IN UTERO EXPOSURE TO ORGANOCHLORINE AND ATRAZINE PESTICIDES AND EARLY MENARCHE IN THE AVON LONGITUDINAL STUDY ON PARENTS AND CHILDREN COHORT

by

GONZA NAMULANDA

(Under the Direction of Luke Naeher)

ABSTRACT

Introduction: Pesticides are toxic substances that can also cause unintended adverse effects, e.g., endocrine disruption, in exposed non-target populations such as humans. Some endocrine disrupting compounds (EDCs) (e.g., the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT)) are identified as persistent organic pollutants while other suspected EDCs (e.g., atrazine) are less persistent in the body. A trend towards early puberty in girls has been observed in the United States and Europe. This decline in the age of onset of puberty has been associated with several factors including exposures to EDCs. The endocrine disrupting effects due to population exposures to pesticides are still not well understood.

Objectives: 1) To characterize the *in utero* exposure of girls in the Avon Longitudinal Study on Parents and Children (ALSPAC) in Bristol, United Kingdom (UK) to organochlorine pesticides and examine the association with early menarche 2) To characterize the *in utero* exposure of girls in ALSPAC to atrazine pesticide and examine the association with early menarche.

Methods: Exposure to 9 organochlorine compounds and 7 atrazine analytes measured in maternal serum or urine collected during pregnancy was characterized using non-parametric survival analysis methods. Logistic regression methods were used to examine the association between *in utero* exposure to these pesticides and early menarche.

Results: Hexacholorbenzene (HCB), β-hexacholorocyclohexane (β-HCH), 2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethane (p,p'-DDT) and 2,2-Bis(4-chlorophenyl)-1,1-dichloroethene (p,p'-DDE) were detected in more than 50% of the study participants. Diaminochlorotriazine (DACT) was the only atrazine analyte detected in more than 50% of the study participants. Overall, there was no association between *in utero* exposure to organochlorines and early menarche. *In utero* exposure to higher levels of the atrazine metabolite DACT in the ALSPAC cohort was associated with an increased odds of early menarche. **Conclusions**: More emphasis needs to be placed on measuring exposure in countries where organochlorine pesticides are still in use and evaluating their associations with adverse health effects. Our results suggest an association between *in utero* exposure to the atrazine metabolite DACT and early menarche in the ALSPAC cohort. To accurately assess atrazine exposure in populations, several metabolites including DACT need to be measured.

INDEX WORDS: Endocrine disrupting compounds, ALSPAC, menarche, early puberty

IN UTERO EXPOSURE TO ORGANOCHLORINE AND ATRAZINE PESTICIDES AND EARLY MENARCHE IN THE AVON LONGITUDINAL STUDY ON PARENTS AND CHILDREN COHORT

by

GONZA NAMULANDA

BS, University of Nairobi, Kenya, 1998

MS, University of Missouri-Columbia, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PUBLIC HEALTH

ATHENS, GEORGIA

2014

IN UTERO EXPOSURE TO ORGANOCHLORINE AND ATRAZINE PESTICIDES AND EARLY MENARCHE IN THE AVON LONGITUDINAL STUDY ON PARENTS AND CHILDREN COHORT

by

GONZA NAMULANDA

Major Professor: Luke Naeher

Committee: Steve Rathbun
Judy Qualters
Mildred Maisonet
John Vena
Mary Alice Smith

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
December 2014

ACKNOWLEDGEMENTS

I would like to acknowledge my advisor and chair of my dissertation committee, Dr Luke Naeher, for his direction and expertise during this doctoral journey. His support, advice and encouragement have always been timely and valuable. I would like to thank him for his mentorship.

I would also like to acknowledge the other members of my dissertation committee. I would like to thank Drs Vena and Rathbun for their support and expertise in environmental epidemiology and statistics. I would like to thank Dr Maisonet for her expertise and her knowledge about the ALSPAC study that she imparted to me. Her passion for the subject matter and the study were evident, and I value the guidance she provided to this work. I would also like to thank Dr. Qualters for her support, expertise and time taken to guide me during this process. From the start, she has been supportive of my plans to go back to school and pursue a doctoral degree. She went out of her way to help me identify a suitable project for my dissertation. As a branch chief and now a division director, she has been a great mentor and excellent professional example to me and others within the division of environmental hazards and health effects since I joined CDC about 8 years ago. I'd like to thank Dr. Smith for the knowledge gained in her classes; it was in her toxicology class that I began the journey of studying atrazine pesticide.

I would also like to acknowledge all current and former professors from UGA, from whom I have learned a lot; Drs. Joel Lee, Jia-Sheng Wang, Marsha Black, Mary Alice Smith, Su-I Hou, Mark Wilson, Steve Valeika, Woncheol Jang, Jay Shelton, Deborah Carroll, Dan Hall,

Xiangrong Yin, Brian Forrester, Monica Taylor-Jones and John Vena, and also, Justine Blanford from Pennsylvania State University.

I would like to acknowledge the Tracking Branch, CDC, for their support and allowing me to pursue my studies. I would also like to acknowledge the Health Studies Branch who made this study and data available to me. In particular, I'd like to thank Ethel Taylor, Colleen Martin and Adrianne Holmes. I would also like to acknowledge Terry Hartman and Michele Marcus from Emory University for their expertise and help with ALSPAC. I would like to acknowledge other collaborators and co-authors of the studies presented in this dissertation for their expertise provided; Andreas Sjodin, David Olson, Dana Flanders and Dana Barr. I also acknowledge the participants of the ALSPAC study and the ALSPAC team.

I would also like to acknowledge my fellow students who have helped me along this journey, particularly Ofia Hodoh. I thank my family – my parents, brothers and sister, and their families – for their support and encouragement.

Finally, most importantly, I would like to acknowledge God – without Him none of this would have been possible. Thank you.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Pesticides play an important role in agriculture and human health. They are used to eliminate pests that compete with humans for food, serve as vectors for disease and destroy crops (Moore, 2007). These toxic substances can also cause unintended adverse effects in exposed non-target populations such as humans. The adverse health effects that have been associated with pesticide exposures include: reproductive damage, birth defects, nervous system damage, cancer and endocrine system interference (Moore, 2007). Endocrine disrupting compounds (EDCs) have structures that mimic naturally occurring hormones, therefore fooling the body into over-responding or responding at inappropriate times (UNEP & WHO, 2012; US EPA, 2011). Some EDCs such as the organochlorine dichlorodiphenyltrichloroethane (DDT) are identified as persistent organic pollutants while other suspected EDCs such as atrazine are less persistent in the body (UNEP & WHO, 2012). The number of compounds being identified as EDCs has increased in the last 10 years (UNEP & WHO, 2012). There has also been an increase in the incidence of disorders such as early puberty that are possibly associated with exposure to EDCs (UNEP & WHO, 2012).

Puberty in girls is notably observed by breast development (thelarche), pubic hair development (pubarche) and menstruation (menarche) (UNEP & WHO, 2012). A trend towards early puberty in girls has been observed during the period of 1940 to 1994 in the United States and Europe (Euling et al., 2008; McDowell, Brody, & Hughes, 2007). This decline in the age of onset of puberty has been associated with genetic factors and other factors including childhood

obesity (UNEP & WHO, 2012). Studies suggest that the declining trend cannot be explained by genetics and obesity alone, and other environmental factors, such as exposures to EDCs, are involved (Mouritsen et al., 2010; UNEP & WHO, 2012). There are some public health implications of altered puberty timing. Early puberty is a risk factor for increased risk of breast cancer, polycystic ovarian syndrome, reduced adult height, and greater likelihood of engaging in childhood risky behaviors such as smoking, unprotected sex, alcohol and drugs (UNEP & WHO, 2012).

Few studies have examined the association between pesticide exposures and puberty. Those that have examined the association between organochlorines and early puberty show conflicting results. Some studies have associated organochlorine exposure with early pubic hair development, early breast development, or early onset of menarche while other studies have not shown these associations (Gladen, Ragan, & Rogan, 2000; Vasiliu, Muttineni, & Karmaus, 2004). The endocrine disrupting effects due to population exposures to pesticides are still not well understood and more epidemiologic studies are needed (Buck Louis et al., 2008). This dissertation reports the results of two studies that characterize *in utero* exposure of children in the Avon Longitudinal Study on Parents and Children (ALSPAC) in Bristol, United Kingdom (UK) to organochlorine and atrazine pesticides, and the association with early menarche.

Though some organochlorines such as DDT were banned over 4 decades ago, they are still detected in humans because of their environmental and biological persistence. The first study characterizes *in utero* exposure of girls in the ALSPAC cohort to these 9 organochlorine compounds measured in maternal serum: hexachlorobenzene (HCB), β- and ϒ-hexachlorocyclohexane (HCH), oxychlordane, trans-nonachlor, p,p'– and o,p'–dichlorodiphenyltrichloroethane (DDT), p,p-1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-

DDE), and Mirex. Some studies suggest that *in utero* exposures to EDCs can affect outcomes later in life such as onset of menarche. Vasiliu et al. (2004) found that an increase of 15µg/L of DDT *in utero* reduced age at menarche by one year. Therefore, our study also examines the association between *in utero* exposure to organochlorine pesticides and early menarche in a nested case-control study of the ALSPAC cohort. For this section we focused on HCB, β-HCH, p,p'-DDT and p,p'-DDE compounds which were detected in over 50% of the study participants. This study and results are presented in Chapter 3.

Atrazine is a commonly used herbicide worldwide (LeBaron, H., McFarland, J. & Burnside, O., 2008). Although still in use in over 70 countries globally, atrazine was banned by the European Union (E.U.) in 2003 because of its presence throughout the environment above the E.U. recommended limit of pesticide residue in drinking water, and the inability to prevent it contaminating the environment when used as a herbicide (Sass & Colangelo, 2006; LeBaron, et al, 2008). The second study characterizes *in utero* exposure of girls in the ALSPAC cohort to these 7 atrazine analytes measured in maternal urine: atrazine, atrazine mercapturate (AM), desethyl atrazine mercapturate (DEAM), diaminochlorotriazine (DACT), desethyl atrazine (DEA), desisopropyl atrazine (DIA), and hydroxyl atrazine (ATZ-OH). To our knowledge, no epidemiologic studies have examined the association between exposure to atrazine and onset of puberty, however animal studies suggest that exposure to atrazine and its metabolites delays the onset of puberty in rats (Laws et al., 2003, Rayner et al., 2004). Therefore, this study also examines the association between *in utero* exposure to the atrazine analytes measured and early menarche in a nested case-control study of the ALSPAC cohort. For this section, we focused on DACT which was the only metabolite detected in over 50% of the study participants. This study and results are presented in Chapter 4.

This dissertation also includes a literature review presented in Chapter 2 which summarizes the current knowledge on organochlorine and atrazine exposure, and association with puberty. The literature review section also provides some background information on the ALSPAC study purpose, data and participants, and information on the prevalence, trends and predictors of early puberty. Chapter 5 summarizes the conclusions of the studies presented in Chapters 3 and 4, and Chapter 6 presents the public health implications of the findings.

REFERENCES

Buck Louis, G. M., Gray, L. E., Marcus, M., Ojeda, S. R., Pescovitz, O. H., Witchel, S. F., . . . Euling, S. Y. (2008). Environmental Factors and Puberty Timing: Expert Panel Research Needs. *Pediatrics, 121(Supplement 3), S192-S207*. doi: 10.1542/peds.1813E

Euling, S., Herman-Giddens, M.E., Lee, P., Selevan, S., Juul, A., Sorensen, T., . . . Swan, S.H. (2008). Examination of US Puberty-Timing Data from 1940 to 1994 for Secular Trends: Panel Findings. *Pediatrics, 121*.

Gladen, B., Ragan, B., & Rogan, W. (2000). Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. *The Journal of Pediatrics, 136(4)*.

Laws, S., Ferrell, J., Stoker, T., & Cooper, R. (2003). Pubertal development in female wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-s-chlorotriazine and hydroxyatrazine. Toxicological Sciences, 76, 190-200

LeBaron, H., McFarland, J. & Burnside, O. (Eds.). The Triazine Herbicides; 50 Years Revolutionizing Agriculture. 2008

McDowell, M. A., Broody, D.J., Hughes, J. (2007). Has Age at Menarche Changed? Results from the National Health and Nutrition Examination Survey (NHANES) 1999–2004. *Journal of Adolescent Health, 40,* 227-231.

Moore, G.S. (2007). *Living with the Earth: Concepts in Environmental Health Science (3rd Ed)*. New York, NY: Taylor & Francis Group, LLC.

Mouritsen, A., Aksglaede, K., Sorensen, K., Mogensen, S., Leffers, H., Main, K., . . . Juul, A. (2010). Hypothesis: exposure to endocrine-disrupting chemicals may interfere with timing of puberty. International Journal of Andrology, 33, 346-359.

Rayner, J., Wood, C., & Fenton, S. (2004). Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. *Toxicol Appl Pharmacol, 195*, 23-34.

Sass, J. B., & Colangelo, A. (2006). European Union bans atrazine, while the United States negotiates continued use. *Int J Occup Environ Health, 12(3),* 260-267.

United Nations Environment Programme (UNEP), & World Health Organization (WHO). (2012). State of the Science of Endocrine Disrupting Chemicals- 2012.

US Environmental Protection Agency (EPA). (2011). Endocrine Primer. Retrieved September 12th, 2013, from http://www.epa.gov/endo/pubs/edspoverview/primer.htm

Vasiliu, O., Muttineni, J., & Karmaus, W. (2004). In utero exposure to organochlorines and age at menarche. *Hum Reprod, 19(7), 1506-1512*. doi: 10.1093/humrep/deh292

CHAPTER 2

LITERATURE REVIEW

BACKGROUND

This section of the literature review provides a background on the ALSPAC study and information about mechanisms, prevalence, trends and predictors of early puberty.

About ALSPAC

The Avon Longitudinal Study of Parents and Children (ALSPAC) originated from a World Health Organization (WHO) meeting in Moscow in 1985 that recommended the establishment of cohort studies in Europe to study modifiable influences on child health (Boyd et al., 2013). ALSPAC was subsequently established in 1991 covering the area of Bristol, South west of England in the United Kingdom (UK). The main aim of this longitudinal cohort study was to understand how genetic and environmental characteristics influence the health and development of parents and their children (Fraser et al., 2013). ALSPAC is based at the University of Bristol who together with UK Medical Research Council and Wellcome Trust provide core funding for the study (Bristol University, 2014). ALSPAC has received funding from several other partners for individual projects. Examples of such partners include United States Centers for Disease Control and Prevention (CDC) who initiated a study to understand factors (including the role of *in utero* exposures to endocrine disrupting compounds) that influence the timing of pubertal development (Rubin et al., 2009).

Participants

Pregnant women resident in a defined geographical area in the South west of England (Figure 2.1) were eligible to participate if their expected delivery date was between and including the dates 1st April, 1991 and 31st December, 1992. The women were recruited through media, recruitment staff visiting community locations and information cards given during routine antenatal care (Boyd et al., 2013). Through these recruitment processes, 13,761 women were initially included in the study, and resulted in 14,062 live births. At age of 7, there was a second phase of recruitment when an additional 456 children based on the original recruitment criteria, were included. During a third phase of recruitment from ages 8 to 18 years, 257 more eligible children were added, bringing the total live births to 14,775. Only 14,701 of these live births were still alive at age one (Boyd et al., 2013).

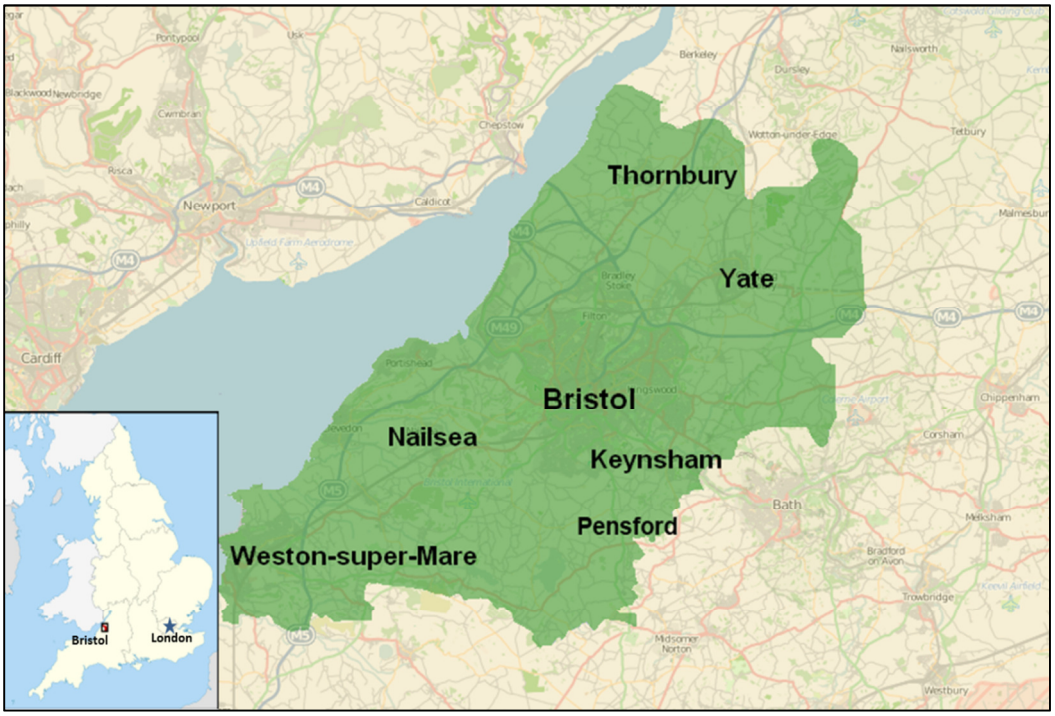


Figure 2.1 Study Recruitment Area: Bristol, South West of England

Source: ALSPAC and Mapwikipedia

Data collected

The ALSPAC study is a rich resource of epidemiological data. Data on study participants, which includes children and their parents, have been collected through self-reported questionnaires, obstetric data abstracted from medical records and several opportunistic clinical assessments. The breadth of data collected is too large to detail in this document, therefore only an overview of the types of data collected will be provided. The ALSPAC study website contains details of all the data that are available through a fully searchable data dictionary (http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/).

The questionnaires collect these demographics data about the child's mother and her partner: personality; life-course characteristics such as occupation and housing conditions; health-related behaviors; and health which covers mental, reproductive, respiratory, musculoskeletal and cardiometabolic health, including medication use (Fraser et al. 2013; Boyd et al., 2013). CDC initiated a study with ALSPAC to understand factors that influence the timing of maturation, and beginning at the age of 8, a 'Changing and Growing' puberty questionnaire was mailed to the children participants each year until age 17, with the exclusion of age 12 due to a delay in administering the questionnaire. This questionnaire collected information about the timing and onset of puberty in enrolled girls and boys. For girls, onset of menarche information including the age and date of first menstrual period were self-reported, and recorded in these questionnaires by the girls or their parents (Rubin et al, 2009). Each questionnaire required the respondent to examine line drawings representing 5 Tanner stages for breast size and pubic hair, and record the drawing that most closely represented the girl's current stage of development (Rubin et al., 2009). The respondent was also asked to record if they had first menstrual period, and the month and year started (Rubin et al., 2009). Other information recorded in the puberty

questionnaire included the girl's height and weight that were used to calculate body mass index (BMI), frequency of physical activity, length of menstrual cycle, and any complications such as prolonged bleeding, cramps, or pelvic pain. For this study, analyses were restricted to singleton live births (Rubin et al., 2009). Data with inconsistencies e.g., a pubertal stage reported in one questionnaire and a lower stage recorded in the following questionnaire, or responding 'yes' to menarche in one questionnaire and 'no' in following questionnaires were deleted (Rubin et al., 2009).

From the number of live births enrolled in the study, 4,643 girls responded to at least one puberty questionnaire and only 4,212 girls had menarche data after exclusions (Rubin et al., 2009). From this number, 3,682 returned at least 2 valid and complete questionnaires between the ages of 8 and 13 (Christensen et al., 2011). A previous study examining the association between *in utero* exposure to perfluoroalkyl chemicals and early menarche in this cohort ordered the 3,682 girls according to reported age at menarche; they determined 11.5 years as the cut-off age defining earlier menarche (Christensen et al., 2011). 338 of these girls had menarche before 11.5 years and were selected as cases. A random sample of 394 girls with menarche at or after 11.5 years, were selected as controls. 218 cases had a maternal serum sample that could be analyzed, and 230 controls had a maternal gestational serum sample that could be analyzed (Christensen et al., 2011; Appendix A). 174 cases and 195 controls also had a maternal gestational urine sample that could be analyzed (CDC, 2012). These cases and controls have been used for subsequent nested case-control studies examining the association between *in utero* exposure to several endocrine disrupting compounds measured in gestational maternal blood or urine and early menarche. The following compounds were measured in maternal serum by the CDC Division of Laboratory Sciences: perfluoroalkyl compounds, persistent pesticides,

polychlorinated biphenyls, and polybrominated diphenyls ethers (CDC, 2012). The following compounds were measured in maternal urine: phytoestrogens, atrazines, and phthalates (CDC, 2012).

Data extracted from mother's medical records include measurements such as weight and blood pressure; pregnancy complications e.g., gestational diabetes; hospital admissions; ultra sound examinations; delivery information e.g., method and place; and postnatal health (Fraser et al., 2013). Opportunistic clinic assessments collect data on mothers and their children. The number and time points of these clinics vary. These assessments collect information about anthropometry; bioimpedance for percent body fat; whole body and hip scans; blood pressure; emotional well-being; cognitive function; and visual acuity. Biological samples were also collected from the mother during pregnancy at recruitment and at several time periods after, and these samples include: serum, plasma, urine, hair, toe nails and placenta (Fraser et al., 2013). Gestational age at which serum or urine samples were collected depended on when during pregnancy the mother was recruited, therefore differed among the women. Data collected from questionnaires, medical records and clinics provided covariate data variables that were linked to puberty and pesticide data used in the studies presented in this dissertation.

The recruitment of the participants of ALSPAC began in 1990 through 1992, and the study participants have been followed-up since then for the last 22– 24 years.

Study strengths and limitations

The main strengths of the study are: the sample size, duration of follow-up, availability of repeat measures, and genome-wide and phenotypic data. Another advantage is being able to link these data to other family members.

Some limitations of the study are: majority of the participants are of white ethnicity, therefore limiting generalizability of study findings; the selection of participants based on pregnancy was arbitrary, and this also excluded women who could not get pregnant, which also limits generalizability of study findings; women recruited but had an early pregnant loss before week 23, were not followed-up.

Puberty

Puberty is an important stage in life where the body matures to become capable of sexual reproduction. Puberty in girls is notably observed by breast development (thelarche), followed by pubic hair development (pubarche) and ends with menstruation (menarche) (UNEP&WHO, 2012). The variability in the onset of puberty in individuals has been attributed to genetic factors, ethnicity and nutrition (Kaprio et al., 1995; UNEP&WHO, 2012). Recently, additional factors have been associated with onset of puberty including socio-economic status, obesity and geographic or environmental factors such as exposure to endocrine disrupting compounds (EDCs) (Parent et al., 2003; UNEP&WHO, 2012).

There has been a secular trend towards earlier onset of puberty among American and European girls (Aksglaede, Olsen, Sorensen, Petersen & Juul, 2008; Euling et al., 2008; Parent et al., 2003; Semiz, 2008). Some studies have suggested that the decline in age at onset of puberty may be explained by other factors such as environmental factors, rather than genetic ones only (Crain et al., 2008; UNEP&WHO, 2012; Mouritsen et al., 2010). Exposure to some EDCs such as DDT, has been associated with early onset of puberty, while exposure to other EDCs such as lead, has been associated with delayed puberty (Denham et al., 2005; Vasiliu, Muttinenu & Karmaus, 2004).

Mechanism of action

The puberty process involves the maturation of the hypothalamic-pituitary-gonadal (HPG) system and the maturation of the hypothalamic-pituitary-adrenal (HPA) androgenesis system (Golub et al., 2008). Breast development and onset of menarche are associated with HPG maturation and pubic hair development with HPA maturation (Buck Louis et al, 2008). These processes begin in-utero and continue after birth until puberty (Golub et al., 2008).

There are two hypotheses that suggest the mechanisms through which EDCs can induce early puberty: 1) causing hypothalamic and/or pituitary maturation (central), or 2) acting directly on the ovary or breast independent of hypothalamic-pituitary axis (peripheral) (Crain et al., 2008; Raiser, Parent, Gerard, Lebrethon & Bourguignon, 2006).

In laboratory studies, DDT initiated hypothalamic maturation in rats (Raiser et al., 2007). Early post natal transient exposure to DDT in infant female rats caused early puberty observed by increased Gonadotropin-releasing hormone (GnRH) secretion and lowered Luteinizing hormone (LH) levels causing early vaginal opening and first estrus (Raiser et al., 2007). In other animal studies, atrazine delayed onset of puberty in female and male rats (Laws, Ferrell, Stoker, & Cooper, 2003; Laws, Ferrell, Stocker, Schmid, & Cooper, 2000; Rayner, Enoch & Fenton, 2005; Rayner, Wood, & Fenton, 2004; Stoker, Guidici, Lawson, & Cooper, 2002; Stoker, Lawson, Guidici, & Cooper, 2000). The study authors suggest that *in utero* and post natal exposure to atrazine delayed vaginal opening and development of mammary glands in the rats by altering the hypothalamic-pituitary activity (Laws et al., 2003).

