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Exposure to phytoestrogens *in utero* and age at menarche in a contemporary British cohort

By

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Master of Public Health

Epidemiology

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Exposure to phytoestrogens *in utero* and age at menarche in a contemporary British cohort

By

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Bachelor of Arts
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2012

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An abstract of
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Abstract

Exposure to phytoestrogens *in utero* and age at menarche in a contemporary British cohort
By Kristin Marks

Phytoestrogens are estrogenic compounds that occur naturally in plants. Phytoestrogens can cross the placenta, and animal studies have found associations between *in utero* exposure to phytoestrogens and markers of early puberty. We investigated the association between *in utero* exposure to phytoestrogens and early menarche (defined as <11.5 years at onset) using data from a nested case-control study within the Avon Longitudinal Study of Parents and Children, a longitudinal study involving families living in the South West of England. Concentrations of six phytoestrogens were measured in maternal urine samples collected during pregnancy. Logistic regression was used to explore associations between tertiles of phytoestrogen concentrations with menarche status, with adjustment for maternal age at menarche, maternal education, pre-pregnancy BMI, child birth order, and duration of breastfeeding. Among 367 mother-daughter dyads, maternal geometric mean (95% confidence interval (CI)) creatinine-corrected concentrations (in μg/g creatinine) were: daidzein 184 (162 – 208), enterodiol 71.5 (64.6 – 79.1), enterolactone 755 (674 – 846), equol 5.65 (4.91 – 6.49), genistein 63.5 (54.9 – 73.4), and *O*-desmethylangolensin (*O*-DMA) 11.2 (9.34 – 13.5). In analyses comparing those in the highest tertile relative to those in the lowest tertile of *in utero* phytoestrogen exposure, only one statistically significant association with onset of menarche was found. Higher *O*-DMA levels were statistically significantly associated with early menarche (odds ratio (OR) = 2.36; CI: 1.26 - 4.43). *O*-DMA is an intestinal bacterial metabolite of daidzein; not all individuals harbor bacteria capable of metabolizing daidzein to *O*-DMA, and *O*-DMA may exhibit different biological actions than its parent compound. These findings suggest that *in utero* exposure to *O*-DMA, but no other phytoestrogens, may be associated with earlier age at menarche.

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CHAPTER I

Background/Literature Review

Pubertal Onset and Age at Menarche

Puberty is a critical time of growth and development. Pubertal indicators yield information on overall health status, past exposures, and may predict future health outcomes (Biro et al, 2001; Golub et al, 2008). Puberty is characterized as a cascade of events leading to the attainment of adult reproductive capacity and involves the maturation of the hypothalamus, anterior pituitary, ovaries, uterus, and breasts. In girls, the pubertal cascade is initiated by activation of the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator, which results in anterior pituitary release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Secretion of FSH and LH stimulates ovarian estradiol (E2) production that initiates breast development, known as thelarche, and uterine-endometrial maturation. The adrenal glands increase secretion of androgens, which results in pubic hair growth, known as pubarche, occurring approximately six months after thelarche.

Approximately two years after pubertal onset, menarche, also known as the first menstrual period, occurs (Crain et al, 2008). Menarche is often used as a marker for timing of puberty. Data gathered over the past 20 years suggest only moderate correlation (0.37-0.38) between menarche and onset of puberty (defined as the age at areolar stage 2 or at pubic hair stage 2, whichever occurred earlier); this correlation has decreased significantly during the last 50 years (0.64-0.86). This finding suggests that there are both similar and unique factors that impact the age at onset of puberty and age at menarche,

thus age at menarche and age at onset of puberty should not be used interchangeably (Biro et al, 2006).

Trends in Age at Menarche

Age at menarche has decreased from the late 19th century to present (Wyshak & Frisch, 1982; Zacharias & Wurtman, 1969), though a secular trend towards earlier menarche in the latter half of the 20th century has been somewhat debated. In 2008, an expert panel was asked to evaluate the weight of the evidence on this topic, and using data from 1940 to 1994, the majority of panelists concluded that data were sufficient to suggest a trend toward earlier menarche in the United States, although the magnitude of the change was not assessed (Euling et al, 2008a).

The average age at menarche in the United States is estimated to be approximately 12.4 years of age (Chumlea et al, 2003; McDowell et al, 2007), with some variation due to race (Wu et al, 2002). The current estimate for age at menarche is almost a year younger than the average age at menarche of American women born in the 1920s (13.3 years), and decreases in age at menarche have been observed across all race/ethnicity groups (McDowell et al, 2007). In the United Kingdom, the median age at menarche is estimated to be slightly higher at 12.9 years of age (Rubin et al, 2009). Globally, age at menarche ranges from approximately 12.0 to 16.1 years (Thomas et al, 2001), and similar trends in decreasing age at menarche have been observed in countries such as China, Russia, and Thailand (Song et al, 2014; Kozlov et al, 2015; Jaruratanasirikul et al, 2014).

Early Menarche

Early menarche is often defined as menarche occurring before 12 years of age (Lakshman et al, 2009; Charalampopoulos et al, 2014; Adgent et al, 2011) or less often, menarche occurring before 11 years of age (Sandler et al, 1984). In the United States, it is estimated that nearly half of black girls reach menarche before 12 years of age, while a quarter of white girls reach menarche by that time. Some aspects of socioeconomic status likely play a role in black-white differentials in the onset of menarche in the U.S. (Braithwaite et al, 2008). It is estimated that less than 10% of all U.S. girls reach menarche before 11 years of age (Chumlea et al, 2003).

