race (selection factor) • Education • Parity • Lactation • Physical activity • Thyroid condition • Body mass index	• Monotonic increase in HR (95%CI) across percentile categories of DDE (<50^{th}, 50^{th}-74^{th}, 75^{th}-89^{th}, ≥90^{th}): 1.0, 1.02 (0.9-1.6), 1.2 (0.9-1.8), 1.3 (0.9-2.1) • HR for continuous DDE: 1.4 (1.0-1.3) • No effect of total PCBs
Yu, 2000, Taiwan [217]	680 PCB-poisoned women and age-matched controls, ages 30-59	• Poisoned by contaminated cooking oil (based on signs and symptoms of illness)	• Descriptive analysis only	• Age(-matched)	• No difference in mean age at menopause between PCB-exposed women and their controls

PBB=polybrominated biphenyls, LOD = limit of detection, HRT=hormone replacement therapy, OC=oral contraceptives, LMP=last menstrual period, HR=hazard ratio

2. SPECIFIC AIMS

SPECIFIC AIM #1: To determine the intra-individual changes and correlations in DDE and total PCB levels over a median of 24.4 years (1978-1982 to 2003-2004)

SPECIFIC AIM #2: To develop a model for the prediction of change in DDE and total PCB levels to be used for estimating organochlorine levels at age 40 (i.e. around the time of menopausal transition)

SPECIFIC AIM #3: To determine whether elevated levels of DDE and PCBs affect the timing of menopause

3. MATERIALS AND METHODS

3.1 STUDY POPULATIONS

3.1.1 Baseline North Carolina Infant Feeding Study, n=877

The baseline North Carolina Infant Feeding Study was conducted in Greenville, Raleigh and Durham cities in North Carolina, on a non-random sample of 877 pregnant women, who had given birth between 1978 and 1982 [1]. Six mothers bore twins and 47 had two study pregnancies during the study time period. For those with two study pregnancies, the first will be considered the "index" pregnancy for the purposes of this dissertation.

At or near delivery, mothers who enrolled in the study were administered a questionnaire that elicited information on age, race, education, pre-pregnancy weight and weight gain during the current pregnancy, medical and pregnancy history, smoking, alcohol use, and breastfeeding history for the previous pregnancies. Maternal and cord bloods and breast milk samples were also collected at that time for measurement of DDE and PCBs. Follow-up visits at 6 weeks, and 3 and 6 months postpartum consisted of questionnaires, collecting data on lactation and weight, as well as another blood sample at 6 weeks post-partum, and periodic milk samples for as long as the child was breastfed. Of the 877 women, 12 had no samples to be analyzed for organochlorines. The median levels of DDE and total PCBs in maternal serum at the birth of the index child were 12.8 and 9.8 ug/L serum, respectively.

Mothers were mostly white (92%), well-educated (53% with a college education or greater and 41% self-identified as professionals), and had a median age of 27 years (range 16 to 41). Eighteen percent smoked and 40% drank alcohol at least once a week. Median pre-pregnancy weight was 125 pounds (range 88-220 pounds). Sixty-two percent were primiparous. Among multiparous women, 62% had breastfed prior to the index pregnancy. Most (88%=704/804) breastfed the index child to some extent: 10% of those 704 continued for less than a month, 8.1% continued a year or more, and the median duration of breastfeeding was 24 weeks.

3.1.2 Follow-up North Carolina Menopause Study, n=512

In 2003-2004, a follow-up North Carolina Menopause Study of participants from the North Carolina Infant Feeding Study was conducted to assess the relationship between organochlorine levels and ovarian senescence. Of the 877 participants at the baseline study, 12 (1.4%) were not eligible because they had no tissue samples, and 139 (15.8%) were not traceable due to missing contact information (TABLE 3.1). Of the remaining 726, 118 (16.3%) could not be found and 96 (13.3%) were lost due to refusal, death or other reasons; thereby, leaving 512 (58.4%) of the baseline participants, including 4 who had twin pregnancies and 34 who had two study pregnancies. TABLE 3.2 presents selected baseline characteristics of participants and non-participants. Aside from non-participants being slightly younger (mean age of 26.6 years compared to 28.1 years for participants) and having a slightly higher proportion of women with less than a high school education and being of Black race, non-participants and participants were similar in other respects, particularly their

organochlorine levels. The participants will constitute the study population for Study Aim 3: evaluating the effect of DDE and PCBs on timing of menopause.

At follow-up, a telephone-based questionnaire was administered to collect data on age, menstrual cycle history and menopause, detailed reproductive and breastfeeding history, medical history, surgery and cancer treatment, use of hormone replacement therapy (HRT) and birth control hormones, smoking, alcohol use, and height and weight (TABLE 3.3).

Blood was also drawn on subset of women, shortly thereafter, for two purposes: 1) measurement of DDE and PCBs and 2) measurement of the ovarian hormones: follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E_2) (TABLE 3.4). The selection probabilities for a blood draw were as follows: all women under age 55 whose menopausal status was unclear due to a hysterectomy with retention of $\geq$1 ovary (n=41), current use of hormone replacement therapy without having had a hysterectomy (n=39), and symptoms of peri-menopause (n=107), plus a 30% random sample of everyone else (n=98), regardless of age. At the time of the blood draw, women were also asked the age and date of their last menstrual period. Twenty-three (12.2%) of those with unclear menopausal status and 18 (18.4%) of the random others refused to donate blood, and an additional 2 (2%) samples from those randomly selected were lost in transit.

Blood samples from 126 women (constituting 82% of a 30% random sample of the 512 follow-up participants) were sent for DDE and PCB analysis. These women will comprise the study population for Study Aims 1 and 2.

A total of 203 blood samples collected from women with unclear menopausal status and half of the group of random others were sent for FSH, LH and E_2 analysis. Twenty-two random blood duplicates were also sent. These ovarian hormones were used to augment the

classification of menopausal status among those whose status was unclear, for the purposes of Study Aim 3. The coefficients of variations for FSH, LH, and E_2 were 3.8%, 4.6%, and 17.9%, respectively. Classification of menopausal status did not change whether the original or duplicate sample was used.

3.2 ORGANOCHLORINE MEASUREMENT

3.2.1 DDE and PCBs in baseline study

At baseline, p,p'-DDE and total PCBs were extracted with a mixture of hexane/ether/ethanol, purified with florisil, and assayed using packed column gas chromatography coupled with electron capture detection. Total PCBs was quantitated as the sum of two peaks on a chromatogram [2]. The coefficients of variation were <12%. Organochlorine levels were recovery-adjusted to account for the less than 100% recovery of chemicals. The limits of detection were variable and dependent on sample volume. Serum measures were not lipid-adjusted (i.e. wet weight) because intra-individual variability in the lipid content was considered minimal compared to those in breast milk. However, due to the high variability in milk fat content between women and over the course of lactation [3], milk samples were lipid-adjusted and expressed as ug/g lipid.

Among participants with DDE/PCB measurements, not all had each type of sample nor had detectable levels, and therefore, to maximize the data usage, values from all tissue (maternal serum, breast milk and cord blood) specimens were combined and expressed as an estimated "composite" measure of organochlorine in milk at birth [1]. First, any sample with a level below the limit of detection, was assigned an estimated value. Assuming that organochlorines were log normally distributed, this imputed value was calculated by estimating the expected value conditional on being less than the quantitation limit [4]. To

allow for comparability to milk at birth, these levels were then multiplied by a scaling factor (median ratio of birth milk sample to the sample in question), which adjusted for the declining milk values over time and for the much lower levels in blood compared to milk. All available rescaled levels were then averaged. The correlation between the estimated and actual amount in available milk was 0.94 for DDE and 0.86 for PCBs [1]. These composite measures supplemented the measures that were directly available in serum and breast milk.

3.2.2 DDE and PCBs in follow-up study

At follow-up, serum measures of p,p'-DDE, congener-specific PCBs and total PCBs (as an aggregate of the 14 measured congeners: PCB-28, 52, 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, and 187) were determined upon extraction using a mixture of ammonium sulfate/ethanol/hexane, purification with florisil, and quantitation using high resolution gas chromatography coupled with mass spectrometry (GC-MS). The coefficients of variation were 5.1% for DDE and 3.5%-11.4% for the various PCB congeners. The limit of detection was 0.2 for PCB-52, 0.03 for PCB-28, and 0.02 for the remaining PCB congeners and DDE. Total lipids were also estimated from total cholesterol, phospholipids and triglycerides using the Phillips equation [5], thereby allowing concentrations to be expressed per gram of lipid. A validation study shows that this approach [5] gives values very similar to those obtained using the standard gravimetric lipid analysis [6]. Hence, both wet weight and lipid-adjusted serum measures were available. Comparison of wet weight to their corresponding lipid-adjusted DDE measures show Pearson and Spearman correlations greater than 0.97, thereby lending support to the aforementioned assumption that intra-

individual lipid variability is minimal in serum as well as suggesting the feasibility of directly analyzing wet weight serum measures.

3.3 METHODOLOGY FOR STUDY AIM 1

The goals of Study Aims 1 were to evaluate the intra-individual changes and correlations between baseline and follow-up serum wet weight DDE and PCBs. Of the 126 women with a follow-up organochlorine measure (TABLE 3.4), 3 were missing baseline serum and were excluded from final analyses.

The distributions, extent of missing values and non-detects in DDE and PCB measures at both baseline and follow-up were explored. At follow-up, there was an appreciable amount of non-detectable levels (52%-98%) in 6 of the 14 PCB congeners (PCB-28, 52, 101, 105, 128, and 183) (TABLE 3.5). Given these congeners comprise a small proportion (<6%) of the sum of all 14 congeners, the remainder of this document will evaluate PCBs as the sum of the remaining 8 congeners (PCB-99, 118, 138, 153, 156, 170, 180, and 187), which have the lowest proportion of non-detects (<18%) and the highest relative abundance (together comprising ~94% of the sum of all PCB congeners measured).

In comparing baseline to follow-up organochlorine measures, some issues need to be considered regarding how to deal with: 1) the changes in quantitation methods since the baseline study, 2) non-detects, and 3) pregnancy related increases in lipids and organochlorines. TABLE 3.6 provides an overview of the following sections (3.3.1 -3.3.4) that address these issues.

3.3.1 Accounting for the change in PCB quantitation methods

There was no appreciable change in the extraction method for either DDE or PCBs, and the recovery rates were very high for the follow-up study (>93% for DDE and >92% for PCBs). Furthermore, DDE quantitation (which involves measurement of a single peak) was the same at baseline and follow-up. However, the PCB quantitation methods used at baseline differed from those used at follow-up and do not produce directly comparable results. To convert baseline PCB concentrations into their follow-up equivalents, 10 baseline milk samples, spanning the range of PCB levels, were reanalyzed by the follow-up GC-MS methodology [7]. Baseline levels were multiplied by the median ratio (0.236127) of concentration using the follow-up method to concentration using the baseline method. To evaluate the validity of this conversion, the observed milk PCB values obtained by the follow-up method was compared to those estimated by converting the baseline method to the follow-up method. The Pearson and Spearman correlations between the observed and estimated levels were very high (0.96 and 0.83, respectively), indicating the feasibility of this conversion factor (FIGURE 3.1).

3.3.2 Handling non-detects

At baseline, 1 (1%) individual had non-detectable serum DDE and 18 (14.6%) had non-detectable serum PCBs. Serum organochlorine concentrations below the limit of detection (LOD) at baseline were assigned estimated values using the aforementioned "composite" measures that reflect the concentration in milk lipids at birth of the index child [1]. These composite measures were converted into their approximate serum equivalent by multiplying by the median ratio of serum to composite concentrations (5.0071367 for DDE and 5.4120890 for PCBs) obtained from members of the baseline population with detectable serum values. To evaluate the validity of this imputation, estimated and actual

concentrations among those with detectable levels in serum at delivery were compared. The mean % difference and standard deviation were 6.1 $\pm$ 36.9 for DDE and 3.8 $\pm$ 24.3 for PCBs. Pearson and Spearman correlations were high between estimated and observed values for both DDE (0.90 and 0.91, respectively) and PCBs (0.91 and 0.84) (FIGURES 3.2 and 3.3).

At follow-up, one individual had non-detectable serum DDE, whereas 28 (22.8%) had at least one non-detectable PCB congener among the 8 most abundant congeners considered. Non-detects at follow-up were assumed to have a log normal distribution and were assigned the limit of detection (LOD) divided by the square root of 2 [8]. To determine whether the method by which non-detects are imputed makes a difference, estimated total PCB values using LOD for non-detects, and alternatively, using 0 for non-detects were compared. Estimated values using our primary method (LOD/square root 2) for handling non-detects will fall between those obtained using LOD and 0. The ratio of PCBs (i.e. sum of 8 congeners) using LOD for non-detects to PCBs using 0 for non-detects show a greater than 10% difference (range 12-156%) for only 6 individuals.

3.3.3 Accounting for pregnancy-related increase in DDE and PCBs

As serum lipids were not available at baseline, our primary goal was to explore changes in wet weight serum organochlorine measures over time. Measures at baseline were acquired at or around the time of delivery; however, short-term changes due to pregnancy are not the changes of interest. Given the characteristic and consistent rise in lipid content across pregnancy [9-15] and the consequential elevation in organochlorines, wet weight serum measures were adjusted to reflect non-pregnant levels. Based on mean serum total lipids of 5.9 g/L among more than 2000 non-pregnant Caucasian women, aged 20-34, participating in

the 1976-80 National Health and Nutrition Examination Survey [16, 17] and 8.5 g/L in a 1977-79 study of 553 pregnant Caucasian women, ages 20-41, at 36 weeks of gestation [14], a 30% increase in total lipids across pregnancy was estimated. To account for this rise, wet weight serum concentrations at baseline were reduced by an equivalent amount (i.e. by multiplying by 0.7).

3.3.4 Secondary analysis of lipid-adjusted serum measures

Though the primary analysis was based on wet weight organochlorines, a secondary analysis was conducted on estimated lipid-adjusted measures. Lipid-adjusted serum concentrations at baseline were estimated by dividing the aforementioned "composite" values by a correction factor (1.5) that accounts for the difference in lipid concentrations between milk and serum. This factor is the approximate ratio of concentrations in milk lipids to those in serum lipids for both DDE [18, 19] and PCBs [19, 20].

3.3.5 Statistical analysis

Descriptive analyses of DDE and PCB measures, at both baseline and follow-up, were conducted, including means, medians, miminum and maximum values, and percentile distributions. Separate analyses were done for wet weight and estimated lipid-adjusted measures. The relative change in levels over the two study periods was evaluated as the ratio of follow-up to baseline concentrations. Non-parametric Spearman correlation coefficients, which are not susceptible to influential observations, as well as graphical plots were used to assess the relationship between baseline and follow-up serum measures.

3.4 METHODOLOGY FOR STUDY AIM 2

The goal of Study Aim 2 is to model the predictors of change in DDE and PCBs, so as to provide a model for exposure estimation at a time of etiologic relevance to menopause (i.e. Study Aim 3). Of the 123 women with both baseline and follow-up organochlorine measures, one individual had missing pregnancy information and was excluded from this analysis.

3.4.1 Predictive model

Although changes in serum DDE and PCB levels over time are driven by complex age- and cohort-related processes, we presume the changes in organochlorine levels during the period under study could be reasonably approximated by an exponential decay, as other similar compounds and biological systems tend to operate on an exponential decay [21, 22]. Thus, the basic predictive model was:

$$[\text{Follow-up}] = [\text{Baseline}] * \exp(-\text{slope} * \text{follow-up time})$$

which can be re-written as

$$\frac{\text{Log [Follow-up]} - \text{Log [Baseline]}}{\text{follow-up time}} = -\text{slope}$$

[Follow-up] and [Baseline] are the wet weight organochlorine concentrations, respectively, at follow-up and baseline, and "follow-up time" represents the years from baseline to follow-up. Slope represents change in log concentrations per unit of follow-up time. To determine whether slope (i.e. the rate of change) is constant or is affected by various determinants, linear regression was used, such that the full model is of the form:

$$\frac{\text{Log [Follow-up]} - \text{Log [Baseline]}}{\text{follow-up time}} = -(\alpha + \beta 1\ (X1) \ldots + \beta k\ (Xk))$$

where α is the intercept and βk is the regression coefficient for the k-th predictor, Xk.

3.4.2 Potential predictors

Initial concentration, lactation duration, body mass index at baseline, percent change in fat mass, and mother's date of birth were evaluated for their possible influence on the rate of change in DDE and PCBs from the baseline to the follow-up study. Univariate statistics, including frequencies, means and medians were generated for these variables. The distributions of each of the variables were examined to check for missing data. One individual with missing pregnancy information was dropped from analysis. Missing observations for weight at baseline (n=3) and at follow-up (n=5) were derived or imputed from other available data. For missing baseline weight, the reported weight at the 6 week and 6 month interviews following the index pregnancy was used. For missing follow-up weight, categories of weight were available at the follow-up interview and the midpoint of each category was taken as the weight). Two individuals were missing age at menarche. One reported her first period in 5th grade and was assigned 11 as her age at menarche. Various coding schemes (continuous and categorical) were explored. Each potential predictor was examined to obtain the fewest categories needed to adequately characterize the relationship between that variable and the slope.

Initial (baseline) DDE and PCB concentrations (ug/L serum) were log-transformed, given their log normal distributions, and were included in the model as a linear variable.

Lactation duration, as assessed from the follow-up questionnaire, was defined as the number of weeks the participant breast-fed twice or more per day. Given the diminishing role of exclusive breast-feeding as lactation proceeds, the effect of breast-feeding on the rate of organochlorine excretion could lessen later in lactation. Thus, lactation duration was treated as a continuous variable and fitted with a piecewise linear model over 3 intervals of lactation duration, with the slope of each interval estimated directly from the data. This was accomplished by defining 3 variables representing the number of weeks of lactation during the 3 intervals: 0-26th, 27th-39th, and >39th lactational week. The number of weeks of breast-feeding for each pregnancy from baseline to follow-up was split among these three variables. Additional cutpoints at the quarter (13 weeks) and the 1 year (52 weeks) time points did not change the results. The number of weeks of breast-feeding for each pregnancy from baseline to follow-up was split among these three variables. For women with multiple lactations between baseline and follow-up, the contribution from each pregnancy was summed. To illustrate, for a woman with one child whose lactation lasted 60 weeks, a value of 26, 13 and 21 weeks was assigned to the first, second and third lactation variables, respectively. If she also had a second child who was breastfed for 30 weeks, then an additional 26 weeks was assigned to the first lactation variable and 4 weeks to the second, giving final values of 52, 17, and 21 for the three lactation variables. To operationalize in modeling, the value (i.e. number of breastfeeding weeks) of each of the three variables would be multiplied by the beta coefficients obtained for the corresponding variable.