Reviews of human puberty studies suggest different hypotheses for the mechanism of action through which early puberty is observed which is associated with the timing, presence and withdrawal of EDC exposure. These reviews suggest that pre-pubertal girls exposed to EDCs

e.g., DDT, in developing countries can initially experience peripheral puberty through the stimulation of hypothalamic maturation while inhibiting pituitary gonadotrophins through a negative feedback effect (Parent et al., 2003; Raiser et al., 2006). Other studies also hypothesize that withdrawal from pre-pubertal EDC exposure can also induce early puberty; after migrating to developed countries, and EDC exposure is withdrawn or absent, the pituitary inhibition disappears allowing central puberty maturation through the hypothalamic-pituitary axis (Krstevska-Konstantinova et al., 2001; Raiser, 2006). Therefore, pre-pubertal girls exposed to EDCs in developing countries and who subsequently move to developed countries have a higher risk of experiencing early puberty (Krstevska-Konstantinova et al., 2001).

In another study, *in utero* exposure to DDT initiated early onset of menarche (Vasiliu et al., 2004). The authors suggested that DDT's estrogenic effect *in utero* programmed the age at menarche, but the mechanism through which early puberty was initiated (central or peripheral) was not discussed (Vasiliu et al., 2004).

Prevalence

Early puberty is defined as any pubertal change (breast development, pubic hair development or menstruation) in girls before age 8 (Golub et al., 2008). Because of regional and racial variations, there is a suggestion to redefine early onset of puberty to; pubertal change before the age of 6 in Black American girls, or age of 7 in White American girls, or age 8 in all other parts of the world (Golub et al., 2008). Few studies have measured the prevalence of early puberty. A population-based study in Denmark estimated the prevalence of pubertal change before age 8 in Danish girls as 0.2%, while in the US, analyses of the third National Health and Nutrition Examination Survey (NHANES III) data concluded that 3.2% of girls had attained early breast development, Tanner Stage 2, before age 8, and 7% attained early menarche, i.e.,

menarche before 10 years of age (Allsworth, Weitzen, & Boardman, 2005; Rosenfield, Lipton, & Drum, 2009; Teilmann, Pedersen, Jensen, Skakkebaek, & Juul, 2005).

Other studies using the NHANES III data reported that 10% of US girls start to menstruate by the age of 11.11 years (Chumlea et al., 2003). In normal weight girls, 1% had menarche before age 9, 3.2% of 8 year olds had Tanner stage 2 breast development, and 5% had stage 3 pubic hair development before age 9.3 years (Rosenfield et al., 2009). The results of these studies varied by ethnicity with non-Hispanic Black and Mexican American girls attaining puberty earlier than non-Hispanic White girls (Rosenfield et al., 2009).

Trends

Studies have shown a decrease in age at menarche (Castellino et al., 2005; McDowell, 2007) and others have shown a decrease in age at thelarche (Aksglaede et al., 2009; Biro, 2010; Castellino et al., 2005; Semiz, Kurt, Kurt, Zencir, & Sevinc, 2008). The average age at onset of menarche for several decades has been 13 years, while 200 years ago, it was 17 years (UNEP&WHO, 2012). The mean age at onset of menarche in US girls decreased to 12.43 years for the period 1988 – 1994, compared to 12.77 years in earlier studies (Chumlea et al., 2003). Girls born in 1980 had a mean age at onset of menarche of 12.34 years, which was 3-6 months earlier than girls born before 1980 (Demerath et al., 2004).

The results of some studies in Europe have supported the observed secular trend towards a decrease in age at onset of puberty while some have not, and instead show a leveling of this age (Bau et al., 2009). A study of 3496 children, 1638 of them females, from Northern Italy (1998-2001) showed that girls began puberty approximately 1 year earlier than the Tanner reference percentiles published in 1976 using data from British girls (Castellino et al., 2005). The 50^{th} percentile age at Tanner Stage 2 breast development for the Italian girls was 10.5 years compared

to 11.2 years from the Tanner reference (Castellino et al., 2005). In another study performed in 2005, Turkish girls attained Tanner Stage 2 breast development at an average age of 10.16, approximately 1 year earlier than Tanner reference data (11.2 years) (Semiz et al., 2008).

A cross-sectional survey of girls in Berlin during 2006-2007 found that the mean age at menarche was 12.8 years, similar to results of studies in Germany conducted during the period 2003-2006 and 20 years ago (Bau et al., 2009). There was a non-statistically significant downward trend in the mean age of menarche in Finland during the 1980s, and a significant difference in age of menarche between regions which leveled by 1989 (Rimpela & Rimpela, 1993). The mean age of menarche in Finland during this period, 1979 to 1989, ranged from 13.4 – 13.3 years (Rimpela & Rimpela, 1993).

Other factors associated with early menarche

There are several factors associated with early onset of puberty. Mother's age at menarche and race have strong associations with onset of menarche in offspring (Rubin, 2009). Daughters of mothers who had an early onset of menarche are more likely to also have an early onset of menarche (Graber, Brooks-Gunn, & Warren, 1995; Rubin, 2009). In a 20 year study of girls in the US, Black girls experienced menarche 3 months earlier than White girls (Freedman et al., 2002). Black girls were also 1.4 times more likely to have menarche before age 11 than White girls (Freedman et al., 2002). Daughters of mothers who were overweight or obese before pregnancy are more likely to have an onset of menarche before age 12 (Keim Branum, Klebanoff, & Zemel, 2009). Childhood characteristics such as pre-pubertal BMI and birth weight are also predictors of onset of menarche. Children with higher pre-pubertal BMI are more likely to have menarche before age 12 (Freedman et al., 2003), while children with increased birth weight have a later age at menarche (Ruder, Hartman, Rovine, & Dorgan, 2010). Socio-

economic factors and family characteristics also play an important role in onset of menarche. Girls from high socio-economic families are more likely to begin menarche earlier than girls from lower socio-economic status (Wronka & Pawlinska-Chmara, 2005).

Public health implications

Early puberty has short-term and long-term health implications later in life. Girls who have early puberty are at greater risk to suffer depression and anxiety (Walvoord, 2010). They also have greater rates of smoking, drinking, drug abuse, delinquent behavior, and earlier sexual experiences (Golub et al., 2008; Walvoord, 2010). Menarche before the age of 12 and early peak growth, a marker for onset of puberty, have been associated with greater risk for breast cancer (Walvoord, 2010). There is also an increased risk for reduced adult height, ovarian cancer and polycystic ovarian syndrome for girls with early menarche (Golub et al., 2008; UNEP&WHO, 2012; Walvoord, 2010).

Summary

There are geographic differences in the trends of onset of puberty. Some studies show a decline in the age at onset of puberty while others do not. There are several factors that are associated with early menarche including exposure to endocrine disrupting compounds. Trends towards early onset of puberty cannot be explained by genetic factors alone.

ORGANOCHLORINE PESTICIDES

This section of the literature review examines literature related to organochlorine pesticides; overview of associated developmental health effects in humans, *in utero* and infant exposures, exposure trends, endocrine disrupting mechanisms of action, and exposure

association with early puberty studies. This literature review will focus on DDT, HCB and HCH, which were detected in over 50% of the participants of this study.

Introduction

Organochlorine pesticides have been used as fungicides and as insecticides on agricultural crops, and to control insects that carry diseases such as malaria and typhus (ATSDR, 2002, 2005, 2013). These compounds are synthetically manufactured and do not occur naturally in the environment (ATSDR, 2002, 2005, 2013). Organochlorine compounds can exist in several forms and isomers. They also break down into several metabolites in the environment or in the body. They can persist in the environment for several years, for example, they can have a half-life of >30 years in soil (ATSDR, 2002). Organochlorine pesticides are lipophilic and bioaccumulate through the food chain (ATSDR, 2002). Exposure to organochlorines has been associated with neurological, reproductive and developmental health effects (ATSDR, 2002, 2005, 2013).

Organochlorine metabolites, forms and developmental health effects

DDT

When DDT enters the body, it can be broken down to 1,1-dichloro-2,2-bis (p-chlorophenyl)ethylene (DDE) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD) (ATSDR, 2002). Primary isomers of DDT are p,p'-DDT, p,p'-DDE, and p,p'-DDD (ATSDR, 2002). Other isomers include the o,p'- isomer. A higher ratio of DDT to DDE in the body usually signifies recent exposure while lower ratios signify less recent exposure (ATSDR, 2002). Smith (1999) calculated a population half-life of DDT in breast milk of 4.2 to 5.6 years using population–based studies published from 1950 to 1996 (Smith, 1999).

The primary target organs for DDT, DDE, and DDD toxicity include the nervous system, the liver and the reproductive system (ATSDR, 2002). DDT has been associated with these developmental and reproductive health outcomes; pre-term birth, miscarriages, spontaneous abortion in pregnant women and reduced height in children (ATSDR, 2002; Gerhard, Daniel, Link, Monga, & Runnebaum, 1988; Saxena, Siddiqui, Seth, & Murti, 1981b; Wasserman et al., 1982).

HCH

HCH exists in several forms depending on the position of the hydrogen in the chemical structure, for example, ϒ-HCH (lindane), β-HCH and α-HCH (ATSDR, 2005). These forms have different toxicities depending on length of exposure. For acute exposures, ϒ-HCH is most toxic while for chronic exposures, β-HCH is the most toxic form (ATSDR, 2005). β- HCH is more persistent that the other isomers of HCH, which are more rapidly metabolized or excreted from the body after exposure (ATSDR, 2005). β- HCH has the longest half-life in the body at 7.2 years in whole blood and 7.6 years in lipids (Jung et al., 1997).

Acute, high exposure to HCH affects the nervous system and chronic exposures produce adverse hematological effects in humans (ATSDR, 2005). There are limited studies on the health effects/developmental effects of HCH in humans. One study suggests that HCH exposure may be associated with intrauterine growth retardation in babies of exposed mothers, while another study showed HCH exposure may result in increased luteinizing hormone in the serum of exposed men (Siddiqui, Srivastava, Mehrotra, Mathur, & Tandon, 2003; Tomczak, Baumann, & Lenert, 1981).

HCB

Primary targets of toxicity for HCB are liver, reproductive and developmental systems (ATSDR, 2013). Some developmental effects associated with HCB exposure include increased

risk for undescended testis and impaired development of locomotive skills (ATSDR, 2002). Boys (mean age of 4.2 years) with undescended testis had three fold higher HCB concentrations in blood compared to boys (mean age 3.5 years) without the condition (ATSDR, 2002). No half-life for HCB in humans has been documented.

HCB, DDT and HCH were banned in the United States in 1966, 1972 and 1976 respectively (ATSDR, 2002, 2005, 2013). DDT was banned in the United Kingdom in 1986 (Rogan & Chen, 2005). Twelve persistent organic pollutants including DDT and HCB were banned globally in a 2004 treaty at the Stockholm Convention on Persistent Organic Pollutants (The Stockholm Convention, 2008). HCH is one of the nine persistent organic pesticides added to the initial list of 12 when the Stockholm Convention was amended in 2009 (The Stockholm Convention, 2008).

In utero and infant exposures

Children are exposed to organochlorine pesticides *in utero* through the placenta and after birth through breastfeeding (Covaci, Jorens, Jacquemyn, & Schepens, 2002; Fukata, Omori, Osada, todaka, & Mori, 2005; Waliszewski, Aguirre, Infanzon, & Siliceo, 2000). Pregnancy involves the transfer of lipids and lipo proteins from maternal tissues through the placenta to the fetus. Studies have shown positive correlations between organochlorines measured in maternal blood, cord blood and placenta (Saxena et al., 1981a).

In several studies, fetal exposure to organochlorines has been assessed in tissues such as umbilical cord, umbilical cord blood/serum and maternal blood/ serum (Covaci et al., 2002; Fukata et al., 2005; Waliszewski et al., 2000). In maternal blood and cord blood samples collected from 44 pregnant women at delivery in a Belgium hospital, there were detectable levels

of p,p'-DDE and HCB organochlorines in the cord blood of newborns (Covaci et al., 2002). DDE cord blood concentrations were positively associated with maternal blood concentrations, and on a lipid basis, were two times lower than in maternal blood (Covaci et al., 2002). Other studies have shown equal concentrations of DDE in maternal and cord blood (Waliszewski et al., 2000). In a study of 64 pregnant mothers admitted for cesarean delivery in Mexico, organochlorine pesticides were measured in adipose tissue, maternal serum and cord blood (Waliszewski et al., 2000). The highest correlation of organochlorines was found between maternal blood serum and cord blood serum concentrations (Waliszewski et al., 2000). HCB, β-HCH and p'p'-DDE had the highest frequency of detection, and p'p'-DDE concentrations were found to be in equilibrium across the three samples (Waliszewski et al., 2000). A more recent study of 32 pregnant women in Japan reported that cord serum is the best tissue sample to assess fetal exposure to persistent compounds (Fukata et al., 2005). In this study, there was a strong correlation between maternal serum and cord serum in some organochlorines, particularly HCB and HCH (Fukata et al., 2005). HCH and p,p' - DDE showed high correlation between cord serum and umbilical cord blood (Fukata et al., 2005). The study also found that on a lipid basis, concentrations were higher in cord blood than in cord serum, and concentrations and detection rates were lower in cord serum compared to maternal serum and cord blood (Fukata et al., 2005).

A study in Poland that measured DDT, HCB and HCH in maternal serum, cord serum and breast milk from 22 mothers, found a correlation between milk and cord serum concentrations. This correlation was weaker than the correlation between maternal and cord serum (Jaraczewska et al., 2006). No statistically significant correlation was found between maternal serum and milk concentrations (Jaraczewska et al., 2006). Another study assessed exposure to organochlorine pesticides in placenta and breast milk samples from Finnish and

Danish women. The results showed that there was a correlation between milk and placenta concentrations and using a lipid base, milk samples had higher concentrations of DDT, DDE and HCB pesticides than placenta samples (Shen, 2007). However, the authors also found that some pesticides concentrations in placenta did not correlate to the milk concentrations and were accumulated in the placenta. This was observed for α-HCH, pentachlorobenzene, pentachloroanisole and methoxychlor (Shen et al., 2007). The results of this study also suggest that the compound of interest will determine in which tissue to do biomonitoring (Shen et al., 2007).

Exposure trends in United Kingdom and Europe

Diet, particularly meat, fish and dairy products, is the primary source of exposure of adults to persistent organochlorines (CDC, 2009). Human exposure to organochlorines is measured in several tissues such as blood, breast milk and adipose tissues (ATSDR, 2002, 2005, 2013). The body burden of organochlorines, particularly DDT, has been decreasing since its ban decades ago (ATSDR, 2002). A 1992 study in Wales measured organochlorines in the adipose tissue of 75 human cadavers (Duarte-Davidson, Wilson, & Jones, 1994). The lipid adjusted mean concentrations for p,p'-DDE, p,p'-DDD and p,p'-DDT were 1,410.5ng/g, 12.5ng/g and 39.7ng/g respectively (Duarte-Davidson et al., 1994). Another study covering England, Wales, Scotland and Northern Ireland during the period of 1997–1998 detected organochlorines in 168 samples of human breast milk (Harris, 1999). The mean concentrations for p,p'-DDE, p,p'-DDT, HCB, β – HCH, γ-HCH and Dieldrin were 430ng/g, 40ng/g, 43ng/g, 68ng/g, 35ng/g, and 48ng/g respectively (Harris, 1999). DDE concentrations showed a decrease from the Duarte-Davidson et al. (1994) study done 5–6 years earlier. DDE concentrations showed a further decrease in a more

recent study done in 2001–2003, which measured organochlorines in 54 breast milk samples from mothers resident in UK (Kalantzi et al., 2004). The mean concentrations for p,p'-DDE , HCB, and β-HCH for this study were, 220ng/g, 20ng/g and, 40ng/g respectively (Kalantzi et al., 2004). HCB concentration also decreased compared to the previous 1997–1998 study.

A cross-sectional study measured organochlorines in about 400 ten year old children in Germany in four cycles during the period 1993–2003 (Link et al., 2005). The concentrations of DDE and HCB showed a statistically significant decrease during this 10 year period, and HCB had a mean decrease of 0.15ug/L (Link et al., 2005).

Endocrine disrupting effects

"An endocrine disruptor is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations." (UNEP & WHO, 2012). "A potential endocrine disruptor is an exogenous substance that possesses properties that might be expressed to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations. " (UNEP & WHO, 2012). Some endocrine disruptors can act directly on hormone receptors as hormone mimics or antagonists. Others can act directly on any number of proteins that control the delivery of a hormone to its normal target cell or tissue (UNEP & WHO, 2012).

Early puberty mechanism of action

Organochlorines have exhibited both estrogenic and androgenic properties in different studies (Kelce et al., 1995; Raiser et al., 2007: Cooper et al., 1989). DDT for example has been shown to be agonistic to the estrogen receptor. O,p-DDT exposure in rats was observed to mimic estrogen by increasing uterine weight, increasing glycogen content and premature vaginal

opening (ATSDR, 2002). The suggested mechanism of action is that DDT exposure causes early maturation of the GnRH mediated through the estrogen receptor (Raiser et al., 2007). LH response to the GnRH is reduced, causing hypothalamic-pituitary maturation before puberty (Raiser et al., 2007). DDT estrogen-like properties are mostly attributed to the o,p- isomer as observed in animal studies (ATSDR, 2002).

Exposure to ϒ-HCH (lindane) caused a delay in vaginal opening in female rats and disrupted ovarian cyclicity (Cooper et al., 1989). The suggested mechanism for delayed puberty is lindane's antiestrogenic activity which blocks the response of estrogen-dependent tissues (Cooper et al., 1989). Lindane has also exhibited weakly estrogenic activity in animal studies. Female rats fed lindane had an increase in glycogen content in the uterus, cervix and vagina, which was an indication of the estrogen action of lindane (Raizada, Misra, Saxena, Datta, & Dikshith, 1980). The suggested mechanism is through rapid excretion of lindane from the reproductive system (Raizada et al., 1980). β-HCH has also exhibited estrogenic activity in exposed weanling female rats (van Velsen, Danse, Leeuwen, Dormans, & van Logten, 1986). Several effects observed in the rats including inhibiting ovulation and hyperplastic lesions in the uterus were indicative of long term exposure to estrogens pointing towards the possible estrogenic action of β-HCH (van Velsen et al., 1986).

Another study observed that β-HCH exhibited estrogenic activity by redistributing the estrogen receptor and inducting synthesis of the cycstolic progesterone receptor in human breast cancer cell lines (ER positive cell lines) (Coosen & van Velsen, 1989). The study also concluded that β-HCH appeared to be estrogenic but does not seem to bind to the estrogen receptor (Coosen & van Velsen, 1989). A further study examining the estrogenic mechanism of action of β-HCH on human breast cancer cell lines showed that β-HCH does not activate the estrogen receptor but

it requires a combinational regulation found in a complex promoter (Steinmetz et al., 1996). The study authors presented this as a novel estrogenic action of β-HCH.

HCB has exhibited estrogenic effects in animal studies. These studies have suggested different mechanisms of action through which these effects are observed. Superovulated female rats exposed to HCB had elevated serum levels of progesterone, and also increased ovarian weights in the lowest dose group (1mg/kg) (Foster, Pentick, McMahon, & Lecavalier, 1992)). The authors suggest that HCB effect on the ovarian production of progesterone is indirect, i.e., HCB may have direct effect on the hypothalamus or pituitary function which suppress gonadotrophins secretion or modify circulating levels of luteinizing hormone or follicle stimulating hormone (Foster et al., 1992). In another study examining the effect of HCB on estrus cycle, female rats exposed to HCB had a significant decrease in circulating levels of estradiol, but no significant differences in progesterone levels (Alvarez et al., 2000). The authors suggest that HCB effect on these levels could be due to its effect on the hypothalamic-pituitary function or that HCB disrupts the ovarian steroid production through specific steroidogenic enzymes (Alvarez et al., 2000).

Studies have also shown p,p'-DDE to be an androgen receptor antagonist (Kelce et al., 1995). In Kelce et al. (1995), p,p'-DDE delayed onset of puberty in male rats. The suggested mechanism of action is through the inhibition of androgen receptor (AR) binding and inhibition of transcriptional activity (Kelce et al., 1995). The study also found that the concentration needed to exhibit these properties (63.6 parts per billion (ppb)) was less than levels that accumulate in the environment or humans (140 ppb in areas where DDT is still in use) (Kelce et al., 1995). Male rats exposed lactationally to ϒ-HCH had reduced testosterone levels at puberty (Dalsenter, Faqi, Merker, & Chahoud, 1997). The testicular weight, sperm, and spermatid number were also

reduced. The suggested mechanisms of action are through lindane's a) direct action on the testis, or b) interference of hormonal regulation of testis (Dalsenter et al., 1997).

Organochlorines and puberty

Age at menarche

As part of a reproductive health study, a cross sectional study in China collected measurements of DDT in blood and recalled age at menarche from newly married women (Ouyang et al., 2005). Women in the highest quartile of DDT and p,p'-DDE concentrations had a lower mean age at menarche compared to women in the lowest quartile concentrations (Ouyang et al., 2005). Vasiliu et al. (2004) used data from several Michigan anglers and fish eaters surveys to evaluate the association between DDE and early menarche. Using backwards extrapolated serum concentrations to estimate DDE concentrations during pregnancy, the study results showed that *in utero* exposure to DDE lowers age at menarche. A DDE increase of 15µg/l, reduced age at menarche by 1 year (Vasiliu et al., 2004). After controlling for BMI at the age at menarche, this association was no longer statistically significant.

Another study found no association between organochlorines and age at menarche (Denham et al., 2005). Mohawk Nation girls aged 10 to 16.9 years were assessed for menarche status and their blood samples assessed for DDE, HCB, Mirex and other compounds. No association was found between p,p'-DDE, HCB or Mirex and menarcheal status (Denham et al., 2005).

Pubic hair and breast development

Krstevska-Konstatinova et al. (2001) found that immigrant girls from developing countries that had earlier breast development had higher plasma DDE. In this Belgian study of

children with precocious puberty, blood samples for girls with Tanner breast stage 2 before the age of 8 were measured for p,p'-DDE. The immigrant girls had higher plasma levels of p,p'-DDE.

Other studies have not shown an association between organochlorine pesticides and breast or pubic hair development (Gladen et al., 2000; Wolff et al., 2008). A North Carolina cohort study measured transplacental and lactational DDE exposures, and Tanner breast and pubic hair development in 316 girls (Gladen et al., 2000). The study results showed that girls with higher *in-utero* p,p'-DDE concentrations tended to mature earlier, but this association was not statistically significant. A New York City study of 9 year old girls did not show any association between breast or pubic hair development and DDE measured in blood (Wolff et al., 2008). In the agricultural region of Menderes in Turkey, p,p'-DDE measured in serum and adipose tissue of 94 girls was not associated with premature breast development (Ozen et al., 2012).

Summary

Several studies have measured exposure to organochlorines in populations, but few of these are in the United Kingdom. Of the studies that have used biomonitoring measurements of organochlorines, few have analyzed *in utero* exposures to organochlorines, and only 2 have examined *in utero* exposures and the association with puberty. Studies that have analyzed the association between puberty and organochlorine exposure show conflicting results of association. The studies also use different measurements of puberty such as pubic hair development, breast development or onset of menarche. More studies are needed to better

understand the association between organochlorine exposure and onset of puberty, and the mechanisms through which EDCs induce early puberty.

Table2.1 Summary of studies associating biomonitoring measurements of organochlorine pesticides (OCPs) with puberty outcomes

Citation	Purpose	Study Population	OCP	Study Design	Findings
Vasiliu, O., Muttineni, J., et al. (2004). In utero exposure to organochlorines and age at menarche. Human Reproduction, Vol 19	To examine in utero exposure to PCBs and DDE on age at menarche in offspring	N=151 girls; Michigan angler cohort fish-eating mothers	DDE	Retrospective cohort; backward extrapolation of serum concentrations at pregnancy; retrospective age at menarche	Reduced age at menarche by 1 year with increase in 15µg/l of DDE in utero
Gladen, B. C., Ragan, N.B, et al. (2000).Pubertal growth and development and prenatal and lactational exposure to PCBs and DDE. The Journal of Pediatrics, Vol 136	To study whether prenatal or lactational exposures to background levels of PCBs or DDE were associated with altered pubertal growth and development in humans	N=316 girls; North Carolina infant feeding study	DDE	Prospective cohort; maternal milk and serum concentrations; pubertal questionnaire	No association with puberty – menarche, breast or pubic hair development
Krestevska-Konstatinova, M., Charlier, C. et al. (2001), Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. Human Reproduction, Vol 16	To evaluate epidemiological and auxological data of foreign children seen with precocious puberty in Belgium	N= 41. 26 immigrant girls and 15 native Belgian girls	DDE	Retrospective auxological study; patients interview and physical examination; serum measurements	Higher levels of plasma DDE in immigrant girls with earlier breast development
Ouyang, F., Perry, M.J. et al. (2005). Serum DDT, age at menarche, and abnormal menstrual cycle length. Occup Environ Med, Vol	To investigate age of menarche and abnormal menstrual cycle length in relation to serum DDT concentrations in a	N=466 Chinese female textile workers	DDT/ DDE	Prospective cohort; Recalled age at menarche; serum analyzed for DDT	Girls in 4th DDT/DDE quartile had lower mean age at menarche

62	cohort of Chinese women.				
Denham, M., Schell, L. et al. (2005). Relationship of Lead, Mercury, Mirex, DDE, HCB, and PCBs to Timing of Menarche Among Akwesasne Mohawk Girls. Pediatrics, Vol 115	To examine the relationship between attainment of menarche and levels of 6 environmental pollutants to which children are commonly exposed at low levels	N=138 girls, 10-16.9 years; Mohawk nation	DDE, HCB, Mirex	Cross-sectional; Interview (menarche yes/ no); blood sample taken.	No association between OCPs and early menarche
Wolff, M.S., Britton, JA. et al. (2008). Environmental Exposures and Puberty in Inner-City Girls. Environmental Research, Vol 107	To examine pubertal stages in relation to hormonally active environmental factors, including exposure biomarkers	N=192 multi ethnic inner city girls in NYC;	DDE	Cross-sectional; Recruited from hospital; serum measurements; pediatrician assessed puberty stage	No association with breast stage or pubic hair development
Ozen, S., Darcan, S. et al. (2012) Effects of pesticides used in agriculture on the development of precocious puberty. Environmental Monitoring and Assessment, Vol 184	To evaluate the effects of pesticides on premature breast development	N=94 girls; Turkey	DDT/ DDE	Case-control; serum and adipose tissue measurements; pediatrician assessed puberty examination	No association with breast development

ATRAZINE

This section of the literature review examines literature related to atrazine pesticide exposures, health effect studies, and endocrine disrupting mechanisms of action.