The trend of decreasing age at menarche is attributed to a variety of factors. The decrease in the average age at menarche observed in the late 19th and early 20th centuries is attributed to improvements in nutrition and decreases in strenuous physical activity (Wyshak & Frisch, 1982). Recent debates surrounding the secular trend towards earlier menarche have focused on environmental factors, like the rising prevalence of childhood obesity and the effects of endocrine disrupting chemicals (Biro et al, 2012; Walvoord, 2010). Over the span of four decades, there has been an astonishing increase in childhood obesity, which many attribute to the imbalance between dietary energy intake and energy expenditure. Comprehensive reviews suggest that other factors could contribute to the obesity epidemic, including epigenetic modifications, exposure to endocrine disrupting chemicals, and the intrauterine environment (McAllister et al, 2009). There are several potential mechanisms that could impact the relationship of pubertal timing in girls with greater body mass, including direct effects of obesity on pubertal timing as well as underlying exposures that impact body mass in addition to the timing of pubertal maturation (Biro et al, 2012).

Obesity

Many studies have linked obesity with earlier puberty and menarche in girls (Biro et al, 2001; Freedman et al, 2002). Studies have found an association between height and adiposity with pubertal development (Britton et al, 2004), and have revealed a significant positive correlation between body mass index and earlier entrance into puberty (Kaplowitz et al, 2001). Studies have also examined the timing of excessive weight gain on pubertal development and found that excessive weight gain in the first 9 months of life was a very strong predictor of early menarche and that a higher body mass index (BMI) Z-score at 36 months of age was associated with earlier puberty (Lee et al, 2007; Ong et al, 2009).

Endocrine Disrupting Chemicals

There is growing concern surrounding the potential effects of endocrine disrupting chemicals (EDCs) on the timing of puberty (Herman-Giddens et al, 1997; Wang et al, 2005; Louis et al, 2008; Hond et al, 2002). EDCs are exogenous compounds that alter the production, action, and metabolism of endogenous hormones, most often mimicking estrogens (Walvoord, 2010; Euling et al, 2008b; Goldman et al, 2000). An EDC may have more than one mode of action and the effects may depend on the dose and duration of the exposure, as well as the developmental stage of the exposed individual (Mouritsen et al, 2010). Pubertal timing is especially sensitive to *in utero* or peripubertal exposure to certain EDCs, suggesting that this may be a 'critical window' of susceptibility (Goldman et al, 2000). EDCs may exert their action through adipocytes or other hormonally responsive tissues, impacting the timing of maturation. Exposure to EDCs may change adipocyte metabolism, and EDCs can mimic estrogens, potentially

impacting the hypothalamic-pituitary-gonadal axis (HPG), (Louis et al, 2008; Grün & Blumberg, 2009a; Grün & Blumberg, 2009b; Mouritsen et al, 2010), thereby potentiating the combination of obesity and environmental chemical exposures (Biro et al, 2012).

Potential Associations with Early Menarche

Maternal Factors

Evidence suggests that maternal maturation has a strong influence on the timing of menarche. Prenatal predictors of menarche by age 11 in the Avon Longitudinal Study of Parents and Children (ALSPAC) in the United Kingdom (n=1,707) included earlier maternal age at menarche and high maternal pre-pregnancy BMI (Rubin et al, 2009). Other cohort studies confirm that maternal age at menarche is associated with age at menarche (Moisan et al, 1990; Behie & O'Donnell, 2015). One cohort study (n=402) found that maternal age at menarche was strongly and significantly correlated with age at menarche (r=0.66) (Tehrani et al, 2014), while a cross-sectional study (n=1,017) found only a weak, but significant correlation between the age at menarche of girls and their mothers (r=0.26), which disappeared in girls with a BMI greater than 25 (Ersoy et al, 2005). As stated, maternal pre-pregnancy body weight has also been observed to be associated with early menarche. A cross-sectional study of mother-daughter pairs (n=2,497 pairs) found that pre-pregnancy overweight/obesity increased the chance of earlier menarche in daughters by 20% and excess gestational weight gain increased the chance of earlier menarche by 13% (Deardorff et al, 2012). A follow up to a longitudinal study (n=597) of mothers and daughters found that compared with those whose mothers had a BMI less than 25, the odds of daughters of obese mothers experiencing early

menarche were 3.1 times greater, though this study was limited by recall as an adult of age at menarche (Keim et al, 2009).

Prenatal Factors

The Barker hypothesis suggests that many human fetuses have to adapt to a limited supply of nutrients, and in doing so, permanently change their physiology and metabolism. These prenatally programmed changes may be the origins of a number of diseases later in life (Barker, 1997). A 2001 longitudinal study set in the Philippines (n=997) provides additional evidence of fetal programming of later health outcomes by showing that future growth and maturation trajectories are established *in utero*. This study found that size at birth predicts age at menarche and rapid postnatal growth potentiates the effects of size at birth and is related independently to earlier pubertal maturation (Adair, 2001). Similarly, a prospective cohort study based in Australia (n=776) found that birth weight and weight gain in childhood are associated with age at menarche, with opposing effects: lower birth weight combined with higher BMI during childhood predicted early age at menarche (Sloboda et al, 2007). Another longitudinal study set in Sweden (n=320) found that girls who were small for gestational age had earlier menarche than girls who were of normal size for gestational age, though controlling for postnatal growth patterns eliminated the effect of birth size (Persson et al, 1999). In a large cohort of British women (n=1,471), it was found that girls who were heavier at birth had later menarche, but those who were heavier at 7 years had earlier menarche (Cooper et al, 1996).

Pre-Pubertal Factors

Pre-adolescent body size is considered to be a predictor of early menarche. Larger body size at 8 years old was associated with an earlier age at menarche in a longitudinal cohort (n=1,707) of mostly Caucasian British girls as well as in a sample (n=1,493) of Australian girls (Rubin et al, 2009; Behie & O'Donnell, 2015). Furthermore, higher relative weight was strongly associated with an increased likelihood of having reached menarche in two nationally representative cross-sectional samples of U.S. girls (n=3,272 and n=1,326), after controlling for age and race (Anderson et al, 2003). A multiethnic cohort study based in California (n=679) found that tall girls and girls with a high BMI experienced earlier menarche when compared to short girls and girls with a low BMI, respectively (Koprowski et al, 1999). A nested case-control study of the correlates of early menarche also found that weight, height, and skinfold thickness were associated with early menarche among Canadian girls (n=640) (Moisan et al, 1990).