Baseline body mass index was included in the model as a categorical predictor. Due to its narrow distribution, cutpoints for body mass index were set at 20 and 23 kg/m^2.

Percent change in body fat (kg) was based on a validated formula for percent body fat, which was then converted to body fat by multiplying by body weight [23]:

$$\text{Body fat} = [((1.46 * \text{BMI}) + (0.14 * \text{age}) - 10) / 100] * \text{weight}$$

Usual weight prior to baseline study pregnancy and current weight were obtained, respectively, from the baseline and follow-up questionnaires. Percentage change in body fat, defined as the difference in body fat from baseline to follow-up divided by baseline body fat, was included as a linear variable.

Mother's date of birth, a marker of secular trend in environmental DDE/PCB levels, was included as a continuous variable with unit in days (i.e. as a SAS date with reference date of January 1, 1960). Though baseline maternal age was another potential predictor, it was not included because it was highly correlated with date of birth (r=0.97).

3.4.3 Statistical analysis

For predictive modeling, linear regression models were fit using SAS (version 9.1). The statistical significance of each variable was assessed using the F test. Spearman correlations comparing actual versus model-predicted concentrations at follow-up were used to assess the predictiveness of each model. To illustrate the model fits, model-predicted changes in organochlorine levels were plotted along with actual changes for the 122 individuals with non-missing questionnaire data.

3.5 METHODOLOGY FOR STUDY AIM 3

The goal of Study Aim 3 is to determine whether the timing of menopause is influenced by DDE and PCB levels, estimated at a common age that is likely to be of

etiologic significance. For the 512 participants in the North Carolina Menopause Study, serum DDE and PCB levels were estimated at age 40, around the time of menopausal transition, using the regression coefficients from the predictive model in Study Aim 2.

3.5.1 Exposure: DDE and PCBs at age 40

Predicted levels at age 40 were obtained using the following equation:

$$[FU] = [BS] * \exp [-(a + \beta1 (\text{init conc}) + \beta2 (\text{lact1}) + \beta3 (\text{lact2}) + \beta4 (\text{lact3}) + \beta5 (\text{bBMI1}) + \beta6 (\text{bBMI2}) + \beta7(\text{fat change})) * \text{time}]$$

where

[FU] = serum organochlorine concentration at age 40 (ug/L)

[BS] = baseline organochlorine serum concentration (ug/L)

init conc = log of initial (baseline) serum concentration (ug/L)

lact1 = number of weeks of breastfeeding during the 0-26 weeks lactation period for every child born from the baseline study until the mother reaches age 40

lact2 = number of weeks of breastfeeding during the 27-39 weeks lactation period . . .

lact3 = number of weeks of breastfeeding during the >39 weeks lactation period . . .

bBMI1 = having a body mass index at baseline of 20-22.9 kg/m^2

bBMI2 = having a body mass index at baseline of $\geq$23 kg/m^2

fat change = percent change in fat mass from baseline to age 40; via linear interpolation of fat mass at baseline and follow-up

time = time span from baseline to age 40 (yrs)

α and $\beta1$ to $\beta6$ = regression coefficients obtained from the predictive model in Study Aim 2

Estimated organochlorine levels were evaluated as both continuous and categorical variables, with similar results. Categories were established using a natural cutpoint close to

the upper 10th percentile for the highest DDE and PCB category, and approximately evenly dividing the remaining concentrations into 4 additional ranges. This produced a lowest referent category that was adequately large (>25%).

3.5.2 Outcome: Age at natural menopause

Natural menopause is defined as having ≥12 months of amenorrhea, not due to surgery, pregnancy, lactation, radiation, chemotherapy, or known medical causes. The onset of menopause is defined as the time of the final menstrual bleeding. However, unless a bleeding free year has already passed, it is not possible to determine whether the last observed menstrual period is the final menstrual period. Events, such as having a hysterectomy or the interview within 12 months of the last observed menstrual period will prevent one from seeing the future, and hence, the true age at menopause. Given these events, individuals would be censored, such that their age at onset of menopause occurs on or after the censored time point.

Censoring was conducted with the goal of correctly classifying menopausal status and measuring the age at menopause. For example, assume the interview occurred 3 months after the last observed menstrual period (LMP). Since a year of amenorrhea has yet to pass, we do not know if this menstrual period is actually the final menstrual period (FMP), defining the onset of menopause. Censoring would occur at the LMP. The same censoring strategy (i.e. censoring at LMP) would apply to women with an ovary-preserving hysterectomy or bilateral oophorectomy within 12 months following the LMP or for women with radiation or chemotherapy treatment within 12 months of the LMP. For the one participant who was currently pregnant, censoring occurred at the time of the interview.

For 203 women (<55 years of age) with unclear menopausal status and a random sample of everyone else (TABLE 3.4), follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E_2) measures were available and used to confirm or augment classification of menopausal status and narrow the time window in which menopause is expected to occur. Classification of menopause proceeded in a 3 step process whereby an individual was assigned a menopausal status based first on her level of FSH (pre-menopausal if FSH<15 and post-menopausal if FSH>40) (TABLE 3.7). For women whose FSH fell within a range (15-40 IU/L) in which status could not be clearly defined, then E_2 levels were used (premenopausal if E_2>100 and post-menopausal if E_2<60). If the E_2 levels fell within a range (60-100 pg/mL) that did not allow resolution of menopausal status, then the ratio of FSH/LH was used (premenopausal if FSH/LH$\leq$1 and postmenopausal if FSH/LH>1). The cutpoints used for each step were selected to take into account the distribution and levels of all three ovarian hormones at different stages of reproductive life [24] as well as literature suggesting optimal cutpoints for FSH [25, 26], E_2 [27] and FSH/LH ratio [28, 29].

The supplementary hormone data allows refinement of the time window in which women's onset of menopause occurs, which therefore increases precision. For women who had radiation or chemotherapy within a year of the last menstrual period, censoring would still begin at the LMP, regardless of the hormone data (TABLE 3.8). The hormone data is not reliable among these women given the varying effects of radiation and chemotherapy: women can either experience premature menopause or can eventually regain their menstrual cycles, and therefore, censoring at any time after the last menstrual period would be erroneous. Likewise, those currently pregnant would be censored at the time of the interview

and those missing a date for the last menstrual period would be censored at the return of menses following the last pregnancy.

If the hormone data indicates pre-menopausal status, then censoring would occur at the time of the follow-up blood draw, rather than at the last menstrual period. If the hormone data suggests postmenopausal status, then censoring would begin at the last menstrual period for those with a bilateral oophorectomy, whereas censoring would occur between the last menstrual period and follow-up blood draw ("interval censored") for those with a hysterectomy but still retain $\geq$1 ovary. For everyone else, natural menopause would occur at the last menstrual period.

In a secondary analysis, assignment of menopause status and age took into account the effect of hormone replacement therapy (HRT) use. Hormone replacement therapy can cause postmenopausal "breakthrough" bleeding, thereby masking the true onset of menopause [30]. Hence, if an episode of hormone replacement therapy began before the last menstrual period and lasted at least until one year prior to LMP, the following guidelines were used to define menopausal status: if FSH/LH/E_2 data suggest she is pre-menopausal, censor at the blood draw, otherwise, extend the left side of the menopause interval, as defined by the rules outlined above, to include the whole HRT episode (if it does not already). For a woman who reported using oral contraceptives for menopausal symptoms, her last episode of oral contraceptive was treated as hormone replacement therapy. Multiple episodes of HRT use separated by a $\leq$1 year gap were treated as one continuous episode.

Some methodologic considerations should be noted in using FSH, LH and E_2 for classifying menopausal status. Given that serum measures were not timed to the menstrual cycle, use of this FSH/LH/E_2 algorithm would better discriminate menopausal status

compared to FSH alone. However, for those taking hormone replacement therapy, it is still possible that menopausal status could be misclassified using this algorithm, given that introduction of exogenous estrogen can artificially decrease the FSH levels, thereby, causing some postmenopausal women to be classified something other than postmenopausal. Among women with a bilateral oophorectomy participating in the National Health and Nutrition Examination Study (NHANES), the same proportion (~85%) of women taking HRT and women not taking HRT had elevated FSH levels defined as more than 20 IU/L. In contrast, 52% of those taking HRT and 67% of those not taking HRT had FSH levels above 40 [31]. In North Carolina Menopause Study, 33 of 39 women who were currently taking hormone replacement therapy had hormone measures. Of these, 25 had FSH>40 IU/L and were assumed to be correctly defined as postmenopausal, whereas 4 individuals had FSH<15 IU/L and were assumed to be correctly defined as premenopausal, given the findings in NHANES. Only the remaining 4 individuals had 15<FSH<40 (12% of those taking HRT), who may have been subject to misclassification of menopausal status.

Among women with unclear menopausal status, ovarian hormone analysis was limited to those less than 55 years of age. Excluding women age$\geq$55 should not bias the estimate of effect however, as menopausal status for any one individual is not misclassified. Rather, for those with unclear menopausal status, age 55 and over, the time window in which her menopausal experience is expected could not be refined (i.e. precision is not improved), because she did not have the benefit of the additional hormone information.

The hormone algorithm in this study has not been validated. However, there was high agreement between the interview and hormone data among those with clearly defined postmenopausal status (i.e. those with a bilateral oophorectomy and $\geq$1 year amenorrhea). Of

the 54 women with ≥1 year of amenorrhea and hormone data, 53 were defined as postmenopausal by hormone. Of the 10 with a bilateral oophorectomy and hormone data, 9 were classified as postmenopausal by hormone. For the one individual who reported having both ovaries removed but whose hormone data (FSH=22, LH=11.14 and estradiol=162.45) suggests she was currently premenopausal, the hormone data was taken to be correct. A duplicate blood sample on this individual did not change her hormonal classification. It is unclear whether the participant misreported the number of ovaries removed.

3.5.3 Covariates

Factors that have been associated with age at menopause but are not on the causal pathway from exposure to outcome and which data were available were included in regression model. Data on these variables were collected at the follow-up study, except for BMI at age 40, which was estimated based on the change in BMI from the baseline to follow-up interview.

Information on cigarette smoking was used to construct a dichotomous indicator variable for smoking status: one group containing those who smoked regularly (at least 1 cigarette per day for ≥3months) during ages 36-45 and the other containing those who either never smoked or smoked only outside of that age range. Alcohol use from ages 40-49 was included as an ordinal categorical variable with 6 categories: 1=one for those who never drank, 2=one for those who rarely drank (<10 drinks per year), and of the remaining participants who had 10 or more drinks for any given year, 4 additional groups were established based on the number of drinks they had per week (3=less than 1, 4=1 to 2, 5=3 to 6 and 6=7 or more drinks per week). Body mass index at age 40 was used as a continuous

predictor and was estimated via linear interpolation of BMI from the baseline study to BMI at the follow-up study. Age at menarche was categorized into ages 9-11, 12-13, and 14-17. Menopause before age 46 in a mother and/or sister was included as a dichotomous indicator of familial/genetic inheritance. First (whole or part of one) ovary removed before age 40 was categorized as yes/no.

3.5.4 Statistical analysis

Age at menopause (in years), the outcome of interest, was directly modeled using linear regression. To account for the interval censoring, models were fit using the LIFEREG procedure in SAS 9.0. Non-parametric survival curves were generated by fitting a model without any exposure or covariates. The overall distribution of age at menopause was examined for symmetry (FIGURE 3.4), and both normal and logistic distributions of the error term were explored. Since the latter gave a better fit to the data, modeling proceeded with the assumption of a logistic distribution.

Separate models for DDE and PCBs, as well as a model that included both chemicals, were fitted, controlling for all the aforementioned predictors of age at menopause. Sensitivity analysis excluding certain medical conditions, juvenile diabetes and lupus, was also conducted to determine whether these would distort the relationship between organochlorines and age at menopause. Regression coefficients and 95% confidence intervals were generated. The regression coefficients were interpreted as the difference in mean age at menopause (years) per unit change in the exposure or covariate value.

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TABLE 3.1 Response rate into North Carolina Menopause Study

Response	**N (% of 877 in NC Infant Feeding Study)**	**N (% of 726 eligibles who were traceable)**
Not eligible (no DDE/PCBs)^	12 (1.4%)	
Not traceable *	139 (15.8%)	
Not found	118 (13.5%)	118 (16.3%)
Refusals	81 (9.2%)	81 (11.2%)
Deceased	10 (1.1%)	10 (1.4 %)
Other	5 (0.6%)	5 (0.7%)
Completed follow-up interview	512 (58.4%)	512 (70.5%)
Total	**877 (100.0%)**	**726 (100.0%)**

^ no tissue sample available for measurement of DDE or PCBs
* no name or other contact information required for tracing

Table 3.2 Baseline characteristics (N and %) of participants and non-participants in the North Carolina Menopause Study

Variables	Non-participants (n=365)*	Participants (n=512)*
Baseline DDE (ug/mL)	Median (range) = 13.0 (1.3-72.1)	Median (range) = 12.6 (1.9-180.0)
Baseline PCB (ug/mL)	Median (range) = 9.3 (4.4-88.8)	Median (range) = 10.0 (4.0-69.6)
Age (yrs)		
16-20	25 (7.4)	14 (2.7)
21-30	249 (73.5)	370 (72.3)
>30	65 (19.2)	128 (25)
Race		
White	308 (89.5)	495 (96.7)
Black	35 (10.2)	15 (2.9)
Other	1 (0.3)	2 (0.4)
Weight (kg)	Mean (SD) = 130. 2 (23.0) Median = 125	Mean (SD) = 130.1 (20.6) Median = 125
Number of years in school		
<12	20 (5.9)	1 (0.2)
12-16	266 (78.5)	374 (73.3)
≥17	53 (15.6)	135 (26.5)
Cigarettes smoked per day when not pregnant		
None	259 (76.4)	434 (85.4)
0-½ pk	24 (7.1)	23 (4.5)
½-1 pk	24 (7.1)	19 (3.7)
≥1 pk	32 (9.4)	32 (6.3)
Beer can per week when not pregnant		
0	281 (83.1)	409(80.4)
1-2	36 (10.7)	70 (13.8)
3-4	13 (3.8)	23 (4.5)
≥5	8 (2.4)	17 (3.3)
Glass of wine per week when not pregnant		
0	254 (75.4)	321 (63.1)
1-2	58 (17.2)	140 (27.5)
3-4	17 (5.0)	34 (6.6)
≥5	8 (2.4)	14 (2.8)
Liquor drink per week when not pregnant		
0	301 (89.3)	410 (80.6)
1-2	29 (8.6)	80 (15.7)
3-4	4 (1.2)	13 (2.6)
≥5	3 (0.9)	7 (1.4)

*some non-participants had missing information on some variables

TABLE 3.3 Data collected in the Menopause Study questionnaire

Menstrual cycle history and menopause

- Age and school grade when had first period
- Any menstrual periods (not spotting) in past 12 months
- Age and date (mon/yr) of last menstrual period
- For those with no periods in past 12 months, the reason for amenorrhea
- Menstrual cycle length and irregularity in 12 months before the last menstrual period
- Night sweats, hot flash, vaginal dryness in the 2 years before the last menstrual period

Pregnancy and breastfeeding history

- Currently pregnant
- Number of times pregnant
- Outcome of current and previous pregnancy
- Age and date (mon/yr) pregnancy ended
- Ever breastfed and number of weeks breastfed current and previous child

Medical and family history

- Age of diagnosis of autoimmune diseases, including juvenile diabetes and lupus
  - Currently taking medications for those diseases (for diabetes, start of insulin use)
- Mother and/or sister had natural menopause at or before age 46

Surgery and cancer treatment

- Ever had a hysterectomy and/or oophorectomy
  - Age, date (mon/yr), and season of surgery
  - Number of ovaries removed at surgery, if any
- Ever had chemotherapy / radiation
  - Age and date (mon/yr) of treatment

TABLE 3.3 (continue)

Birth control hormones and hormone replacement therapy (HRT)

Ever used oral contraceptives, Norplant or Depo-Provera for any reason

Age, date (mon/yr), and season started and ended use for each episode of use

Did the start of use occur before or after the last menstrual period

For those using birth control >35 years of age, reason for use (including menopause)

Ever used female hormones other than birth control

Age, date and season started and ended use for each episode of use

Did the start of use occur before or after the last menstrual period

Did a menstrual period occur within 12 months before started use and was it regular

Smoking

Ever smoked regularly (≥1 cigarette / day for ≥3 months)

On average, smoked regularly in past 3 months

Age started smoking regularly & age stopped smoking for ≥1 year, per smoking episode

Number of cigarettes smoked per day between ages 30-49

Alcohol use

Ever drank in your forties

Number of years drank ≥10 drinks per year between ages 40-49

Number of alcoholic drinks per week between ages 40-49

Height and weight

Height (feet and inches)

Current weight (pounds); if unknown, then categories of weight

TABLE 3.4 Derivation of serum subsets for organochlorine and hormone analysis

Group	# women	DDE/PCB (Study Aim 1)			Hormones (Study Aim 2)		
		% chosen	# chosen	# assayed	% chosen	# chosen	# assayed
1	41	30%	12	11	100%	41	36
2	39	30%	12	10	100%	39	33
3	107	30%	32	28	100%	107	95
4	325	30%	98	77	15%	49	39
Total	512		154	126		236	203

TABLE 3.5 Concentrations of PCB congeners as proportion of total PCBs at follow-up, n=126*

PCB congener	Mean concentration, wet weight	Mean as % of total mean concentration	Mean concentration, lipid adjusted	Mean as % of total mean concentration	N (%) non-detects
PCB128	0.0002	0.02	0.0190	0.02	125 (99.2)
PCB101	0.0023	0.29	0.3571	0.29	123 (97.6)
PCB52	0.0031	0.39	0.5397	0.45	124 (98.4)
PCB105	0.0083	1.04	1.1206	0.92	87 (69.0)
PCB183	0.0115	1.44	1.6556	1.37	66 (52.4)
PCB28	0.0174	2.18	2.8817	2.38	112 (88.9)
PCB99	**0.0359**	**4.50**	**5.4563**	**4.50**	**22 (17.5)**
PCB156	**0.0367**	**4.61**	**5.6111**	**4.63**	**11 (8.7)**
PCB187	**0.0510**	**6.40**	**7.6770**	**6.33**	**3 (2.4)**
PCB170	**0.0552**	**6.92**	**8.3778**	**6.91**	**2 (1.6)**
PCB118	**0.0678**	**8.50**	**10.2135**	**8.43**	**3 (2.4)**
PCB138	**0.1180**	**14.81**	**17.9524**	**14.81**	**2 (1.6)**
PCB180	**0.1714**	**21.51**	**26.0714**	**21.51**	**0 (0)**
PCB153	**0.2183**	**27.38**	**33.2730**	**27.45**	**1 (0.8)**
Total	0.7970	100.00	121.2063	100.00	