Introduction

Atrazine herbicide is used in over 70 countries worldwide; most frequently in the United States, Brazil, Argentina, Mexico and China (LeBaron, H., McFarland, J. & Burnside, O., 2008). Atrazine is not naturally occurring, and is manufactured in the laboratory (ATSDR, 2003). It is applied on crops, golf courses and residential lawns to control weeds (ATSDR, 2003; EPA, 2012). The European Union (E.U.) banned the use of atrazine in 2003 because of its presence throughout the environment above the E.U. recommended limit of pesticide residue in drinking water of 0.1 parts per billion (ppb), and the inability to prevent it contaminating the environment when used as a herbicide (Sass & Colangelo, 2006). Atrazine is designated as a restricted use pesticide in the United States, and is not available to the general public for use (EPA, 2012).

Atrazine metabolites and exposure studies

Exposure to atrazine can occur orally, by inhalation or via dermal contact (ATSDR, 2003; EPA, 2012). Water run-off from crops and lawn applications can get into ground and surface waters and contaminate drinking water wells (ATSDR, 2003; EPA, 2012). Atrazine is the most frequently detected pesticide in surface water in the U.S. (EPA, 2012). Atrazine concentrations in air after application to crops can affect workers and those living near these areas (ATSDR, 2003; EPA, 2012). Exposure can also occur when coming into contact with

contaminated soils (ATSDR, 2003). The general population can be exposed to atrazine through contaminated drinking water seasonally when it is applied to crops (CDC, 2009).

Once atrazine is absorbed in the body, it is quickly broken down and excreted primarily in urine and to a lesser extent in the feces (ATSDR, 2003; Catenacci, Barbieri, Bersani, Cottica, & Maroni, 1993). Approximately 50% of atrazine is excreted in the first 8 hours, and it is completely excreted in 24–48 hours (Catenacci et al., 1993). In the environment, atrazine can persist in ground water for long periods (half-life >6 months) (ATSDR, 2003). Atrazine exposure is mostly detected by analyzing urine for the parent compound or one or more of its metabolites: diaminochlorotriazine (DACT), desisopropyl atrazine (DIA), desethylatrazine (DEA), atrazine mercapturate, desethylatrazine mercapturate and hydroxyl atrazine (ATZ-OH) (ATSDR, 2003).

In utero and infant exposures

Atrazine has been detected in maternal blood, umbilical cord plasma and breast milk in animal and human studies. A study of about 200 pregnant women in northern Manhattan or South Bronx, NY City collected umbilical cord blood samples from the women in their third trimester, as close to delivery as possible, and a sample of maternal blood 2 days postpartum (Whyatt et al., 2003). Atrazine was detected in 23% of the maternal blood samples and 20% of umbilical cord plasma samples (Whyatt et al., 2003). This exposure represented low-level, urban, residential exposure in minorities (Whyatt et al., 2003). In another study, 10 breast milk samples collected from a maternity hospital in France were measured for atrazine, DEA and DIA (Balduini et al., 2003). Atrazine was detected in 3 samples (2 of them below the limit of detection), while the other metabolite concentrations were below the limit of detection in all samples (Balduini et al., 2003).

In an animal study, atrazine and metabolites were measured in dam, fetus and neonate plasma, tissues (e.g., mammary and adrenal tissues), and milk samples (Fraites et al., 2011). DACT was the principle metabolite detected in the plasma and tissues of dam and fetus gestational exposure (Fraites et al., 2011). DACT was also the principle metabolite detected in milk, plasma and tissues of dam and neonate postnatal and lactational exposure (Fraites et al., 2011). Another animal study suggested that rat neonates may also be exposed to atrazine through lactational exposure (Rayner et al., 2004).

Occupational exposures

In a study of occupational exposure to atrazine during its industrial production in Italy, atrazine metabolites were measured in the following percentages in the workers urine: DACT (80%), DIA (10%), DEA (8%), and unmodified atrazine (1–2%) (Catenacci et al., 1993). Other occupational studies showed equal amounts of DIA and DACT excreted in urine of railway workers in Finland (Ikonen, Kangas, & Savolainen, 1988) or atrazine mercapturate as the principle metabolite most detected in the urine of farm pesticide applicators in Wisconsin (Perry et al., 2000) .

Farm household exposures

In other exposure studies, only one atrazine metabolite, atrazine mercapturate, was predominantly measured. A study by Curwin et al. (2007) examined pesticide exposure and farm and non-farm households in Iowa as part of the on-going National Institutes of Health (NIH) and EPA funded Agricultural Health Study (AHS). Atrazine mercapturate above the level of detection was detected in 23% of the urine samples, however when the concentrations below the

detection level were considered, atrazine mercapturate was detected in about 80% of the urine samples (Curwin et al., 2007). Another study, also part of the AHS, measured urinary metabolites of several pesticides, including atrazine, and exposure risk factors in farmworker children from 6 counties in North Carolina (Arcury et al., 2007). Atrazine mercapturate was detected in only 6.7% of the urine samples (Arcury et al., 2007).

A longitudinal study also within the AHS examined atrazine exposure in farm and non-farm men, and the background levels of non-persistent pesticides for 2 years in Iowa (Bakke et al., 2009). Urinary atrazine mercapturate levels were measured pre- and post-application during different farming seasons, and daily exposure information was recorded for each participant (Bakke et al., 2009). Atrazine above the level of detection was detected in 13% of farmers pre application and 42% of farmers post application, and in 5% of non-farmers pre application and 8%of non-farmers post application (Bakke et al., 2009).

Atrazine biomarkers

Barr et. al. (2007) showed that atrazine exposure studies measuring only atrazine mercapturate metabolite in urine may be underestimating exposure to atrazine. In their study, eight atrazine metabolites including atrazine mercapturate were measured in urine from 5 occupationally exposed, 8 low-level (non-occupationally) exposed and 11 environmentally exposed participants in Georgia (Barr, Panuwet, Nguyen, Udunka, & Needham, 2007). For the occupationally exposed, DACT (51%) was the predominantly detected metabolite followed by DEA (31%) (Barr et al., 2007). Atrazine mercapturate was detected in only 12% of the samples (Barr et al., 2007). For the low-level exposed, DEA was the most prominently detected metabolite (33%) followed by DACT (28%) (Barr et al., 2007). Atrazine mercapturate was

detected in 6% of the samples. For the environmental exposures, DACT was the predominant metabolite, and it was detected in 77% samples, while DEA and atrazine mercapturate were detected in 15% and 2% of the samples respectively (Barr et al., 2007). This study shows that different ratios of metabolites measured in urine correspond to the type of exposure. The study results also demonstrate that measuring atrazine mercapturate alone may misrepresent atrazine exposure in populations (Barr et al., 2007). However, the authors also state that "measuring atrazine compound or atrazine mercapturate in urine is the unequivocal indication that a person was exposed to atrazine and not an environment degradate".

Human health effect studies

To our knowledge, no studies have been done examining the association between atrazine exposure and puberty outcomes in humans. However, several studies have examined the association between atrazine exposure and other developmental and reproductive health outcomes. Atrazine exposure has been linked to these adverse birth outcomes: intrauterine growth retardation (IUGR)(Munger et al., 1997) , low birth weight (Munger et al., 1997; Ochoa-Acuna, Frankenberger, Hahn, & Carbajo, 2009), preterm delivery (Villanueva, Durand, Coutte´, Chevrier, & Cordier, 2005), small for gestational age (Ochoa- Acuna et al., 2009; Villanueva et al., 2005), spontaneous abortions (Arbuckle, Lin, & Mery, 2001), and several birth defects (Winchester, Huskins, & Ying, 2008), specifically abdominal wall defects (Mattix, Winchester, & Scherer, 2007). However, none of these studies used biomonitoring measurements of atrazine and its metabolites, and instead used proxy measures, for example, measurements in well water or surface water.

A more recent study examined the association between biomonitoring levels of atrazine and adverse birth outcomes in France. A prospective cohort study of urinary biomarkers of prenatal exposure to atrazine and adverse birth outcomes showed an association between atrazine exposure and small head circumference (SHC) and fetal growth restricted (FGR) (Chevrier et al., 2011). Women from Brittany, France, were enrolled in the study before the 19th week of gestation and urine samples collected (Chevrier et al., 2011). Live birth singletons were classified as FGR if their birth weight was below the 5th percentile of expected birth weight for the cohort (Chevrier et al., 2011). SHC was classified as head circumference below the 5th percentile of birth head circumference distribution for France (Chevrier et al., 2011). Congenital malformations were diagnosed by pediatricians (Chevrier et al., 2011). Twelve triazine pesticide compounds including atrazine and its metabolites were measured in the urine (Chevrier et al., 2011). Exposure to atrazine or one of its metabolites was significantly associated with SHC and FGR (Chevrier et al., 2011). Atrazine was also weakly associated with male genital anomalies observed in only 5 cases (Chevrier et al., 2011). Associations were not examined by specific metabolites.

Mechanisms of action

Atrazine is a suspected endocrine disruptor that affects the endocrine and reproductive systems (ATSDR, 2003). Most of these effects have been observed in animal studies (ATSDR, 2003). Several studies have been carried out on amphibians, rodents and other animals, and they have produced contradicting results regarding atrazine adverse effects and its mechanism of toxicity.

In several studies, atrazine delayed the onset of puberty in female and male rats (Laws et al., 2003; Laws et al., 2000; Rayner et al., 2005; Rayner et al., 2004; Stoker et al., 2002; Stoker et al., 2000). Female rats exposed prenatally to atrazine displayed a significant delay in puberty, i.e., delayed vaginal opening and mammary gland development (Rayner et al., 2005). In other studies, post-natal exposure to atrazine also delayed vaginal opening in female rats (Laws et al., 2003; Laws et al., 2000; Rayner et al., 2004). The results of these studies support the hypothesis that atrazine can delay puberty by altering the hypothalamic-pituitary activity (Laws et al., 2000). In Laws et al. (2003) DACT was suggested as the active metabolite of atrazine, and additionally, the findings showed that DACT can be as potent as the parent compound in delaying puberty (Laws et al., 2003). The suggested mechanism of action is altering hypothalamic-pituitary activity by suppressing luteinizing hormone secretion resulting in a delay in pubertal development (Laws et al., 2003).

Some studies have suggested that atrazine induces aromatase formation and converts testosterone to estradiol (Hayes et al., 2002; Roy, Chakraborty & Chakraborty, 2009). Atrazine exposure to African clawed frogs, Xenopus laevis resulted in gonadal abnormalities, hermaphroditism and demasculinized larynges (Hayes et al., 2002). The authors of this paper suggest that atrazine caused a disruption in steroidogenesis by inducing aromatase and promoted the conversion of testosterone to estrogen, resulting in hermaphroditism and demasculinized larynges (Hayes et al., 2002). Other studies have not been able to replicate these results (Du Preez et al., 2007; Kloas et al., 2008; Oka et al., 2008).

Previous studies have shown that atrazine exposure caused an earlier onset and increased incidence of mammary gland tumors in rats by causing changes in the ovarian function as reported by Cooper et al. (2007), in a review and summary of their studies on atrazine and the

reproductive function (Cooper et al., 2007). The studies support the hypothesis that the brain is the main target of atrazine exposure, where atrazine exposure disrupts the hypothalamic regulation of the pituitary therefore disrupting luteinizing hormone (LH) secretion (Cooper et al., 2007). Studies done later suggest that atrazine adverse effect on hypothalamic catecholamine systems is unlikely to be restricted to this particular region, but could affect brain catecholamine systems more generally and thus affect pathways critical to the control of movement and complex cognitive functions (Rodriguez, Thiruchelvam, & Cory-Slechta, 2005). In a study by Rodriguez et al. (2005), male rats showed increased locomotor activity after 3 and 6 months of atrazine exposure (Rodriguez et al., 2005). The results of this study may indicate that atrazine has an effect on the brain monoamine systems and thus on behavioral functions (Rodriguez et al., 2005).

Summary

Few studies have measured atrazine exposure in the general population, and only one of these in Europe. Few studies have examined the association between atrazine exposure and adverse health outcomes in humans. Those that have are predominantly retrospective studies. These retrospective studies used a proxy measure for atrazine exposure, for example drinking water concentrations, to determine exposure. These proxy measures do not ascertain human exposure, and such studies are usually pilot or preliminary studies whose results need further validation, for example, using biomonitoring pesticide measurements to assess exposures. This next validation step has only been examined by one study in France, presented above, that measured atrazine levels in the urine of pregnant women and associated these with birth

outcomes. To our knowledge, no studies have examined the association between atrazine exposure and puberty outcomes in humans.

Table2.2 Summary of atrazine exposure and metabolite profile studies

Citation	Purpose	Population	Urine Metabolites	Statistics Reported
Occupational Exposures				
Ikonen, R., J. Kangas., et. al. (1988).Urinary atrazine metabolites as indicators for rat and human exposure to atrazine. Toxicology letters, Vol 44	Show differences in rat and human metabolism of atrazine by measuring urinary excretion of metabolites using a biological monitoring strategy	N=6 railway workers weeding railway; Finland	DIA (50%) DACT (50%)	Mean
Catenacci, G., Barbieri, F., et al. (1993)Biological monitoring of human exposure to atrazine. Toxicology Letters, Vol 69	Investigate the human exposure to atrazine during industrial production by means of assessment of ambient exposure and determination of atrazine dealkylated metabolites in urine	N=6 atrazine manufacturing workers; Italy	DACT (80%) DIA (10%) DEA (8%) ATZ (2%)	Mean
Barr, D., Panuwet, P. et al. (2007). Assessing exposure to atrazine and its metabolites using biomonitoring. Environmental health perspectives, Vol 115	To evaluate multiple metabolites of atrazine in persons exposed in occupational and environmental scenarios; to evaluate if one metabolite was sufficient to estimate atrazine exposure, or several accurately assess exposure	N = 24. 5 applicators, 8 non-applicators, 11 volunteers; GA, US	DACT (51; 48; 77%) DEA (31; 43; 15%) DIA (5; 2; 6%) AM (12; 8; 2%)	% detected
Agricultural Exposures				
Perry M.J., Christiani,	Compare three lab	N = 120. 99 farm	DEA 37 %	Mean;

D.C. et al. (2000). Urinalysis of atrazine exposure in farm pesticide applicators. Toxicology and Industrial health, Vol 16	methods for detection of atrazine and related metabolites in urine	applicators and 21 non-applicators in WI	(0% in non-applicators); AM 80% (50% in non-applicators) Triazines – 100% (18 % in non-applicators)	Range
Curwin, B., Hein, M.J. et al. (2007). Urinary Pesticide Concentrations Among Children, Mothers and Fathers Living in Farm and Non-Farm Households in Iowa. Ann. Occup. Hyg, Vol 51	To measure urinary pesticide levels among farm and non-farm families in Iowa and ascertain what factors influence these levels	N = 212. 114 farm and 98 non-farm in IA	AM (23%)	Geometric mean; Range
Arcury, T., Grzywacz, J.G. et al. (2007). Pesticide Urinary Metabolite Levels of Children in Eastern North Carolina Farmworker Households. Environmental Health Perspectives, Vol 15	To provide urinary concentrations of pesticide metabolites in children living in farm households in eastern NC and the number of metabolites for each child; delineate the risk associated with this exposure	N = 60 farmworker children in NC	AM (6.7%)	Median; Geometric mean
Bakke, B., De Roos, A., et al. (2009) Exposure to atrazine and selected non-persistent pesticides among corn farmers during a growing season. Journal of exposure science and environmental epidemiology, Vol 19	To estimate farmers exposure to atrazine and provide background levels of selected non-persistent pesticides	N = 38. 28 farm and 10 non-farm men in IA	AM 58% (6% of non-farm men)	10th & 90th percentiles; Geometric mean
Mendas, G., Vuletic, M. (2012). Urinary metabolites as biomarkers of human exposure to atrazine:	To investigate the levels of ATZ and AM in agricultural workers during ATZ application	N= 11 agricultural workers	ATZ 0% AM 30% pre- & 100% post	Median; Range

Atrazine mercapturate in agricultural workers. Toxicology Letters, Vol 210			application	
Population Exposures				
Chevrier, C., Limon, G., et al. (2011). Urinary biomarkers of prenatal atrazine exposure and adverse birthoutcomes on the PELAGIE birth cohort. Environmental Health Perspectives, Vol 119	To assess the association between urinary metabolites of prenatal exposure to atrazine and adverse birth outcomes, taking into account other herbicides	N = 579 pregnant women; France	ATZ (2%) AM (4%) DEAM (10%) ATZ-OH (10%) DACT (7%) DEA-OH (5%) DIA (1%)	50th & 95th percentiles

REFERENCES

Adair, L. (2001). Size at Birth Predicts Age at Menarche. *Pediatrics, 107*.

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2002). Toxicological Profile for DDT, DDE, and DDD.

Agency for Toxic Substances Disease Registry, A (ATSDR). (2003). Toxicological Profile for Atrazine. Retrieved October 26th, 2013, from http://www.atsdr.cdc.gov/toxprofiles/tp153.pdf

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2005). Toxicological Profile for Alpha-, Beta-, Gamma-, and Delta-Hexachlorocyclohexane.

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2013). Draft Toxicological Profile for Hexachlorobenzene.

Aksglaede, L., Olsen, L.W., Sorensen, T., Petersen, J., Juul, A. (2008). Forty Years Trends in Timing of Pubertal Growth Spurt in 157,000 Danish School Children. *PLoS ONE, 3(7).*

Aksglaede, L., Sorensen, K., Petersen, J. H., Skakkebaek, N. E., Juul, A. (2009). Recent Decline in Age at Breast Development: The Copenhagen Puberty Study. *Pediatrics, 123.*

Allsworth, J., Weitzen., S., Boardman, L. (2005). Early Age at Menarche and Allostatic Load: Data from the Third National Health and Nutrition Examination Survey. *Annals of Epidemiology, 15(6).*

Alvarez, L., Randi, A., Alvarez, P., Piroli, G., Chamson-Reig, A., Lux-Lantos, V., & Kleiman de Pisarev, D. (2000). Reproductive effects of hexachlorobenzene in female rats. Journal of Applied Toxicology, 20, 81-87.

Arbuckle, T., Lin, Z., & Mery, L. (2001). An Exploratory Analysis of the Effect of Pesticide Exposure on the Risk of Spontaneous Abortion in an Ontario Farm Population. *Environ Health Perspect, 109*, 851-857.

Arcury, T., Grzywacz, J., Barr, D., Tapia, J., Chen, H., & Quandt, S. (2007). Pesticide Urinary Metabolite Levels of Children in Eastern North Carolina Farmworker Households. *Environ Health Perspect, 15(7).*

Bakke, B., De Roos, A., Barr, D., Stewart, P., Blair, A., Freeman, L., & al., e. (2009). Exposure to atrazine and selected non-persistent pesticides among corn farmers during a growing season. *Journal of Exposure Science and Environmental Epidemiology, 19*, 544-554.

Balduini, L., Matoga, M., Cavalli, E., Seilles, E., Riethmuller, D., Thomassin, M., & Guillaume, Y. (2003). Triazine herbicide determination by gas chromatography-mass spectrometry in breast milk. *Journal of Chromatography B*, 389-395.

Barr, D., Panuwet, P., Nguyen, J., Udunka, S., & Needham, L. (2007). Assessing exposure to atrazine and its metabolites using biomonitoring. *Environ Health Perspect, 115(10).*

Bau, A. M., Ernert, A., Schenk, L., Wiegand, S., Martus, P., Gruters, A., Krude, H. (2009). Is there a further acceleration in the age at onset of menarche? A cross-sectional study in 1840 school children focusing on age and bodyweight at the onset of menarche. *European Journal of Endocrinology, 160*, 107-113.

Biro, F. M., Galvez, M. P., Greenspan, L.C., Succop, P.A., Vangeepuram, N., Pinney, S.M., … Wolff, M.S. (2010). Pubertal Assessment Method and Baseline Characteristics in a Mixed Longitudinal Study of Girls. *Pediatrics, 126*.

Boyd, A., Golding, J., Macleod, J., Lawlor, D. A., Fraser, A., Henderson, J., … Smith, G. (2013). Cohort Profile: The 'Children of the 90s' - The Avon Longitudinal Study of Parents and Children. *International Journal of Epidemiology, 42(1).*

Bristol University. (2014). Avon Longitudinal Study of Parents and Children. Retrieved May 5th, 2014 from http://www.bristol.ac.uk/alspac/researchers/

Castellino, N., Bellone, S., Rapa, A., Vercellotti, A., Binotti, M., Petri, A. Bona, G. (2005). Puberty onset in Northern Italy: A random sample of 3597 Italian children. *J. Endocinol. Invest., 28*, 589-594.

Catenacci, G., Barbieri, F., Bersani, A., Cottica, D., & Maroni, M. (1993). Biological monitoring of human exposure to atrazine. *Toxicol Lett, 69*, 217-222.

Centers for Disease Control and Prevention, National Center for Health Statistics (CDC). (2009). The Fourth National Report on Human Exposure to Environmental Chemicals.

Centers for Disease Control and Prevention, Health Studies Branch. (2012) ALSPAC Puberty Project Documentation.

Chevrier, C., Limon, G., Monfort, C., Rouget, F., Garlantezec, R., Petit, C., … Cordier, S. (2011). Urinary biomarkers of prenatal atrazine exposure and adverse birthoutcomes on the PELAGIE birth cohort. *Environ Health Perspect, 119(7).*

Christensen, K., Maisonet, M., Rubin, C., Holmes, A., Calafat, A., Kato, K., & al., e. (2011). Exposure to polyfluoroalkyl chemicals during pregnancy is not associated with offspring age at menarche in a contemporary British cohort. Environment International, 37, 129 - 135.

Chumlea, W. C., Schubert, C., Roche, A.F., Kulin, H. E., Lee, P.A., Himes, J.H., Sun, S.S. (2003). Age at Menarche and Racial Comparisons in US Girls. *Pediatrics, 111.*

Cooper, R.L., Laws S.C., Das, P.C., Narotsky, M.G., Goldman, J.M., Tyrey, E.L., & Stoker, T.E. (2007). Atrazine and reproductive function: mode and mechanism of action studies. *Birth Defects Research, 80*, 98-112.

Cooper, R. G., Chadwick, R., Rehnberg, G., Goldman, J., Booth, K., Hein, J., & McElroy, W. (1989). Effect of lindane on hormonal control of reproductive function in the female rat. Toxicology and Applied Pharmacology, 99(3), 384-394.

Covaci, A., Jorens, P., Jacquemyn, Y., & Schepens, P. (2002). Distribution of PCBs and organochlorine pesticides in umbilical cord and maternal serum. *The Science of the Total Environment, 298*, 45-53.

Crain, D., Janssen, S., Edwards, T.M., Heindel, J., Ho, S., Hunt, P., … Guillette, L.J. (2008). Female reproductive disorders: the roles of endocrine disrupting compounds and developmental timing. *Fertility and Sterility, 90*, 911–940.

Curwin, B., Hein, M., Sanderson, W., Stirley, C., Heederick, D., Kromhout, H., .., Alavanja, M.C. (2007). Urinary Pesticide Concentrations Among Children, Mothers and Fathers Living in Farm and Non-Farm Households in Iowa. *Ann. Occup. Hyg, 51(1)*, 53–65.

Dalsenter, P., Faqi, A., Merker, J., & Chahoud, I. (1997). Reproductive toxicity and toxicokinetics of lindane in the male offspring of rats exposed during lactation. Human and Reproductive Toxicology, 16, 146-153.

Demerath, E., Towne, B., Chumlea, C., Sun, S., Czerwinski, S., Remsberg, K., Siervogel, R. (2004). Recent Decline in Age at Menarche: The Fels Longitudinal Study. American *Journal of Human Biology, 16*, 453-457.

Denham, M., Schell, L. M., Deane, G., Gallo, M. V., Ravenscroft, J., & DeCaprio, A. P. (2005). Relationship of lead, mercury, mirex, dichlorodiphenyldichloroethylene,

hexachlorobenzene, and polychlorinated biphenyls to timing of menarche among Akwesasne Mohawk girls. *Pediatrics, 115(2)*, e127-134. doi: 10.1542/peds.2004-1161

Du Preez, L., Kunene, N., Everson, G., Carr, J., Giesy, J., Gross, T., . . . Van Der Kraak, G. (2007). Reproduction, larval growth , and reproductive development in African clawed frogs (Xenopus laevis) exposed to atrazine. *Chemosphere, 71*.

Duarte-Davidson, R., Wilson, S., Jones, K. (1994). PCBs and other Organochlorines in Human Tissue Samples from the Welsh Population: I-Adipose. *Environmental Pollution, 84*, 69 - 77.

Environmental Protection Agency (EPA). (2012). Atrazine background fact sheet. Retrieved October 26th, 2013, from http://www.epa.gov/pesticides/factsheets/atrazine_background.htm

Euling, S., Herman-Giddens, M.E., Lee, P., Selevan, S., Juul, A., Sorensen, T., . . . Swan, S.H. (2008). Examination of US Puberty-Timing Data from 1940 to 1994 for Secular Trends: Panel Findings. *Pediatrics, 121*.