Race and Ethnicity

Race/ethnicity is likely associated with an increased risk of early menarche, potentially mediated through socioeconomic status or obesity, though some studies suggest an independent effect of race (Rubin et al, 2009). In multiple large cross-sectional and cohort studies, the average age at menarche for black girls is about half a year earlier than the average age at menarche for white girls (Biro et al, 2001; Wu et al, 2002; Freedman et al, 2002), and the prevalence of early menarche has been reported to be 1.4- to 2-fold higher among black girls than white girls (Braithwaite et al, 2008; Freedman et al, 2002). Age at menarche among Hispanic girls is less studied, and the cross-sectional studies conducted disagree on whether average age at menarche among

Hispanic girls is more similar to that of black girls or white girls (Wu et al, 2002; Britton et al, 2004).

Maternal Smoking

Maternal smoking during pregnancy is also thought to have an influence on the timing of menarche. Maternal cigarette smoking on most days during gestation was associated with a 40% increased chance of earlier menarche when compared to no maternal cigarette smoking during gestation in a longitudinal cohort study of Australian girls (n=1,493) (Behie & O'Donnell, 2015). Another large longitudinal cohort study (n=1,707) found that smoking during the third trimester was a predictor of menarche before age 11 (Rubin et al, 2009). One cohort study with predominantly black participants (n=1,556) found that the mean age at menarche was a few months earlier among girls whose mothers smoked a pack or more of cigarettes daily during pregnancy, compared to girls whose mothers did not smoke (Windham et al, 2004).

Early Menarche/Early Puberty as a Risk Factor

Menarche is an important developmental milestone that can yield information that may assist in predicting future health outcomes (Biro et al, 2001).

Reproductive Outcomes

Early menarche is considered a risk factor for breast cancer and other reproductive cancers, such as ovarian cancer, though the effect appears to be weak and the increased risk seems to be quite small when compared to modifiable risk factors (Walvoord, 2010; Lacey et al, 2009; Kelsey et al, 1993; Moorman et al, 2009; Vo & Carney, 2007). A prospective cohort study (n=1,513) on the age at menarche and

subsequent reproductive events found that women with very early menarche (<11 years old), compared to all other women, reported a greater percentage of no pregnancies, and of those who were ever pregnant, a greater percentage of women with early menarche reported no live births (Sandler et al, 1984).

Chronic Diseases

Early menarche is associated with an increased risk of adult obesity (Golub et al, 2008; Biro et al, 2003); findings from a Finnish birth cohort (n=3,404) suggest that early menarche is associated with a higher BMI at 14 and 31 years old (Laitinen et al, 2001). Early menarche is also associated with type 2 diabetes, as has been observed in large American and British cohort studies (n=10,702 and n=13,259), and this association appears to be mediated through excessive adult adiposity (He et al, 2009; Lakshman et al, 2008). A Chinese cross-sectional study (n=9,097) that examined the effect of age at menarche on metabolic risk factors for cardiovascular diseases found that early menarche is significantly associated with increased body fatness, insulin insensitivity, and blood lipid levels (Feng et al, 2008). Furthermore, in a systematic review and meta-analysis of nine articles, it was found that, compared with other women, those who had early menarche had higher risks of hypertension, incident cardiovascular disease, incident coronary heart disease, cardiovascular disease mortality, and all-cause mortality (Charalampopoulos et al, 2014).

Psychological Effects

Lastly, there is some evidence that suggests there are psychological effects of early puberty. Numerous studies have shown that girls with early puberty suffer from higher rates of depression (Conley & Rudolph, 2009; Kaltiala-Heino et al, 2003; Siegel et

al, 1999; Stice et al, 2001; Ge et al, 2003) and anxiety (Reardon et al, 2009; Blumenthal et al, 2009). Girls with early pubertal development seem to have increased rates of smoking (van Jaarsveld et al, 2007), delinquent behavior, and earlier sexual experiences (Johansson & Ritzen, 2005; Ostovich & Sabini, 2005). These data suggest that the biologic and social transformation that accompany puberty make very young adolescents more at risk for the development of maladaptive coping mechanisms, presumably because they are developmentally underprepared to effectively deal with these changes (Walvoord, 2010).

Endocrine Disrupting Chemicals

EDCs can be both natural and man-made, and research suggests that EDCs may pose the greatest risk during prenatal and early postnatal development when organ and neural systems are forming (National Institute of Environmental Health Sciences, 2015). Most EDCs have estrogenic and/or anti-androgenic actions, while few have androgenic or anti-estrogenic actions (Daxenberger et al, 2001). It is thought that heavier exposure to estrogenic/anti-androgenic chemicals may have the strongest puberty-inducing effects in females, where endogenous pre-pubertal estradiol levels are higher and effects of EDCs may therefore be more noticeable because they exceed a threshold level for effects (Mouritsen et al, 2010).

Previous studies have examined the effects of *in utero* exposure to various EDCs and some studies have found an effect on pubertal development, particularly age at menarche, whereas others have not observed an association. A cohort study (n=151) of *in utero* exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethlene (DDE) on age at menarche found that increased exposure to DDE significantly decreased

age at menarche, while exposure to PCBs was not associated (Vasiliu et al, 2004). Furthermore, a cohort study (n=327) of polybrominated biphenyls (PBBs) found that girls who were breastfed and exposed to high levels of PBBs *in utero* experienced earlier menarche than those breastfed and exposed to low levels of PBBs *in utero* or those who were not breastfed (Blanck et al, 2000). *In utero* exposure to diethylstilbestrol (DES), a synthetic estrogen used during pregnancy in the 1950s and 1960s, was borderline associated with early menarche in a combined cohort study (n=5,856) (Hatch et al, 2011). A nested case-control study (n=448) of *in utero* exposure to polyfluoroalkyl chemicals (PFCs) found no association with age at menarche (Christensen et al, 2011).