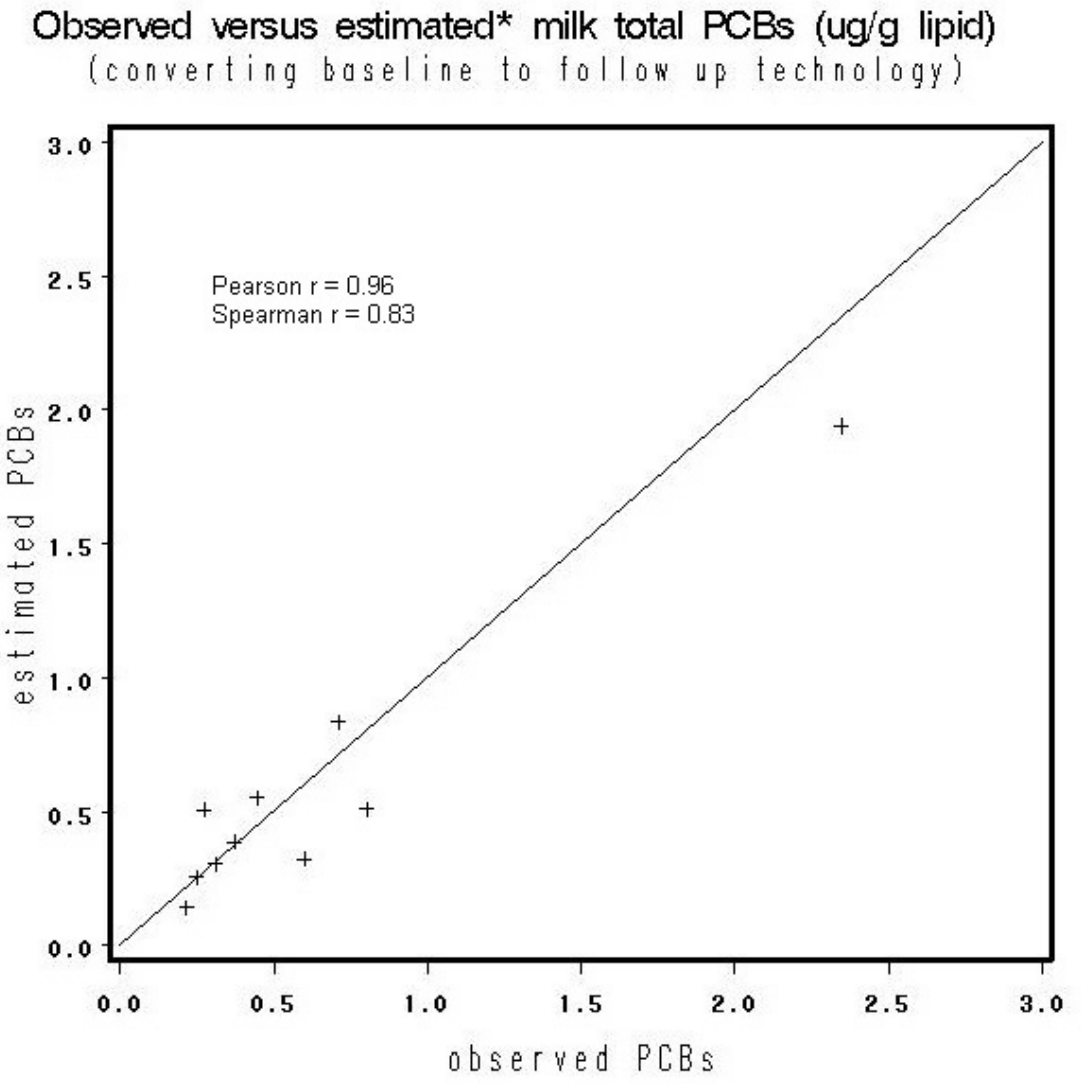
* Assume those below the limit of detection have a value of zero

TABLE 3.6 Formulas for transforming baseline organochlorine measures (into measures that are comparable to those at follow-up)

Unit of analysis	Organo-chlorine	Detectable (D) or non-detectable (ND) sample	Baseline measure used	Account for change in quantitation method	Convert composite to serum equivalent for non-detects	Account for lipid rise during pregnancy	Account for difference in milk lipid and serum lipid
Wet weight (ug/L serum)	DDE	D	Serum			x 0.7	
		ND	Composite		x 5.0071367	x 0.7	
	PCB	D	Serum	x 0.236127		x 0.7	
		ND	Composite	x 0.236127	x 5.4120890	x 0.7	
Lipid-adjusted (ug/g lipid)	DDE	D	Composite				/ 1.5
		ND	Composite				/ 1.5
	PCB	D	Composite	x 0.236127			/ 1.5
		ND	Composite	x 0.236127			/ 1.5

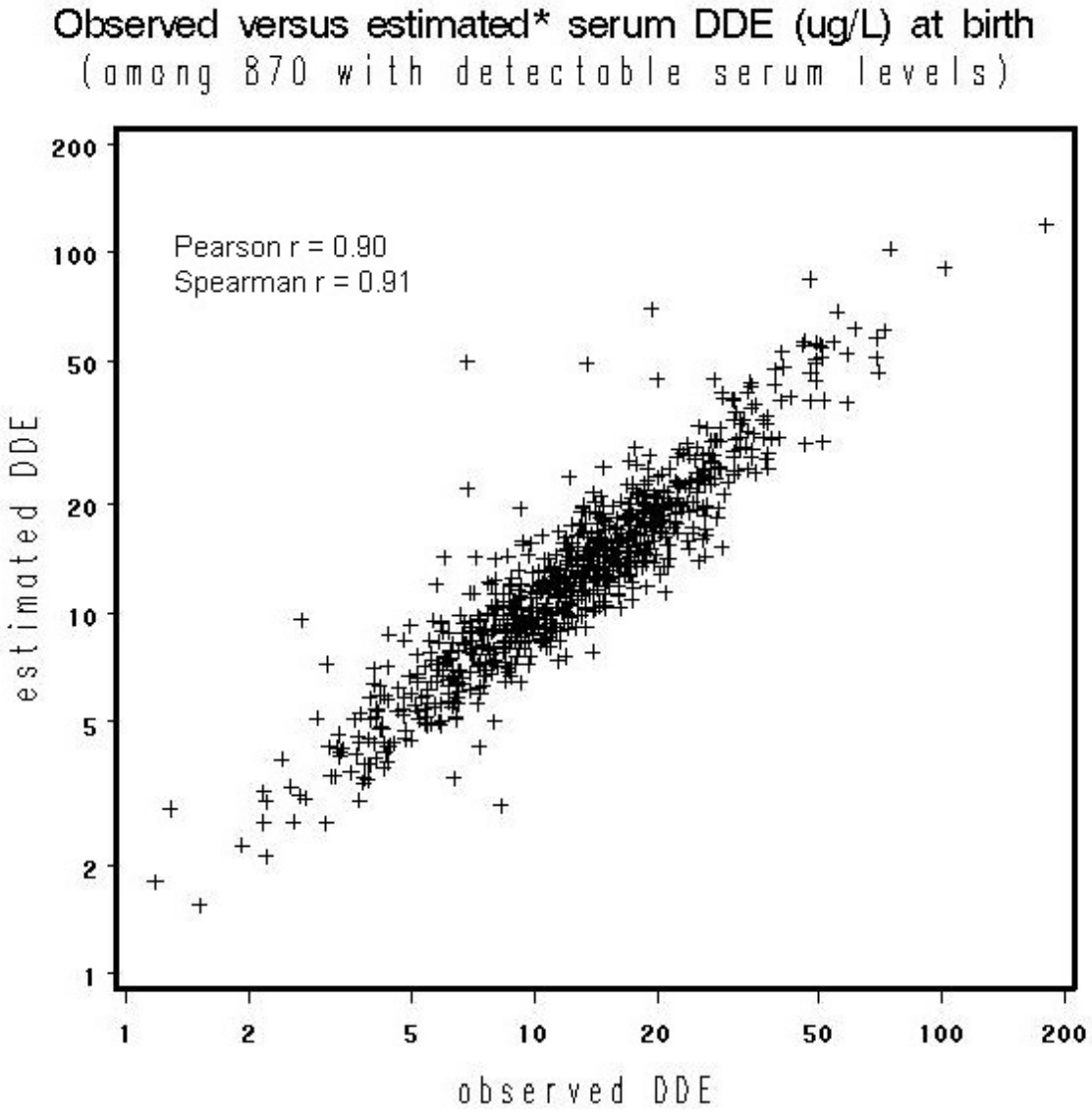
Comp = "composite" measure representing milk at birth , x=multiplication , /=division

FIGURE 3.1 Comparison of PCBs using the follow-up quantitation method to PCBs using baseline converted to follow-up method, n=10



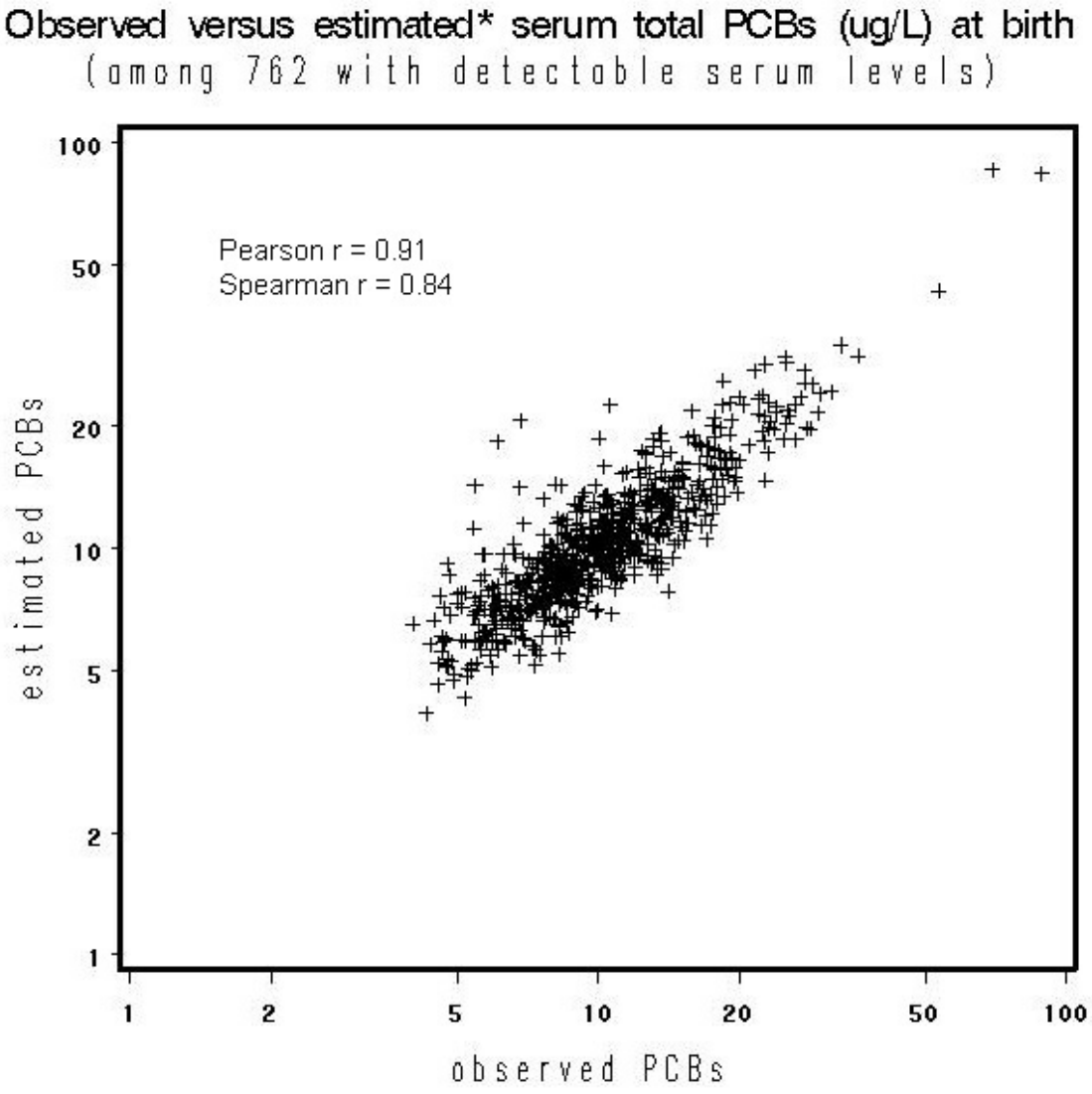
*Estimated PCBs derived from converting values using baseline quantitation method to values that would have been obtained using follow-up quantitation method

FIGURE 3.2 Comparison of actual serum DDE at birth to those estimated by converting composite measures, n=870 with detectable serum levels



*Estimated serum DDE from milk composte measures
(by multiplying with median ratio=5.0071367 of serum to milk composite)

FIGURE 3.3 Comparison of actual serum PCBs at birth to those estimated by converting composite measures, n=762 with detectable serum levels



*Estimated serum total PCBs from milk composte measures
(by multiplying with median ratio=5.4120890 of serum to milk composite)

TABLE 3.7 Hormone algorithm for defining menopausal status (at the blood draw)

Step	Hormone	Premenopaual	Postmenopausal
1	FSH <15 IU/L	X	
	FSH >40		X
2	15≤FSH≤40 and		
	E_2 <60 pg/mL		X
	E_2 >100	X	
3	15<FSH≤40 and 60≤E_2≤100 and		
	FSH/LH ratio ≤1	X	
	FSH/LH ratio >1		X

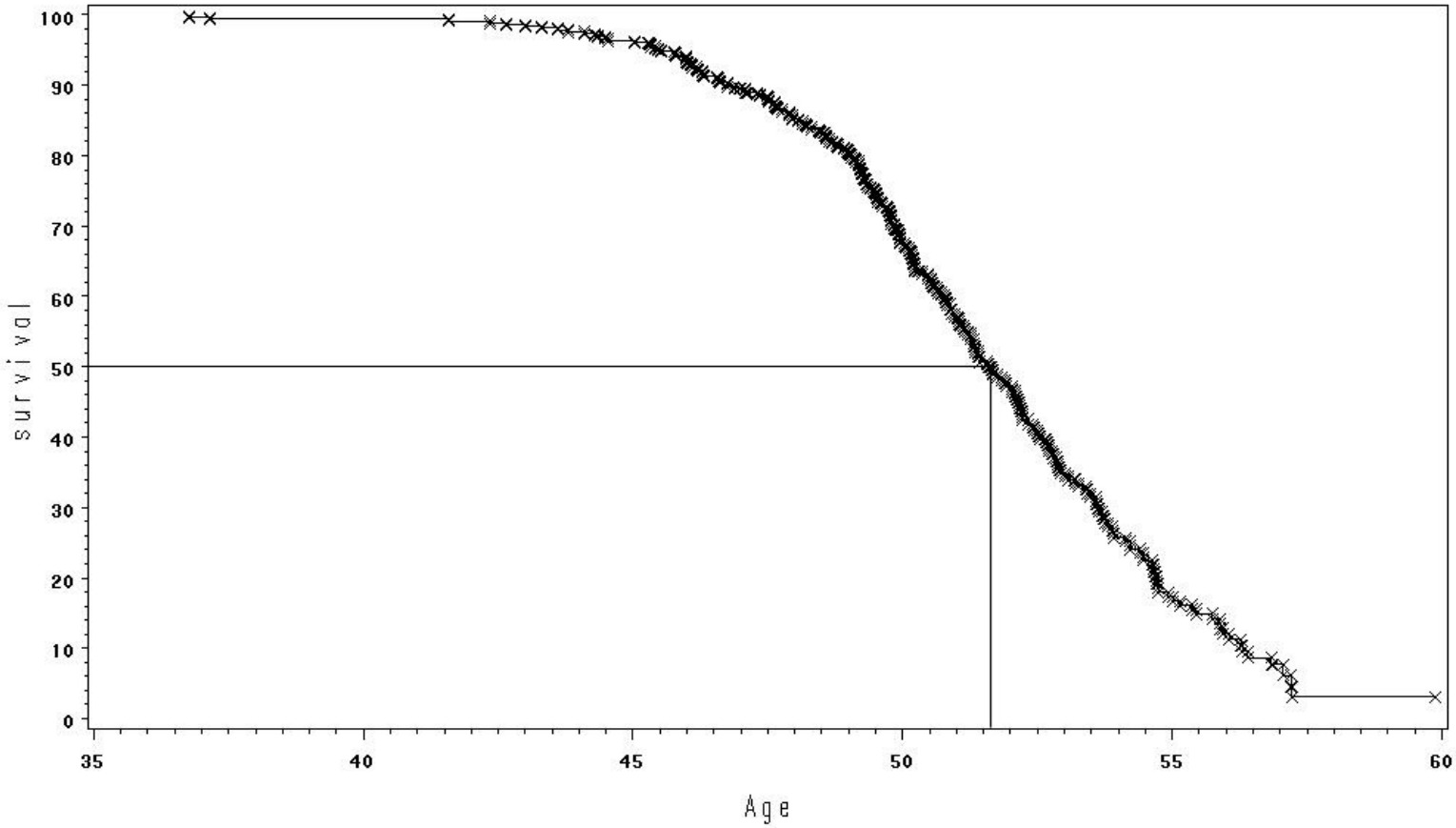
TABLE 3.8 Menopausal status and censoring for survival analysis

Categories	Age at menopause
Currently pregnant	Censor at interview
Missing date of LMP*	Censor at return of menses following last pregnancy
Radiation or chemotherapy began within 12 months prior to or following the LMP and before any hysterectomy AND	
- post-menopausal by FSH/LH/E_2	Censor at LMP
- pre-menopausal by FSH/LH/E_2	Censor at LMP
- no FSH/LH/E_2	Censor at LMP
Hysterectomy before LMP + 12 months with any part of an ovary maintained AND	
- post-menopausal by FSH/LH/E_2	Interval censor between LMP and blood draw
- pre-menopausal by FSH/LH/ E_2	Censor at blood draw
- no FSH/LH/ E_2	Censor at LMP
Both ovaries removed before LMP + 12 months AND	
- post-menopausal by FSH/LH/E_2	Censor at LMP
- pre-menopausal by FSH/LH/E_2	Censor at blood draw^
- no FSH/LH/E_2	Censor at LMP
LMP <12 months prior to interview AND	
- post-menopausal by FSH/LH/E_2	Natural menopause at LMP
- pre-menopausal by FSH/LH/E_2	Censor at blood draw
- no FSH/LH/E_2	Censor at LMP
LMP ≥12 months prior to interview AND	
- post-menopausal by FSH/LH/E_2	Natural menopause at LMP
- pre-menopausal by FSH/LH/E_2	Censor at blood draw
- no FSH/LH/E_2	Natural menopause at LMP

^ Interview data suggests she is post-menopausal, while her hormone data (FSH=22, LH=11.14 and E_2=162.45) suggests pre-menopausal. Assume her hormone data is correct.