Foster, W., Pentick, J., McMahon, A., & Lecavalier, P. (1992). Ovarian toxicity of hexachlorobenzene (HCB) in the superovulated female rat. Journal of biochemical toxicology, 7(1), 1-4.

Fraites, M., Narotsky, M., Best, D., Stoker, T., Davis, L., Goldman, J., . . . Cooper, R.L.. (2011). Gestational atrazine exposure: effects on male reproductive development and metabolite distribution in the dam, fetus and neonate. *Reproductive Toxicology, 32*, 52-63.

Fraser, A., Macdonald-Wallis, C., Tilling, K., Boyd, A., Golding, J., Smith, G., … Lawlor, D. (2013). Cohort Profile: The Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. *International Journal of Epidemiology, 42(1)*.

Freedman, D. A., Khan, L., Serdula, M., Dietz, W., Srinivasan, S., & Berenson, G. (2003). The relation of menarcheal age to obesity in childhood and adulthood: the Bogalusa heart study. *Pediatrics, 110(4).*

Fukata, H., Omori, M., Osada, H., Todaka, E., & Mori, C. (2005). Necessity to measure PCBs and organochlorine pesticide concentrations in human umbilical cords for fetal exposure assessment. *Environ Health Perspect, 113(3),* 297-303.

Gerhard, I., Daniel, V., Link, S., Monga, B., & Runnebaum, B. (1988). Chlorinated Hydrocarbons in Women with Repeated Miscarriages. *Environmental Health Perspectives, 6(10).*

Gladen, B., Ragan, B., & Rogan, W. (2000). Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. *The Journal of Pediatrics, 136(4).*

Golub, M., Colluman, G., Foster, P., Kimmel, C. A., Rajpert-De Meyts, E., Reiter, E., … Toppari, J. (2008). Public Health Implications of Altered Puberty Timing. *Pediatrics, 121*.

Graber, J., Brooks-Gunn, J., & Warren, M. (1995). The antecedents of menarcheal age: heredity, family environment, and stressful life events. *Child Development, 66,* 346-359.

Harris, C. A., O'Hagan, S., Merson, G. HJ. (1999). Organochlorine pesticide residues in human milk in the United Kingdom 1997 - 8. *Human & Experimental Toxicology, 18,* 602 - 606.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A.A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicise atrazine at low ecologically relevant doses. *Proceedings of the national academy of sciences of the United States of America, 99(8).*

Ikonen, R., Kangas, J., & Savolainen, H. (1988). Urinary atrazine metabolites as indicators for rat and human exposure to atrazine. *Toxicol Lett, 44*, 109-112.

Jaraczewska, K., Lulek, J., Covaci, A., Voorspoels, S., Kaluba-Skotarczak, A., Drews, K., & Schepens, P. (2006). Distribution of polychlorinated biphenyls, organochlorine pesticides and polybrominated diphenyl ethers in human umbilical cord serum, maternal serum and milk from Wielkopolska region, Poland. *Sci Total Environ, 372(1),* 20-31. doi: 10.1016/j.scitotenv.2006.03.030

Jung, D., Becher, H., Edler, L., Flesch-Janys, D., Gurn, P., Konietzko, J., … Papke, O. (1997). Elimination of β-hexachlorocyclohexane in occupationally exposed persons. *Journal of Toxicology & Environmental Health, 51*, 23-34.

Kalantzi, O. M., Martin, F., Thomas, G., Alcock, R., Tang, H., Drury, S., … Jones, K.C. (2004). Different Levels of Polybrominated Diphenyl Ethers (PBDEs) and Chlorinated Compounds in Breast Milk from Two U.K. Regions. *Environmental Health Perspectives, 112(10).*

Kaprio, J., Rimpela, A., Winter, T., Viken, R., et al. (1995). Common genetic influences on BMI and age at menarche. *Hum Biol, 67*, 739-753.

Keim, S. A., Branum, A., Klebanoff, M., & Zemel, B. (2009). Maternal body mass index and daughters' age at menarche. *Epidemiology, 20(5),* 677-681.

Kelce, W., Stone, C., Laws, S., Earl Gray, L., Kemppainen, J., Wilson, E. (1995). Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature, 375(15).*

Kloas, W., Lutz, I., Springer, T., Krueger, H., Wolf, J., Holden, L., & Hosmer, A. (2008). Does atrazine influence larval development and sexual differentiation in Xenopus laevis? *Toxicological Sciences, 107(2).*

Krstevska-Konstantinova, M., Charlier, C., Craen, M., Du Caju, M., Heinrichs, C., de Beaufort, C., . . . Bourguignon, J. P. (2001). Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. *Hum Reprod, 16(5),* 1020-1026.

Laws, S.C., Ferrell, J.M., Stoker, T.E., Schmid, J., & Cooper, R.L. (2000). The effects of atrazine in female wistar rats: An evaluation of the protocol for assessing pubertal development and thyroid function. *Toxicological Sciences, 58*, 366-376.

Laws, S., Ferrell, J., Stoker, T., & Cooper, R. (2003). Pubertal development in female wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-s-chlorotriazine and hydroxyatrazine. *Toxicological Sciences, 76*, 190-200.

Link, B., Gabrio, T., Zoellner, I., Piechotowski, I., Paepke, O., Herrmann, T., … Wuthe, J. (2005). Biomonitoring of persistent organochlorine pesticides, PCDD/PCDFs and dioxin-like PCBs in blood of children from South West Germany (Baden-Wuerttemberg) from 1993 to 2003. *Chemosphere, 58,* 1185-1201.

LeBaron, H., McFarland, J. & Burnside, O. (Eds.). The Triazine Herbicides; 50 Years Revolutionizing Agriculture. 2008

Mattix, K., Winchester, P., & Scherer, L. (2007). Incidence of abdominal wall defects is related to surface water atrazine and nitrate levels. *Journal of Pediatric Surgery, 42*.

McDowell, M. A., Broody, D.J., Hughes, J. (2007). Has Age at Menarche Changed? Results from the National Health and Nutrition Examination Survey (NHANES) 1999–2004. *Journal of Adolescent Health, 40,* 227-231.

Munger, R., Isacon, P., Hu, S., Burns, T., Hanson, J., Lynch, C., … Hausler, W. (1997). Intrauterine Growth Retardation in Iowa Communities with Herbicide-contaminated Drinking Water Supplies. *Environmental Health Perspectives, 105(3),* 308–314.

Ochoa- Acuna, H., Frankenberger, J., Hahn, L., & Carbajo, C. (2009). Drinking-water herbicide exposure in Indiana and prevalence of small for gestational age and preterm delivery. *Environ Health Perspect, 117(10).*

Oka, T., Tooi, O., Mitsui, N., Miyahara, M., Ohnischi, Y., Takase, M., … Iguchi, N.S. (2008). Effect of atrazine in metamorphosis and sexual differentiation in Xenopus laevis. *Aquatic toxicology, 87(8),* 215-226.

Ouyang, F., Perry, M., Venners, S., Chen, C., Wang, B., Yang, F.,… Wang, X. (2005). Serum DDT, age at menarche, and abnormal menstrual cycle length. *Occupational & Environmental Medicine, 62*, 878–884.

Ozen, S., Darcan, S., Bayindir, P., Karasulu, E., Simsek, D. G., & Gurler, T. (2012). Effects of pesticides used in agriculture on the development of precocious puberty. *Environmental Monitoring and Assessment, 184(7)*, 4223-4232. doi: http://dx.doi.org/10.1007/s10661-011-2257-6

Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, N., Toppari, J., Bourguignon, J.P. (2003). The Timing of Normal Puberty and the Age Limits of Sexual Precocity: Variations around the World, Secular Trends, and Changes after Migration. *Endocrine Reviews, 24(5)*, 668-693.

Perry, M.J., Christiani, D.C., Mathew, .J, Degenhardt, D., Tortorelli, J., Strauss, J., & Sonzogni, W.C. (2000). Urinalysis of atrazine exposure in farm pesticide applicators. *Toxicology and Industrial health, 16(7),* 285-290.

Raiser, G., Toppari, J., Parent, A., & Bourguignon, J. (2006). Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: A review of rodent and human data. *Molecular and Cellular Endocrinology*, *254-255*, 187-201.

Raiser, G., Parent, AS., Gerard, A. Lebrethon, MC. Bourguignon, JP. (2007). Early Maturation of Gonadotropin-Releasing Hormone Secretion and Sexual Precocity after Exposure of Infant Female Rats to Estradiol or Dichlorodiphenyltrichloroethane. *Biology of Reproduction, 77,* 734-742.

Raizada, R., Misra, P., Saxena, I., Datta, K. K., & Dikshith, T. (1980). Weak estrogenic activity of lindane in rats. Journal of Toxicology and Environmental Health, 6, 483-492.

Rayner, J., Enoch, R., & Fenton, S. (2005). Adverse effects of prenatal exposure to atrazine during a critical period of mammary gland growth. *Toxicological Sciences, 87(1),* 255-266.

Rayner, J., Wood, C., & Fenton, S. (2004). Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. *Toxicol Appl Pharmacol, 195*, 23-34.

Rimpela, A., & Rimpela, M. (1993). Towards an equal distribution of health? Socioeconomic and regional differences of the secular trend of the age of menarche in Finland from 1979 to 1989. *Acta Paediatr, 82*, 87-90.

Rodriguez, V., Thiruchelvam, T., & Cory-Slechta, D. (2005). Sustained exposure to the widely used herbicide atrazine: altered function and loss of neurons in brain monoamine systems. *Environ Health Perspect, 113(6).*

Rogan, W. J., & Chen, A. (2005). Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). *The Lancet, 366(9487),* 763-773. doi: 10.1016/S0140-6736(05)67182-6

Rosenfield, R. L., Lipton, R.B., Drum, M.L. (2009). Thelarche, Pubarche, and Menarche Attainment in Children With Normal and Elevated Body Mass Index. *Pediatrics, 123*.

Roy, J., Chakraborty, S., Chakraborty, T.R. (2009). Estrogen-like endocrine disrupting chemicals affecting puberty in humans - a review. *Med Sci Monit, 15(6).*

Rubin, C., Maisonet, M., Kieszak, S., Monteilh, C., Holmes, A. K., Flanders, D., . . . al., e. (2009). Timing of maturation and predictors of menarche in girls enrolled in a contemporary British cohort. *Paediatr Perinat Epidemiol, 23,* 492-504.

Ruder, E., Hartman, T., Rovine, M., & Dorgan, J. (2010). Birth characteristics and age at menarche: results from the dietary intervention study in children (DISC). *Cancer Causes Control, 21,* 1379-1386.

Sass, J. B., & Colangelo, A. (2006). European Union bans atrazine, while the United States negotiates continued use. *Int J Occup Environ Health, 12(3),* 260-267.

Saxena, M., Siddiqui, M.K.J., Bhargava, A.K., Krishna Murti, C.R. Kutty, D. (1981). Placental Transfer of Pesticides in Humans. *Archives of Toxicology, 48*, 127-134.

Saxena, M., Siddiqui, M.K.J., Seth, T.D., & Murti, K. (1981). Organochlorine Pesticides in Specimens from Women Undergoing Spontaneous Abortion, Premature or Full-Term Delivery. *Journal of Analytical Toxicology, 5*.

Semiz, S, Kurt, F., Kurt, DT., Zencir, M., Sevinc, O. (2008). Pubertal development of Turkish children. *Journal of Pediatric Endocrinology & Metabolism, 21*, 951-961.

Shen, H., Main, K. M., Virtanen, H. E., Damggard, I. N., Haavisto, A. M., Kaleva, M., . . . Schramm, K. W. (2007). From mother to child: investigation of prenatal and postnatal exposure to persistent bioaccumulating toxicants using breast milk and placenta biomonitoring. *Chemosphere, 67(9)*, S256-262. doi: 10.1016/j.chemosphere.2006.05.106

Siddiqui, M.K.J., Srivastava, S., Mehrotra, P.K., Mathur, N., Tandon, I. (2003). Persistent chlorinated pesticides and intra-uterine foetal growth retardation: a possible association. *Int Arch Occup Environ Health, 76*, 75-80.

Smith, D. (1999). Worldwide Trends in DDT Levels in Human Breast Milk. *International Journal of Epidemiology, 28,* 179-188.

Steinmetz, R., Young, P., Caperell-Grant, A., Gize, E., Madhukar, B., Ben-Jonathan, N., & Bigsby, R. (1996). Novel estrogenic action of the pesticide residue β-hexachlorocyclohexane in human breast cancer cells. Cancer Research, 56, 5403-5409.

Stoker, T., Guidici, D., Lawson, G., & Cooper, R. (2002). The effects of atrazine metabolites on puberty and thyroid function in the male wistar rat. *Toxicological Sciences, 67*, 198-206.

Stoker, T., Lawson, G., Guidici, D., & Cooper, R. (2000). The effect of atrazine on puberty in male wistar rats: an evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicological Sciences, 58*, 50-59.

Teilmann, G., Pedersen, C., Jensen, TK., Skakkebaek, N., Juul, A. (2005). Prevalence and Incidence of Precocious Pubertal Development in Denmark: An Epidemiologic Study Based on National Registries. *Pediatrics, 116.*

The Stockholm Convention. (2008). The 12 initial POPs under the Stockholm Convention. Retrieved February 15th, 2014

Tomczak, S., Baumann, K., Lehnert, G. (1981). Occupational Exposure to Hexachlorocyclohexane IV. *Int Arch Occup Environ Health, 48*, 283-287.

United Nations Environment Programme (UNEP), & World Health Organization (WHO). (2012). State of the Science of Endocrine Disrupting Chemicals- 2012.

van Velsen, F., Danse, L., Leeuwen, F., Dormans, J. P., & van Logten, M. (1986). The subchronic oral toxicity of the β-isomer of hexachlorocyclohexane in rats. Fundamental and Applied toxicology, 6, 697-712.

Vasiliu, O., Muttineni, J., & Karmaus, W. (2004). In utero exposure to organochlorines and age at menarche. *Hum Reprod, 19(7), 1506-1512*. doi: 10.1093/humrep/deh292

Villanueva, C., Durand, G., Coutte´, M.-B., Chevrier, C., & Cordier, S. (2005). Atrazine in municipal drinking water and risk of low birthweight, preterm delivery, and small-for-gestational-age status. *Occup Environ Med, 62*, 400-405.

Waliszewski, S., Aguirre, A., Infanzon, R., & Siliceo, J. (2000). Carry-over of persistent organochlorine pesticides through placenta to fetus. *salud pública de méxico, 42(5).*

Walvoord, E. (2010). The Timing of Puberty: Is it Changing? Does it Matter? *Journal of Adolescent Health, 47*, 433-439.

Wasserman, M., Ron, M., Bercovici, B., Wasserman, D,Cucos, S., Pines, A. (1982). Premature Delivery and Organochlorine Compounds: Polychlorinated Biphenyls and some Organochlorine Insecticides. *Environmental Research, 28*, 106-112.

Whyatt, R., Barr, D., Camann, D., Kinney, P., Barr, J., Andrews, H., . . . Perera, F. (2003). Contemporary-use pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns. *Environmental Health Perspectives, 111(5),* 749-756.

Winchester, P., Huskins, J., & Ying, J. (2008). Agrichemicals in surface water and birth defects in the United States. *Acta Pædiatrica, 98*, 664-669.

Wolff, M. S., Britton, J. A., Boguski, L., Hochman, S., Maloney, N., Serra, N., & Liu, Z. (2008). Environmental Exposures and Puberty in Inner-City Girls. *Environmental Research, 107(3)*, 393.

Wolff, M.S., Zeleniuch-Jacquotte, A., Dubin, N., Toniolo, P. (2000). Risk of Breast Cancer and Organochlorine Exposure. *Cancer Epidemiology, Biomarkers & Prevention, 9*, 271-277.

Wronka, I., & Pawlinska-Chmara, R. (2005). Menarcheal age and socio-economic factors in Poland. *Annals of Human Biology, 32(5),* 630-638.

CHAPTER 3

IN UTERO EXPOSURE TO ORGANOCHLORINE PESTICIDES AND EARLY MENARCHE IN THE AVON LONGITUDINAL STUDY OF PARENTS AND CHILDREN COHORT[1]

[1] To be submitted to Journal of Clinical Endocrinology and Metabolism

ABSTRACT

Introduction: Some organochlorine pesticides have been banned or restricted in most countries for decades. However, because of their biological and long environmental persistence, human exposure to these compounds remains a concern. Some organochlorines can exhibit estrogenic or anti-estrogenic properties and therefore act as endocrine disrupting compounds (EDCs). *In utero* exposure to EDCs can affect outcomes later in life such as onset of menarche. This study measures the *in utero* exposure of girls to organochlorine pesticides and examines the association with early menarche in a nested case-control study of the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort in the United Kingdom.

Methods: Serum collected during pregnancy in 1991–1992 from mothers of 218 girls who reported age at menarche before age 11.5 years (cases) and 230 girls who reported menarche at or after 11.5 years (controls) were measured for 9 organochlorine pesticides and metabolites using gas chromatography isotope dilution high resolution mass spectrometry. The association between *in utero* serum pesticide concentrations and early menarche was analyzed using logistic regression. Multivariate logistic regression models controlled for mother's age at menarche, mother's prenatal BMI, or child's duration of breastfeeding.

Results: Hexachlorobenzene (HCB), β-Hexachlorocyclohexane (β-HCH), 2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethane (p,p'-DDT) and 2,2-Bis(4-chlorophenyl)-1,1-dichloroethene (p,p'-DDE) were detected in more than 50% of the study participants. HCB was the most frequently detected organochlorine (100%) followed by p,p'-DDE (99.8%), β-HCH (98%), and p,p'-DDT (88%). Overall, there was no association between *in utero* exposure to HCB, β-HCH, p,p'-DDT, or p,p'-DDE and early menarche.

Conclusions: This is the first study to examine the association between *in utero* exposure to HCB or β-HCH and early menarche. *In utero* exposure to organochlorine pesticides is not associated with early menarche in the ALSPAC cohort.

INTRODUCTION

Several organochlorine pesticides have been banned or restricted in most countries for decades because of their persistence in the environment and their association with adverse effects in humans and wildlife (CDC, 2009). However, they are still in use especially in developing countries, mainly to control vector-borne diseases (UNEP&WHO, 2012). Human exposure to organochlorine pesticides occurs primarily through diet, particularly consumption of fatty foods such as meat, fish, and dairy products (CDC, 2009). Organochlorine pesticides can be transferred to the developing fetus through the placenta and to newborns through breast milk (CDC, 2009; Saxena, Siddiqui, Bhargava, Krishnamurti, & Kutty, 1981).

It has been suggested that the *in utero* environment, critical for fetal development, may affect health outcomes later in life such as onset of menarche (Adair, 2001; Kaprio, Arja, Torsten, Richard, & al., 1995). There has been a secular trend towards early puberty in American and European girls (Euling et al., 2008; McDowell, Brody, & Hughes, 2007). Variability in the onset of puberty has been associated with a host of genetic and non-genetic factors including exposures to endocrine disrupting compounds (EDCs). EDCs can mimic, block, or cause over- or under-production of naturally occurring hormones (EPA, 2011).

Animal studies suggest that organochlorine pesticides can exhibit weak estrogenic or anti-estrogenic properties associated with early or delayed onset of puberty (Clement & Okey, 1972; Cooper et al., 1989; Gellert, Heinrichs, & Swerdloff, 1972). Early postnatal transient exposure to the estrogenic effect of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) in infant female rats initiated early puberty through the hypothalamic-pituitary axis (Raiser, Parent, Gerard, Lebrethon, & Bourguignon, 2007). In another study, female rats exposed to ϒ-hexachlorocyclohexane (ϒ-HCH/Lindane) experienced delayed puberty due to lindane's anti-

estrogenic activity which either blocked the response of estrogen-dependent secretion of pituitary hormones or acted directly on the hypothalamic-pituitary axis (Cooper et al., 1989). Other organochlorines such as β-HCH and hexachlorobenzene (HCB) have also exhibited some estrogenic activity in animal studies though the suggested mechanisms of action on their effects on puberty or their effects on the hypothalamic-pituitary axis are not documented or not well understood (Alvarez et al., 2000; Coosen & van Velsen, 1989; Foster, Pentick, McMahon, & Lecavalier, 1992; van Velsen, Danse, Leeuwen, Dormans, & van Logten, 1986; Warner et al., 2013).

Epidemiologic studies have shown conflicting results of the association between exposure to organochlorine pesticides and early puberty in humans. Some studies have associated organochlorine pesticides exposures with early pubic hair development, breast development, or onset of menarche while others have found no association (Blank HM et al., 2000; Gladen, Ragan, & Rogan, 2000; Vasiliu, Muttineni, & Karmaus, 2004). Some of these study results support the hypothesis that *in utero* exposure to the estrogenic effect of organochlorine pesticides can result in early menarche (Vasiliu et al., 2004). Early puberty is a risk factor for adverse adult health outcomes such as pre- and post-menopausal breast cancer, ovarian cancer, and polycystic ovarian syndrome (Gail et al., 1989; Golub et al., 2008; Moorman, Palmieri, Akushevich, Berchuck, & Schildkraut, 2009; Warner et al., 2013). Endocrine disrupting effects due to population exposures to organochlorine pesticides are still not well understood and more epidemiologic studies are needed (Buck Louis et al., 2008). This nested case-control study measures *in utero* exposure to organochlorine pesticides and examines the association with early onset of menarche measured prospectively.

STUDY DESIGN AND METHODS

Study population

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective observational birth cohort study designed to investigate how genotype and environmental characteristics influence health and development in children and parents (Fraser et al., 2013). The study recruited pregnant women living in Bristol area, South West of England in the United Kingdom (UK) with an expected date of delivery from 1st April, 1991 to 31st December, 1992 (Fraser et al., 2013). A total of 14,775 live births were included in the ALSPAC cohort (Boyd et al., 2013). Information about these mothers, their partners, and their children has been collected repeatedly since pregnancy, and they have been followed for the last 22–24 years (Fraser et al., 2013; Boyd et al., 2013). Menarche status and age and date at first menstrual period were self-reported and recorded in the 'Growing and Changing' questionnaire; this questionnaires was mailed to participants each year from age 8 to 17, with the exception of age 12 due to a delay in administering the questionnaire (Rubin et al., 2009). The ALSPAC study website contains details of all data that are available through a fully searchable data dictionary (ALSPAC, 2014).

The selection of cases and controls is described in detail by Christensen et al. (2011). Briefly, from the cohort of live births, 3682 singleton girls returned at least 2 Growing and Changing questionnaires from the ages of 8 to 13 years (Christensen et al., 2011). Of these, 218 had earlier menarche, defined as first period before 11.5 years, and had at least one maternal gestational serum sample that could be analyzed (cases) (Christensen et al., 2011). A random sample of 230 controls was selected from those who had menarche at 11.5 years or older and had at least one maternal gestational serum sample that could be analyzed (Christensen et al., 2011; Appendix A). Human subject protection was assessed and approved by the ALSPAC Law and

Ethics Committee, the Local Research Ethics Committees, and Centers for Disease Control and Prevention (CDC) Institutional Review Board (Christensen et al., 2011).

Exposure assessment

Maternal serum samples collected during pregnancy and banked at the University of Bristol were sent to the National Center for Environmental Health, CDC, Atlanta, GA in 2008 for analysis. The following organochlorine pesticides were measured by gas chromatography isotope dilution high resolution mass spectrometry method as previously described (Sjodin et al., 2004): Hexachlorobenzene (HCB), Isomers of Hexachlorocyclohexane (i.e., β-Hexachlorocyclohexane (β-HCH) and ϒ- Hexachlorocyclohexane (ϒ-HCH/Lindane)), Chlorodane-related pesticides (i.e., Oxychlorodane (Oxychlor) and trans-Nonachlor (T-Nona)), Mirex, and Isomers of DDT (i.e., 2,2-Bis(4-chlorophenyl)-1,1-dichloroethene (p,p'-DDE); 2-(4-chlorophenyl)-2-(2-chlorophenyl)-1,1,1-trichloroethane (o,p'-DDT); and 2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethane (p,p'-DDT)). Lipid adjusted measurements (ng/g) were determined, and each serum sample had an individual limit of detection (LOD) based on the sample's weight and lipid concentration.

We performed statistical analyses using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA). The organochlorine pesticide concentrations measured in maternal serum included some values reported below the LOD; therefore, we used survival analysis methods to account for these values with data transformed for left censoring using SAS. We used non-parametric Kaplan Meier methods using the LIFETEST procedure to generate maximum likelihood estimates for the median and the 25th and 75th percentiles. We did not assess exposure for organochlorine analytes detected in less than 50% of the samples.

Early menarche and exposure association

For organochlorines detected in more than 50% of samples, we substituted values less than the LOD with LOD/ √2. Since pesticide concentrations were not normally distributed, we also natural log transformed the values before analysis. The serum sample weights for this study ranged from 0.12g to 1.23g, and the smaller samples (less than 0.4g) tended to have higher LODs (i.e., less ability to detect low levels of pesticide concentrations) (CDC, 2009), which could bias analyses when substituting values <LOD with LOD/√2 using the high LODs. Therefore, we plotted lipid-adjusted LOD levels against the sample weight, selected an LOD cut-off of 13ng/g, and removed any record with lipid adjusted LOD ≥ 13ng/g (Figure 1) (Sjodin et al., 2014). Using this criteria, we removed 13 records from the analysis of p,p'-DDT which was the only pesticide evaluated that had values <LOD of greater than or equal to 13 ng/g.