Phytoestrogens

Phytoestrogens are estrogenic compounds that occur naturally in plants, with the most common source of phytoestrogens being soybean products (Kim & Park, 2012), though red clover, flax, licorice, hops, alfalfa, ginseng, and evening primrose oil are also common phytoestrogen-containing foods, among others (Ososki & Kennelly, 2003). Phytoestrogens have a nonsteroidal structure and can behave as estrogen mimics (Setchell, 1998). Isoflavones, found in soybeans and other soy products, are the most extensively studied class of phytoestrogens. The major isoflavones are genistein and daidzein, and metabolites equol and *O*-desmethylangolensin. Another well-known class of phytoestrogens, called lignans, includes enterodiol and enterolactone (Kim & Park, 2012). A litany of health benefits such as a decreased risk of osteoporosis, heart disease, breast cancer, and menopausal symptoms (Kim, 2008; Messina et al, 2002; Messina et al, 2004; Cassidy et al, 2006; Cooke, 2006) are frequently attributed to soy foods because they contain phytoestrogens, but there is growing concern that phytoestrogens are also

EDCs with the potential to cause adverse health effects as well (Patisaul & Jefferson, 2010).

As an estrogen mimic, it is suspected that phytoestrogen exposure can affect sexual development, including altered pubertal timing. Estrogens act through two subtypes of receptors found in target tissues, known as estrogen receptor α (ERα) and estrogen receptor β (ERβ) (Kim & Park, 2012). ERα is most abundant in the uterus, ovaries, mammary glands, bones, and hypothalamus (Couse et al, 1997; Brandenberger et al, 1997), while ERβ expression is highest in ovarian granulosa cells and the gastrointestinal tract, and to a lesser extent in mammary glands, hypothalamus, and pituitary glands (Brandenberger et al, 1997; Enmark, 1997). The two phenolic rings in isoflavones, similar to estrogens, allows these compounds to bind to estrogen receptors and exert estrogenic effects on the target organs that possess ERα/ERβ (Kim & Park, 2012; Sarkar & Li, 2002). For example, the binding affinity of genistein for ERβ is 87%, while that for ERα is 4% (Kuiper et al, 1998).

Animal Studies

Studies in animal models demonstrate several adverse effects of phytoestrogens on the reproductive system, including premature pubertal onset (Lee et al, 2009; Bateman & Patisaul, 2008), reduced fertility (Jefferson et al, 2005; Nagao et al, 2001), altered estrous cyclicity (Bateman & Patisaul, 2008; Kouki et al, 2003), and disrupted pituitary responsiveness to gonadotropin releasing hormone (GnRH) (Faber & Hughes, 1993). The effects of phytoestrogens are observed to be quite different according to time, dosage and route. One study of perinatal exposure to genistein found no effect on the age of pubertal onset in rats (Takagi et al, 2004). Other studies in rodents have shown that exposure to

high doses of isoflavone *in utero* and through diet in early life induced the acceleration of pubertal onset in female animals (Casanova et al, 1999; Takashima-Sasaki et al, 2006). Moreover, additional studies have found that short-term neonatal exposure to high doses of isoflavone expedite vaginal opening in female rats (Bateman & Patisaul, 2008; Kouki et al, 2003; Lewis et al, 2003), and high doses of genistein in the pre-pubertal period lead to early vaginal opening in female rats (Lee et al, 2009), affirming the importance of timing, dosage, and route of exposure.

Phytoestrogens in utero

Exposure to phytoestrogens is mostly dietary, but it is known that phytoestrogens can cross the placental barrier in humans. In one study (n=53) of Californian women undergoing amniocentesis, 96% of second trimester amniotic fluid samples had quantifiable amounts of dietary phytoestrogens (Foster et al, 2002). Another study of 194 women found high correlations for isoflavone levels between maternal urine and blood samples and umbilical cord blood, suggesting that isoflavone can be transferred from the maternal to fetal compartment (Nagata et al, 2006). It is also suggested that the metabolic and/or excretion rates of phytoestrogens are different between mother and fetus, and once phytoestrogens are transferred to the fetus, they are metabolized/excreted at a slower rate in the fetus than in the mother (Todaka et al, 2005).

Biological Plausibility

Factors triggering the physiological onset of puberty are poorly understood (Tena-Sempere, 2010), which hampers investigations into the causes of premature maturation. Still, it is suspected that exposures to EDCs ubiquitously found in food and the environment may play a role. In theory, hormones or substances with hormone-disrupting

properties may interfere with pubertal development by actions at different levels, including alterations to neuroendocrine signals, the hypothalamic-pituitary axis, the gonads, and peripheral target organs such as breasts, hair follicles, and genitals. At the onset of puberty, activation of the HPG axis is initiated by changes in hypothalamic expression of several neurotransmitters. The factors responsible for this activation are not known, although peripheral factors, such as leptin, are thought to be involved (Mouritsen et al, 2010).

Soy Exposure in Childhood

Phytoestrogens in soy-based infant formula are one area of concern due to the dose and timing of exposure during the perinatal and neonatal periods, although potential endocrine disrupting effects of soy-based infant formula have not been extensively studied as prospective data is difficult to find. The urinary concentration of total isoflavones among infants exclusively fed soy-based formula is approximately 500 times the concentration of those fed cows' milk formula (Cao et al, 2008), and plasma isoflavone concentrations by bodyweight are an order of magnitude higher in soy-based formula-fed infants than in adults consuming diets containing soy protein (Setchell et al, 1997). The results of animal experiments are often not applicable to the human infant due to longer periods, higher doses, and irrelevant routes of exposure, plus the neonatal rodent is not equivalent to the human newborn in terms of developmental stages, therefore results must be interpreted with caution (Leung & Otley, 2009). Given those limitations, exposure of neonatal animals to the isoflavones present in soy-based infant formula can cause subtle alterations in sex organ development (Delclos et al, 2001;

Sharpe et al, 2002; Tan et al, 2006), brain maturation (Faber & Hughes, 1991), and immune system function (Yellayi et al, 2002).