* LMP – last menstrual period

FIGURE 3.4 Survival curve of age at menopause (no covariates), n=512



4. MANUSCRIPT 1. DDE AND PCBS: INTRA-INDIVIDUAL CHANGES, CORRELATIONS AND PREDICTORS

4.1 Abstract

BACKGROUND: Dichlorodiphenyldichloroethane (DDE) and polychlorinated biphenyls (PCBs) are widespread environmental contaminants that have been postulated to increase the risk for neurodevelopmental delay, miscarriage, early menopause, and other health outcomes. Studies assessing the effect of organochlorine exposure often can only measure organochlorine levels once, such as at study enrollment. A single measure might not reflect the exposure levels at an etiologically relevant time period.

OBJECTIVE: Our aim was to model the temporal changes in DDE and PCBs and the predictors of those changes over a follow-up period spanning approximately 25 years..

METHODS: Interview data and DDE/PCB measures were collected from 123 women enrolled in a baseline study from 1978-1982 and followed up in 2003-2004. Baseline and follow-up organochlorine levels were compared using Spearman correlations (r_s) and predictors of the rate of change in log concentration were evaluated using linear regression models.

RESULTS: While serum concentrations dramatically declined (median follow-up to baseline concentration ratio was 16% for DDE and 45% for PCBs), baseline and follow-up measures were strongly correlated for DDE (r_s=0.72) and moderately correlated for PCBs (r_s=0.43). Prediction of follow-up PCB levels was substantially improved (r_s=0.75) with data on initial

concentration, length of lactation, baseline body mass index and percent change in body fat, while DDE prediction improved slightly (r_s=0.83) with data on lactation and baseline body mass index.

CONCLUSION: Our results suggest that a single organochlorine measure provides considerable information on relative ranking at distant times. Furthermore, predictors such as initial concentration, length of lactation, baseline body mass index and percent change in body fat can increase the predictive power, particularly for PCBs.

4.2 Introduction

Extensive use of the pesticide, dichlorodiphenyltrichloroethane (DDT), beginning in the 1940s and the industrial chemical, polychlorinated biphenyls (PCBs) beginning in 1929, have led to their widespread distribution in the environment. Despite their ban since the 1970s, PCBs and the DDT metabolite, dichlorodiphenyldichloroethane (DDE), remain ubiquitous, raising concerns over their environmental and public health impact. Many studies have evaluated associations of these compounds with human health effects, including neurodevelopmental delay [1, 2], miscarriage [3, 4], and earlier menopause [5, 6], although clear associations have not been established.

Studies assessing the effect of these organochlorines on risk of chronic diseases or diseases with long latency periods often can only measure levels once; for example, they may be measured at enrollment into a prospective study of disease incidence. While a single measure reflects exposure accumulation and excretion of a compound up to the time of sampling, it may not provide an accurate picture of exposure level at a time of etiologic relevance.

The objective of this paper is to examine the ability to predict changes in organochlorine levels from baseline forward in time over a period of decades. Few studies to date have assessed long-term intra-individual changes in DDE or PCB concentrations and the predictors of those changes. These longitudinal studies [7-12] span approximately 2 to 10 years, and most have limited covariate data. Our study uses information collected from women who had enrolled in a baseline study of organochlorines (from 1978-1982) and were followed for approximately 25 years.

4.3 Methods

Study population

From 1978-1982, pregnant women (n=877) from North Carolina were recruited into the baseline study of organochlorines and child development [13]. Around the time of delivery, a questionnaire including demographics, pre-pregnancy weight, reproductive and medical history was administered; maternal and cord bloods, placenta and breast milk were also collected for organochlorine determination. Additional samples were collected at subsequent study visits, including blood at the 6 week visit and milk periodically as long as the child was breast-fed.

In 2003-2004, women were traced and invited into a follow-up study on menopause. Of 719 traceable women, 513 (71.3%) participated. Additional data including reproductive and lactation history (covering all pregnancies), height and current weight were obtained via a telephone-administered questionnaire.

A sample of 126 individuals, constituting 82% of a 30% random sample of follow-up participants who were selected for a blood draw, had DDE/PCB assessment. Of these, 123 had a serum organochlorine measure at delivery from the baseline study and will be the subjects for the current study. One individual had missing pregnancy data on the follow-up questionnaire and was excluded from our predictive modeling. Informed consent and Institutional Review Board approval were obtained for both baseline and follow-up studies.

Laboratory Assays

At baseline, p,p'-DDE and PCBs were assayed using packed column-gas chromatography with electron capture detection. PCBs were quantified as the sum of two

peaks on a chromatogram [14]. The coefficients of variation were <12% [14]. Due to the highly variable fat content in breast milk, milk concentrations were expressed as grams of organochlorine per gram of lipid, while concentrations in other tissues were reported per gram of tissue.

At follow-up, organochlorines were assayed using gas chromatography coupled with mass spectrometry (GC-MS); coefficients of variation were 5.1% for DDE and 3.5%-11.4% for various PCB congeners. Fourteen congeners were quantitated; for this study, we focused on the total of 8 congeners (PCB# 99, 118, 138, 153, 156, 170, 180, and 187) having the lowest proportion of non-detects (<18%, compared to 52-98% for the remaining 6 congeners: PCB-28, 52, 101, 105, 128, and 183) and the highest relative abundance (together comprising ~94% of total PCBs measured). Total lipids were also estimated from total cholesterol, phospholipids and triglycerides using the Phillips et al. approach [15], thereby allowing concentrations to be expressed per gram of lipid. A validation study shows that this approach gives values very similar to those obtained using the standard gravimetric lipid analysis [16].

Accounting for change in PCB quantitation methods

Though the assays have changed since the baseline study, DDE quantitation (which involves measurement of a single peak) was the same. However, PCB quantitation methods used at baseline differed from those used at follow-up and do not produce directly comparable results. To convert baseline PCB concentrations into their follow-up equivalents, we used data from 10 baseline milk samples reanalyzed by GC-MS [17]. Baseline levels were multiplied by the median ratio (0.236127) of concentration using the follow-up method to concentration using the baseline method.

Handling non-detects

One individual at baseline had non-detectable serum DDE and 18 (14.6%) had non-detectable serum PCBs. Serum organochlorine concentrations below the limit of detection (LOD) at baseline were assigned estimated values using measurements from all available specimens at baseline. The concentrations from each specimen were previously scaled to reflect the concentration in milk lipids at birth of the index child and then averaged to create a composite measure for each woman [13]. For this analysis, these composite measures were converted into their approximate serum equivalents by multiplying by the median ratio of serum to composite concentrations (5.0071367 for DDE and 5.4120890 for PCBs) obtained from members of the baseline population with detectable serum values. To evaluate the validity of this imputation, we looked at estimated and actual amounts among those with detectable concentrations in serum at delivery: mean % difference and standard deviation were 6.1 $\pm$ 36.9 for DDE and 3.8 $\pm$ 24.3 for PCBs. Pearson and Spearman correlations were high between estimated and observed values for both DDE (0.90 and 0.91, respectively) and PCBs (0.91 and 0.84). At follow-up, one individual had non-detectable serum DDE, whereas 28 (22.8%) had at least one non-detectable PCB congener. Non-detects at follow-up were assigned LOD divided by the square root of 2 [18]. Assuming DDE and PCBs are log-normally-distributed, non-detects would be more accurately represented by this imputation approach as opposed to using LOD or 0 as a substitute for non-detects. Nevertheless, since the non-detectable congeners were usually the minor ones, there were only six individuals in which the imputation (using extremes of LOD or 0) made a >10% difference in their estimated total PCBs.

Accounting for pregnancy-related increases

As serum lipids were not available at baseline, our primary goal was to explore changes in wet weight serum organochlorine measures over time. Measures at baseline were acquired at or around the time of delivery; however, short-term changes due to pregnancy are not the changes of interest. Given the characteristic rise in lipid content across pregnancy [19, 20] and the resulting elevation in organochlorines, wet weight serum measures were adjusted to reflect estimated non-pregnant levels. Based on mean serum total lipids of 5.9 g/L among more than 2000 non-pregnant Caucasian women, aged 20-34, participating in the 1976-80 National Health and Nutrition Examination Survey [21, 22] and 8.5 g/L in a 1977-79 study of 553 pregnant Caucasian women, ages 20-41, at 36 weeks of gestation [20], we estimated a 30% increase in total lipids across pregnancy. To account for this rise, wet weight serum concentrations at baseline were reduced by an equivalent amount.

Secondary analysis: Lipid-adjusted serum measures

Though our primary analysis was based on wet weight organochlorines, we conducted a secondary analysis on estimated lipid-adjusted measures. Lipid-adjusted serum concentrations at baseline were estimated by dividing the aforementioned "composite" milk values by a correction factor (1.5) that accounts for the difference in lipid concentrations between milk and serum. This factor is the approximate ratio of concentrations in milk lipids to those in serum lipids for both DDE [23, 24] and PCBs [23, 25].

Predictive modeling

Although changes in serum DDE and PCB levels over time are driven by complex processes, we posit that the time course of such changes during the period under study can be reasonably approximated by an exponential decay. Thus, our basic predictive model was:

$$[\text{Follow-up}] = [\text{Baseline}] * \exp(-\text{slope} * \text{follow-up time})$$

which can be re-written as

$$\frac{\text{Log [Follow-up]} - \text{Log [Baseline]}}{\text{follow-up time}} = -\text{slope}$$

where [Follow-up] and [Baseline] are the concentrations, respectively, at follow-up and baseline, and "follow-up time" represents years from baseline to follow-up. Slope represents change in log concentrations per unit of follow-up time. The slope can be affected by various determinants, so we modeled it using linear regression; the full model is then of the form:

$$\frac{\text{Log [Follow-up]} - \text{Log [Baseline]}}{\text{follow-up time}} = -(\alpha + \beta 1\ (X1) \ldots + \beta k\ (Xk)$$

where α is the intercept and βk is the regression coefficient for the k-th predictor, Xk.

Lactational transfer from mother to infant is an important route of organochlorine excretion [13, 26]. In this study, lactation duration, from the follow-up questionnaire, is defined as the number of weeks she breast-fed twice or more per day. Given the diminishing role of exclusive breast-feeding as lactation proceeds, the effect of breast-feeding on the rate of organochlorine excretion could lessen later in lactation. Thus, lactation duration was treated as a continuous variable but modeled in a piecewise linear fashion with the points where the slope changes prespecified as 26 and 39 weeks of lactation. This was

accomplished by defining 3 variables representing the number of weeks of lactation during the 3 intervals: 0-26th, 27th-39th, and >39th week of lactation. The number of weeks of breast-feeding for each pregnancy from baseline to follow-up was split among these three variables. For women with multiple lactations between baseline and follow-up, the lactation contribution from each pregnancy was summed. To illustrate, for a woman with one child whose lactation lasted 60 weeks, a value of 26, 13 and 21 weeks was assigned to the first, second and third lactation variables, respectively. If she also had a second child who was breastfed for 30 weeks, then an additional 26 weeks was assigned to the first lactation variable and 4 weeks to the second, giving final values of 52, 17, and 21 for the three lactation variables. Regressing on these 3 variables produces estimates for the 3 slopes, such that the contribution to overall slope of organochlorine decline is: slope contribution= $\beta 1$(interval 1) + $\beta 2$(interval 2) + $\beta 3$(interval 3).

Previous studies on PCBs and dioxins show that high initial concentration was associated with faster decline over time [27, 28]. We included the log of initial (baseline) concentration of serum DDE or PCBs as a linear variable in the model.

Body mass index (BMI) may be associated with an individual's ability to metabolize organochlorines and other xenobiotics, with heavier individuals having slower metabolism [29]. Baseline BMI was included in the model as a categorical predictor, with cutpoints at 20 and 23 kg/m^2, due to its narrow distribution.

Since fat is where organochlorines are sequestered, an increase in body fat without an increase in organochlorines might dilute organochlorine concentrations. Thus, we included a measure of percent change in body fat (kg) based on a validated formula for percent body fat, which we then converted to body fat by multiplying by body weight [30]:

$$\text{Body fat} = [((1.46 * \text{BMI}) + (0.14 * \text{age}) - 10) / 100] * \text{weight}$$

Usual weight prior to baseline study pregnancy and current weight were obtained, respectively, from the baseline and follow-up questionnaires. Percentage change in body fat, defined as the difference in body fat from baseline to follow-up divided by baseline body fat, was included as a linear variable.

Mother's date of birth reflects the secular trend in environmental DDE/PCB levels, and hence, the exposure potential. Calendar date of birth (measured in days with a SAS reference date of January 1, 1960) was included as a linear variable in the model. Another possible predictor is baseline maternal age; organochlorine metabolism may change with age. However, since age at baseline is highly correlated with date of birth (r=0.97), it was not included.

Statistical analysis

Linear regression models were fit using SAS (version 9.1). Each predictor was examined to obtain the fewest categories needed to adequately characterize the relationship between that variable and the slope. The predictors were added to the model in successive order of their assumed strength of effect. The statistical significance of each potential predictor was assessed using the F test. Spearman correlations comparing actual versus model-predicted concentrations at follow-up were used to assess the predictiveness of each model. To illustrate the model fits, model-predicted changes in organochlorine levels were plotted along with actual changes for the 122 individuals with non-missing questionnaire data.

4.5 Results

The mean age of our study sample at baseline was 29 years. Other descriptive information is shown in Table 4.1. Among the 122 women with pregnancy data, there were 207 births during the study period (i.e. from baseline to follow-up, including baseline pregnancy) and 39% of these are the only birth during the study period. Ninety percent of the babies were breast-fed, and 52% were breast-fed for more than 26 weeks. Approximately 90% of women had a pre-pregnancy body mass index of less than 25 kg/m^2 at baseline, and all but two had increased (65% with at least a 50% increase) their body fat mass by the time of follow-up.

At baseline, the median wet weight concentrations were 8.5 ug/L for DDE and 1.5 ug/L for PCBs (TABLE 4.2). At follow-up, medians fell to 1.2 ug/L and 0.7 ug/L, respectively; expressing each follow-up concentration as a percent of her baseline, medians were 16% for DDE and 45% for PCBs. There was a dramatic decline in DDE levels, with 90% of women having follow-up concentrations $\leq$30% of baseline, whereas the variability in individual changes was greater for PCBs. Changes over time in lipid-adjusted measures were of similar magnitude.

The correlations between the two wet weight measures taken more than 20 years apart were relatively strong (FIGURES 4.1 and 4.2). DDE at baseline was highly correlated with DDE at follow-up (Spearman r = 0.72), whereas the correlation for PCBs was lower, but still sizeable (r_s= 0.43). Correlations were similar for lipid-adjusted measures (0.73 and 0.50, respectively).

Higher initial concentration, lactation before 39 weeks, and an increase fat mass was associated with an increase in the rate of elimination of wet weight DDE and PCBs, whereas

higher baseline BMI tended to decrease the rate of decay (TABLE 4.3). Similar results were found for lipid-adjusted values (not shown). Mother's date of birth is not presented, as its effect was not significant for either DDE or PCBs. Table 4.4 shows the statistical significance of successive additional terms in the model for the slope and the correlation of the resulting predicted follow-up concentration with observed concentration. Lactation duration and baseline BMI were the only statistically significant predictors of DDE slope, whereas for PCBs, initial concentration and percent change in body fat were also significant. Predicted follow-up concentrations from the full model were highly correlated with actual follow-up concentrations for both DDE (Spearman r=0.83) and PCBs (Spearman r=0.75) (TABLE 4.4, FIGURES 4.3 and 4.4).

Figures 4.5 and 4.6 illustrates the predicted changes in DDE and PCB levels, and the effect of lactation and baseline BMI. Overall, there is a rapid decline in DDE and shallower reduction in PCB. The effect of the heaviest versus lightest category of baseline BMI was considerable for both DDE and PCBs. The drop in concentrations as a result of breast-feeding one child for one year versus no breast-feeding was evident for both compounds, although weaker for DDE. Although not shown, the effect of initial concentration (75^{th} versus 25^{th} percentile) on PCB decline was also notable, but the effect of an increase in fat mass was comparatively small.

4.6 Discussion

We found a substantial decline in intra-individual DDE levels over an approximate 25 year time span (1978-1982 to 2003-2004), with a smaller drop in PCB concentrations. These declines correspond with the reduction in environmental levels since the banning of DDT in

1972 and PCBs in 1977 in the United States [31-34]. The larger decline in DDE compared to PCBs is similar to those of previous studies conducted among Swedish men from 1991-2001 [11] and Great Lakes fisheaters from 1982-1989 [8]. In shorter studies, with medians 25.4 months [10] and 5 years of follow-up [7], the differences in the decline between DDE and PCBs were less obvious. Peak production and the restrictions on usage occurred later for PCBs than for DDT and may partly explain the smaller decline in PCBs. Differences between regions and sampling time since organochlorine restrictions may also explain the discrepancies between studies.

Our baseline levels of DDE were highly correlated with those at follow-up (Spearman r=0.7). Correlations were lower for PCBs. Higher correlations for DDE than for PCBs were also found in the Wolff et al. study with a median of 25.4 months of follow-up (0.95 versus 0.83, respectively) [10] and the Hoyer et al. study with a maximum of 7 years follow-up (0.79 versus 0.64, respectively) [7]. These differences became less evident when the specific congener PCB-153 was assessed; DDE and PCB-153 correlations were respectively 0.92 and 0.90 in a 1991-2001 study by Hagmar et al. [11] and 0.79 and 0.68 in the Hoyer et al. [7] study. The latter study also showed that correlations varied by PCB congener. Individual congeners could not be evaluated in our study since they were not measured at baseline, and the fact that total PCBs is a mix of congeners with different rates of decomposition may partly explain the lower correlations in PCBs.