Through a review of literature, we identified a priori, covariates that could be associated with the exposure or the outcome (Biro et al., 2010; Freedman et al., 2003; Longnecker, Klebanoff, Gladen, & Berendes, 1999; McDowell et al., 2007; Ruder, Hartman, Rovine, & Dorgan, 2010; Wronka & Pawlinska-Chmara, 2005; Rubin et al., 2009). We assessed the following 9 covariates for potential confounding and effect modification: Mother's age at delivery (<20, 20–24, 25–29, 30–39, or ≥40 years), Mother's pre-natal body mass index (BMI) (<18.5 (underweight), 18.5–24.9 (normal), 25–29.9 (over-weight), or ≥30 (obese)), Mother's age at menarche (8–11, 12–14, or ≥15 years), Mother's education (Certificate of secondary education (CSE)/None, Vocational, O-level, A-level, or Degree), Mother's race (White or non-White), Child's birth weight (<2500 or ≥2500g); Child's birth order (first born, second born, or third born or later), Child's BMI at age 7; Duration of breast feeding (never, < 6 , or ≥6 months), and gestation trimester when serum sample was collected (≤ 12, 13–28, or ≥29 weeks).

We used ANOVA or linear regression methods to assess the association between potential confounders and pesticide exposure, and logistic regression methods to assess the association between potential confounders and early menarche and effect modification. We used logistic regression to analyze the association between early menarche and organochlorine pesticide exposure. Potential confounders with p-value (p) <0.25 (Mickey & Greenland, 1989) and potential effect measure modifiers with interaction term $p < 0.05$ were included in the multivariate logistic model (Appendices B & C). We performed two additional multivariate analyses of the associations between early menarche and organochlorine pesticide exposure with organochlorine pesticide exposure categorized as either below the median or at or above the median and in quartiles based on the distribution of the controls.

RESULTS

There were significant differences in the distribution of cases and controls within mother's age at menarche and mother's prenatal BMI categories. Cases were more likely than controls to have a mother who had onset of menarche between the ages of 8 and 11 years (32.5% compared to 15.2% of controls) or was overweight or obese before pregnancy (21.3% and 9.6%, respectively compared to 10.2% and 5.9% of controls) (Table 1). Cases were also more likely to be first born children (53.9% compared to 45.6% of controls) or have a mother who was of non-white race (3.3% compared to 0.5% of controls). Cases had significantly higher pre-pubertal BMIs at age 7 compared to controls (p <0.0001).The median age at menarche for cases was 11 years and for controls was 12.75 years.

HCB was the most frequently detected pesticide above the LOD (100% of study participants) while Mirex was the least detected pesticide (0.45% of study participants) (Table 2). For the organochlorines detected in more than 50% of the samples, p,p'-DDE had the highest

median concentration at 311.0ng/g in the total study population, while p,p' -DDT had the lowest median concentration at 11.2ng/g (Table 2). Cases had higher median values than controls for all organochlorines except β-HCH, but none of these differences were significant.

In utero exposure to organochlorine pesticides was not significantly associated with early menarche for any of the evaluated organochlorine pesticides (Table 3). Covariates included in the multivariate models varied by organochlorine pesticide (Table 3). In the unadjusted models, the odds ratios (OR) for pesticide-early menarche association were <1 for all organochlorine pesticides except p,p'-DDT which had an OR (95% confidence interval (CI)) = 1.01 (0.70, 1.45). In the adjusted multivariate models, all odds ratios were <1. None of the associations were statistically significant. For the additional multivariate analyses, the results with exposure categorized as < or ≥ median and for the highest quartile (≥75th percentile) remained similar (Appendices D & E).

DISCUSSION

The objectives of this study were to characterize the in utero exposure of girls in the ALSPAC cohort to HCB, β-HCH, p,p'-DDT and p,p'-DDE, and examine associations between exposure to organochlorine pesticides and early menarche. We did not find any association between in utero exposure to HCB, β-HCH, p,p'-DDT, or p,p'-DDE and early menarche. To our knowledge, this is the first study that has examined the association between in utero exposures to HCB or β-HCH and early meanrche, although two previous studies examined in utero exposure to DDE and onset of puberty (Vasiliu et al., 2004; Gladen et al., 2000).

Puberty

Our study results are consistent with Gladen et al. (2000) who examined *in utero* exposure to background levels of organochlorines (DDE) and pubertal development. Gladen et al. (2000) evaluated general population exposures in 316 girls born between 1978 and 1982 as part of the North Carolina Infant Feeding Study, and did not find an association between *in utero* exposure to DDE and pubertal development. The lipid adjusted median DDE concentration for the Gladen et al. (2000) study population was 2,400 ppb. In comparison, our study results represent general population exposures in children born from 1991 through 1992 in UK. The lipid adjusted median DDE concentration for our study population was 311ng/g (ppb). Another study by Vasiliu et al. (2004) found that an increase of 15µg/l of DDE *in utero* reduced age at menarche by one year; however, after controlling for BMI at age of menarche, this association was no longer significant. In comparison to our study, they evaluated children born between 1950 and 1980 with potentially higher DDE exposures than the general population due to high maternal consumption of contaminated fish in the Great Lakes. This was also a smaller study (N = 151), with only 22 girls with age at onset of menarche from 9–11 years. The median *in utero* DDE concentration for girls who attained menarche between the ages of 9 and 11 years was 7µg/l. In other studies not using *in utero* exposures, a Belgian study of girls undergoing treatment for early breast development found that girls who immigrated to Belgium from developing countries had higher plasma levels of DDE compared to non-immigrant girls (Krstevska-Konstantinova et al., 2001), suggesting an association between pre-pubertal exposure to DDE and early puberty. In another study, married Chinese textile workers aged 20 – 34 years in the highest quartile of total DDT in serum had a younger mean age at menarche compared to women in the lowest quartile (Ouyang et al., 2005). Other epidemiological studies found no

association between DDE, HCB or Mirex measured in girls' blood and menarcheal status, pubic hair development, or breast development (Denham et al., 2005; Ozen et al., 2012; Wolff et al., 2008).

Exposure

DDT was banned in the UK in 1986 (Rogan & Chen, 2005). Twelve persistent organic pollutants (POPs) including DDT and HCB were banned globally in a 2004 treaty at the Stockholm Convention on Persistent Organic Pollutants, and an additional nine, including HCH, were added to the treaty in 2009 (The Stockholm Convention, 2008). Although organochlorine pesticides are still detected in humans, and their associations with adverse health effects remain a concern, UK population exposure studies with serum samples collected 2001–2003 showed more than 50% decrease in p,p'-DDT and p,p'-DDE, median concentrations and more than 60% decrease in HCB and β-HCH median concentrations compared to our study (Kalantzi et al., 2004; Thomas, 2006; Appendix F). This decreasing trend has also been observed in other developed countries such as the United States where these pesticides are no longer in use or have been restricted (CDC, 2009). However, future research should focus on measuring exposure in countries where organochlorine pesticides are still in use and evaluating associations between exposure to these pesticides and adverse health effects.

Study strengths and limitations

This study is subject to some limitations. A previous study compared the cases and controls included in this study to those who did not respond to two Growing and Changing puberty questionnaires that were excluded from the study. The parents of those who did not respond to the questionnaires were of lower educational attainment (72.1% had a CSE compared

to 86.6% of respondents) and had a lower age at delivery (69.9% older than 25 years compared to 85.1% of respondents) (Christensen et al., 2011). About 6% of non-respondents were of non-white race compared to 3.8% of respondents (Christensen et al., 2011). These differences may affect the results of our study.

The puberty questionnaires were mailed to all participants each year during ages 8–17 years, with the exception of age 12. Since the puberty questionnaires relied on self-reported age at menarche information, onset of menarche reported during the ages 11–13 years could have been affected by recall bias due to this recall period (Koo & Rohan, 1997).

We removed 13 records from the analysis of p,p'-DDT as detailed in the methods section of this paper. Some of the serum samples for the participants in this study were small, and the LODs for these small samples tended to be large, which would bias the substitution of values <LOD with LOD/√2. The removal of some records could bias the study results; however, when all the samples of p,p'-DDT were included in the analyses, the results were similar. Some covariate data and lipid adjusted values of the organochlorine concentrations were missing for some cases and controls. Records with missing data were excluded from our analyses which could bias our results.

Strengths of this study include serum measurements of several organochlorine pesticides and metabolites and the ability to link them to demographic and health-related characteristics of the mother and child, collected prospectively and repeatedly over the follow-up period of 22–24 years.

CONCLUSION

This is the first study to examine the association between *in utero* exposure to HCB or β-HCH and early menarche. *In utero* exposure to organochlorine pesticides is not associated with early menarche in the ALSPAC cohort. More emphasis needs to be placed on measuring exposure in countries where organochlorine pesticides are still in use and evaluating their associations with adverse health effects.

Much of the research currently available focuses on the health effects of exposure to a single chemical. Since combinations of pesticides are sometimes used, populations are usually exposed to a mixture of EDCs with differing effects and mechanisms of action. Future work should include examining the effects of multiple pesticide exposures on onset of puberty.

REFERENCES

Adair, L. (2001). Size at Birth Predicts Age at Menarche. Pediatrics, 107.

Alvarez, L., Randi, A., Alvarez, P., Piroli, G., Chamson-Reig, A., Lux-Lantos, V., & Kleiman de Pisarev, D. (2000). Reproductive effects of hexachlorobenzene in female rats. Journal of Applied Toxicology, 20, 81-87.

Avon Longitudinal Study of Parents and Children (ALSPAC). (April 21st 2014). ALSPAC Data Dictionary. Retrieved June 20th, 2014, from http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/

Biro, F., Galvez, M. P., Greenspan, P. A., Succop, P. A., Vangeepuram, N., Pinney, S. M., . . . Wolff, M. S. (2010). Pubertal Assessment Method and Baseline Characteristics in a Mixed Longitudinal Study of Girls. Pediatrics, 126.

Blank HM, Marcus M, Tolbert PE, Rubin C, Henderson AK, Hertzberg VS, . . . Cameron L. (2000). Age at Menarche and Tanner Stage in Girls Exposed In Utero and Postnatally to Polybrominated Biphenyl. Epidemiology, 11(6).

Boyd, A., Golding, J., Macleod, J., Lawlor, D. A., Fraser, A., Henderson, J., . . . Smith, G. D. (2013). Cohort Profile: The 'Children of the 90s' - The Avon Longitudinal Study of Parents and Children. International Journal of Epidemiology, 42(1).

Buck Louis, G. M., Gray, L. E., Marcus, M., Ojeda, S. R., Pescovitz, O. H., Witchel, S. F., . . . Euling, S. Y. (2008). Environmental Factors and Puberty Timing: Expert Panel Research Needs. Pediatrics, 121(Supplement 3), S192-S207. doi: 10.1542/peds.1813E

Centers for Disease Control and Prevention (CDC). (2009). The Fourth National Report on Human Exposure to Environmental Chemicals.

Christensen, K., Maisonet, M., Rubin, C., Holmes, A., Calafat, A., Kato, K., & al., e. (2011). Exposure to polyfluoroalkyl chemicals during pregnancy is not associated with offspring age at menarche in a contemporary British cohort. Environment International, 37, 129 - 135.

Clement, J., & Okey, A. (1972). Estrogenic and anti-estrogenic efects of DDT administered in the diet to immature female rats. Canadian Journal of Physiology and Pharmacology, 50, 971-975.

Cooper, R. G., Chadwick, R., Rehnberg, G., Goldman, J., Booth, K., Hein, J., & McElroy, W. (1989). Effect of lindane on hormonal control of reproductive function in the female rat. Toxicology and Applied Pharmacology, 99(3), 384-394.

Coosen, R., & van Velsen, F. (1989). Effects of the β-isomer of hexachlorocyclohexane on estrogen-sensitive human mammary tumor cells. Toxicology and Applied Pharmacology, 101(2), 310-318.

Denham, M., Schell, L. M., Deane, G., Gallo, M. V., Ravenscroft, J., DeCaprio, A. P. (2005). Relationship of Lead, Mercury, Mirex, Dichlorodiphenyldichloroethylene, Hexachlorobenzene, and Polychlorinated Biphenyls to Timing of Menarche Among Akwesasne Mohawk Girls. Pediatrics, 115(2), e127-e134. doi: 10.1542/peds.2004-1161

Euling, S. Y., Herman-Giddens, M. E., Lee, P. A., Selevan, S. G., Juul, A., Sorensen, T., . . . Swan, S. H. (2008). Examination of US Puberty-Timing Data from 1940 to 1994 for Secular Trends: Panel Findings. Pediatrics, 121.

Foster, W., Pentick, J., McMahon, A., & Lecavalier, P. (1992). Ovarian toxicity of hexachlorobenzene (HCB) in the superovulated female rat. Journal of biochemical toxicology, 7(1), 1-4.

Fraser, A., Macdonald-Wallis, C., Tilling, K., Boyd, A., Golding, J., Smith, G. D., . . . Lawlor, D. A. (2013). Cohort Profile: The Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. International Journal of Epidemiology, 42(1).

Freedman, D. A., Khan, L., Serdula, M., Dietz, W., Srinivasan, S., & Berenson, G. (2003). The relation of menarcheal age to obesity in childhood and adulthood: the Bogalusa heart study. Bmc Pediatrics, 3.

Gail, M., Brinton, L. A., Byar, D., Corle, S., Green, S., Schairer, C., & Mulvihill, J. (1989). Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. Journal of the National Cancer Institute, 81(24).

Gellert, R., Heinrichs, L., & Swerdloff, R. (1972). DDT Homologues: Estrogen-Like Effects on the Vagina, Uterus and Pituitary of the Rat Endocrinology, 91(4).

Gladen, B. C., Ragan, N. B., & Rogan, W. J. (2000). Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. J Pediatr, 136(4), 490-496.

Golub, M. S., Colluman, G. W., Foster, P., Kimmel, C. A., Meyts, E. R., Reiter, E. O., . . . Toppari, J. (2008). Public Health Implications of Altered Puberty Timing. Pediatrics, 121.

Kalantzi, O., Martin, F., Thomas, G., Alcock, R. E., Tang, H. R., Drury, S. C., . . . Jones, K. C. (2004). Different Levels of Polybrominated Diphenyl Ethers (PBDEs) and Chlorinated Compounds in Breast Milk from Two U.K. Regions. Environmental Health Perspectives, 112(10).

Kaprio, J., Arja, J., Torsten, W., Richard, V., & al., e. (1995). Common genetic influences on BMI and age at menarche. Human Biology, 67, 739-753.

Koo, M., & Rohan, T. (1997). Accuracy of short-term recall of age at menarche. Annals of Human Biology, 24(1), 61-64.

Krstevska-Konstantinova, M., Charlier, C., Craen, M., Du Caju, M., Heinrichs, C., de Beaufort, C., . . . Bourguignon, J. P. (2001). Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. Human reproduction (Oxford, England), 16(5), 1020-1026.

Longnecker, M., Klebanoff, M., Gladen, B., & Berendes, H. (1999). Serial Levels of Serum Organochlorines During Pregnancy and Postpartum. Archives of Environmental Health, 54(2), 110-114.

McDowell, M. A., Brody, D. J., & Hughes, J. (2007). Has Age at Menarche Changed? Results from the National Health and Nutrition Examination Survey (NHANES) 1999–2004. Journal of Adolescent Health, 40, 227-231.

Mickey, R., & Greenland, S. (1989). The impact of confounder selection criteria on effect estimation. Am J Epidemiol, 129(1), 125-137.

Moorman, P., Palmieri, R., Akushevich, L., Berchuck, A., & Schildkraut, J. (2009). Ovarian cancer risk factors in African-American and White women. American Journal of Epidemiology, 170(5).

Ozen, S., Darcan, S., Bayindir, P., Karasulu, E., Simsek, D. G., & Gurler, T. (2012). Effects of pesticides used in agriculture on the development of precocious puberty. Environmental Monitoring and Assessment, 184(7), 4223-4232. doi: http://dx.doi.org/10.1007/s10661-011-2257-6

Raiser, G., Parent, A. S., Gerard, A., Lebrethon, M. C., & Bourguignon, J. (2007). Early Maturation of Gonadotropin-Releasing Hormone Secretion and Sexual Precocity after

Exposure of Infant Female Rats to Estradiol or Dichlorodiphenyltrichloroethane. Biology of Reproduction, 77, 734-742.

Rogan, W. J., & Chen, A. (2005). Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). The Lancet, 366(9487), 763-773. doi: 10.1016/S0140-6736(05)67182-6

Rubin, C., Maisonet, M., Kieszak, S., Monteilh, C., Holmes, A. K., Flanders, D., . . . Marcus, M. (2009). Timing of maturation and predictors of menarche in girls enrolled in a contemporary British cohort. Paediatr Perinat Epidemiol, 23, 492-504.

Ruder, E., Hartman, T., Rovine, M., & Dorgan, J. (2010). Birth characteristics and age at menarche: results from the dietary intervention study in children (DISC). Cancer Causes Control, 21, 1379-1386.

Saxena, M., Siddiqui, M., Bhargava, A., Krishnamurti, C., & Kutty, D. (1981). Placental transfer of pesticides in Humans. Arch Toxicol, 48, 127-134.

Sjodin, A., Jones, R., Lapeza, C., Focant, J., McGahee III, E., & Patterson Jr, D. (2004). Semi-Automated High-Throughput Extraction and Cleanup Method for the Measurement of Polybrominated Diphenyl Ethers, Polybrominated Biphenyls and Polychlorinated Biphenyls In Human Serum. Anal. Chem., 76, 1921-1927.

Sjodin, A., Schecter, A., Jones, R., Wong, L.-Y., Colacino, J., Malik-Bass, N., . . . Calafat, A. (2014). Polybrominated Diphenyl Ethers, 2, 2, 4, 4, 5, 5-Hexachlorobiphenyl (PCB-153), and p,p′□Dichlorodiphenyldichloroethylene (p,p□DDE) Concentrations in Sera Collected in 2009 from Texas Children. Environmental Science and Technology, 48, 8196-8202.

The Stockholm Convention. (2008). The 12 initial POPs under the Stockholm Convention. Retrieved February 15th, 2014

Thomas, G. W., M. Hodson, S. Jones, K. (2006). Organohalogen Chemicals in Human Blood from the United Kingdom. Environmental Pollution, 141, 30-41.

UNEP&WHO . (2012). State of the Science of Endocrine Disrupting Chemicals- 2012.

US Environmental Protection Agency. (2011). Endocrine Primer. Retrieved March 19th, 2014, from http://www.epa.gov/endo/pubs/edspoverview/primer.htm

van Velsen, F., Danse, L., Leeuwen, F., Dormans, J. P., & van Logten, M. (1986). The subchronic oral toxicity of the β-isomer of hexachlorocyclohexane in rats. Fundamental and Applied toxicology, 6, 697-712.

Vasiliu, O., Muttineni, J., & Karmaus, W. (2004). In utero exposure to organochlorines and age at menarche. Hum Reprod, 19(7), 1506-1512. doi: 10.1093/humrep/deh292

Warner, E., Colditz, G. A., Palmer, J., Partrifge, A., Rosner, B., & Tamimi, R. (2013). Reproductive factors and risk of premenopausal breast cancer by age at diagnosis: Are there differences before and after age 40? Breast Cancer Research & Treatment, 142, 165-175.

Wolff, M. S., Britton, J. A., Boguski, L., Hochman, S., Maloney, N., Serra, N., & Liu, Z. (2008). Environmental Exposures and Puberty in Inner-City Girls. Environmental Research, 107(3), 393.

Wronka, I., & Pawlinska-Chmara, R. (2005). Menarcheal age and socio-economic factors in Poland. Annals of Human Biology, 32(5), 630-638.

Table 3.1 Study population characteristics

Characteristic	Cases (N = 218) N	%	Controls (N = 230) N	%	p-value [a]
Mother's Ethnicity					0.06
White	205	96.7	218	99.5	
Other Ethnicity	7	3.3	1	0.5	
Missing	6		11		
Mother's age at delivery (yrs)					0.43
<20	1	0.5	7	3.1	
20–24	43	19.9	41	17.9	
25–29	83	38.4	81	35.4	
30–39	86	39.8	96	41.9	
>40	3	1.4	4	1.8	
Missing	2		1		
Mother's Education[b]					0.88
CSE/None	31	14.8	26	11.9	
Vocational	17	8.1	15	6.9	
O-Level	67	31.9	73	33.3	
A-Level	61	29.1	66	30.1	
Degree	34	16.2	39	17.8	
Missing	8		11		
Mother's prenatal BMI					0.01
<18.5	7	3.6	11	5.4	
18.5–24.9	129	65.5	161	78.5	
25–29.9	42	21.3	21	10.2	
>30	19	9.6	12	5.9	
Missing	21		25		
Mother's age at menarche (yrs)					0.0004
8–11	63	32.5	30	15.2	
12–14	119	61.3	153	77.3	
≥15	12	6.2	15	7.6	
Missing	24		32		
Child's Birth order					0.07
First born	110	53.9	98	45.6	
Second born	57	27.9	83	38.6	
Third born or later	37	18.1	34	15.8	
Missing	14		15		
Child's BMI at age 7	Median 17.2	Quartile 15.9–18.9	Median 15.8	Quartile 14.9–16.9	<0.0001

Child's age at menarche	11	10.8–11.3	12.75	12.2–13.3

[a] Comparison between cases and controls using logistic regression

[b] CSE = certificate of secondary education, O-level = ordinary level, A-level = advanced level

Table 3.2 Lipid adjusted gestational serum concentrations (ng/g)

	Total		**Cases**		**Controls**		
Organochlorine	**N (%) > LOD[a]**	**Median (IQR)**	**N (%) > LOD**	**Median (IQR)**	**N (%) > LOD**	**Median (IQR)**	**p-value[b]**
HCB	426 (100)	50.2 (37.9–63.4)	208 (100)	50.65 (37.4–63.7)	218 (100)	49.95 (38.3–63.4)	0.94
β - HCH	429 (98.1)	47.15 (34.6–62.5)	211 (98.1)	45.85 (33.5–59.9)	218 (98.2)	47.5 (37.3–63.2)	0.17
ϒ - HCH	446 (20.6)	–	218 (19.3)	–	228 (21.9)	–	
p, p- DDE	428 (99.8)	311.0 (192.5–499.0)	210 (99.5)	314.0 (184.0–522.0)	218 (100)	309.5(200.0–484.0)	0.63
p, p'- DDT	424 (88)	11.2 (8.1–16.5)	210 (89.1)	11.6 (7.8–16.7)	214 (86.9)	11.0 (8.52–16.2)	0.52
o, p'- DDT	447 (1.3)	–	218 (1.4)	–	229 (1.3)	–	
T-Nona	429 (30.1)	–	209 (30.6)	–	220 (29.6)	–	
Oxychlor	447 (28)	–	218 (25.7)	–	229 (30.1)	–	
Mirex	447 (0.45)	–	218 (0)	–	229 (0.87)	–	

[a] Each biological sample had an individual limit of detection based on the sample's weight and lipid concentration

[b] Comparison between cases and controls using Wilcoxon Rank Sum Test

Table 3.3 Association between lipid adjusted organochlorine gestational serum concentrations (ng/g) and early menarche

Organochlorine	Unadjusted OR (95% CI) [a]	Adjusted OR (95% CI)[a, f]	p-value
HCB	0.96 (0.62–1.47)	0.93 (0.58–1.50)	0.77[b]
β-HCH	0.86 (0.61–1.21)	0.95 (0.64–1.41)	0.79[c]
p,p' - DDE	0.91 (0.70–1.19)		0.49[d]
p,p' - DDT	1.01 (0.70–1.45)	0.93 (0.63–1.38)	0.73[e]

[a] Odds of early menarche for a unit increase of logged organochlorine pesticide concentration
[b] Adjusted for mother's age at menarche
[c] Adjusted for mother's age at menarche and duration of breastfeeding
[d] Unadjusted p value. No potential confounders or effect measure modifiers were identified
[e] Adjusted for mother's prenatal BMI
[f] When adjusting for confounders observations with missing data were excluded. Pesticide (number of cases, number of controls used in adjusted models): HCB (184, 188); β-HCH (151, 151); PP, DDT (183, 179)

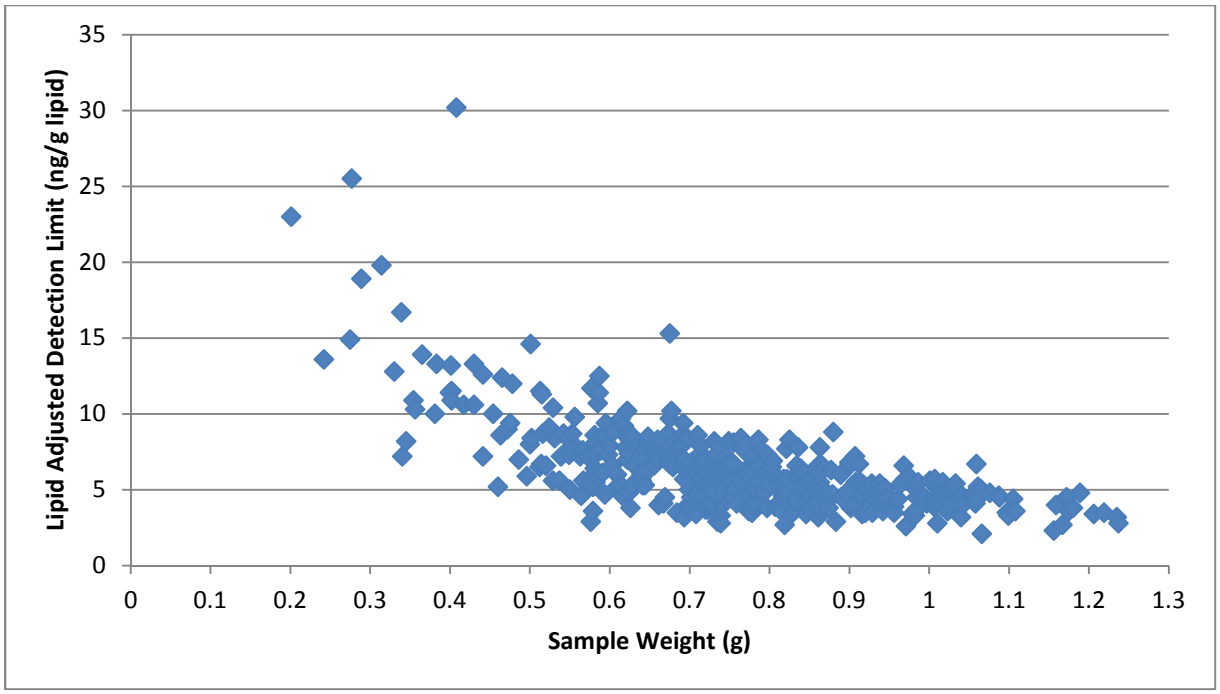


Figure 3.1 Lipid adjusted detection limit for all organochlorine pesticides (ng/g) Vs. Sample weight (g)

CHAPTER 4

IN UTERO EXPOSURE TO ATRAZINE ANALYTES AND EARLY MENARCHE IN THE AVON LONGITUDINAL STUDY OF PARENTS AND CHILDREN COHORT[2]

[2] To be submitted to Journal of Clinical Endocrinology and Metabolism

ABSTRACT

Introduction: Although banned by the European Union in 2003, atrazine continues to be used in over 70 countries globally. Atrazine, a broad spectrum herbicide, is a suspected endocrine disrupting compound (EDC), although most of these effects have been observed only in animal studies. Some studies suggest that *in-utero* exposure to EDCs is associated with early onset of menarche. We used a nested case-control study design to measure *in utero* exposure to atrazine in children in the Avon Longitudinal Study of Patents and Children (ALSPAC) cohort and analyzed the association with early menarche.