Studies on the association between soy-based infant formula and age at menarche are inconclusive. A retrospective cohort study (n=811) based on a 16-week infant feeding study from 1965-1978 found no association between soy-based formula and self-reported age at menarche, when compared to cows' milk-based formula; this study is limited by potential recall bias as age at menarche was ascertained through a phone interview conducted in adulthood (Strom et al, 2001). A prospective cohort study (n=2,028) in British girls found a 53% increased risk of early menarche among those fed soy-based formula (introduced to soy formula at ≤4 months of age and sustained use at 6 months of age, as reported by a parent on an infant feeding questionnaire at 6 months of age), as compared to cows' milk-based formula (Adgent et al, 2011). Even fewer studies have examined the effects of soy consumption among pre-pubertal girls on age at menarche. A cross-sectional study (n=339) of soy intake in girls ages 12-18 years old near two Seventh-day Adventist universities in California and Michigan found that amongst a population with relatively high soy intake (mean: 12.9 servings/week) as measured by a food frequency questionnaire, quartile of soy consumption was not significantly associated with age at menarche (Segovia-Siapco et al, 2014).

Current State of the Evidence

Given the current trends in the decreasing age at menarche, and the evidence suggesting EDCs may be associated with an earlier age at menarche, it is important to explore further naturally-occurring EDCs like phytoestrogens to better understand the effects of EDCs on puberty, using age at menarche as an indicator of pubertal timing.

Animal studies have found that phytoestrogens such as genistein can exert endocrine disrupting effects. Among other reproductive effects, studies of animals exposed *in utero* to high levels of phytoestrogens have demonstrated earlier vaginal opening, a sign of pubertal onset in rodents.

There have been no human studies published to date which investigate the association between *in utero* phytoestrogen exposure and age at menarche. This study aims to examine the relationship between *in utero* phytoestrogen concentrations and the timing of menarche, using human data and representative levels of phytoestrogen exposure. This study is particularly well-suited to answer this question as the Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective study of a well-characterized population and contains comprehensive data on numerous potential confounders. Furthermore, this study uses objective biomarker data for phytoestrogen exposure measured by a lab with extensive experience and excellent quality control measures in place.

CHAPTER II

Introduction

Puberty is a critical time of growth and development. Timing and patterning of developmental milestones, such as age at menarche, yield information on overall health status, past exposures, and may predict future health outcomes (Biro et al, 2001; Golub et al, 2008). Studies have found that early menarche, often defined as menarche occurring before the age of 12 (Lakshman et al, 2009; Charalampopoulos et al, 2014; Adgent et al, 2011), is associated with maternal, prenatal, and early life factors such as race (Biro et al, 2001; Wu et al, 2002; Braithwaite et al, 2008; Freedman, 2002; Britton et al, 2004), maternal age at menarche (Rubin et al, 2009; Moisan et al, 1990; Behie & O'Donnell, 2015; Tehrani et al, 2014; Ersoy et al, 2005), maternal pre-pregnancy body mass index (Rubin et al, 2009; Deardorff et al, 2012; Keim et al, 2009), maternal smoking during pregnancy (Rubin et al, 2009; Behie & O'Donnell, 2015; Windham et al, 2004), size at birth (Adair, 2001; Cooper et al, 1996; Sloboda et al, 2007; Persson et al, 1999), and pre-adolescent body size (Rubin et al, 2009; Moisan et al, 1990; Behie & O'Donnell, 2015; Anderson et al, 2003; Koprowski et al, 1999). Early menarche has been associated with many unfavorable health outcomes, including reproductive cancers (Walvoord, 2010; Lacey et al, 2009; Kelsey et al, 1993; Moorman et al, 2009; Vo & Carney, 2007), adult obesity (Golub et al, 2008; Biro et al, 2003; Laitinen et al, 2001), type 2 diabetes (He et al, 2009; Lakshman et al, 2008), and cardiovascular disease (Feng et al, 2008; Charalampopoulos et al, 2014), and early puberty has been linked with higher rates of depression (Conley & Rudolph, 2009; Kaltiala-Heino et al, 2003; Siegel et al, 1999; Stice et al, 2001; Ge et al, 2003) and anxiety (Reardon et al, 2009; Blumenthal et al, 2009).

Age at menarche decreased from the late 19th century (Wyshak & Frisch, 1982; Zacharias & Wurtman, 1969), and a secular trend towards earlier development of secondary sexual characteristics was reported among girls in the United Kingdom (Rubin et al, 2009). Recent estimates for age at menarche (12.4 years) are almost a year younger than the average age at menarche of American women born in the 1920s (13.3 years), and decreases in age at menarche have been observed across all race/ethnicity groups (McDowell et al, 2004). While improvements in nutritional status since the 19th century and the increasing prevalence of childhood obesity may be responsible in part for this trend, exposure to environmental chemicals may also contribute to altered timing and patterns of pubertal development (Biro et al, 2001; Biro et al, 2012; Freedman et al, 2002; Christensen et al, 2011).

Endocrine disrupting chemicals (EDCs) are chemicals that may affect the body's endocrine system and cause adverse developmental, reproductive, neurological, and immune effects in humans and animals. EDCs can be natural or man-made, and research suggests that EDCs may pose the greatest risk during prenatal and early postnatal development when organ and neural systems are forming (National Institute of Environmental Health Sciences, 2015). Most EDCs have estrogenic and/or anti-androgenic actions (Daxenberger et al, 2001), which are thought to have puberty-inducing effects in females (Mouritsen et al, 2010). Previous studies examined the associations of *in utero* exposure to various EDCs with pubertal development, particularly age at menarche, with some studies finding an association while others did not. Most studies were limited by the use of retrospectively-collected age at menarche data (Vasiliu et al, 2004; Blanck et al, 2000; Hatch et al, 2011; Christensen et al, 2011).