A number of previous studies have used occupational cohorts or individuals exposed to very high, acute doses to estimate half-lives of organochlorines, with estimates being approximately 6-10 years for DDE [1, 35] and a few weeks to over 10 years for individual PCB congeners [36]. We examined a group of women with protracted, albeit declining,

environmental exposures to organochlorines, with attention to the influence of individual factors such as lactation and weight fluctuations on DDE and PCB levels. Based on the predicted curves (FIGURES 4.5 and 4.6), the apparent half-lives we predict during this time period are approximately 10 years for DDE and 25 years for PCBs, although they vary for women with different levels of the predictors. Since exposure is ongoing, although declining, in our study population, half-life estimation is expected to be longer compared to the aforementioned studies of occupational cohorts.

Breast-feeding was important in the elimination of organochlorines in previous studies of approximately 2-year [13] and 4-year follow-up [37], and our study indicates that the effect of lactation still exists approximately 25 years later. In our study, lactation beyond 9 months had much less impact on DDE and PCB levels than the earlier months. This change in effect across windows is expected, given that breast-feeding is likely to diminish in the later months as solid foods become an important component of diet. The impact of one year of lactation was more dramatic for PCBs than for DDE (FIGURES 4.5 and 4.6).

We found an association between higher initial concentration and faster elimination of PCBs, consistent with studies among those occupationally exposed [28, 38]. A study of 701 women environmentally exposed to PCBs (median 31 months follow-up) also reported similar findings; those whose PCBs had decreased were over 3 times more likely to have higher initial concentrations than those who had maintained their levels [9]. A possible explanation for this phenomenon that is most likely in our population is higher induction of enzymes at higher doses [27]. Although faster elimination of DDT from adipose tissue was associated with higher initial concentration in an experimental study of 3 individuals taking varying high level doses of DDT [39], similar data for DDE is not available.

Baseline BMI had a strong effect on the rate of change of both DDE and PCBs. This is consistent with BMI-dependent variation in DDE levels in previous studies [10, 12], showing a positive association or correlation between a single BMI measure and DDE half life. Sweeney et al. also found a small positive association between baseline BMI and an increase in PCB levels [9], although another study did not [10]. It is possible that the effect of higher body mass index on the rate of decline may be associated with reduced metabolism by xenobiotic-metabolizing enzymes, such as cytochrome P450 (CYP). CYPs of the 1A, 2B and 3A family are most likely involved in the metabolism of various PCB congeners [32, 40, 41]. Obesity has been associated with decreased activity in CYP 3A4 in several studies [29].

Studies assessing the influence of weight change on organochlorine change have been few, particularly for long term trends [7, 11, 42-44]. Increased fat as a result of weight gain may lead to the dilution of organochlorine concentrations, thereby resulting in a negative association between body fat and serum organochlorine levels [45]. Our finding of a significant effect of fat mass change for PCBs agrees with other studies (with 5 or more years of follow-up) that use relative change in body mass index [11] or absolute weight change [7] as the predictors of interest.

Mother's date of birth, and consequently age, was not a significant predictor of change for either DDE or PCBs, which mirrors the lack of association found in other studies between age at study entry and change in DDE [11] or PCB levels [9]. As postulated by several theoretical models, the exposure patterns over time are a result of complex interactions between cohort-related exposures and breast-feeding patterns as well as age-dependent growth and weight/fat content, dietary intake and composition, and metabolism [45, 46]. However, given the small variation in birth year and age in our study population

and the fact that everyone was born prior to the ban, we would need a much larger sample size to more fully assess the effect of birth cohort and age.

Although the factors considered in our study help to explain a large portion of the variation in DDE and PCB levels over time, other potentially important covariates such as changes in diet, particularly fish consumption, were not measured. A longitudinal study [34] conducted from 1980-1995 and a serial cross-sectional study from 1973-1993 [47] of Great Lakes fisheaters and "non-eaters" showed fish consumption in the previous year to be predictive of PCB body burden. Likewise, whale blubber consumption in the Faroes Islands have been associated with highly chlorinated PCBs [48].

A limitation of this study is that baseline and follow-up samples were analyzed by different labs at different times using different assays. The most important difference, however, is the change in PCB quantitation methods. To obtain comparable PCB values from the old and new techniques, we used conversion factors derived from a set of 10 breast milk samples spanning the range of organochlorine values. Though derived from a small sample, our conversion factor seemed reasonable, given the similarity in values and high Spearman and Pearson correlations (0.83 and 0.96, respectively) between the original and converted values.

We analyzed both estimated wet-weight and lipid-adjusted measures. These measures involved various assumptions, including those regarding methodology, lipid concentration and non-detects. Similar results for both analyses, nevertheless, provide further assurance of our outcome and conclusions.

"Weeks of lactation" were modeled as three periods of lactation duration, and additional cutpoints (at 13 weeks and 52 weeks) did not significantly alter the results.

However, "weeks of lactation" alone does not fully capture the extent to which organochlorines are being transferred, as there is no data on the amount of milk expressed per breast-feeding session or the duration of weaning for all relevant pregnancies. Additionally, for women who fed multiple children, we assumed the effect of lactation would not vary across pregnancies; however, it is not possible to evaluate this assumption given the small sample size and the lack of organochlorine measurement at each pregnancy.

While our findings agree with those of previous studies, there are limits to the generalizability. Our study population consists of women who were pregnant at baseline, highly educated and mostly Caucasian (95%). These participants may be different from those not studied in terms of their exposure potential, dietary pattern, BMI, and metabolism. More importantly, our results may not be generalizable to other regions of the world with vastly different exposures, and the findings can be extrapolated beyond the time frame of our study (1978-1982 to 2003-2004) only with caution. Although our assumption of an exponential decay is plausible, the shape of the concentration curve between the two measurements cannot be determined given only two measures. Should the shape of the concentration curve be better captured by a different form of decay model, then the predicted organochlorine values during the period under study can be under- or over-estimated, depending on the duration of follow-up. Multiple measures would be required to more fully evaluate the shape. Nevertheless, our data may be useful to those conducting studies on the role of these organochlorines in the development of chronic diseases and have only one DDE/PCB measure, at perhaps, an etiologically irrelevant time period.

We have focused on predicting future levels based on past measurements. Others have attempted to predict past exposure from current concentrations [49, 50]. While our

model coefficients cannot be directly used for backward estimations (since the coefficient for the predictor, initial (baseline) concentration would be rendered meaningless), our study is useful in identifying a handful of important predictors to consider in such estimation models. A single measure of DDE is highly predictive of a woman's relative exposure over a time span of approximately 25 years, and can be further improved with data on lactation and baseline BMI. A single measure of PCBs is also predictive of future levels. Though initial concentration and percent body fat change contribute to the prediction of only PCB value, lactation duration and baseline BMI are the predominant contributors to both DDE and PCBs and information on both of these can be obtained retrospectively.

To our knowledge, this study which addresses intra-individual variations and influences on body burden of DDE and PCBs over time in a general population with relatively low level environmental exposure, has the longest duration between serial organochlorine measures. Longer term trends and predictions are of interest for public health assessment as well as studies of diseases with long latency periods, where the levels of etiologic relevance may be decades apart from the time of measurement. Future studies to replicate or confirm our findings (particular in other epochs and populations) are warranted and would benefit from multiple organochlorine measures and additional information on other potential predictors.

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TABLE 4.1 Characteristics of study population, n=123[a]

Variable	N	(%)
Age at baseline (years)		
19 – 24	24	(19)
25 – 29	54	(44)
30 – 34	39	(32)
35 – 37	6	(5)
No. of births (baseline to follow-up)		
1	48	(39)
2	56	(46)
3	17	(14)
6	1	(1)
No. of babies breast-fed (baseline to follow-up)[b]		
0	12	(10)
1	45	(37)
2	48	(39)
3	16	(13)
6	1	(1)
No. weeks of lactation for each birth (baseline to follow-up)[b]		
0 – 13	49	(24)
14 – 26	50	(24)
27 – 39	31	(15)
40 – 52	54	(26)
53 – 165	23	(11)
Pre-pregnancy BMI (kg/m^2)		
16.6 – 19.9	47	(38)
20 – 24.9	65	(53)
25 – 29.9	9	(7)
30 – 32.9	2	(2)
% change in body fat		
-6 – 0	2	(2)
1 – 24	13	(11)
25 – 49	27	(22)
50 – 99	49	(40)
100 – 149	18	(14)
150 – 294	14	(11)

[a] One individual had missing pregnancy and lactation data

[b] Among the 207 births delivered to 122 mothers from baseline to follow-up

TABLE 4.2 DDE and PCB[a] concentrations in serum, n=123

Organochlorine		Percentile					
	Min	10	25	50	75	90	Max
Wet weight measures							
DDE (ug/L serum)							
Baseline	1.3	3.8	5.8	8.5	12.5	19.8	41.0
Follow-up	0.01	0.5	0.7	1.2	2.3	3.9	11.0
Relative change[b]	0.002	0.07	0.10	0.16	0.23	0.29	0.62
PCBs (ug/L serum)							
Baseline	0.6	0.9	1.2	1.5	2.1	2.7	5.2
Follow-up	0.2	0.4	0.5	0.7	1.0	1.2	2.2
Relative change[b]	0.09	0.25	0.33	0.45	0.65	0.80	1.43
Lipid-adjusted measures							
DDE (ug/g lipid)							
Baseline	0.3	0.7	1.8	1.7	2.5	3.7	7.4
Follow-up	0.002	0.1	0.1	0.2	0.4	0.5	1.9
Relative change[b]	0.002	0.06	0.08	0.12	0.18	0.22	0.56
PCBs (ug/g lipid)							
Baseline	0.1	0.2	0.2	0.3	0.4	0.5	0.7
Follow-up	0.03	0.1	0.2	0.1	0.1	0.2	0.3
Relative change[b]	0.08	0.21	0.29	0.37	0.51	0.59	0.96

[a] PCB concentration comprises PCB-99, 118, 138, 153, 156, 170, 180, and 187

[b] Relative change = (follow-up concentration / baseline concentration) among all 123 participants

FIGURE 4.1 Correlation of baseline with follow-up DDE (ug/L serum), n=123

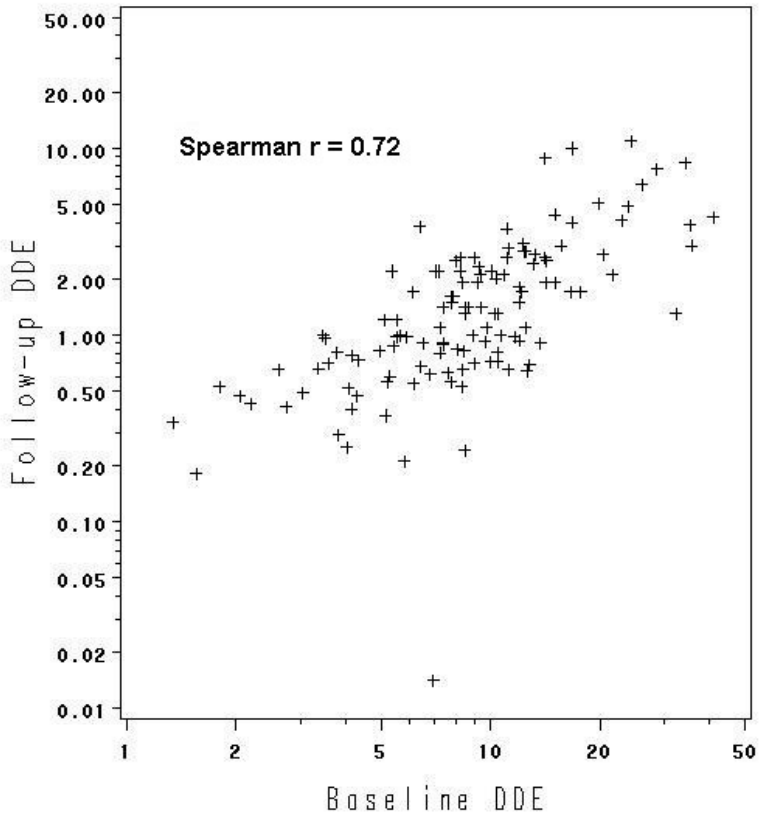


FIGURE 4.2 Correlation of baseline with follow-up PCBs (ug/L serum), n=123

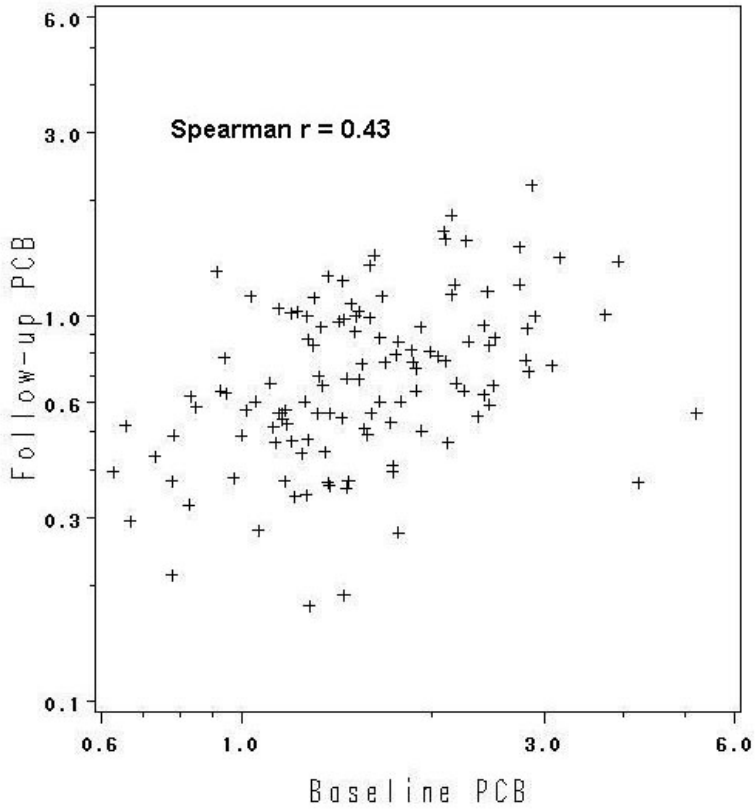


TABLE 4.3 Full model coefficients and standard error for slope of DDE and PCB decline*, n=122

Predictors	Regression coefficient (S.E.)			
	DDE		PCB	
Intercept	-0.061983	(0.0100)	-0.012182	(0.0036)
Log of initial concentration (ug/L)	-0.004270	(0.0036)	-0.016655	(0.0031)
Total weeks of lactation in each interval of lactation				
0-26th	-0.000202	(0.0001)	-0.000201	(0.0001)
27th-39th	-0.000295	(0.0003)	-0.000427	(0.0001)
>39th	0.000052	(0.0001)	-0.000086	(0.0001)
Baseline BMI (kg/m^2)				
16.6-19.9	referent		referent	
20-22.9	0.005467	(0.0053)	0.002533	(0.0029)
≥23	0.031238	(0.0063)	0.011727	(0.0036)
Percent change in body fat	-0.008504	(0.0046)	-0.006323	(0.0025)

TABLE 4.4 Description of successive models for slope, n=122

		Statistical significance (p-value) of successive terms in model for slope		Spearman correlation for observed and predicted follow-up concentration	
Model[a]	Predictors	DDE	PCB	DDE	PCB
I	Intercept	n/a	n/a	0.72	0.43
II	Log of initial concentration (ug/L)	0.75	<0.01	0.73	0.46
III	Weeks of lactation	0.05	<0.01	0.75	0.67
IV	Baseline BMI	<0.01	0.01	0.82	0.72
V	Percent change in body fat	0.07	0.01	0.83	0.75