Methods: Cases were girls who reported menarche before 11.5 years while controls were girls who reported menarche at or after 11.5 years. Atrazine and 6 metabolites were measured in maternal gestational urine samples (collected during pregnancy 1991–1992) from 174 cases and 195 controls using on-line solid phase extraction-isotope dilution-high performance liquid chromatography-tandem mass spectrometry. The association between *in utero* pesticide exposure and early menarche was evaluated using multivariate logistic regression.

Results: Diaminochlorotriazine (DACT), Desethyl atrazine (DEA), atrazine parent compound and desisopropyl atrazine (DIA) were detected in 58%, 6.2%, 0.8% and 0.5% of the participants respectively. DACT was the only metabolite detected in more than 50% of the study participants. For analysis, we classified DACT into the following categories: < limit of detection (LOD); LOD to <median; and ≥median. Our results suggest *in utero* exposure to DACT increases the odds of early menarche in girls. The adjusted odds ratio (95% Confidence Interval) for girls with DACT exposures greater than or equal to the median was 1.82 (1.02, 3.25) compared to girls with DACT exposures lower than the limit of detection.

Conclusion: This is the first study to examine the association between atrazine exposure and its relation to onset of puberty in humans. *In utero* exposure to the atrazine metabolite DACT in the ALSPAC cohort was associated with an increased odds of early menarche. To accurately assess atrazine exposure in populations, several metabolites including DACT need to be measured.

INTRODUCTION

Atrazine herbicide is used in more than 70 countries worldwide; most frequently in United States (US), Brazil, Argentina, Mexico and China (LeBaron, H., McFarland, J. & Burnside, O., 2008). Atrazine is used to control broadleaf and grassy weeds in agricultural crops, mainly corn (EPA, 2012). Human exposure to atrazine can occur through several routes. Water run-off from crops and lawn applications can get into ground and surface waters and contaminate drinking water wells (Munger et al., 1997). Atrazine dispersed in air through spraying can affect agricultural workers and those living nearby (ATSDR, 2003). Exposure can also occur through contact with contaminated soils or ingestion of contaminated agricultural products (ATSDR, 2003). Developing fetuses and children can also be exposed to atrazine *in utero* or thorough breast milk (Balduini et al., 2003; Whyatt et al., 2003). Atrazine has been banned from use by the European Union (E.U.) since 2003 because of its persistent contamination of drinking and ground water above the E.U. recommended limit of 0.1 parts per billion (ppb) (Sass & Colangelo, 2006). However, several countries still use this herbicide including the US where it is a restricted use pesticide (ATSDR, 2003).

In water, atrazine breaks down to the following metabolites: desethyl atrazine (DEA), desisopropyl atrazine (DIA) and diaminochlorotriazine (DACT) (EPA, 2012). In areas where atrazine is used, the general population can be exposed to atrazine or any of its metabolites through contaminated drinking water (Munger et al., 1997; Ochoa-Acuna, Frankenberger, Hahn, & Carbajo, 2009; Villanueva, Durand, Coutte´, Chevrier, & Cordier, 2005). Atrazine is broken down rapidly in the body and eliminated primarily in urine, within 24-48 hours (Catenacci, Barbieri, Bersani, Cottica, & Maroni, 1993). Although it does not bioaccumulate appreciably in humans, atrazine and its metabolites can persist in groundwater and contaminate surface and

drinking water sources (ATSDR, 2003). This persistent contamination of drinking water can result in continuous exposure in humans and therefore can be of concern (UNEP & WHO, 2012).

Atrazine and its metabolites are suspected endocrine disruptors, although most of these effects have been observed in animal studies. Atrazine exposure has been shown to cause hermaphroditism and demasculinized larynges in frogs by inducing aromatase formation and converting testosterone to estradiol (Hayes et al., 2002), although these results have not been replicated (Du Preez et al., 2007; Kloas et al., 2008; Oka et al., 2008). In rats, atrazine caused a disruption in estrus cycle by impairing the ovulatory surge of luteinizing hormone, and also induced an earlier onset and increased incidence of mammary gland tumors by disrupting the ovarian function (Cooper et al., 2007; Wetzel, Luempert, Breckenridge, Tisdel, & Stevens, 1994). Prenatal and lactational atrazine exposure in female rats resulted in delayed puberty in the offspring, possibly by altering hypothalamic-pituitary activity (Laws, Ferrell, Stoker, & Cooper, 2003; Laws, Ferrell, Stoker, Schmid, & Cooper, 2000; Rayner, Wood, & Fenton, 2004). Further, DACT, the active metabolite of atrazine, has been demonstrated to be as potent as atrazine in delaying puberty (Laws et al., 2003).

A secular trend towards earlier onset of puberty among girls in Europe and the United States has been reported in several studies (Aksglaede, Olsen, Sorensen, Petersen, & Juul, 2008; Euling et al., 2008; McDowell, Brody, & Hughes, 2007; Parent et al., 2003). Early puberty is a risk factor for childhood risky behaviors (e.g., smoking, alcohol consumption and drug abuse) and adult diseases (e.g., breast and ovarian cancer) (Gail et al., 1989; Moorman, Palmieri, Akushevich, Berchuck, & Schildkraut, 2009; vanJaarsveld, Fidler, Simon, & Wardle, 2007; Walvoord, 2010). Various factors have been associated with earlier onset of puberty including; genetic and socio-economic characteristics, and environmental exposures (e.g., endocrine

disrupting compounds (EDCs)). EDCs have the potential to interfere with hormone action and produce adverse effects including early puberty (UNEP & WHO, 2012). The endocrine disrupting effects due to environmental-level exposures to pesticides are still not well understood (Buck Louis et al., 2008). To our knowledge, no epidemiologic studies have examined the association between atrazine exposure and onset of puberty. This study uses a nested case-control study design to measure *in utero* exposure of cases and controls to atrazine and its metabolites, and examines the association with early menarche.

MATERIALS AND METHODS

Study population

Pregnant women living in the Bristol area, south west of England, United Kingdom, with an expected date of delivery from 1st April, 1991 to 31st December, 1992, were recruited to participate in the Avon Longitudinal Study of Parents and Children (ALSPAC) (Fraser et al., 2013). A total of 14,775 live births were included in the ALSPAC cohort (Boyd et al., 2013). Details about the recruitment and study participants have already been published (Boyd et al., 2013; Fraser et al., 2013). Beginning at the age of 8, a puberty questionnaire titled 'Growing and Changing' was mailed to the participants each year until age 17 (except age 12) to collect information about the timing and onset of puberty in enrolled girls and boys. Onset of menarche information including the age and date of first menstrual period were self-reported and recorded prospectively in these questionnaires by the girls and their parents (Rubin et al., 2009). The ALSPAC study website contains details of all the data that are available through a fully searchable data dictionary (ALSPAC, 2014).

The selection of cases and controls for the nested case-control study has been previously described (Christensen et al., 2011). In brief, 3682 singleton girls returned at least 2 Growing and Changing questionnaires from the ages 8 to 13 years. From this number 218 were identified as having menarche before 11.5 years of age, and had one maternal gestational serum sample available for analysis. A subset of 174 cases also had maternal gestational urine samples available for analysis. A random sample of 394 girls that had menarche at or after 11.5 years of age were selected; 230 of these had one maternal gestational serum sample and 195 had maternal gestational urine samples available for analysis. Only the 174 cases and 195 controls with analyzable maternal gestational urine samples were included in this study. Human subject protection was assessed and approved by the ALSPAC Law and Ethics Committee, the Local Research Ethics Committees, and Centers for Disease Control and Prevention (CDC) Institutional Review Board (Christensen et al., 2011).

Exposure assessment

Banked urine samples from Bristol University were transferred to CDC's Division of Laboratory Sciences within the National Center for Environmental Health in 2008 for analysis. The following atrazine analytes were measured using on-line solid phase extraction-isotope dilution-high performance liquid chromatography-tandem mass spectrometry method (Panuwet et al., 2008; Panuwet et al., 2010): Atrazine, DACT, DEA, DIA, atrazine mercapturate, desethyl atrazine mercapturate, and hydroxyl atrazine. The limits of detection (LOD) of the method were: atrazine, 0.05ng/mL; DACT, 0.19ng/mL; DEA, 0.10ng/mL; DIA, 0.12ng/mL; atrazine mercapturate, 0.08ng/mL; desethyl atrazine mercapturate, 0.09ng/mL; and hydroxyl atrazine, 0.14ng/mL.

Only DACT which had more than 50% of sample measurements over the LOD was included in the analyses. To account for the left censored values (<LOD), maximum likelihood estimates for the median and 25th and 75th percentiles were calculated using non-parametric survival analysis methods using the LIFETEST procedure in SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA) after transforming the data for left censoring.

Statistical analysis

We classified the exposure variable into the following 3 categories: <LOD; LOD to <median; and ≥median. The median was determined using the distribution of controls ≥LOD. We selected covariates a priori from the literature (Freedman et al., 2003; Freedman et al., 2002; Ruder, Hartman, Rovine, & Dorgan, 2010; Winchester, Huskins, & Ying, 2008; Wronka & Pawlinska-Chmara, 2005; Rubin et al., 2009) and evaluated them for their potential to confound the outcome–exposure association or modify the effect of association measured. The following covariates were evaluated: Mother's age at delivery (<20, 20–24, 25–29, 30–39, or ≥40 years), mother's pre-natal body mass index (BMI) (<18.5 (underweight), 18.5–24.9 (normal), 25–29.9 (over-weight), or ≥30 (obese)), mother's age at menarche (8–11, 12–14, or ≥15 years), mother's education (Certificate of secondary education (CSE)/None, Vocational, O-level, A-level, or Degree), child's birth weight (<2500 or ≥2500g), child's birth order (first born, second born, or third born or later), child's BMI at age 7, child's duration of breast feeding (never, < 6 , or ≥6 months), gestation trimester when urine sample was collected (<12, 13–≤28, or ≥29 weeks) and month urine sample was collected (December–February, March–May, June–August, or September–November) or (March–August and September–February).

We used logistic regression methods in SAS to evaluate the covariate – exposure and the covariate – outcome association. Covariates with p value (p) <0.25(Mickey & Greenland, 1989) for both associations were considered potential confounders, and covariates with interaction term p <0.05, potential effect measure modifiers (Appendices G & H).

We used logistic regression to analyze the association between early menarche and atrazine exposure, and in the adjusted multivariate model, we included potential confounders and/or effect measure modifiers. Additionally, we performed multivariate analyses with exposure dichotomized as <LOD or ≥LOD.

RESULTS

Child's birth order, mother's prenatal BMI and mother's age at menarche significantly differed between cases and controls (Table 1). Cases were more likely to be first born children than controls (61.5% vs. 52%), have a mother who was overweight (BMI 25–29.9) or obese (BMI>30) (18.2% and 10.4%, respectively vs. 7.9% and 4% of controls), or have a mother who had onset of menarche between the ages 8–11 years (32.9% vs. 13.4% of controls) (Table 1). Cases had significantly higher BMIs at age 7 compared to controls (p <0.0001). Cases were also more likely to have a mother with either no education or a CSE (15.5% vs. 9.4% of controls). The median age at menarche for cases was 11 years compared to 12.8 years for the controls (Table 1).

DACT was the only analyte detected above the LOD in over 50% of the participants (58.3%) (Table 2). For this analyte, the median concentration for cases was 0.27ng/mL compared to 0.24ng/mL for controls. Other less frequently detected analytes were DEA (6.2%), desethyl

atrazine mercapturate (3.3%), atrazine mercapturate (1.4 %), and DIA (0.5%). Atrazine was detected in only 0.8% of the study participants.

We observed an increase in the odds of early menarche with higher exposure to DACT. In the unadjusted model assessing the association between DACT and early menarche, the odds ratio (95% confidence interval (CI)) was 1.01 (0.61, 1.67) for the LOD - <median category and 1.34 (0.83, 2.18) for the ≥median category compared to the reference category (< LOD) (Table 3). In the multivariate model adjusted for child's BMI at age 7 and mother's prenatal BMI, the odds ratio (95% CI) was 1.17 (0.62, 2.21) for the LOD - <median category and 1.82 (1.02, 3.25) for the ≥median category compared to the reference category (Table 3). The adjusted association between DACT exposure ≥median and early menarche compared to the reference was statistically significant. For the multivariate analyses with DACT exposure dichotomized as 'Yes' if at or above the LOD and 'No' if below the LOD, the adjusted odds ratio (95% CI) was 1.613 (0.95, 2.75) and was not statistically significant (Appendix I).

DISCUSSION

This is the first study to analyze the association between exposure to atrazine and metabolites and onset of menarche in humans. Our results show a statistically significant association between *in utero* exposure to the atrazine metabolite DACT at higher levels and early menarche.

The profile of the metabolites detected in the present study population indicates environmental exposure to atrazine as opposed to occupational exposures or acute low-level non-occupational exposures. As reported by Barr et al. (2007), DACT is the metabolite predominantly detected (77%) in environmental exposures, followed distantly by DEA (15%),

DIA (6%) and then atrazine mercapturate (2%). In our study, DACT was detected at 58.3%, followed by DEA (6.2%). Atrazine mercapturate and DIA were detected in 1.4% and 0.5% of our study participants respectively. The environmental exposure profile observed in ALSPAC participants is consistent with exposure to atrazine and its metabolites through drinking water or food (Barr, Panuwet, Nguyen, Udunka, & Needham, 2007).

In our study, a single sample of urine was collected during pregnancy from each mother, which given the short half-life of atrazine, would most likely only describe exposure in the past 24–48 hours, and may not be an accurate representation of exposure throughout the whole period of pregnancy. However, studies have shown that atrazine use can result in persistently contaminated drinking water supplies. If exposure to atrazine in this population occurred regularly through drinking water as indicated by the environmental exposure profile, the measurement of atrazine in this study may be an accurate indication of usual or daily exposure. This assumption does not take into account that some of the mothers may have other sources of drinking water, e.g., bottled water. Another factor that may not be taken into consideration is the water filtration systems that may be used at the public drinking water supply system or in the home. Although the use of only one urine sample may introduce error to the classification of exposure, this classification error is likely to be non-differential for cases and controls.

In countries where atrazine is still in use, e.g., the US, the metabolite mainly measured in exposure studies has been atrazine mercapturate (Arcury et al., 2007; Bakke et al., 2009). A more recent study in France measured exposure to atrazine and 7 metabolites in urine samples from 579 pregnant women in the PELAGIE cohort, and examined the association with several adverse birth outcomes including fetal growth restriction and small head circumference (Chevrier et al., 2011). DACT was detected in 7% of the participants, while DEA and DIA were detected in 10%,

and 4% of the participants respectively. Atrazine mercapturate was detected in 4% of the study participants (Chevrier et al., 2011). As suggested by Barr et. al. (2007), and also observed in the metabolite profile of our study and the study in France, measuring atrazine mercapturate alone may misrepresent atrazine exposure in some populations. Other metabolites including DACT, DEA and DIA among others need to be measured as well. However, Barr et. al. (2007) also state that "measuring atrazine compound or atrazine mercapturate in urine is the unequivocal indication that a person was exposed to atrazine and not an environment degradate".

Our study results conflict with animal studies suggesting that atrazine and DACT may delay the onset of puberty by altering the hypothalamic-pituitary activity. In most of these studies the doses used to induce the observed outcomes are typically very high (≥50 mg/kg daily) and do not compare to environmental levels that human populations are usually exposed to. It is possible that at lower doses of DACT, early hypothalamic-pituitary maturation could be observed thereby inducing early onset of puberty. Another suggested endocrine disrupting mechanism of action associated with atrazine exposure is aromatase induction, which results in an increase in estrogen (Hayes et al., 2000). It is suggested that this may contribute to some estrogen-mediated toxicities observed in animal studies (Sanderson et al., 2001). Animal studies also suggest that atrazine does not bind to the estrogen receptor and that the hypothalamic function is the main target of atrazine exposure (Cooper et al., 2007). More studies at background levels of atrazine metabolites need to be done to determine health outcomes, including onset of puberty, and mechanisms of action at doses that populations are most likely exposed to.

Some strengths of this study are the measurement of several atrazine metabolites in urine; the ability to link to several characteristics of the child and mother; and the length of follow-up

of the ALSPAC cohort, currently 22-24 years. A disadvantage of this study is only having one urine sample during the pregnancy period. The puberty questionnaires relied on self-reported age at menarche, and because the questionnaire was not sent to participants at age 12, onset of menarche reported during the ages 11–13 years could have been affected by recall bias due to this recall period (Koo & Rohan, 1997). Data for mothers prenatal BMI and childhood BMI at age 7 were missing for some cases and controls, therefore, our multivariate models adjusting for these covariates excluded observations with missing data. The exclusion of cases and controls with missing covariate data from our analyses may bias our results.

CONCLUSION

Our results suggest an association between *in utero* exposure to the atrazine metabolite DACT and early menarche in the ALSPAC cohort. To accurately assess atrazine exposure in populations, several metabolites including DACT need to be measured.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). (2003). Toxicological Profile for Atrazine.

Aksglaede, L., Olsen, L. W., Sorensen, T., Petersen, J., & Juul, A. (2008). Forty Years Trends in Timing of Pubertal Growth Spurt in 157,000 Danish School Children. PLoS ONE [Electronic Resource], 3(7).

Arcury, T., Grzywacz, J., Barr, D., Tapia, J., Chen, H., & Quandt, S. (2007). Pesticide Urinary Metabolite Levels of Children in Eastern North Carolina Farmworker Households. Environ Health Perspect, 15(7).

Avon Longitudinal Study of Parents and Children (ALSPAC). (April 21st 2014). ALSPAC Data Dictionary. Retrieved June 20th, 2014, from http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/

Bakke, B., De Roos, A., Barr, D., Stewart, P., Blair, A., Freeman, L., . . . Vermeulen, R. (2009). Exposure to atrazine and selected non-persistent pesticides among corn farmers during a growing season. Journal of Exposure Science and Environmental Epidemiology, 19, 544-554.

Balduini, L., Matoga, M., Cavalli, E., Seilles, E., Riethmuller, D., Thomassin, M., & Guillaume, Y. C. (2003). Triazine herbicide determination by gas chromatography-mass spectrometry in breast milk. Journal of Chromatography B, 389-395.

Barr, D., Panuwet, P., Nguyen, J., Udunka, S., & Needham, L. (2007). Assessing exposure to atrazine and its metabolites using biomonitoring. Environ Health Perspect, 115(10).

Boyd, A., Golding, J., Macleod, J., Lawlor, D. A., Fraser, A., Henderson, J., . . . Smith, G. D. (2013). Cohort Profile: The 'Children of the 90s' - The Avon Longitudinal Study of Parents and Children. International Journal of Epidemiology, 42(1).

Buck Louis, G. M., Gray, L. E., Marcus, M., Ojeda, S. R., Pescovitz, O. H., Witchel, S. F., . . . Euling, S. Y. (2008). Environmental Factors and Puberty Timing: Expert Panel Research Needs. Pediatrics, 121(Supplement 3), S192-S207. doi: 10.1542/peds.1813E

Catenacci, G., Barbieri, F., Bersani, A., Cottica, D., & Maroni, M. (1993). Biological monitoring of human exposure to atrazine. Toxicol Lett, 69, 217-222.

Chevrier, C., Limon, G., Monfort, C., Rouget, F., Garlantezec, R., Petit, C., . . . Cordier, S. (2011). Urinary biomarkers of prenatal atrazine exposure and adverse birthoutcomes on the PELAGIE birth cohort. Environ Health Perspect, 119(7).

Christensen, K., Maisonet, M., Rubin, C., Holmes, A., Calafat, A., Kato, K., & al., e. (2011). Exposure to polyfluoroalkyl chemicals during pregnancy is not associated with offspring age at menarche in a contemporary British cohort. Environment International, 37, 129 - 135.

Cooper, R. L., Lawson, G., Das, P. C., Narotsky, M. G., Goldman, J. M., Tyrey, E., & Stoker, T. E. (2007). Atrazine and reproductive function: mode and mechanism of action studies. Birth Defects Research, 80, 98-112.

Du Preez, L., Kunene, N., Everson, G., Carr, J., Giesy, J., Gross, T., . . . Van Der Kraak, G. (2007). Reproduction, larval growth , and reproductive development in African clawed frogs (Xenopus laevis) exposed to atrazine. Chemosphere, 71.

Enoch, R. R., Stanko, J. P., Greiner, S. N., Youngblood, G. L., Rayner, J. L., & Fenton, S. E. (2007). Mammary gland development as a sensitive end point after acute prenatal

exposure to an atrazine metabolite mixture in female long-evans rats. Environ Health Perspect, 115(4), 541-547. doi: http://dx.doi.org/10.1289/ehp.9612

Environmental Protection Agency (EPA). (2012). Atrazine background fact sheet. Retrieved October 26th, 2013, from http://www.epa.gov/pesticides/factsheets/atrazine_background.htm

Euling, S. Y., Herman-Giddens, M. E., Lee, P. A., Selevan, S. G., Juul, A., Sorensen, T., . . . Swan, S. H. (2008). Examination of US Puberty-Timing Data from 1940 to 1994 for Secular Trends: Panel Findings. Pediatrics, 121.

Fraser, A., Macdonald-Wallis, C., Tilling, K., Boyd, A., Golding, J., Smith, G. D., . . . Lawlor, D. A. (2013). Cohort Profile: The Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. International Journal of Epidemiology, 42(1).

Freedman, D. A., Khan, L., Serdula, M., Dietz, W., Srinivasan, S., & Berenson, G. (2003). The relation of menarcheal age to obesity in childhood and adulthood: the Bogalusa heart study. Bmc Pediatrics, 3.

Freedman, S., Khan, L., Serdula, M., Dietz, W., Srinivasan, S., & Berenson, G. (2002). Relation of Age at Menarche to Race, Time Period, and Anthropometric Dimensions: The Bogalusa Heart Study. Pediatrics, 110(4).

Gail, M., Brinton, L. A., Byar, D., Corle, S., Green, S., Schairer, C., & Mulvihill, J. (1989). Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. Journal of the National Cancer Institute, 81(24).

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., & Stuart, A. A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicise atrazine at low

ecologically relevant doses. Proceedings of the national academy of sciences of the United States of America, 99(8).

Kloas, W., Lutz, I., Springer, T., Krueger, H., Wolf, J., Holden, L., & Hosmer, A. (2008). Does atrazine influence larval development and sexual differentiation in Xenopus laevis? Toxicological Sciences, 107(2).

Koo, M., & Rohan, T. (1997). Accuracy of short-term recall of age at menarche. Annals of Human Biology, 24(1), 61-64.

Laws, S. C., Ferrell, J. M., Stoker, T. E., & Cooper, R. L. (2003). Pubertal development in female wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-s-chlorotriazine and hydroxyatrazine. Toxicological Sciences, 76, 190-200.

Laws, S. C., Ferrell, J. M., Stoker, T. E., Schmid, J. E., & Cooper, R. L. (2000). The effects of atrazine on female wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. Toxicological Sciences, 58, 366-376.

LeBaron, H., McFarland, J. & Burnside, O. (Eds.). The Triazine Herbicides; 50 Years Revolutionizing Agriculture. 2008.

McDowell, M. A., Brody, D. J., & Hughes, J. (2007). Has Age at Menarche Changed? Results from the National Health and Nutrition Examination Survey (NHANES) 1999–2004. Journal of Adolescent Health, 40, 227-231.

Mickey, R., & Greenland, S. (1989). The impact of confounder selection criteria on effect estimation. Am J Epidemiol, 129(1), 125-137.

Moorman, P., Palmieri, R., Akushevich, L., Berchuck, A., & Schildkraut, J. (2009). Ovarian cancer risk factors in African-American and White women. American Journal of Epidemiology, 170(5).