One potential class of naturally-occurring EDCs of interest is phytoestrogens. Phytoestrogens are estrogenic compounds that occur naturally in plants, with the most common dietary source of phytoestrogens being soybean products (Kim & Park, 2012). Exposure to phytoestrogens is mostly dietary, but it is known that phytoestrogens can cross the placental barrier in humans (Foster et al, 2002). Phytoestrogens have a nonsteroidal structure and can behave as estrogen mimics (Setchell, 1998). As an estrogen mimic, it is suspected that phytoestrogen exposure can affect sexual development, including altered pubertal timing (Kim & Park, 2012).

Studies in animal models found the effects of phytoestrogens to be quite different according to time, dosage and route. Studies in rodents found that exposure to high doses of phytoestrogens (isoflavones) *in utero* and through diet in early life accelerated pubertal onset in female animals (Casanova et al, 1999; Takashima-Sasaki et al, 2006). In humans, the effect of soy-based infant formula on pubertal development has been studied to some extent, though this has yielded mixed results regarding an association with age at menarche (Adgent et al, 2011, Strom et al, 2001). However, there have been no human studies published to date that investigated an association between *in utero* phytoestrogen exposure and age at menarche. Our aim was to examine the association between *in utero* phytoestrogen concentrations and the timing of menarche, using human data and representative levels of phytoestrogen exposure.

Methods

The Avon Longitudinal Study of Parents and Children (ALSPAC) is an ongoing prospective birth cohort of 14,541 pregnancies. ALSPAC enrolled pregnant women with

an expected delivery date between April 1st, 1991 and December 31st, 1992 from three health districts in the county of Avon, Great Britain. Information has been collected on these parents and children through interviews, mailed questionnaires, and clinic visits. Details on ALSPAC recruitment and study methods have been described elsewhere (Boyd et al, 2013).

A nested case-control study was conducted within the ALSPAC cohort to explore associations of prenatal maternal concentrations of various endocrine disrupting chemicals and age at menarche among the daughters (Figure 1). A 'Growing and Changing' questionnaire was developed to collect information on the offspring's pubertal development and distributed to participants annually between the ages of 8–17 years (1999–2008), with the exception of age 12 (2003). Menarche was determined through self-report of menarche status, and, if it had occurred yet, age at menarche (month and year of occurrence). From the original base population of 14,062 live births, case and control series were selected from singleton (n=11,820) female subjects (n=5,756) who had completed at least two puberty staging questionnaires between the ages of 8 and 13 (5 possible questionnaires returned; n=3,682). Girls meeting eligibility criteria were ordered according to reported age at menarche when the 13-year old data became available. A cut-off of 11.5 years was established as defining 'early' menarche. Eligible cases could complete any two questionnaires in the series, provided that one was completed after menarche, while controls had to complete the 13-year old questionnaire in order to ascertain that menarche had not occurred by the cut-off of 11.5 years. Of the girls who reported menarche before the age of 11.5 (n=338), 59.8% (n=202) had at least one prenatal maternal urine sample available, and were considered potential cases.

Among girls who reported menarche at or after the age of 11.5, a random sample of 394 was chosen as potential controls, and of these, 61.2% (n=241) had at least one maternal urine sample available. After evaluating the integrity of the maternal urine samples, 85.1% (n=172) of potential cases and 80.9% (n=195) of potential controls had analyzable samples.

Phytoestrogens (enterolactone, daidzein, genistein, enterodiol, *O*-desmethylangolensin, and equol) were measured in 367 stored maternal first morning void urine samples collected at a median gestational age of 12 weeks (interquartile range 8–17 weeks). The maternal urine samples were analyzed at the National Center for Environmental Health, Centers for Disease Control and Prevention (Atlanta, GA) using high-performance liquid chromatography–tandem mass spectrometry. The analytical methods were described elsewhere (Rybak et al, 2008). Phytoestrogens were assessed individually and by class. Classes included isoflavones (genistein and daidzein) and lignans (enterodiol and enterolactone). Each phytoestrogen concentration was divided into tertiles by using cut points based on the distribution among the controls.

Potential confounders to be considered in the analyses were identified *a priori* based on previously published literature and biological plausibility. Covariates were collected at various points throughout enrollment in the cohort. We considered the following as covariates: child ethnic background (white/non-white); maternal education (ordinally classified as less than O-level (ordinary level), O-level, or greater than O-level); maternal age at menarche (categorized as 8–11 years, 12–15 years, or missing); maternal pre-pregnancy body mass index(BMI) (kg/m^2), prenatal vegetarian diet (yes/no), prenatal smoking (any/none), maternal age at delivery (years), child birth order

(categorized as first born, second born, or third born or later), child birth weight (grams), breastfeeding duration (ordinally classified as not breastfed, <3 months, 3–5 months, ≥6 months), use of infant soy formula (any/none), vegetarian diet during childhood (yes/no), and childhood BMI Z-score at age 8 (if missing for age 8, used age 7, 9, or 10). Missingness was below 6% for each covariate, with the exception of maternal pre-pregnancy BMI (8.2%), childhood BMI Z-score (10.9%), and maternal age at menarche (14.2%).

All data analysis was performed using SAS 9.3 (Cary, NC). Descriptive statistics were calculated for the sample comprised of mother-daughter dyads for which exposure and outcome data were available across at least 4 of the 6 phytoestrogens; chi square and Fisher's exact tests were used to compare groups by menarche status. Geometric means were calculated for each phytoestrogen for the total sample and by menarche status, and the Wilcoxon rank sum test was used to compare groups by menarche status. Additionally, medians and interquartile ranges were calculated by class of phytoestrogen (isoflavones and lignans) for the total sample across covariate groups. Differences were assessed using the Wilcoxon rank sum test or the Kruskal-Wallis test.