[a] Each model includes all variables from the preceding model

FIGURE 4.3 Plot of predicted versus observed follow-up DDE (ug/L), n=122

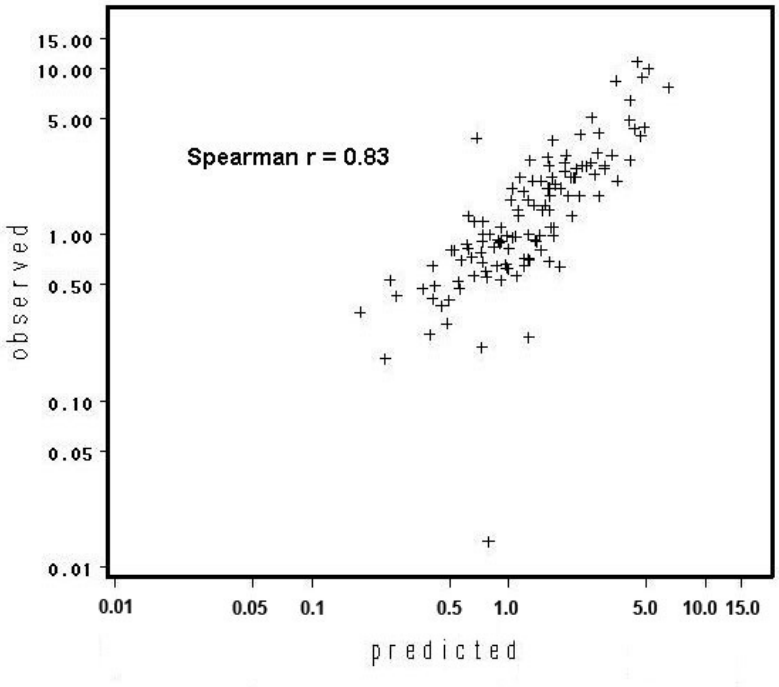


FIGURE 4.4 Plot of predicted versus observed follow-up PCB (ug/L), n=122

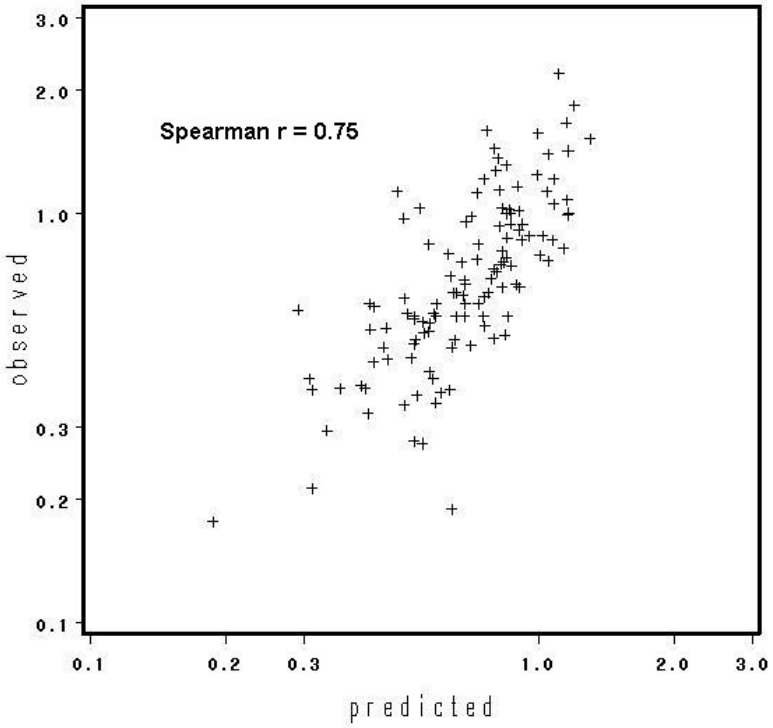
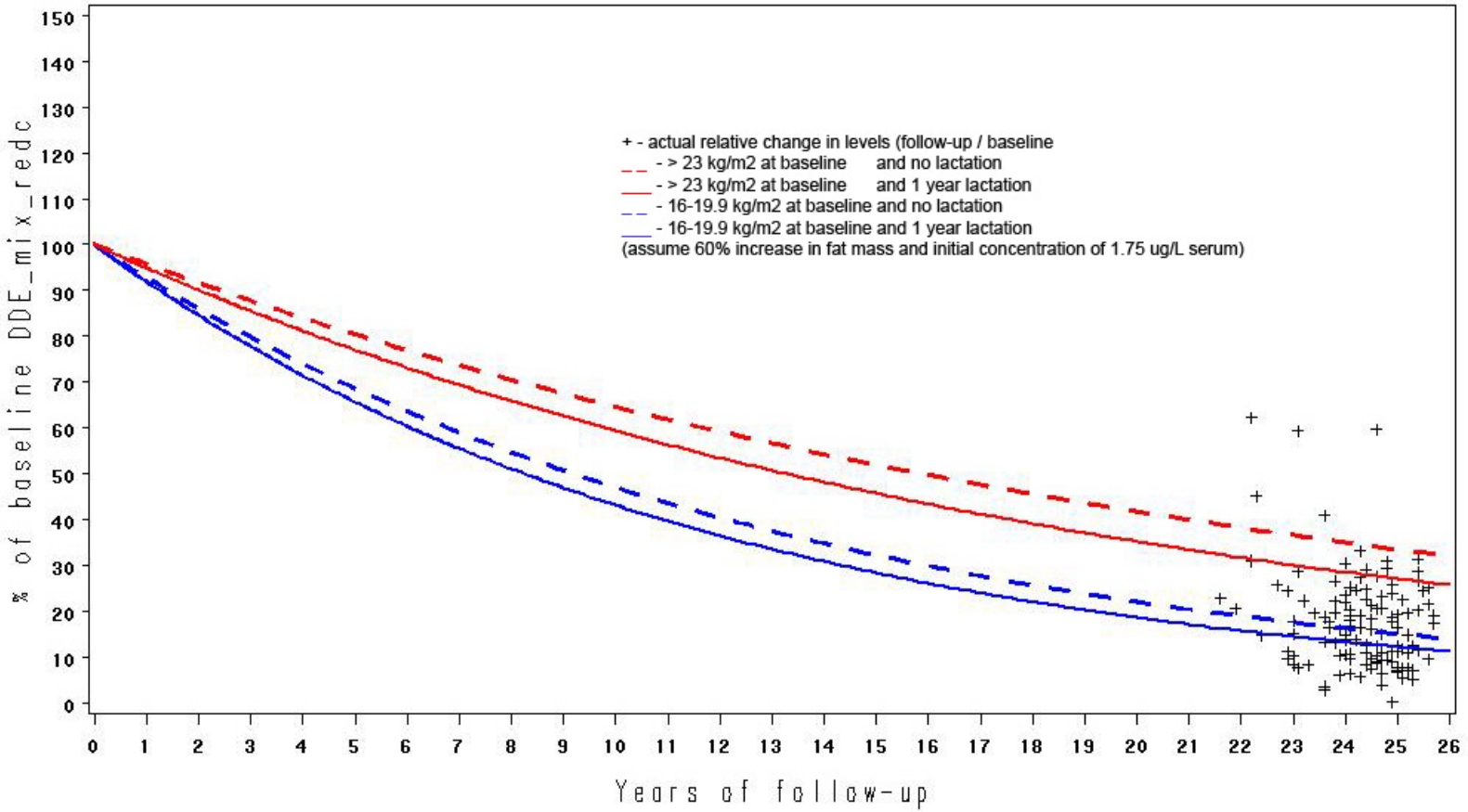
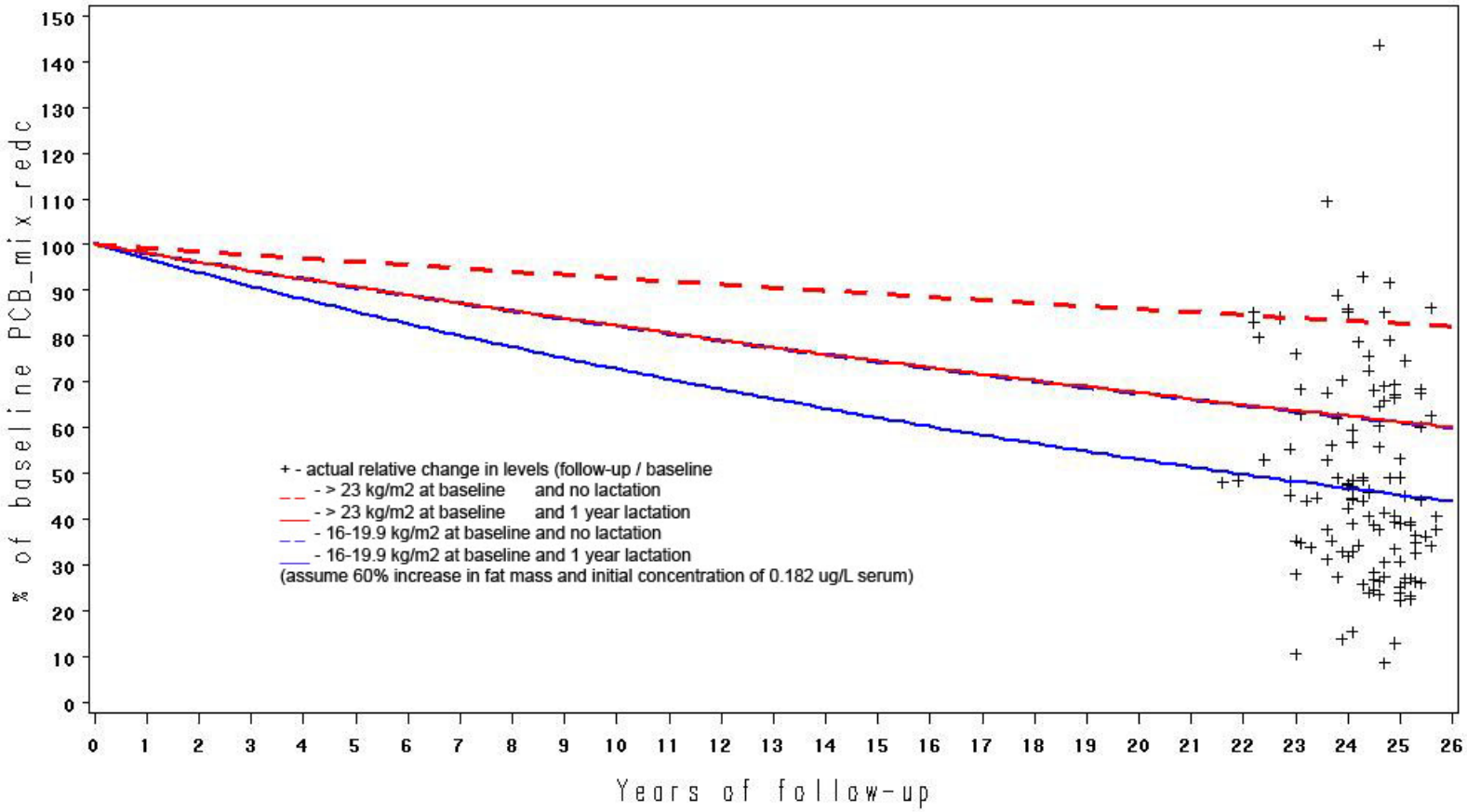


FIGURE 4.5 Predicted DDE curve: follow-up concentration as % of baseline, by years of follow-up, n=122



Crosses depict the actual relative change (follow-up/baseline) in levels. The illustrative lines show the predicted drop in DDE levels over time using specific scenarios: each assumes a 60% increase in fat mass and an initial DDE concentration of 1.75 ug/L serum. Assuming baseline BMI of >=23 kg/m^2, DDE levels at 26 year of follow-up would be 28% of baseline for those with 1 year of lactation (solid red) and 35% of baseline for those with no lactation (dashed red). Assuming those with a baseline BMI of 16-19.9 kg/m^2, DDE levels at 26 year of follow-up would be 12% of baseline for those with 1 year of lactation (solid blue) and 15% of baseline for those with no lactation (dashed blue).

FIGURE 4.6 Predicted PCB curve: follow-up concentration as % of baseline, by years of follow-up, n=122



Crosses depict the actual relative change (follow-up/baseline) in levels. The illustrative lines show the predicted drop in PCB levels over time using specific scenarios: each assumes a 60% increase in fat mass and an initial DDE concentration of 0.182 ug/L serum. Assuming baseline BMI of >=23 kg/m^2 at baseline, PCB levels at 26 year of follow-up would be 62% of baseline for those with 1 year of lactation (solid red) and 83% of baseline for those with no lactation (dashed red). Assuming baseline BMI of 16-19.9 kg/m^2, PCB levels at 26 year of follow-up would be 47% of baseline for those with 1 year of lactation (solid blue) and 62% of baseline for those with no lactation (dashed blue).

5. MANUSCRIPT 2. DDE/PCBS AND TIMING OF MENOPAUSE

5.1 Abstract

BACKGROUND: There are limited and inconsistent data on the effects of DDE and PCBs on the timing of menopause, and previous studies are based on measurements at ages that vary and may not be etiologically relevant.

OBJECTIVE: Our aim is to evaluate the effect on age at menopause of circulating DDE and PCBs, estimated at a standardized age of 40, an age postulated to be sensitive to the effects on ovarian aging.

METHODS: Subjects were drawn from a previous study of organochlorines and child development. Mothers of the children were re-contacted at an average age of 52.4 years. All mothers had organochlorine measurements at ages 26-41 in the original study, and a random sample had current measurements. Based on this, serum DDE and PCB levels (ug/L) at age 40 for all women were estimated using a predictive model. Interview and ovarian hormone data were used to classify menopausal status and define the age at menopause.

RESULTS: There was no trend in the unadjusted or adjusted effect estimates across the five categories of DDE and PCB exposure, and the estimates were imprecise. Compared to the lowest exposure category, menopause occurred -0.02, 0.4, 0.7, -0.3 years later across ascending categories of DDE, and -0.4, 0.4, 0.8, and 0.6 years later across PCB categories.

CONCLUSION: Our data do not suggest an effect of relatively low level exposure of DDE and PCBs on the age of menopause.

5.2 Introduction

Age at menopause is an important reproductive parameter, with early age at menopause being a possible indicator of a damaged ovarian follicular pool or altered endocrine feedback loop [1, 2]. In addition to the direct effect on fertility, early age at menopause may influence the risk of heart disease [3] and osteoporosis [4]. Various factors have been reported to hasten menopause, with smoking most consistently associated with earlier age at menopause (~1.5-2 yrs). The association is usually seen among current rather than former smokers, suggesting that the menopausal transition period may be particularly vulnerable to the effects of ovarian toxicants [5-7]. Removal of an ovary before age 48 [8] and family history of early menopause (before age 46) [9, 10] also have similar or larger effects. Other factors, such as alcohol use, body mass index or weight, and early age at menarche, have also been associated with the timing of menopause, but reported effects have been small or inconsistent [10-12].

More recently, p,p'-DDE, a breakdown product of the pesticide dichlorodiphenyltrichloroethane (DDT), and the industrial mixture of polychlorinated biphenyls (PCBs) have been suggested to influence the timing of menopause. Four epidemiologic studies assessing the relationship between DDE/PCB levels and age at natural menopause have been published, showing inconsistent results [13-16]. Organochlorines were measured at widely divergent ages (range 21-74 years) among participants in these studies. The objective of our study is to examine whether exposure to environmental levels of DDE and PCBs influences the age at onset of menopause. In contrast to previous studies, we estimated organochlorine exposure levels at a standardized age posited to be of etiologic relevance.

5.3 Methods

Study population

Our study population consists of women from North Carolina who were originally enrolled in 1978-82 in a baseline study of the effects of organochlorines on the development of their infants [17]. Participants were administered a questionnaire that elicited demographics, reproductive and medical history; maternal and cord blood samples, as well as placenta and breast milk samples, were collected for DDE and PCB determination.

Of the 877 original participants, 512 (58.4%) were recruited into this 2003-4 follow-up study. Sixteen percent lacked information for tracing, 13.5% could not be found, 9.2% refused to participate, 1.4% did not have a DDE/PCB measure, and 1.7% had other reasons for non-participation. Follow-up data on demographics, medical and reproductive histories, behavioral factors, menstrual cycle function and menopausal status were collected via a telephone interview. All women under age 55 whose menopausal status was unclear (i.e. 41 who had a hysterectomy but still retained $\geq$1 ovary, 39 women currently taking hormone replacement therapy (HRT) without a hysterectomy, and 107 women with symptoms of peri-menopause) plus a 15% random sample of everyone else (regardless of age) were selected for a blood draw for measurement of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E_2), to ascertain or confirm their menopausal status. Informed consent was obtained at both the baseline and follow-up study.

Outcome: Age at natural menopause

Interview and ovarian hormone (FSH, LH and E_2) data were combined to determine a participant's menopausal status and age at natural menopause. At the follow-up interview,

each woman was asked the month and year of her last menstrual period (LMP). Those with no period in the 12 months prior to the interview were asked to identify the reason her periods had stopped; choices included natural menopause, ovarian surgery, chemotherapy or radiation, use of hormone replacement therapy (HRT) or oral contraceptives (OC), pregnancy, breastfeeding or other reason. The age and/or date of all occurrences of these events were also asked.

For premenopausal and some postmenopausal women, the exact age at natural menopause could not be determined, and therefore, their age at natural menopausal was censored (see TABLE 5.1). Among everyone with FSH/LH/E_2 data (n=203), classification of menopausal status was augmented using the following algorithm. Women with FSH < 15 IU/L were considered premenopausal, whereas those with FSH > 40 IU/L, post-menopausal. For women with FSH levels between 15 and 40 IU/L, those with estradiol $\geq$100 pg/ml were considered premenopausal while those with estradiol $\leq$60 pg/ml were considered post-menopausal. For the remaining women (FSH between 15 and 40 and estradiol between 60 and 100 pg/ml), those with a FSH:LH ratio above 1 were deemed postmenopausal and those with a ratio $\leq$1, premenopausal. Cutpoints were selected to take into account the reported distribution and levels of these hormones at different stages of reproductive life [18] as well as mimimize misclassification of menopause [19-21].

Secondary analysis

In a secondary analysis, we evaluated alternative rules for assigning menopause status and age. These alternative rules accounted for the postmenopausal "breakthrough" bleeding caused by HRT use, which can mask the true onset of menopause [22]. If an episode of HRT

began before LMP and lasted at least until one year prior to LMP, the following guidelines were used to define menopausal status: if FSH/LH/e2 data suggest she is pre-menopausal, censor at the blood draw, otherwise, extend the left side of the menopause interval (as defined by the rules in TABLE 5.1) to include the whole HRT episode (if it does not already). For a woman who reported using oral contraceptives for menopausal symptoms, her last episode of oral contraceptive was treated as HRT. Multiple episodes of HRT use separated by a gap of a year or less were treated as one continuous episode.

Exposure: DDE and total PCBs at age 40

We are interested in the effect on timing of menopause of baseline DDE and PCBs at an etiologically relevant time period in women's reproductive life. Analogous to smoking [5], we propose that a relevant time period is just prior to the onset of menopause, so we chose age 40. However, baseline measurements were assessed in women at different ages (18-41 years, mean of 27), so the baseline exposure levels are not equally reflective of the etiologically relevant concentration. We would expect a woman who was near age 40 at baseline to have a measured organochlorine level relatively close in magnitude to the value that would have been measured at age 40, while the measured value in a woman who was substantially younger than age 40 at baseline may have changed considerably by the time she reached age 40. To make comparable these divergently measured exposure levels, we used a predictive model to obtain, for each woman, their estimated organochlorine level at age 40.

Among a random sample of 123 participants with both baseline and follow-up measures, changes in organochlorine concentrations were approximated using an exponential

decay model, whereby the slope represented the change in log concentration from baseline to follow-up per year of follow-up time (see Manuscript 1).

$$\frac{\text{Log [Follow-up]} - \text{Log [Baseline]}}{\text{follow-up time}} = -\text{slope}$$

Slope was allowed to be influenced by various determinants, including initial (baseline) concentration, lactation duration, baseline BMI, and percent change in body fat from baseline to follow-up. Model-predicted levels at follow-up and actual follow-up levels were highly correlated: Spearman correlation coefficient of 0.83 for DDE and 0.75 for PCBs. This model was used to predict organochlorine levels at age 40 for our entire sample. Further, the estimated values at age 40 were highly correlated with those estimated at age 20 (0.92 for DDE and 0.89 for PCBs) and 30 (0.97 for DDE and 0.94 for PCBs).

Estimated organochlorine levels at age 40 were categorized into 5 groups. We used a natural cutpoint close to the upper 10^{th} percentile for the highest category, and approximately evenly divided the remaining concentrations into 4 additional ranges (TABLE 5.2).

Covariates

Factors that have been associated with age at menopause and for which information was available were included in the model. Information on cigarette smoking was used to construct a dichotomous variable for smoking status, with one group containing those who smoked regularly (at least 1 cigarette per day for $\geq$3months) during ages 36-45 and the other containing those who either never smoked or smoked only outside of that age range. Alcohol use from ages 40-49 was included as an ordinal variable with 6 categories: 1=those who never drank, 2= those who rarely drank (<10 drinks per year), and of the remaining

participants who had 10 or more drinks for any given year, 4 additional groups were established based on the number of drinks they had per week (3=less than 1, 4=1 to 2, 5=3 to 6 and 6=7 or more drinks per week). Body mass index at age 40 was estimated via linear interpolation of the BMI from the baseline study to BMI at the follow-up study. Body mass index at age 40 was used as a continuous predictor. Age at menarche was categorized into ages 9-11, 12-13, and 14-17. Menopause before age 46 in a mother and/or sister was included as an indicator of familial/genetic inheritance. First (whole or part of one) ovary removed before age 46 was categorized as yes/no.