Munger, R., Isacon, P., Hu, S., Gburns, T., Hanson, J., Lynch, C., . . . Hausler, W. (1997). Intrauterine Growth Retardation in Iowa Communities with Herbicide-contaminated Drinking Water Supplies. Environmental Health Perspectives, 105(3), 308–314.

Ochoa-Acuna, H., Frankenberger, J., Hahn, L., & Carbajo, C. (2009). Drinking-water herbicide exposure in Indiana and prevalence of small for gestational age and preterm delivery. Environ Health Perspect, 117(10).

Oka, T., Tooi, O., Mitsui, N., Miyahara, M., Ohnischi, Y., Takase, M., & al., e. (2008). Effect of atrazine in metamorphosis and sexual differentiation in Xenopus laevis. Aquatic toxicology, 87(8), 215-226.

Panuwet, P., Nguyen, J., Kuklenyik, P., Udunka, S., Needham, L., & Barr, D. (2008). Quantification of atrazine and its metabolites in urine by on-line solid-phase extraction–high-performance liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem, 391, 1931-1939.

Panuwet, P., Restrepo, P., Magsumbol, M., Jung, K. M., Montesano, A., Needham, L., & Barr, D. (2010). An improved high-performance liquid chromatography–tandem mass spectrometric method to measure atrazine and its metabolites in human urine. Journal of Chromatography B, 878, 957-962.

Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, A., Toppari, J., & Bourguignon, J. P. (2003). The Timing of Normal Puberty and the Age Limits of Sexual Precocity: Variations

around the World, Secular Trends, and Changes after Migration. Endocrine Reviews, 24(5), 668-693.

Rayner, J. L., Wood, C., & Fenton, S. E. (2004). Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. Toxicol Appl Pharmacol, 195, 23-34.

Rubin, C., Maisonet, M., Kieszak, S., Monteilh, C., Holmes, A. K., Flanders, D., . . . Marcus, M. (2009). Timing of maturation and predictors of menarche in girls enrolled in a contemporary British cohort. Paediatr Perinat Epidemiol, 23, 492-504.

Ruder, E., Hartman, T., Rovine, M., & Dorgan, J. (2010). Birth characteristics and age at menarche: results from the dietary intervention study in children (DISC). Cancer Causes Control, 21, 1379-1386.

Sass, J. B., & Colangelo, A. (2006). European Union bans atrazine, while the United States negotiates continued use. Int J Occup Environ Health, 12(3), 260-267.

United Nations Environment Programme, & World Health Organization (UNEP&WHO). (2012). State of the Science of Endocrine Disrupting Chemicals- 2012.

vanJaarsveld, C., Fidler, J., Simon, A., & Wardle, J. (2007). Persistent Impact of Pubertal Timing on Trends in Smoking, Food Choice, Activity, and Stress in Adolescence. Psychosomatic Medicine, 69, 798-806.

Villanueva, C., Durand, G., Coutte´, M.-B., Chevrier, C., & Cordier, S. (2005). Atrazine in municipal drinking water and risk of low birthweight, preterm delivery, and small-for-gestational-age status. Occup Environ Med, 62, 400-405.

Walvoord, E. (2010). The Timing of Puberty: Is it Changing? Does it Matter? Journal of Adolescent Health, 47, 433-439.

Wetzel, L., Luempert, L., Breckenridge, C., Tisdel, M., & Stevens, J. (1994). Chronic effects of atrazine on estrus and mammary tumor formation in female sprague-dawley and fischer 344 rats. J Toxicol Environ Health, 43, 169-182.

Whyatt, R. M., Barr, D. B., Camann, D. E., Kinney, P. L., Barr, J., Andrews, H. F., . . . Perera, F. (2003). Contemporary-use pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns. Environmental Health Perspectives, 111(5), 749-756.

Winchester, P., Huskins, J., & Ying, J. (2008). Agrichemicals in surface water and birth defects in the United States. Acta Pædiatrica, 98, 664-669.

Wronka, I., & Pawlinska-Chmara, R. (2005). Menarcheal age and socio-economic factors in Poland. Annals of Human Biology, 32(5), 630-638.

Table 4.1 Study population characteristics

Characteristic	Cases (N=174) N	%	Controls (N=195) N	%	p-value[a]
Mother's Ethnicity					0.88
White	167	98.2	189	98.4	
Other Ethnicity	3	1.8	3	1.6	
Missing	4		3		
Mother's Education[b]					0.45
CSE/None	26	15.5	18	9.4	
Vocational	12	7.1	14	7.3	
O-Level	56	33.3	61	31.9	
A-Level	48	28.6	64	33.5	
Degree	26	15.5	34	17.8	
Missing	6		14		
Mother's age at delivery (yrs)					0.36
<20	1	0.6	6	3.1	
20–24	32	18.5	28	14.4	
25–29	72	41.6	74	38	
30–39	65	37.6	84	43.1	
>40	3	1.7	3	1.5	
Missing	1				
Mother's prenatal BMI					0.004
<18.5	5	3.3	11	6.2	
18.5–24.9	109	69.0	148	81.8	
25–29.9	28	17.7	15	8.3	
>30	16	10.4	7	3.9	
Missing	16		14		
Mother's age at menarche (yrs)					0.0003
8–11	50	32.9	22	13.4	
12–14	92	60.5	126	76.8	
≥ 15	10	6.6	16	9.8	
Missing	22		31		
Child's Birth order					0.03
First born	102	61.5	98	52.1	
Second born	36	21.7	65	34.6	
Third born or later	28	16.9	25	13.3	
Missing	8		7		
Child's BMI at age 7	Median 17	Quartile 15.7–18.9	Median 15.9	Quartile 15.2–16.8	<0.0001

Child's age at menarche	11	10.7–11.3	12.8	12.3–13.3

[a] Comparison of cases and controls using logistic regression
[b] CSE = certificate of secondary education, O-level = ordinary level, A-level = advanced level

Table 4.2 Gestational urine concentrations (ng/mL)

	Total	**Cases**		**Controls**		**p-value**
Analyte	**N (%) > LOD**	**N (%) > LOD**	**Median (quartiles)**	**N (%) > LOD**	**Median (quartiles)**	
Diaminochlorotriazine (DACT)	369 (58.3)	174 (60.3)	0.27 (0.16–)	195 (56.4)	0.24 (0.16–)	0.31[a]
Atrazine	369 (0.8)	174 (0.6)		195 (1)		
Atrazine mercapturate	369 (1.4)	174 (0.6)		195 (2.1)		
Desethyl atrazine mercapturate	369 (3.3)	174 (2.3)		195 (4.1)		
Desethyl atrazine (DEA)	369 (6.2)	174 (4.6)		195 (7.7)		
Desisopropyl atrazine (DIA)	369 (0.5)	174 (0.6)		195 (0.5)		
Hydroxyl atrazine	369 (1.1)	174 (0.6)		195 (1.5)		

[a] Comparison between cases and controls using Wilcoxon Rank Sum test

Table 4.3 Association between DACT gestational urine concentrations (ng/mL) and early menarche

DACT	Unadjusted OR (95% CI)[a]	Adjusted OR (95% CI) [a, d, e]
< LOD[b]	1 Reference	1 Reference
LOD – < Median[c]	1.01 (0.61–1.67)	1.17 (0.62–2.21)
≥ Median[c]	1.34 (0.83–2.18)	1.82 (1.02–3.25)

[a] Odds of early menarche by DACT exposure category compared to reference (<LOD)
[b] DACT LOD = 0.19 ng/mL
[c] Median = 0.4 ng/mL
[d] Adjusted for childhood BMI at age 7 and Mother's prenatal BMI
[e] When adjusting for confounders, observations with missing data were excluded. 127 cases and 150 controls were used in the adjusted model

CHAPTER 5

SUMMARY AND CONCLUSIONS

Pesticides are used to eliminate pests that compete with humans for food, serve as vectors for disease and destroy crops (Moore, 2007). However, these toxic substances can also cause unintended adverse effects in exposed non-target populations such as humans. Humans are mainly exposed to organochlorine pesticides through diet especially through consumption of fatty foods such as fish and dairy products (CDC, 2009). Humans can also be exposed to other pesticides such as atrazine through contaminated drinking water (ATSDR, 2003). The developing fetus and children can be exposed to pesticides *in utero* or through breast feeding (Balduini et al., 2003; Saxena, Siddiqui, Bhargava, Krishnamurti, & Kutty, 1981; Whyatt et al., 2003). The adverse health effects that have been associated with pesticide exposures include: reproductive damage, birth defects, nervous system damage, cancer and endocrine system interference (Moore, 2007).

The *in utero* environment is critical for development of the fetus. Exposures within this developmental period have been shown to be programming events that can affect health outcomes later in life, e.g., onset of puberty (Adair, 2001; Kaprio et al., 1995). There has been an increase in endocrine related disorders such as early puberty, and these changes are happening at a rate faster than would be explained by genetic factors only (UNEP & WHO, 2012). There is also an increase in the number of compounds being classified as endocrine disrupting compounds (EDCs) (UNEP & WHO, 2012). These compounds have the ability to mimic naturally occurring hormones or fool them to over-respond, or respond at inappropriate times

(UNEP & WHO, 2012). Some EDCs such as the organochlorine dichlorodiphenyltrichloroethane (DDT) are identified as persistent organic pollutants while other suspected EDCs such as the atrazine, are less persistent in the body (UNEP & WHO, 2012). Several studies have shown that organochlorines are associated with early puberty (early breast development, pubic hair development or menarche) (Krstevska-Konstantinova et al., 2001; Ouyang et al., 2005; Vasiliu, Muttineni, & Karmaus, 2004). Only two studies have examined the association between *in utero* exposures to organochlorines and puberty (Gladen, Ragan, & Rogan, 2000; Vasiliu et al., 2004). Vasiliu et al. (2004) showed that *in utero* exposure to organochlorines is associated with early menarche. There are no previous studies examining the association between atrazine exposure and onset of puberty in humans. Early puberty is a risk factor for childhood risky behaviors (e.g., smoking, alcohol consumption and drug abuse) and adult diseases (e.g., breast and ovarian cancer) (Gail et al., 1989; Moorman, Palmieri, Akushevich, Berchuck, & Schildkraut, 2009; vanJaarsveld, Fidler, Simon, & Wardle, 2007; Walvoord, 2010).

The main aims of this dissertation were to characterize the *in utero* exposure of girls in the Avon Longitudinal Study of Parents and Children (ALSPAC) to organochlorine and atrazine pesticides, and examine the association with early menarche.

IN UTERO EXPOSURE TO ORGANOCHLORINE PESTICIDES AND EARLY MENARCHE

The aims of this study were to characterize the *in utero* exposure of girls in the ALSPAC cohort to 9 organochlorine compounds and using a nested case-control study design, examine the association between this exposure and early menarche. 218 cases (menarche before 11.5 years) and 230 controls (menarche at or after 11.5 years) were included in this study.

The following 9 organochlorine compounds were measured in maternal serum collected during pregnancy (1991–1992): hexachlorobenzene (HCB), β- and ϒ- hexachlorocyclohexane (HCH), oxychlordane, trans-nonachlor, p,p'– and o,p'– dichlorodiphenyltrichloroethane (DDT), p,p-1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE), and Mirex. Only HCB, β-HCH, p,p'-DDT and p,p'-DDE were detected in over 50% of the study participants. HCB was the most frequently detected pesticide (100%), followed by followed by p,p'-DDE (99.8%), β-HCH (98%), and p,p'-DDT (88%). Mirex was the least detected pesticide (0.45%). In the four pesticide compounds detected in >50% of the study participants, DDT metabolite p,p'-DDE had the highest median concentration level at 309.27ng/g, while p,p'-DDT had the lowest median concentration (11.2ng/g).

Using logistic regression, we measured the association between HCB, β-HCH, p,p'-DDT and p,p'-DDE and early menarche. None of the pesticide associations with early puberty were statistically significant. For the adjusted models, all odds ratios were <1, and none of the associations were statistically significant.

The results of this study show that although organochlorines have been banned for decades, because of their biological and environmental persistence, they are still detectable in humans. The exposure characterized in this study represents exposure to background levels of organochlorine pesticides. This is the first study to examine the association between *in utero* exposure to HCB or HCH and early menarche, although previous studies have examined the association between *in utero* exposure to DDT/DDE and early puberty. It appears that *in utero* exposure to organochlorine pesticides in the ALSPAC cohort is not associated with early menarche.

IN UTERO EXPOSURE TO ATRAZINE AND EARLY MENARCHE

The main aims of this study were to characterize the *in utero* exposure of girls in the ALSPAC cohort to 7 atrazine analytes and using a nested case-control study design, examine the association of this exposure with early menarche. 174 cases (menarche before 11.5 years) and 195 controls (menarche at or after 11.5 years) were included in this study.

These 7 atrazine analytes were measured in maternal urine collected during pregnancy (1991–1992): atrazine, atrazine mercapturate, desethyl atrazine mercapturate, diaminochlorotriazine (DACT), desethyl atrazine (DEA), desisopropyl atrazine (DIA), and hydroxyl atrazine. Only DACT was detected in over 50% of the participants. The median concentration of DACT was 0.27ng/mL in cases and 0.24ng/mL in controls.

Using logistic regression, we examined the association between *in utero* exposure to DACT and early menarche. Childhood BMI at age 7 and mother's prenatal BMI were included as confounders in the multivariate model. No effect measure modifiers were included. DACT was associated with increased odds of early menarche (adjusted OR = 1.82; CI = (1.02, 3.25)) in girls exposed *in utero* to DACT ≥median compared to the reference group (<LOD). This association was statistically significant.

The results of this study show that humans are exposed to several metabolites of the atrazine compound. The metabolite detection profile shows that these study participants were most likely exposed to atrazine environmentally, i.e., through contaminated food or drinking water. The results also show that measuring only atrazine compound or atrazine mercapturate alone may underestimate exposure. This is the first study to examine the association between

atrazine exposure and puberty in humans. DACT exposure *in utero* was associated with increased odds of early menarche.

REFERENCES

Adair, L. (2001). Size at Birth Predicts Age at Menarche. *Pediatrics, 107*.

Agency for Toxic Substances Disease Registry, A (ATSDR). (2003). Toxicological Profile for Atrazine. Retrieved October 26th, 2013, from http://www.atsdr.cdc.gov/toxprofiles/tp153.pdf

Balduini, L., Matoga, M., Cavalli, E., Seilles, E., Riethmuller, D., Thomassin, M., & Guillaume, Y. (2003). Triazine herbicide determination by gas chromatography-mass spectrometry in breast milk. *Journal of Chromatography B*, 389-395.

Centers for Disease Control and Prevention, National Center for Health Statistics (CDC). (2009). The Fourth National Report on Human Exposure to Environmental Chemicals.

Gladen, B., Ragan, B., & Rogan, W. Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. *The Journal of Pediatrics, 136(4)*.

Gail, M., Brinton, L. A., Byar, D., Corle, S., Green, S., Schairer, C., & Mulvihill, J. (1989). Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. Journal of the National Cancer Institute, 81(24).

Kaprio, J., Rimpela, A., Winter, T., Viken, R., et al. (1995). Common genetic influences on BMI and age at menarche. *Hum Biol, 67*, 739-753.

Krstevska-Konstantinova, M., Charlier, C., Craen, M., Du Caju, M., Heinrichs, C., de Beaufort, C., . . . Bourguignon, J. P. (2001). Sexual precocity after immigration from developing countries to Belgium: evidence of previous exposure to organochlorine pesticides. *Hum Reprod, 16(5),* 1020-1026.

Moorman, P., Palmieri, R., Akushevich, L., Berchuck, A., & Schildkraut, J. (2009). Ovarian cancer risk factors in African-American and White women. American Journal of Epidemiology, 170(5).

Moore, G.S. (2007). *Living with the Earth: Concepts in Environmental Health Science (3rd Ed)*. New York, NY: Taylor & Francis Group, LLC.

Ouyang, F., Perry, M., Venners, S., Chen, C., Wang, B., Yang, F.,… Wang, X. (2005). Serum DDT, age at menarche, and abnormal menstrual cycle length. *Occupational & Environmental Medicine, 62*, 878–884.

Saxena, M., Siddiqui, M.K.J., Bhargava, A.K., Krishna Murti, C.R. Kutty, D. (1981). Placental Transfer of Pesticides in Humans. *Archives of Toxicology, 48*, 127-134.

United Nations Environment Programme (UNEP), & World Health Organization (WHO). (2012). State of the Science of Endocrine Disrupting Chemicals- 2012.

vanJaarsveld, C., Fidler, J., Simon, A., & Wardle, J. (2007). Persistent Impact of Pubertal Timing on Trends in Smoking, Food Choice, Activity, and Stress in Adolescence. Psychosomatic Medicine, 69, 798-806.

Vasiliu, O., Muttineni, J., & Karmaus, W. (2004). In utero exposure to organochlorines and age at menarche. *Hum Reprod, 19(7), 1506-1512*. doi: 10.1093/humrep/deh292

Whyatt, R., Barr, D., Camann, D., Kinney, P., Barr, J., Andrews, H., . . . Perera, F. (2003). Contemporary-use pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns. Environmental Health Perspectives, 111(5), 749-756.

CHAPTER 6

PUBLIC HEALTH IMPLICATIONS

The results presented this dissertation provide information on how the general population in Bristol, United Kingdom (UK) is exposed to organochlorine and atrazine pesticide compounds, and how these concentrations *in utero* are associated with early menarche in girls in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. There are some implications of these results on public health.

ORGANOCHLORINE PESTICIDES

DDT was banned in the UK in 1986 (Rogan & Chen, 2005). Twelve persistent organic pollutants including DDT and HCB were banned globally in a 2004 treaty at the Stockholm Convention on Persistent Organic Pollutants (The Stockholm Convention, 2008). HCH is one of the nine persistent organic pesticides added to the initial list of 12 when the Stockholm Convention was amended in 2009 (The Stockholm Convention, 2008). Despite these bans – some for decades, organochlorine pesticides are still detected in humans, and their associations with adverse health effects remain a concern. However, due to these public health efforts, there has been a reduction in the body burdens of these organochlorines over time. For example, the median concentrations of the organochlorines measured in biological samples collected in the UK 10–12 years after our study, show more than 50% decrease in p,p'-DDT and p,p'-DDE, and more than 60% decrease in HCB and β-HCH compared to our study results (Kalantzi et al., 2004; Thomas, Wilkinson, Hodson, & Jones, 2006).

Organochlorines are environmentally and biologically persistent, and therefore are still detected in the environment and in humans. Populations are exposed to organochlorines mainly through diet. Education on limiting or avoiding exposure through consumption of fatty foods such as fish and dairy products need to continue, especially for pregnant or nursing mothers and in areas where these pesticides are still in use. Education about these pesticides, their associated adverse health effects and ways to avoid exposure needs to be targeted to populations in countries where they are still in use. More efforts need to focus on measuring exposure in populations where organochlorines are still in use and examining their associations with adverse health outcomes.

Countries where these pesticides are still in use should weigh the advantages and disadvantages of using them. Safer alternatives, for example, using less toxic and persistent alternatives, or using other methods e.g., integrated pest management (IPM) principles can also be explored. IPM is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment (EPA, 2014). IPM can include judicious use of pesticides or using pesticides from natural sources (EPA, 2014).

ATRAZINE

Atrazine has been banned from use in the European Union (E.U.) since 2003 however; it is still in use in several countries worldwide including the United States (U.S.). In several exposure studies including the National Health and Nutrition Examination survey (NHANES), atrazine mercapturate has been the predominantly measured atrazine metabolite. The results presented in this dissertation show that this may result in underestimating atrazine exposure in populations because populations are exposed to several atrazine metabolites. Exposure to

atrazine manifests in different profile distributions of DACT, DEA and DIA among other metabolites that indicate the type of exposure. For the general population or environment exposure, DACT is the predominant metabolite followed by DEA and DIA. Exposure studies therefore should include measurement of DACT, DIA and DEA among other atrazine metabolites to give a more accurate characterization of the atrazine compounds populations are exposed to.

The U.S. is one of the 70 or more countries that use atrazine. The U.S. Environmental Protection Agency (EPA) currently sets a maximum concentration limit (MCL) for atrazine in drinking water at 3 ppb (EPA, 2012). Studies have shown that some metabolites of atrazine e.g., DACT, can be as potent as atrazine in inducing adverse effects (Laws, Ferrell, Stoker, & Cooper, 2003). However, EPA does not regulate these metabolites in drinking water. Countries that use atrazine should explore including several metabolites such as DACT, DEA, DIA to other compounds regulated in drinking water. More studies need to be done to establish the MCLs for these metabolites.

Atrazine is currently classified as a non-persistent pesticide though studies show that it can be persistent in ground water therefore persistently contaminating surface and drinking water. This persistent contamination of drinking water can result in continuous exposure in populations which is a concern. Re-classifying atrazine to “pseudo persistent” should be explored.

In areas where atrazine is used, populations should be provided with information on how to prevent or reduce atrazine exposure, especially in the months where it is possible to have elevated levels in drinking water (e.g., above the EPA MCL in US populations). Such options

can include drinking bottled water or investing in a water filter that is capable of removing these compounds from drinking water. This should be encouraged especially to protect susceptible sub-populations such as the fetus and nursing children.

This is the first study that has examined the association between atrazine exposure and onset of menarche in humans. Our study results suggest an association between DACT and early menarche. These results conflict with animal studies which show that DACT delayed puberty in rats; however, the doses used in these studies were very high (>50mg/kg) and not typically what populations would be exposed to. It is possible that a different outcome of onset of puberty could be observed at lower doses. More studies need to be done using background concentrations levels of these atrazine compounds that populations are most likely to be exposed to, and examining their associations with adverse outcomes including early onset of puberty.

EARLY MENARCHE

Our study results show that there were significant differences in the distribution of cases and controls within Mother's age at menarche, Mother's prenatal BMI, or birth order categories and with Child's BMI at age 7. Cases were more likely to have mothers who were overweight or obese. Cases were also more likely to have higher pre-pubertal BMI at age 7. Body size is modifiable. Behavioral intervention can be targeted towards reducing BMI for women before becoming pregnant and children before onset of puberty.

REFERENCES

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2002). Toxicological Profile for DDT, DDE, and DDD.

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2005). Toxicological Profile for Alpha-, Beta-, Gamma-, and Delta-Hexachlorocyclohexane.

Agency for Toxic Substances and Disease Registry CDC (ATSDR). (2013). Draft Toxicological Profile for Hexachlorobenzene.

Environmental Protection Agency. *Atrazine background fact sheet*. 2012 [cited 2013 October 26th]; Available from: http://www.epa.gov/pesticides/factsheets/atrazine_background.htm.

Environmental Protection Agency. *Integrated pest management (IPM) principles*. 2014 [cited 2013 October 26th]; Available from: http://www.epa.gov/opp00001/factsheets/ipm.htm

Kalantzi, O. M., Martin, F., Thomas, G., Alcock, R., Tang, H., Drury, S., … Jones, K.C. (2004). Different Levels of Polybrominated Diphenyl Ethers (PBDEs) and Chlorinated Compounds in Breast Milk from Two U.K. Regions. *Environmental Health Perspectives, 112(10)*.

Laws, S., Ferrell, J., Stoker, T., & Cooper, R. (2003). Pubertal development in female wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-s-chlorotriazine and hydroxyatrazine. *Toxicological Sciences, 76*, 190-200.