To assess the association between potential confounders and earlier age at menarche, logistic regression models were used, with an inclusion criterion of $p \leq 0.30$ for confounders. Next, associations of potential confounders with total phytoestrogen concentration (after natural log transformation) was assessed using a linear model, again using a criterion of $p \leq 0.30$ for confounders. Those variables associated with both earlier age at menarche and phytoestrogen concentration using these guidelines were considered potential confounders, and included in unconditional multivariate logistic models to

assess associations of maternal phytoestrogen concentration with earlier age at menarche. Maternal education, maternal age at menarche, maternal vegetarian diet, and childhood BMI were all considered as potential effect modifiers, but there was no evidence of effect modification.

Please note that the study website contains details of all the data that is available through a fully searchable data dictionary (University of Bristol, 2015). Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. The U.S. Centers for Disease Control and Prevention (CDC) Institutional Review Board assessed and approved human subject protection. Mothers provided informed consent at time of enrollment.

Results

In the ALSPAC cohort, girls were predominantly born to white mothers who were relatively highly educated (Table 1). Cases were more likely to have mothers who had an earlier age at menarche; almost one-third of case mothers reported menarche between 8 and 11 years of age, compared to less than 15% of control-mothers. Mothers of cases were more than twice as likely to have an overweight or obese pre-pregnancy BMI, and cases were more than twice as likely to have a childhood BMI that was more than one standard deviation above the mean. Cases were 18% more likely to be the first born child, and 19% more likely to have never been breastfed or breastfed for less than 3 months. The median age of menarche among cases was 11.0 years, while the median age of menarche among controls was almost two years later at 12.8 years. Geometric mean enterodiol concentrations were statistically significantly 20% lower among cases than

among controls while geometric mean *O*-desmethylangolensin (*O*-DMA) concentrations were borderline statistically significantly 45% higher among cases than among controls (Table 2).

The median isoflavone (sum of genistein and daidzein) concentration was 258.48 µg/g creatinine (IQR: 120.46 – 549.23 µg/g creatinine), but this varied slightly by maternal and child characteristics (Table 3). Isoflavone concentration statistically significantly differed by maternal age at delivery (p-value=0.03), with the highest concentrations observed among mothers under 20 years (627.11 µg/g creatinine) and the lowest among mothers 30 years or older (214.80 µg/g creatinine). Isoflavone concentration also differed by maternal age at menarche (p-value=0.14), though not statistically significantly, with the highest concentrations being among mothers who did not report age at menarche (313.84 µg/g creatinine) and mothers reporting age at menarche between 8 and 11 years (280.87 µg/g creatinine), while the lowest concentrations were among mothers with an age at menarche of 12 years or older (237.52 µg/g creatinine). There was little variation in isoflavone concentration by child ethnic background, maternal education, maternal pre-pregnancy BMI, prenatal vegetarian diet, prenatal smoking, child birth order, and child birth weight.

The median lignan (sum of enterodiol and enterolactone) concentration was 1006.28 µg/g creatinine (interquartile range (IQR): 536.10 - 1682.06 µg/g creatinine), though this also varied somewhat by maternal and child characteristics (Table 3). As maternal education increased, lignan concentration also increased (p-value=0.005); for example, mothers with greater than O-level education had a median lignan concentration of 1,098.15 µg/g creatinine, while mothers with less than O-level education had a median

lignan concentration of 717.54 µg/g creatinine. Lignan concentration also differed by maternal pre-pregnancy BMI (p-value=0.003), with the highest concentrations among normal weight mothers (1,082.38 µg/g creatinine) and the lowest among obese mothers (583.68 µg/g creatinine). Maternal age at delivery differed by lignan concentration (p-value=0.003), with the lowest lignan concentrations among mothers under 20 years (456.79 µg/g creatinine) and the highest among mothers 30 years or older (1,214.75 µg/g creatinine). Also, the mothers of white girls had higher lignan concentrations compared to the mothers of non-white girls (medians of 1,035.04 µg/g creatinine and 382.03 µg/g creatinine, respectively; p-value<0.0001). As maternal age at menarche increased, median lignan concentration also increased, though not significantly (p-value=0.09); mothers with an age at menarche between 8 and 11 years had a median concentration of 813.35 µg/g creatinine while those with an age at menarche of 12 years or older had a median concentration of 1,045.79 µg/g creatinine. There was little variation in lignan concentrations by prenatal vegetarian diet, prenatal smoking, child birth order, and child birth weight.

Because maternal age at menarche was only associated with two phytoestrogens and therefore might not be a confounder, we modeled the association between maternal phytoestrogen concentration and early menarche without controlling for maternal age at menarche (Supplementary Table 1). We found no meaningful difference in the results when controlling for maternal age at menarche versus not controlling for maternal age at menarche.

The results of the multivariate analyses were similar to those from the unadjusted analyses (Table 4). In the multivariable model, the adjusted association of early menarche

with mothers' *O*-DMA concentration was statistically significant at odds ratio (OR)=1.19 (95% CI: 1.03 – 1.36; p-trend: 0.01) when treated as continuous, and an OR=2.36 (95% CI: 1.26 – 4.43; p-trend: 0.008) when comparing those in the third tertile of *O*-DMA concentration to those in the first tertile. The unadjusted association of early menarche with mothers' enterodiol concentration was statistically significant at OR=0.79 (95% CI: 0.64 – 0.98; p-trend: 0.03) when treated as continuous, and an OR=0.58 (95% CI: 0.35 – 0.96; p-trend: 0.03) when comparing those in the third tertile of enterodiol concentration to those in the first tertile; though this association was attenuated following adjustment (OR=0.61, 95% CI: 0.34 – 1.11; p-trend: 0.09). No other statistically significant associations were observed between phytoestrogen concentration and early menarche.