Statistical analysis

Age at menopause, the outcome event, was modeled using linear regression. Our data were censored; menopause for an individual occurred either at a particular age, after a certain age (right censoring) or between two ages (interval censoring). In order to account for the censoring, models were fit using the LIFEREG procedure in SAS version 9.1 (Cary, North Carolina). Error distributions were assumed to be logistic, as this provided a better fit.

Separate models for DDE and PCBs, as well as a model that included both organochlorines, were fitted, controlling for all the aforementioned predictors of age at menopause. Sensitivity analysis excluding certain medical conditions, juvenile diabetes and lupus, was also conducted to determine whether these would distort the relationship between organochlorines and age at menopause.

5.5 Results

The mean and median ages at menopause were computed, taking into account censoring, and were 51.6 years and 51.7 years, respectively; quartiles were 49.5 and 54.5. Computation of mean age at menopause without taking into account censoring (that is, estimation of mean age at menopause only among women who reported natural menopause) would lead to a downward bias in the reported age, since women who would have had later menopause (i.e. those who are right censored) would be excluded. To illustrate, among the 188 women who reported natural menopause in our study, the mean age at menopause was 49.2 years.

Table 5.1 shows that by the time of the follow-up interview, 286 (55.9%) participants could be classified as post-menopausal; of these, 251 could be assigned an exact age at menopause, while the remaining 35 could not. Of the 512 women, 51 women had an ovary-conserving hysterectomy prior to one year past LMP; of these, 25 could be classified as post-menopausal based on ovarian hormones, 11 were pre-menopausal, and 15 had uncertain status. Though not shown, 103 (20.1%) participants had taken hormone replacement therapy or oral contraceptives for menopausal purposes.

The mean age of our study population was 52.4 years (TABLE 5.2). Median baseline concentrations were 8.5 ug/L for DDE (range 0.8-1.26; skewness=5.5) and 1.5 ug/L (range 0.2-11.5; skewness=3.3) for PCBs. The estimated median levels at age 40 were 3.6 ug/L for DDE (range 0.3-67.3; skewness=6.1) and 1.1 ug/L for PCBs (range 0.1-5.9; skewness=1.9). Approximately a third of participants had smoked, with 13% smoking during ages 36-45. Most women (60%) drank alcohol at least once a week and 23% drank more than 3 drinks per week. The mean estimated BMI at age 40 was 24.2 kg/m^2 (range = 17 to 47 kg/m^2) and

the median age at menarche was 12. About 18% had a family history of early menopause whereas only 3% had a single ovary removed by age 40.

Overall, survival curves for the various categories of DDE and PCBs largely appear to overlap, although there was some suggestion of an increase in the age at menopause for the upper 2 categories of PCBs (FIGURES 5.1 and 5.2). After controlling for the other covariates, no discernible effect of either DDE or PCBs was seen (TABLE 5.3). There was no pattern to the estimates and the confidence intervals all included zero. Similar results were found if the alternative definitions of menopause, accounting for HRT, were used (data not shown). Removing the 10 women with lupus and/or juvenile diabetes did not change the conclusions (not shown).

As shown in Table 5.3, smoking between the ages of 36-45 accelerated the timing of menopause by 8 months. Furthermore, although the data is not shown, the effect of smoking on age at menopause was dose dependent (11 months earlier menopause for those smoking a cigarette pack or more a day, on average, compared to 7 months earlier for those smoking less than a pack per day), and smoking only outside of ages 36-45 had little effect (0.8 months later menopause). As also shown in Table 5.3, age at menopause was influenced by family history of early menopause (1.8 years earlier) and ovary removal before age 40 (1.5 yrs earlier).

5.6 Discussion

Our data do not provide evidence for the influence of DDE and PCBs on timing of menopause. Akkina et al. suggested an effect for DDE; Hispanic women (ages 42-74) with the highest quintile of exposure (>23.6 ppb) had an adjusted mean age at natural menopause

of 1.7 years earlier (p=0.13) compared to those in the lowest quintile (<5.5 ppb) [14]. However, there was no pattern to the relationship across the remaining quintiles of exposure. In contrast, the Cooper et al. study found a dose-dependent effect of DDE among 1407 breast cancer cases and controls (median age of 50, range 21-74), with the upper 10^{th} percentile of DDE exposure ($\geq$2.77ug/gm lipid) having an approximately 1 year earlier median adjusted age at menopause compared to those in the bottom 50^{th} percentile 9<0.9 ug/gm) [13]. The magnitudes of exposure studied by Cooper et al. tended to be substantially higher than those of women in our study. Using the cutpoints for categorizing (lipid-adjusted) serum DDE in the Cooper et al. study, few people in this study were classified in the upper exposure categories (TABLE 5.4). Therefore, this study may have little power to evaluate whether exposures of the magnitude studied by Cooper et al. in their highest exposure groups were associated with early menopause.

Our lack of a PCB effect was similar to that in the Cooper et al. study [13] as well as two other studies [15, 16]. One study [16] was conducted among 356 PCB-poisoned Taiwanese women, ages 30-59, and their 312 age-matched neighborhood controls, ages 30-59, and did not adjust for any covariates or potential confounders. The other study [15] of 874 women (average age 36.2 years, range 24-60) had a preponderance (55%) of women with PCB levels at or below the limit of detection (5 ppb).

Some women with uncertain menopausal status (due to HRT use or surgical menopause) were excluded from analysis in two studies [13, 14]; this may bias the results if those excluded were systematically different from participants with respect to their age at menopause and exposure levels. Our methods allowed all recruited women to be included in our analysis, thereby avoiding any such biases and maintaining the size of our study. The use

of data on endogenous hormone levels as a supplement to interview data further improved our assessment of current menopausal status.

DDE and PCBs could influence the timing of menopause via a multitude of plausible mechanisms. Repeated exposure to p,p'-DDE has been shown to decrease estrogen production in porcine granulosa cells [23]. Whether this might influence age at menopause is unknown. p,p-DDE also has anti-androgenic properties [24]. The reported effects of androgens varies, and according to recent reports, may depend on the stage of development of the follicle [25-27]. While androgens have been reported to enhance follicular atresia in immature rats [28, 29] and reduce the number of large follicles [30], recent data in animals, including rhesus monkeys, show that androgens, such as testosterone, stimulate the transition from primary to secondary follicles and growth of preantral and small antral follicles [27, 31, 32] in a dose-dependent fashion [25, 26], and that the stimulatory effect is abrogated by flutamide, an anti-androgen [26]. Menopause is linked to the exhaustion of these early primordial follicles [33], so if androgens increase the rate of their development, early menopause might ensue. The anti-androgenic effects of p,p'-DDE would therefore be hypothesized to delay menopause.

PCB congeners, on the other hand, have estrogenic (i.e. PCB-52, 99, 153) and/or anti-estrogenic properties (i.e. PCB-105, 118) [34] and may lead to premature menopause via the binding and activation of aryl hydrocarbon receptor (AhR)--the same mechanism through which smoke-related toxicants [35] and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may exert their effects (TCDD) [36]. AhR binding induces the estrogen-metabolizing enzyme, cytochrome p4501A1 [37]. Some PCBs (i.e. PCB-118, 138, 156, and 170) have AhR binding capabilities and hence dioxin-like toxicity [38, 39]. Chronic low dose exposure to

TCDD has been reported to hasten reproductive senescence in female rats via induction of a dose-dependent loss of normal cyclicity and estradiol levels, despite a lack of depletion to follicular reserve [36, 40]. Evidence in humans is not clear; one epidemiologic study of TCDD showed an inverted U-shape dose response relationship between TCDD and risk of early menopause among women accidentally exposed to high levels of TCDD [41].

An advantage of the current study is that we were able to standardize DDE and PCB levels to a common age posited to have heightened sensitivity to ovarian toxicants. We estimated organochlorine values at age 40, around the time of menopausal transition. Theoretical models based on biologic data suggest that the rate of atresia in the resting pool of primordial follicles during reproductive life, rather the number of oocytes one is born with, has a greater influence on hastening the age at menopause [42]. It is possible that exposures to DDE and PCBs could increase the rate of atresia during menopausal transition [2, 43]. This hypothesis is supported by the literature on smoking and age at menopause, which shows a greater impact of smoking near the time of menopause than of smoking earlier in life [5-7]. However, as long as the same age is used for all participants, choosing a different age for exposure assessment is not likely to change the results, as the estimated values at age 40 were highly correlated with those estimated at age 20 and 30.

We did not evaluate PCB congeners grouped by their various biologic and estrogenic properties [44] nor did we assess the effect of other DDT metabolites due to their relatively low abundance, and this may have contributed to our not finding an effect. However, we were able to detect effects for smoking, family history of early menopause, and ovary removal that are consistent with that seen in previous studies [7-9, 11, 45, 46]. Similar

findings using the two definitions for menopause, with and without consideration of HRT use, further reassure us of our results.

This study has some limitations. Given our limited ability to find and enroll the participants from the baseline study (58% enrolled), selection bias is possible. Participants were slightly older (mean age of 28.1 years versus 26.6 years), with a slightly higher proportion having over 17 years of education (26.5% versus 15.6%) and of White race (96.7% versus 89.5%). However, it is reassuring that the median baseline organochlorine values for participants and non-participants were similar for both DDE (8.5 and 8.6 ug/L serum, respectively) and PCBs (1.5 ug/L serum each). Self-reported age at menopause can be poorly recalled, and validity of recall can decrease with increasing number of years since LMP [47]. In our study, poor recall is unlikely given the relatively short interval between LMP and the follow-up interview or blood draw, among 251 women with natural menopause (median 2.7 years, range 0-25 years; 90% have <9 years interval).

Our hormone measures were not timed to the menstrual cycle; however, use of an algorithm that encompasses all three ovarian hormones (FSH, LH and E_2) would increase the discriminating power for determining menopausal status, as compared to FSH alone. However, for those taking HRT, it is still possible that menopausal status could be misclassified using this algorithm, given that introduction of exogenous estrogen can artificially decrease the FSH levels, thereby, causing some postmenopausal women to be classified something other than postmenopausal. Among women with a bilateral oophorectomy participating in the National Health and Nutrition Examination Study (NHANES), the same proportion (~85%) of women taking HRT and women not taking HRT had elevated FSH levels defined as more than 20 IU/L [46]. In contrast, 52% of those taking

HRT and 67% of those not taking HRT had FSH levels above 40. In the North Carolina Menopause Study, 33 of 39 women who were currently taking HRT had hormone measures. Of these, 25 had FSH>40 IU/L and were assumed to be correctly defined as postmenopausal, whereas 4 individuals had FSH<15 IU/L and were assumed to be correctly defined as premenopausal, given the findings in NHANES. Only the remaining 4 individuals (12% of those taking HRT) had 15<FSH<40, and thus, may have been subject to misclassification of menopausal status. Exclusion of these 4 women did not alter the study conclusions.

Our hormone algorithm showed high agreement between the interview and hormone data, particularly in the classification of those who were clearly postmenopausal. Fifty-three of the 54 women with $\geq$1 year of amenorrhea and hormone data were classified as postmenopausal by hormone, while 9 out of 10 women with a bilateral oophorectomy and hormone data were classified as postmenopausal by hormone. For those with less than a year of amenorrhea, hormonally-defined menopausal status may be less clear, particularly among those undergoing transition and having symptoms of perimenopause (e.g. irregular cycles, night sweat, hot flash, vaginal dryness). Ignoring the hormone data, and instead, censoring at the last menstrual period for all women with less than one year of amenorrhea did not change the conclusions of this study (not shown).

Aside from tobacco smoke, research on the role of environmental exposures on the timing of menopause has been limited. Our study is the first to evaluate DDE and PCB exposure levels at a standardized and likely etiologically relevant time period of reproductive life. While our data does not provide evidence for an effect of relatively low level exposures of DDE and PCBs, it does not negate a possible effect at higher doses. Further studies with

exposures at a more etiologically relevant time period and among higher exposed populations are warranted.

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TABLE 5.1 Menopausal status and censoring for survival analysis 1, n=512

Categories	Age at menopause	N (%)
Currently pregnant	Censor at interview	1 (0.1)
Missing date of LMP*	Censor at return of menses following last pregnancy	1 (0.1)
Radiation or chemotherapy began within 12 months prior to or following the LMP and before any hysterectomy AND		
- post-menopausal by FSH/LH/e2[#]	Censor at LMP	1 (0.1)
- pre-menopausal by FSH/LH/e2	n/a	0 (0.0)
- no FSH/LH/e2	Censor at LMP	5 (1.0)
Hysterectomy before LMP + 12 months with any part of an ovary maintained AND		
- post-menopausal by FSH/LH/e2	Interval censor between LMP and blood draw	25 (4.9)
- pre-menopausal by FSH/LH/e2	Censor at blood draw	11 (2.1)
- no FSH/LH/e2	Censor at LMP	15 (2.9)
Both ovaries removed before LMP + 12 months AND		
- post-menopausal by FSH/LH/e2	Censor at LMP	9 (1.8)
- pre-menopausal by FSH/LH/e2	Censor at blood draw	1 (0.1)^
- no FSH/LH/e2	Censor at LMP	40 (7.8)
LMP <12 months prior to interview AND		
- post-menopausal by FSH/LH/e2	Natural menopause at LMP	66 (12.9)
- pre-menopausal by FSH/LH/e2	Censor at blood draw	36 (7.0)
- no FSH/LH/e2	Censor at LMP	115 (22.5)
LMP ≥12 months prior to interview AND		
- post-menopausal by FSH/LH/e2	Natural menopause at LMP	53 (10.4)
- pre-menopausal by FSH/LH/e2	Censor at blood draw	1 (0.1)
- no FSH/LH/e2	Natural menopause at LMP	132 (25.8)

*LMP – last menstrual period

[#] FSH/LH/e2 refers to follicle stimulating hormone, luteinizing hormone and estradiol

^ Interview data suggests she is post-menopausal, but she was categorized as pre-menopausal based on her hormone data (FSH=22, LH=11.14 and E_2=162.45).

Table 5.2 Characteristics of Menopause Study population, n=512

	n	(%)
Age at follow-up study		
43.0 – 44.9	15	(2.9)
45.0 – 49.9	125	(24.4)
50.0 – 54.9	249	(48.6)
55.0 – 59.9	110	(21.5)
60.0 – 66.3	13	(2.5)
Estimated DDE at age 40 (ug/mL serum)		
0.3 – 1.9	133	(26.0)
2.0 – 4.2	169	(33.0)
4.3 – 6.5	112	(21.9)
6.6 – 8.9	47	(9.2)
9.0 – 67.3	51	(9.9)
Estimated PCB at age 40 (ug/mL serum)		
0.12 – 0.79	128	(25.0)
0.80 – 1.13	162	(31.6)
1.14 – 1.47	114	(22.3)
1.48 – 1.79	53	(10.4)
1.80 – 5.91	55	(10.7)
Smoking		
Never smoke	332	(64.8)
Smoke only outside ages 36-45	115	(22.4)
Smoke during ages 36-45	65	(12.8)
Drinking during her 40s		
Never drank	96	(18.7)
Drank <10 times per year	115	(22.5)
Drank >10 per year but < 1 drink per week	105	(20.5)
Drank 1-2 drinks per week	78	(15.2)
Drank 3-6 drinks per week	87	(17.0)
Drank 7 or more drinks per week	31	(6.1)
Estimated BMI at age 40 (kg/m^2)		
17.2 – 19.9	68	(13.3)
20.0 – 24.9	266	(51.9)
25.0 – 29.9	122	(23.8)
30.0 – 47.3	55	(10.9)
Age at menarche		
9-11	114	(22.3)
12-13	293	(57.2)
14-17	104	(20.3)
Missing	1	(0.2)
Family history of menopause before age 46 (in mother and/or sister)		
No	420	(82.0)
Yes	92	(18.0)
First whole ovary removed by age 40		
No	497	(97.1)
Yes	15	(2.9)