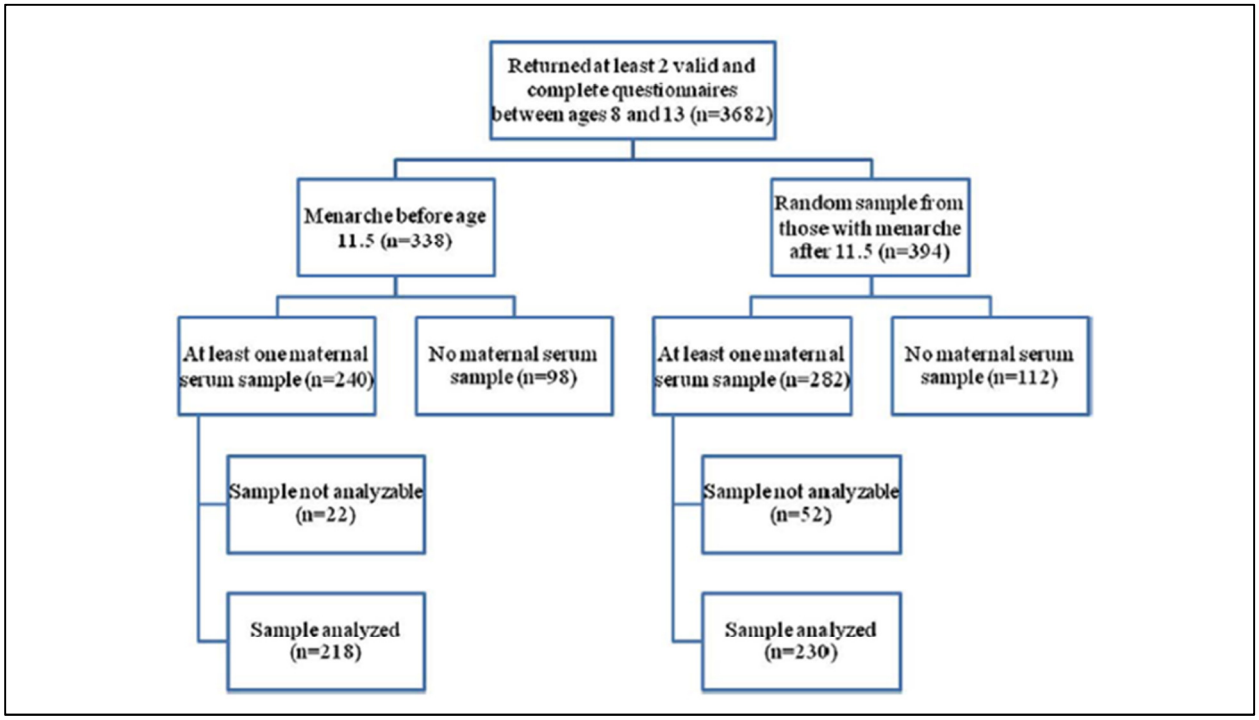
Rogan, W. J., & Chen, A. (2005). Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). *The Lancet, 366(9487),* 763-773. doi: 10.1016/S0140-6736(05)67182-6

The Stockholm Convention. (2008). The 12 initial POPs under the Stockholm Convention. Retrieved February 15th, 2014

Thomas, G. O., Wilkinson, M., Hodson, S., & Jones, K. C. (2006). Organohalogen chemicals in human blood from the United Kingdom. *Environ Pollut, 141(1)*, 30-41. doi: 10.1016/j.envpol.2005.08.027

APPENDICES

APPENDIX A: CASES AND CONTROLS ELIGIBILITY AND EXCLUSIONS CRITERIA



APPENDIX B: EVALUATION OF POSSIBLE CONFOUNDERS TO THE EARLY MENARCHE-ORGANOCHLORINE EXPOSURE ASSOCIATION

Covariate	Covariate-exposure relationship. Is the covariate associated with the exposure in non-cases? p-value[a]	Covariate-outcome relationship. Is covariate associated with outcome while controlling for exposure? p-value[b]	Confounder status Based on p < 0.25 for both columns 2 and 3
Gestation trimester (weeks) 1 = <12 2 = 13 - </=28 3 = >/= 29	HCB: 0.0059 B_HCH: 0.0064 PP_DDE: 0.2648 PP_DDT: 0.8700	HCB: 0.8627 B_HCH:0.9210 PP_DDE:0.9498 PP_DDT:0.8637	No No No No
Maternal pre-natal BMI 1 = <18.5 2 = 18.5-24.9 3 = 25-29.9 4 = >30	HCB: 0.4301 B_HCH: 0.6530 PP_DDE: 0.6439 PP_DDT: 0.0519	HCB: 0.0061 B_HCH:0.0035 PP_DDE:0.0043 PP_DDT:0.0170	No No No Yes
Age at delivery (yrs) 1 = <20 2 = 20-24 3 = 25-29 4 = 30-39 5 = >40	HCB: <.0001 B_HCH:<.0001 PP_DDE:<.0001 PP_DDT:<.0001	HCB: 0.7675 B_HCH:0.6223 PP_DDE:0.6573 PP_DDT:0.7722	No No No No
Education 1 = CSE/None 2 = Vocational 3 = O-Level 4 = A-Level 5 = Degree	HCB: 0.2163 B_HCH:0.0189 PP_DDE:0.5478 PP_DDT: 0.1323	HCB: 0.4802 B_HCH:0.4444 PP_DDE:0.4211 PP_DDT:0.4977	No No No No
Maternal age at menarche (yrs) 1 = 8 – 11 2 = 12 – 14 3 = ≥ 15	HCB: 0.0108 B_HCH:0.1060 PP_DDE:0.4583 PP_DDT:0.6625	HCB: 0.0003 B_HCH:0.0003 PP_DDE:0.0004 PP_DDT:0.0015	Yes Yes No No
Birth weight	HCB: 0.2054	HCB: 0.3701	No

1 = <2500g 2 = >/= 2,500g	B_HCH:0.0100 PP_DDE:0.0007 PP_DDT: 0.1435	B_HCH:0.4227 PP_DDE:0.4178 PP_DDT: 0.3570	No No No
Childhood BMI at age 7 Continuous variable	HCB: 0.9767 B_HCH:0.5486 PP_DDE:0.3014 PP_DDT:0.9718	HCB: <0.0001 B_HCH:<0.0001 PP_DDE:<0.0001 PP_DDT:<0.0001	No No No No
Birth order 1 = first born 2 = 2nd born 3 = third or later	HCB: 0.3102 B_HCH:0.5039 PP_DDE:0.5000 PP_DDT: 0.3336	HCB: 0.4590 B_HCH:0.5093 PP_DDE:0.4945 PP_DDT:0.5865	No No No No
Duration of breastfeeding 1 = Never 2 = < 6 months 3 = >/= 6 months	HCB:0.3703 B_HCH:0.1611 PP_DDE:0.8667 PP_DDT:0.5027	HCB: 0.2511 B_HCH: 0.2473 PP_DDE: 0.2441 PP_DDT: 0.1300	No Yes No No

[a]Using ANOVA or logistic regression
[b]Using logistic regression

APPENDIX C: EVALUATION OF POSSIBLE EFFECT MEASURE MODIFIERS (EMM) OF THE ORGANOCHLORINE-EARLY MENARCHE ASSOCIATION

Organochlorine	Covariate	Stratified OR (95% CI)	p-value[b]	Interaction term p-value[b]	Sample size	EMM? Interaction term p-value <0.05
	Gestation trimester					
HCB	1 2 3	0.51 (0.23 – 1.13) 1.26 (0.63 – 2.50) 1.25 (0.51 – 3.07)	0.0980 0.5107 0.6252	0.0949	184 155 109	No
B_HCH	1 2 3	0.38 (0.18 – 0.79) 0.87 (0.46 – 1.66) 1.34 (0.77 – 2.35)	0.0093 0.6789 0.3045	0.0070	184 155 109	Yes
PP_DDE	1 2 3	0.72 (0.46 – 1.12) 1.08 (0.69 – 1.67) 1.01 (0.57 – 1.78)	0.1446 0.7345 0.9822	0.2603	184 155 109	No
PP_DDT	1 2 3	0.84 (0.45 – 1.57) 1.57 (0.68 – 1.96) 1.09 (0.47 – 2.48)	0.5855 0.5895 0.8220	0.6494	179 148 104	No
	Maternal pre-natal BMI					
HCB	1 2 3 4	2.89 (0.09 – 93.68) 0.72 (0.39 – 1.32) 1.01 (0.36 – 2.82) 0.86 (0.14 – 5.61)	0.5489 0.2813 0.9919 0.8880	0.8817	18 285 62 31	No
B_HCH	1 2 3 4	0.79 (0.06 – 10.48) 0.79 (0.52 – 1.21) 0.81 (0.28 – 2.37) 0.53 (0.12 – 2.34)	0.8584 0.2781 0.7000 0.4077	0.6016	18 285 62 31	No
PP_DDE	1 2 3 4	0.59 (0.13 – 2.76) 0.85 (0.61 – 1.19) 1.77 (0.75 – 4.22) 0.65 (0.21 – 1.99)	0.5084 0.3480 0.1945 0.4479	0.6002	18 285 62 31	No
PP_DDT	1 2 3 4	1.36 (0.12 – 14.81) 0.81 (0.52 – 1.28) 2.73 (0.77 – 9.69) 0.92 (0.26 – 3.22)	0.8030 0.3740 0.1210 0.8959	0.7418	17 276 61 30	No

	Age at delivery[a]					
HCB	1 = <25 2 = 25 - 30 3 = >30	0.95 (0.25 – 3.58) 1.11 (0.46 – 2.68) 0.88 (0.49 – 1.57)	0.9384 0.8117 0.6563	0.6327	92 164 189	No
B_HCH	1 2 3	1.06 (0.45 – 2.48) 0.88 (0.46 – 1.68) 0.75 (0.44 – 1.27)	0.8932 0.6935 0.2789	0.3817	92 164 189	No
PP_DDE	1 2 3	0.85 (0.39 – 1.83) 0.90 (0.52 – 1.57) 0.91 (0.62 – 1.34)	0.6778 0.7159 0.6314	0.9281	92 164 189	No
PP_DDT	1 2 3	0.42 (0.13 – 1.40) 1.24 (0.64 – 2.38) 1.09 (0.67 – 1.79)	0.1596 0.5261 0.7224	0.3196	87 155 187	No
	Birth weight					
HCB	1 2	0.68 (0.16 – 2.94) 0.99 (0.64 – 1.57)	0.6094 0.9909	0.6278	25 423	No
B_HCH	1 2	0.62 (0.11 – 3.69) 0.89 (0.63 – 1.26)	0.6026 0.5087	0.7023	25 423	No
PP_DDE	1 2	0.92 (0.35 – 2.41) 0.93 (0.70 – 1.24)	0.8698 0.6383	0.9812	25 423	No
PP_DDT	1 2	0.66 (0.65 – 4.21) 0.99 (0.66 – 1.48)	0.207 0.9512	0.2708	24 407	No
	Maternal education					
HCB	1 2 3 4 5	0.66 (0.24 – 1.76) 1.12 (0.21 – 5.88) 0.96 (0.39 – 2.44) 1.08 (0.42 – 2.74) 0.92 (0.38 – 2.22)	0.4049 0.8946 0.9460 0.8765 0.8497	0.6159	57 32 140 127 73	No
B_HCH	1 2 3 4 5	0.98 (0.48 – 2.00) 0.99 (0.27 – 3.68) 0.96 (0.49 – 1.89) 0.65 (0.34 – 1.26) 0.63 (0.19 – 2.02)	0.9620 0.9935 0.9096 0.2002 0.4369	0.4077	57 32 140 127 73	No
PP_DDE	1 2 3 4 5	10.81 (0.43– 1.54) 0.82 (0.28 – 2.36) 0.74 (0.44 – 1.23) 0.88 (0.49 – 1.59)	0.5243 0.7080 0.2421 0.6791 0.4324	0.3223	57 32 140 127 73	No

		1.31 (0.67 – 2.58)				
PP_DDT	1 2 3 4 5	0.73 (0.25 – 2.12) 0.45 (0.12 – 1.77) 1.17 (0.58 – 2.35) 1.04 (0.51 – 2.14) 1.35 (0.61 – 2.99)	0.5569 0.2526 0.6679 0.9149 0.4562	0.2153	55 32 134 123 72	No
	Maternal Age at Menarche					
HCB	1 2 3	0.53 (0.19 – 1.43) 1.15 (0.66 – 2.02) 0.4 (0.41 – 4.45)	0.2101 0.6198 0.4640	0.6299	93 272 27	No
B_HCH	1 2 3	0.47 (0.19 – 1.13) 1.01 (0.67 – 1.52) 0.32 (0.04 – 2.99)	0.0930 0.9760 0.3195	0.5247	93 272 27	No
PP_DDE	1 2 3	0.47 (0.19 – 1.13) 1.01 (0.67 – 1.52) 0.31 (0.06 – 1.69)	0.0930 0.9760 0.1769	0.7900	93 272 27	No
PP_DDT	1 2 3	0.93 (0.44 – 1.98) 1.14 (0.71 – 1.83) 0.85 (0.14 – 5.28)	0.8499 0.5775 0.8618	0.8433	90 266 25	No
	Childhood BMI at age 7					
HCB				0.7717		No
B_HCH				0.7482		No
PP_DDE				0.2759		No
PP_DDT				0.1535		No
	Birth order					
HCB	1 2 3	0.90 (0.42 – 1.93) 1.33 (0.66 – 2.69) 0.74 (0.28 – 1.99)	0.7869 0.4325 0.5542	0.7249	208 140 71	No
B_HCH	1 2 3	0.85 (0.49 – 1.46) 0.98 (0.54 – 1.79) 0.77 (0.36 – 1.66)	0.5565 0.9582 0.5080	0.7697	208 140 71	No
PP_DDE	1 2 3	0.79 (0.53 – 1.21) 1.07 (0.65 – 1.74) 0.84 (0.45 – 1.58)	0.2875 0.7961 0.5905	0.8860	208 140 71	No
PP_DDT	1 2 3	0.94 (0.53 – 1.69) 1.02 (0.52 – 1.99) 1.09 (0.44 – 2.70)	0.8383 0.9576 0.8608	0.9300	203 133 70	No
	Duration of breastfeeding					
HCB	1	1.04 (0.29 – 3.71)	0.9492	0.1187	82	No

	2 3	2.08 (0.71 – 6.12) 0.59 (0.30 – 1.17)	0.1838 0.1290		105 167	
B_HCH	1 2 3	1.19 (0.55 – 2.59) 2.18 (0.87 – 5.43) 0.57 (0.32 – 0.99)	0.6533 0.0954 0.0486	0.0502	82 105 167	No
PP_DDE	1 2 3	0.88 (0.43 – 1.77) 1.16 (0.63 – 2.14) 0.89 (0.57 – 1.39)	0.7118 0.6296 0.6232	0.7739	82 105 167	No
PP_DDT	1 2 3	1.43 (0.54 – 3.77) 1.99 (0.86 – 4.64) 0.89 (0.47 – 1.68)	0.4743 0.1087 0.7215	0.1898	77 102 162	No

[a] Age categories collapsed because of small sample sizes
[b] Using logistic regression

APPENDIX D: ASSOCIATION BETWEEN ORGANOCHLORINE GESTATIONAL SERUM CONCENTRATIONS (ng/g) AND EARLY MENARCHE (EXPOSURE CATEGORIZED AS < MEDIAN OR ≥MEDIAN USING THE DISTRIBUTION OF THE CONTROLS)

Pesticide	Unadjusted OR (95%CI)[a]	Adjusted OR (95% CI)[a, f]	p-value
HCB	1.04 (0.71 – 1.52)	0.98 (0.65 – 1.49)	0.93[b]
B_HCH	0.93 (0.64 – 1.36)	0.90 (0.57 – 1.43)	0.66[c]
PP_DDE	1.02 (0.7 – 1.49)		0.92[d]
PP_DDT	1.12 (0.74 – 1.64)	1.00 (0.66 – 1.53)	0.98[e]

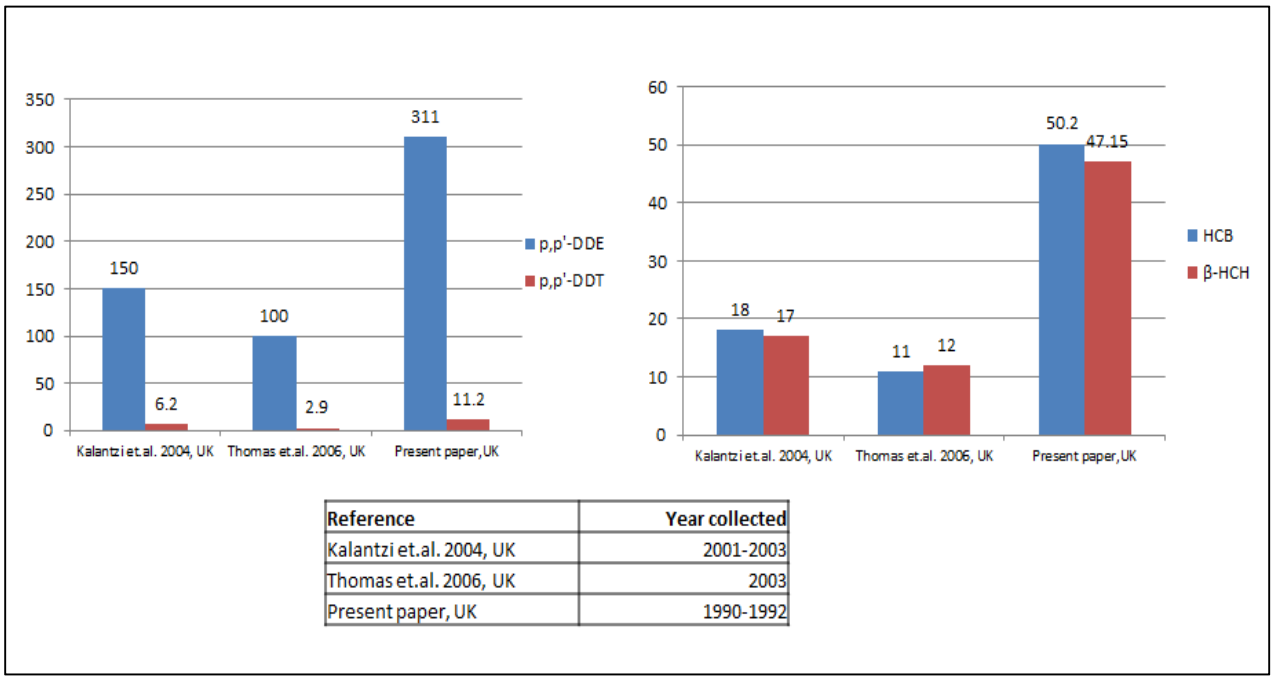
[a] Odds of early menarche for a unit increase of logged organochlorine pesticide concentration
[b] Adjusted for mother's age at menarche
[c] Adjusted for mother's age at menarche and duration of breastfeeding
[d] Unadjusted p value
[e] Adjusted for mother's prenatal BMI
[f] When adjusting for confounders observations with missing data were excluded

APPENDIX E: ASSOCIATION BETWEEN ORGANOCHLORINE GESTATIONAL SERUM CONCENTRATIONS (ng/g) AND EARLY MENARCHE (EXPOSURE CATEGORIZED IN QUARTILES <25th, ≥ 25th - <50TH, ≥ 50th - <75th, AND ≥ 75th PERCENTILES USING THE DISTRIBUTION OF CONTROLS)

Pesticide	Unadjusted OR (95% CI) [a]	Adjusted OR (95% CI) [a, e]
HCB		
Quartile 1	1 Reference	1 Reference
Quartile 2	0.81 (0.47 – 1.4)	0.97 (0.54 – 1.76) [b]
Quartile 3	0.92 (0.54 – 1.55)	0.97 (0.55 – 1.71) [b]
Quartile 4	0.96 (0.55 – 1.68)	0.97 (0.53 – 1.78) [b]
B_HCH		
Quartile 1	1 Reference	1 Reference
Quartile 2	0.81 (0.47 – 1.4)	0.97 (0.54 – 1.76)
Quartile 3	0.92 (0.54 – 1.55)	0.97 (0.55 – 1.71)
Quartile 4	0.96 (0.55 – 1.68)	0.97 (0.53 – 1.78)
PP_DDE		
Quartile 1	1 Reference	
Quartile 2	0.73 (0.43 – 1.24)	
Quartile 3	0.75 (0.44 – 1.27)	
Quartile 4	1.025 (0.6 – 1.75)	
PP_DDT		
Quartile 1	1 Reference	1 Reference
Quartile 2	0.86 (0.50 – 1. 74)	0.85 (0.48 – 1.52) [d]
Quartile 3	1.08 (0.62 – 1.89)	0.96 (0.52 – 1.71) [d]
Quartile 4	0.98 (0.57 – 1.70)	0.89 (0.49 – 1.62) [d]

[a] Odds of early menarche for a unit increase of logged organochlorine pesticide concentration
[b] Adjusted for mother's age at menarche
[c] Adjusted for mother's age at menarche and duration of breastfeeding
[d] Adjusted for mother's prenatal BMI
[e] When adjusting for confounders observations with missing data were excluded

APPENDIX F: COMPARISON OF LIPID ADJUSTED ORGANOCHLORINE MEDIAN VALUES MEASURED IN POPULATIONS IN THE UK (ng/g)



Reference	Year collected
Kalantzi et.al. 2004, UK	2001-2003
Thomas et.al. 2006, UK	2003
Present paper, UK	1990-1992

APPENDIX G: EVALUATION OF POSSIBLE CONFOUNDERS TO THE EARLY MENARCHE-DACT EXPOSURE ASSOCIATION

Covariate	**Covariate – exposure relationship p-value** [a]	**Covariate – outcome relationship p-value** [a]	**Confounder status Based on p <0.25 for both column 2 and 3**
Gestation trimester (weeks) 1 = <12 2 = 13 - </=28 3 = >/= 29 Has underlying continuous variable	0.2656	0.5267	No
Maternal pre-natal BMI 1 = <18.5 2 = 18.5-24.9 3 = 25-29.9 4 = >30 Has underlying continuous variable	0.0489	0.0004	Yes
Age at delivery (yrs) 1 = <20 2 = 20-24 3 = 25-29 4 = 30-39 5 = >40 Has underlying continuous variable	0.6722	0.5590	No
Education 1 = CSE/None 2 = Vocational 3 = O-Level 4 = A-Level 5 = Degree	0.8428	0.0834	No
Maternal age at menarche (yrs) 1 = 8 – 11 2 = 12 – 14	0.8034	0.0003	No

3 = >/= 15 Has underlying continuous variable			
Birth weight 1 = <2500g 2 = >/= 2,500g Continuous variable	0.8981	0.7647	No
Childhood BMI at age 7 Continuous variable	0.1148	<0.0001	Yes
Birth order 1 = first born 2 = 2nd born 3 = third or later	0.1571	0.4290	No
Duration of breastfeeding 1 = Never 2 = < 6 months 3 = >/= 6 months	0.1637	0.7859	No
Exposure Month 1 1 =Dec, Jan, Feb 2 =Mar, Apr, May 3 =Jun, Jul, Aug 4 =Sep, Oct, Nov	0.6756	0.6333	No
Exposure Month 2 (Spring/summer Vs other months) 1 =Mar – Aug 2 =Sep - Feb	0.2585	0.4816	No
Maternal Race 1 = White 2 = non-White	0.2512	0.8950	No
Childs race 1 = White 2 = non-White	0.9229	0.6508	No

[a] Using logistic regression

APPENDIX H: EVALUATION OF POSSIBLE EFFECT MEASURE MODIFIERS (EMM) OF THE DACT-EARLY MENARCHE ASSOCIATION

Covariate	Stratified OR (95% CI)	p-value[b]	Interaction term p-value[b]	Sample size	EMM? Interaction p <0.05
Gestation trimester (weeks) 1 = <12 2 = 13 - </=28 3 = >/= 29 Has underlying continuous variable	1= 0.92 (0.65 – 1.30) 2 = 0.79 (0.53 – 1.17) 3 = 0.88 (0.42 – 1.85)	0.6439 0.2354 0.7388	0.8623	188 138 43	No
Maternal pre-natal BMI 1 = <18.5 2 = 18.5-24.9 3 = 25-29.9 4 = >30 Has underlying continuous variable	1= 0.67 (0.18 – 2.52) 2= 0.84 (0.62 – 1.14) 3= 0.59 (0.28 – 1.29) 4= 3.14 (0.89 – 11.11)	0.5491 0.2668 0.1878 0.0756	0.1130	16 250 42 23	No
Age at delivery (yrs)[a] 1= <25 2= 25 - 30 3= >30 Has underlying continuous variable	1= 1.25 (0.69 – 2.25) 2= 0.71 (0.48 – 1.05) 3= 0.87 (0.59 – 1.26)	0.4489 0.0866 0.4606	0.4705	67 146 155	No
Education 1 = CSE/None 2 = Vocational 3 = O-Level 4 = A-Level 5 = Degree	1= 0.73 (0.34 – 1.57) 2= 0.89 (0.37 – 2.11) 3= 1.05 (0.68 – 1.63) 4= 0.77 (0.48 – 1.22) 5= 0.75 (0.42 – 1.33)	0.4128 0.7860 0.8189 0.2693 0.3239	0.6743	44 26 117 112 60	No
Maternal age at menarche (yrs) 1 = 8 – 11 2 = 12 – 14 3 = >/= 15 Has underlying	1= 0.89 (0.47 – 1.67) 2= 0.93 (0.68 – 1.26) 3= 0.66 (0.25 – 1.73)	0.7075 0.6203 0.3931	0.6955	72 218 26	No

continuous variable					
Birth weight 1 = <2500g 2 = >/= 2,500g Continuous variable	 1= 1.47 (0.35 – 5.81) 2= 0.85 (0.67 – 1.09)	 0.6196 0.2098	0.4806	 14 355	No
Childhood BMI at age 7 Continuous variable			0.0895		No
Birth order 1 = first born 2 = 2nd born 3 = third or later	 1= 0.82 (0.59 – 1.15) 2= 0.69 (0.42 – 1.13) 3= 1.25 (0.68 – 2.29)	 0.2507 0.1369 0.4697	0.3780	 200 101 53	No
Duration of breastfeeding 1 = Never 2 = < 6 months 3 = >/= 6 months	 1= 0.99 (0.55 – 1.78) 2= 1.14 (0.69 – 1.86) 3= 0.55 (0.36 – 0.84)	 0.9788 0.6029 0.0059	0.0518	 67 87 133	No
Exposure Month 1 1 =Dec, Jan, Feb 2 =Mar, Apr, May 3 =Jun, Jul, Aug 4 =Sep, Oct, Nov Has underlying continuous variable	 1= 0.73 (0.46 – 1.15) 2= 1.01 (0.65 – 1.58) 3= 0.67 (0.41 – 1.12) 4= 1.21 (0.68 – 2.14)	 0.1754 0.9563 0.1245 0.5218	0.4123	 102 109 83 73	No
Exposure Month 2 (Spring/summer Vs other months) 1 =Mar – Aug 2 =Sep - Feb Has underlying continuous variable	 1= 0.84 (0.60 – 1.17) 2= 0.89 (0.62 – 1.27)	 0.2970 0.5094	0.8161	 192 175	No
Maternal Race 1 = White 2 = non-White	 1= 0.86 (0.67 – 1.09) 2= 2.00 (0.37 – 10.92)	 0.2196 0.4236	0.3326	 356 6	No

[a] Age at delivery categories collapsed because of small numbers
[b] Using logistic regression

APPENDIX I: ASSOCIATION BETWEEN DACT GESTATIONAL URINE CONCENTRATIONS (ng/mL) AND EARLY MENARCHE (EXPOSURE AS A DICHOTOMOUS VARIABLE)

Analyte	Unadjusted OR (95%CI)[a]	Adjusted OR (95% CI) [a, b, c]	p-value
DACT	1.18 (0.78 – 1.78)	1.613 (0.95 – 2.75)	0.0790

[a] Odds of early menarche by DACT exposure

[b] Adjusted for childhood BMI at age 7 and duration of breastfeeding

[c] When adjusting for confounders observations with missing data were excluded