FIGURE 5.1 Survival curves for the effect of DDE (ug/L serum) at age 40, n=512

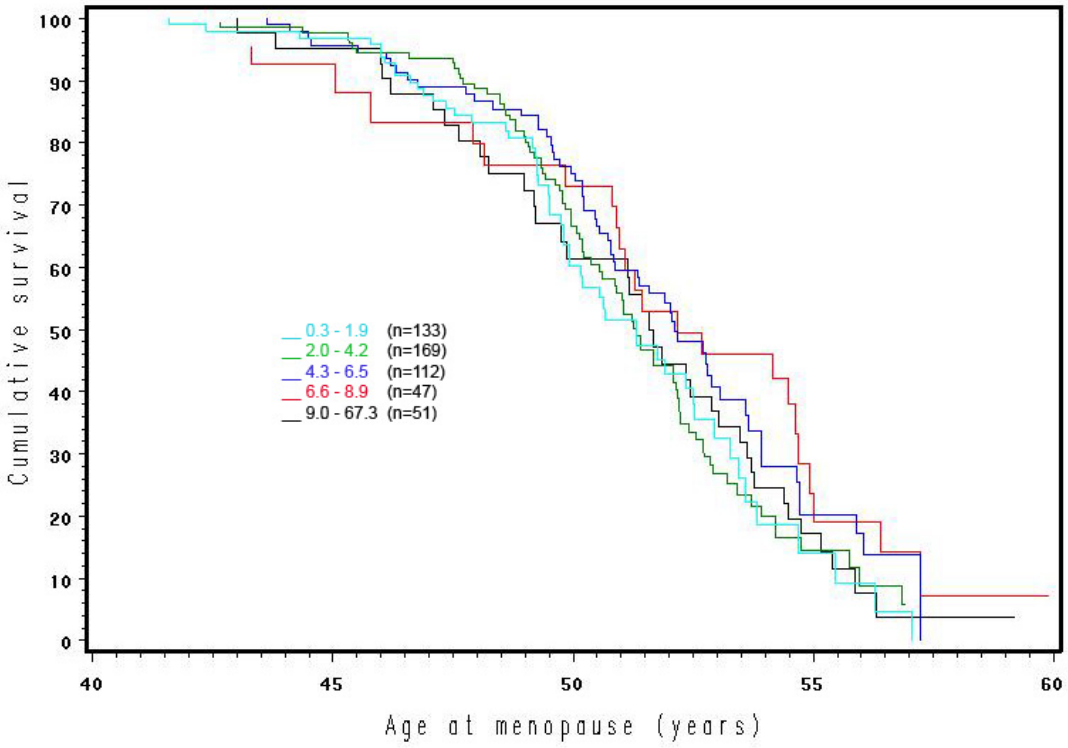

FIGURE 5.2: Survival curves for the effect of PCBs (ug/L serum) at age 40, n=512

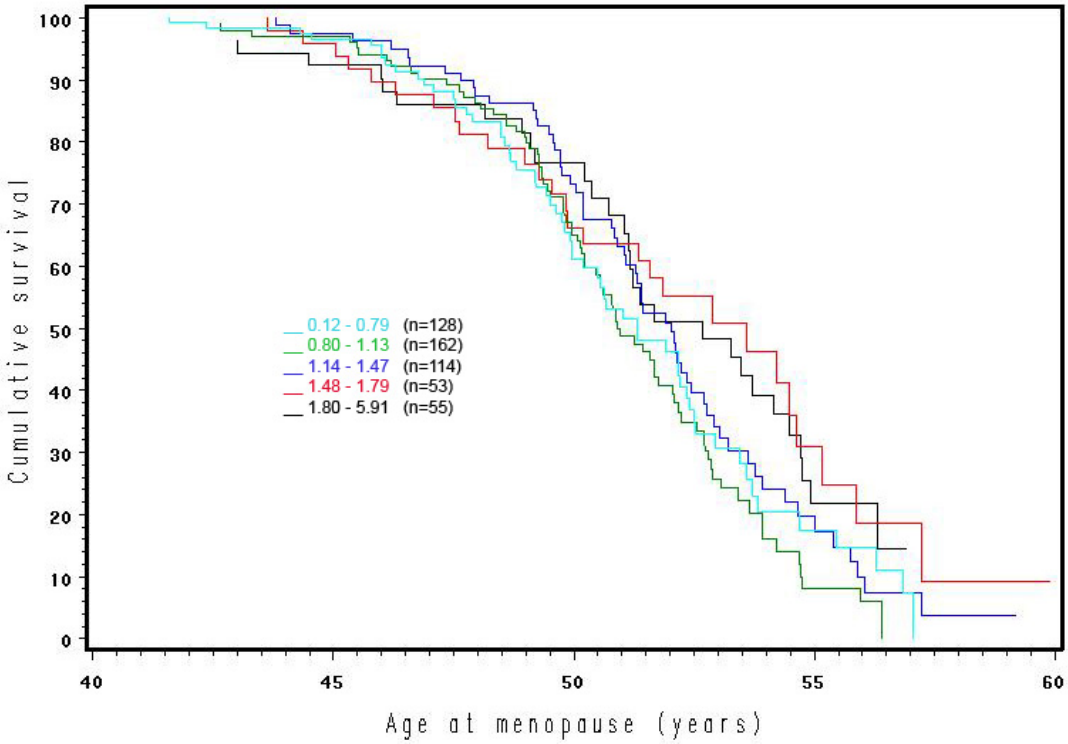

TABLE 5.3 Regression coefficients for effect of DDE and PCBs at age 40, controlling for covariates, n=511

Variable	β (95% CI)
DDE at age 40 (ug/L serum)	
0.3 – 1.9	referent
2.0 - 4.2	-0.023 (-1.101, 1.055)
4.3 – 6.5	0.435 (-0.843, 1.712)
6.6 – 8.9	0.675 (-0.982, 2.332)
9.0 – 71.7	-0.257 (-1.846, 1.332)
PCB at age 40 (ug/L serum)	
0.12 – 0.79	referent
0.80 – 1.13	-0.433 (-1.529, 0.662)
1.14 – 1.47	0.379 (-0.873, 1.631)
1.48 – 1.79	0.786 (-0.869, 2.441)
1.80 – 5.62	0.588 (-0.977, 2.153)
Smoking (between ages 36-45)	
No	referent
Yes	-0.677 (-1.858, 0.504)
Alcohol use	0.074 (-0.177, 0.326)
BMI at age 40	-0.005 (-0.097, 0.087)
Age at menarche	
9-11	Referent
12-13	0.059 (-0.834, 0.951)
>13	1.125 (-0.038, 2.288)
Family history (mom and/or sister)	
No	referent
Yes	-1.852 (-2.806, -0.898)
First ovary removed (by age 40)	
No	referent
Yes	-1.537 (-3.733, 0.659)

TABLE 5.4 Comparison of DDE & PCB levels in the Menopause Study and the Cooper et al. study

	Cooper et al. Study [13]*		Menopause Study^	
DDE (ug/g lipid)	**N**	**Adjusted HR (95% CI)**	**N**	**Adjusted β (95% CI)**
<0.62	699	1.0	282	Referent
0.62-1.36	311	1.2 (0.9, 1.6)	176	0.917 (0.129, 1.705)
1.37-2.76	200	1.3 (0.9, 1.8)	46	0.721 (-0.566, 2.008)
>2.76	138	1.4 (0.9, 2.1)	8	-0.653 (-3.563, 2.258)
PCB (ug/g lipid)	**N**	**Adjusted HR (95% CI)**	**N**	**Adjusted β (95% CI)**
<0.36	675	1.0	496	Referent
0.36-0.52	332	1.0 (0.7, 1.4)	14	1.363 (-1.309, 4.035)
0.53-0.73	190	1.1 (0.8, 1.5)	1	42.443 (-157615, 157699)
>0.73	151	0.9 (0.6, 1.3)	1	-5.589 (-19.842, 8.666)

* HR is hazard ratio

^ DDE levels at age 40 were estimated with adjustment for 6.39 g lipid (as estimated for Caucasian women, ages 36-44 in NHANES study); β is the difference in age at menopause (yr); model adjusted for smoking (never and smoke outside ages 36-45 versus smoking during ages 36-45), ovary removal by age 40, family history before age 46, age at menarche, BMI at age 40, and alcohol use in her 40s

6. DISCUSSION

6.1 Summary of findings

This research was partly motivated by the inconsistent results found in previous literature pertaining to DDE/PCBs and age at menopause, and the need to address the limitation in exposure assessment in these studies. DDE and PCBs, in previous studies, were measured in women with widely divergent ages and oftentimes measured after the onset of menopause, a time that is not of etiologic relevance. In addressing this limitation, the goals of this research were to evaluate the long-term dynamics of intra-individual levels of DDE and PCBs and, based on this information, assess the impact on timing of menopause from these organochlorines, estimated at an age posited to be of etiologic significance.

This study shows a substantial decline in organochlorine levels from 1978-1982 to 2003-2004, this decline was more dramatic for DDE than PCBs. The overall and differential declines between these organochlorines were also seen in other studies and may reflect, in part, the environmental impact of an earlier ban on DDE as compared to PCBs.

A single organochlorine measure at a follow-up period of approximately 25 years is highly correlated with and predictive of baseline values, particularly for DDE, which is consistent with other studies having a maximum of 10 years of follow-up [1-3]. Hence, a single measure of DDE, as compared to PCBs, would be a more meaningful indicator of exposure at distant times. Though it is unclear why the correlation is higher for DDE than

PCBs, the fact p,p'-DDE is a single chemical whereas PCBs are a mix of congeners with variable rates of decomposition may partly explain this finding.

The results of this study show that prediction of organochlorines can be improved with the addition of a few easily collected variables. Baseline BMI and lactation improves the prediction of DDE slightly, whereas these factors along with initial concentration and change in fat mass substantially improved the prediction of PCBs. The discrepancy in the explanatory capacity of these variables across organochlorine compounds may reside in the fact that temporal measures of DDE are already highly correlated with one another and little variability remains to be explained by these additional factors. Furthermore, the results demonstrate the feasibility of using these predictive models for exposure estimation.

This study shows that the cumulative effect of lactation is still evident decades later and is the first to attempt to differentiate the impact of different intervals of lactation duration. The results indicate the earlier weeks (<39 weeks) of lactation is more important than later lactation and highlights the need to consider the likely diminishing role of breastfeeding as lactation proceeds.

Higher body mass index is associated with a slower rate of DDE and PCB elimination, which is similar to the findings of some previous studies [3-5]. In contrast, an increase in fat mass was associated with a faster decline in concentrations. These results suggest that different aspects of weight function differently in the elimination of serum organochlorines, and therefore, should be distinguished. Conceptually, body mass index may be a marker of metabolism, whereas fat mass change may be a reflection of passive dilution of organochlorines.

Initial (baseline) concentration was an important predictor of PCB decline, similar to the findings from studies of occupational cohorts [6, 7], and may likely be a function of greater enzyme induction at higher doses [8]. In contrast, mother's date of birth, which was highly correlated with maternal age, was not an important predictor for either organochlorines. However, given the small variations in birth year and age, the impact of birth cohort and maternal age cannot be more fully assessed.

There was no evidence for an effect of DDE and PCBs on age at menopause, in both unadjusted and adjusted models. The lack of a DDE effect was inconsistent with a prior study by Cooper et al., which reported an earlier age at menopause with DDE exposure [9]. The Cooper et al. study had a wide age spread (ages 21-74) with a sizeable proportion of women who had organochlorines measured after the onset of menopause. While it is possible that there is no biological effect of DDE, it is unclear whether the exposure levels in this study may be too low to see an effect. The magnitudes of exposure studied by Cooper et al. in the highest exposure categories tended to be substantially higher than those of women in our study. Therefore, this study may be underpowered to assess whether these higher magnitudes of exposure were associated with altered age at menopause. Since age at menopause is a risk factor for various estrogen-related health outcomes (breast and endometrial cancers), an impact on age at menopause from DDE and PCB exposure, would suggest a biological effect of these organochlorines on the health outcomes.

One assumption of these analyses was that estimation of organochlorine levels at age 40 would provide a more biologically-relevant exposure metric than simply using baseline measures. We decided to standardize exposures to values expected to be observed at 40 years of age, around the time of menopausal transition and an age that is likely to be sensitive

to the effects of ovarian toxicants. Choosing a different age for exposure estimation would be unlikely to alter the results of this study as the estimated values at age 40 were highly correlated with those at age 20 and 30.

On the other hand, smoking between ages 36-45, family history of early menopause, and unilateral oophorectomy had large effects and hastened menopause by 8 to 20 months. Though these results are somewhat imprecise, they are consistent with previous literature.

6.2 Strengths and limitations of Study Aims 1 and 2

A major strength of the research on intra-individual changes and predictors of change in DDE and PCBs is the very long period of follow-up (approximately 25 years) between organochlorine measurements. Previous prospective studies in populations also exposed to generally low environmental levels have had less than 10 years in duration between measurements.

Previous studies have not simultaneously assessed the effect of different facets of weight measures (i.e. baseline BMI versus percent change in fat mass). The results of this study support the notion that these measures may represent different processes that alter serum organochlorine concentrations. It should be noted that despite the narrow range of BMI at baseline, the effect of a single measure of body mass index was impressive. Whether this effect would be stronger at higher BMI remains unclear.

In contrast to previous studies, lactation was treated as three continuous variables, with each representing a different interval of lactation duration ($<26^{th}$, 27^{th}-39^{th}, and $>39^{th}$ week of lactation). A single continuous measure defined as "weeks of lactation," does not

fully capture the extent to which organochlorines are being transferred, and the results of this study indicate that different intervals of lactation reflect different elimination potential.

In this study, baseline serum organochlorine measures were not directly used, but were transformed to account for the change in PCB quantitation technology, the lipid rise during pregnancy as well as non-detects. However, validation of the conversion factors used at each stage of the transformation revealed very high correlations and similarity in values between estimated and observed measures.

While the predictive models for DDE and PCBs performed well, as indicated by the high correlations between predicted and observed follow-up values, a limitation of having only two organochlorine measures and little variability in the number of years of follow-up (21-26 years) is the inability to assess the exact shape of the predicted curve. As with other similar compounds (dioxins and polybrominated biphenyls) [10, 11], DDE and PCBs are assumed to have an exponential decay. Nevertheless, if the shape of the concentration curve is better captured by a different form of decay model, then the predicted organochlorine values during the period under study can be under- or over-estimated, depending on the duration of follow-up. Multiple serial organochlorine measures would be necessary to evaluate the shape of the curve between the two study periods.

There are limits to the generalizability of the study results. The study population consisted mostly of highly educated, Caucasian women residing in North Carolina, who may have vastly different exposure potentials, dietary habits, BMI, and rates of metabolism (for example, because of genetic differences) than those not studied. More importantly, as measurements were taken after the bans on DDE and PCBs, extrapolation to time periods

prior to this study's time period should be done with extreme caution, as the exposure pattern and potential may be very different in the past, particularly prior to the bans.

6.3 Strengths and limitations of Study Aim 3

A major strength of this research on the role of DDE and PCBs in the timing of menopause is the use of a predictive model to assess organochlorine levels at a common age that is likely to be of etiologic significance. In addition to the more appropriate time window of exposure assessment, an advantage to this approach is that exposure measures taken in women at various ages at baseline can be made more comparable with respect to the exposure measure of interest.

Another strength of this study is the use of ovarian hormone data to supplement the classification of menopausal status, and in combination with censoring, further define the time period of menopausal experience. Given the hormone data, all participants were able to be included, thereby maintaining the size and precision of the study and avoiding the potential selection bias that may have been introduced in previous studies, which excluded women with unclear menopausal status (i.e. those currently taking hormone replacement therapy or those who had a hysterectomy or oophorectomy).

Hormone measures, however, were not timed to the menstrual cycle. Given this scenario, our algorithm that incorporates all three hormones would be a better discriminator of menopausal status than FSH alone [12], as high FSH levels may be indicative of either menopause or a spike during ovulation. Nevertheless, among those taking hormone replacement therapy, misclassification of menopausal status can still occur. These exogenous estrogens can cause a drop in FSH levels [13]. Our analysis indicate that only 4

individuals with FSH levels between 15 IU/L and 40 IU/L are likely to be subject to misclassification. Excluding these women from analysis did not change the study conclusions.

Another aspect of HRT use can cause misclassification of menopausal status. Hormone replacement therapy can mask the onset of menopause by causing false bleeding that may be mistaken for pre-menopausal menstrual bleeding. However, the results were similar when a sensitivity analysis was conducted, in which censoring began at the start of HRT use.

Optimal hormonal cutpoints were obtained from several studies that evaluated the ability of these cutpoints to discriminate menopausal status among those who had clearly defined menopausal status (those with a bilateral oophorectomy or having regular cycles) [12, 14-16]. In our study, the hormone data performed well with regards to correctly classifying (as post-menopausal) women who had a bilateral oophorectomy or had $\geq$1 year of amenorrhea. However, validation of the algorithm has not been conducted among those with less clearly defined status (those with a simple hysterectomy, having irregular cycles, or are within 5 years of their natural menopause). In our study, there were 12 individuals with less than a year of amenorrhea and hormone data, and who did not take HRT, did not have a hysterectomy or oophorectomy, did not report irregular menstrual cycles, and did not have radiation / chemotherapy: 8 were classified as post-menopausal by hormone and 4 as pre-menopausal by hormone. All 8 of the hormally-defined post-menopausal women and one of the hormonally-defined pre-menopausal women did, however, report having hot flashes, vaginal dryness, and/or night sweats in the past 2 years or self-report having had natural

menopause. In addition, ignoring the hormone data and censoring (at the LMP) those with less than a year of amenorrhea did not change the conclusion of this study.

Menopausal age was self-reported and can be subject to errors in recall. The validity of recall can decrease as the time since the last menstrual period increases [17]. However, the follow-up interviews were conducted near the time of menopause (ages 43-66) and poor recall is likely minimal. Furthermore, the ability to recall is not expected to be associated with DDE and PCB levels.

There was a sizeable proportion (42%) of women at baseline who did not participate in the follow-up study. Of these, 70% could not be found or could not be traced due to missing contact information. Although it is unclear whether exclusion of these women is associated with age at menopause, it is reassuring that their DDE and PCB levels were similar to those of participants.

6.4 Implications

DDE and PCBs are ubiquitous environmental pollutants, and therefore, their potential health effects are of great concern. The impact of the bans on environmental levels of DDE and PCBs is partly reflected in the concomitant drop in serum organochlorines over the two study time periods. Nevertheless, persistence of these compounds and their reported, though not established, associations with various health outcomes [18-21] would necessitate the continued public health observance of individual body burden.

High correlations between measures at distant times, particularly for DDE, would be of substantial interest to investigators of etiologic studies using only a single organochlorine measure at a time not relevant to disease causation or pathogenesis. Furthermore, this study

contributes to the relatively small body of literature on organochlorine exposure estimation [22] [23] by identifying additional factors (body mass index, initial concentration, and change in body fat) to be considered in the predictive modeling of exposure levels. Assessing exposures at critical time window(s) is essential for determining the true underlying relationship between exposure and disease.

This study also contributes to the science on the health effects of organochlorines. This study does not provide evidence for an effect of relatively low level exposures to organochlorines on age at menopause. However, it does not negate the potential for an effect at higher concentrations. The study results demonstrate a strong effect for family history of early menopause before age 46 and ovary removal before age 40, in addition to the well-established association between current smoking and earlier age at menopause. Given that early menopause can increase the risk of infertility, heart disease, and osteoporosis, it would be important to investigate these factors further.

6.5 Future directions

This study was limited to the investigation of two serum organochlorine measures and an assumed exponential rate of decay. Better assessment of the shape of the concentration curve could help to improve the prediction of organochlorine concentrations. Multiple serial measures of DDE and PCBs, as well as their predictors, would be beneficial in this regard. The strong effect of body mass index, despite the general lack of obese individuals at baseline, provides us with clues to the important mechanisms underlying the maintenance and elimination of organochlorines. Further investigations are warranted using a wider range of body mass index, with particular consideration for heavier or obese individuals.

Additionally, given the low variability in age and parity in this study, another question left unanswered is whether these factors have an effect on the rate of change in levels. Future studies should also consider the dual role of fat in the metabolism and dilution of these organochlorines, as well as the variation in effect as lactation progresses. While the predictors identified in this study explain a large portion of the variation in organochlorine concentrations over time, other potentially important factors, such as fish consumption, should be considered in future analyses.

Given the importance of age at menopause on the risk of subsequent health outcomes, additional investigations are warranted with consideration of critical time window(s) of exposure. Although we estimated levels at one common age of 40, there is potential heterogeneity between subjects in their etiologically relevant period of exposure. Studies among populations with low level exposures as well higher level exposures would further elucidate the role of these organochlorines. The large effect of family history of early menopause and unilateral oophorectomy also deserves further attention. Understanding the genetic and environmental contributions to age at menopause would be important, as public health resources could be better targeted to address the needs of women faced with an earlier or later menopause.

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