Discussion

Although study participants had nearly ubiquitous exposure to all phytoestrogens, *in utero* phytoestrogen exposure as estimated from maternal urinary phytoestrogen concentration did not appear to be associated strongly with age at menarche. The strongest and only significant estimated association was seen for *O*-DMA, which was associated with increased odds of earlier age at menarche (i.e., higher maternal urinary concentration of *O*-DMA was associated with the girls' earlier age at menarche). *O*-DMA is an intestinal bacterial metabolite of daidzein, and about 90% of individuals harbor bacteria capable of metabolizing daidzein to *O*-DMA; *O*-DMA is less structurally similar to 17β-estradiol than its parent compound and therefore may exhibit different biological actions than daidzein. The underlying bacteria that metabolize daidzein to *O*-DMA may have a distinct physiological role; urinary excretion of *O*-DMA is a marker of harboring intestinal bacteria capable of C-ring cleavage, and therefore the role of the phenotype

may extend beyond daidzein metabolism (Frankenfield, 2011). The directions of associations differed by the phytoestrogen examined, with different results seen for *O*-DMA compared to enterodiol. This may have been due to differences in the mechanism of actions of the phytoestrogens studied, or may have been due to chance, given the modest estimated associations.

To our knowledge, this is the first published study of associations of *in utero* phytoestrogen exposure with age at menarche. Although there were few statistically significant associations found in this cohort between phytoestrogen levels and age at menarche, there is biological plausibility for such an association. Exposures during pregnancy are extremely relevant to pubertal development, since this represents the period of initial organ development, including the brain, endocrine system, and reproductive tract. Furthermore, the fetus is more susceptible to such exposures due to smaller size, lack of a complete blood-brain barrier, and absence of metabolizing enzymes (Todaka et al, 2005). Studies have found that phytoestrogens can cross the placental barrier in humans, and one study (n=53) of Californian women undergoing amniocentesis found that 96% of second trimester amniotic fluid samples contained quantifiable amounts of dietary phytoestrogens (Foster et al, 2002). Based on evidence from animal studies, the mechanisms of action may be particularly relevant for *in utero* exposure to phytoestrogens, as opposed to early life exposures (Takagi et al, 2004; Takashima-Sasaki et al, 2006; Casanova et al, 1999). Studies in rodents have found that isoflavones administered through diet or subcutaneous injection can lead to early vaginal opening (akin to early menarche in humans), irregular estrous cyclicity, and decreased GnRH activation (GnRH coordinates reproductive maturation and function) (Takagi et al,

2004; Takashima-Sasaki et al, 2006; Casanova et al, 1999; Kouki et al, 2003; Lewis et al, 2003; Bateman & Patisaul, 2008; Lee et al, 2009; Nagao et al, 2001).

Urinary phytoestrogen concentrations during 1991–1992 among mothers of girls participating in the ALSPAC were much higher for all phytoestrogens except equol when compared to 2003–2006 National Health and Nutrition Examination Survey (NHANES) data for white women between 20 and 39 years old (CDC, 2012) (Supplementary Table 2). It should be noted though that these samples were taken more than a decade apart.

To our knowledge, no previous studies investigated *in utero* phytoestrogen exposure; therefore, we looked to previous studies on the effect of *in utero* exposure to other potentially endocrine disrupting chemicals, which produced mixed results, as have previous studies on early life phytoestrogen exposure to soy infant formula. For example, a cohort study (n=151) assessing *in utero* exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethlene (DDE) with age at menarche found that increased exposure to DDE was statistically significantly associated with an earlier age at menarche, while exposure to PCBs was not associated (Vasiliu et al, 2004). A nested case-control study (n=448) found no association with *in utero* exposure to polyfluoroalkyl chemicals (PFCs) and age at menarche (Christensen et al, 2011). Furthermore, a cohort study (n=327) evaluating polybrominated biphenyls (PBBs) on timing of menarche found that girls who were breastfed and exposed to high levels of PBBs *in utero* experienced earlier menarche than those breastfed and exposed to low levels of PBBs *in utero* or those who were not breastfed (Blanck et al, 2000). *In utero* exposure to diethylstilbestrol (DES), a synthetic estrogen used during pregnancy in the 1950s and 1960s, was borderline associated with early menarche in a combined cohort

study (n=5,856) (Hatch et al, 2011). Unlike our study and the studies of PCBs, DDE, and PFCs, the studies of PBB and DES both examined populations with unintentional exposure to high levels of an endocrine disrupting chemical; it is unlikely that these levels would be seen in the general population.

Similarly, studies on the association between soy-based infant formula and age at menarche are inconclusive. A retrospective cohort study (n=811) based on a 16-week infant feeding study from 1965–1978 found no association between soy-based formula and self-reported age at menarche, when compared to cows' milk-based formula; this study is limited by potential recall bias as age at menarche was ascertained through a phone interview conducted in adulthood (Strom et al, 2001). A prospective cohort study (n=2,028) in British girls found a 53% increased risk of early menarche among those fed soy-based formula (introduced to soy formula at ≤4 months of age and sustained use at 6 months of age, as reported by a parent on an infant feeding questionnaire at 6 months of age), as compared to cows' milk-based formula (Adgent et al, 2011). While it is difficult to compare across classes of endocrine disrupting chemicals and at different times of exposure, previous studies have yet to suggest a clear association between *in utero* and early life exposure to endocrine disrupting chemicals and age at menarche.

Strengths of this study are the inclusion of multiple phytoestrogen biomarkers, substantial covariate data available on mothers and children from multiple time points over gestation and childhood, and outcome data generally collected in the year that the outcome occurred. Limitations of this study include a single spot urine measurement of phytoestrogen exposure which was not collected at a uniform time of gestation, missing information on age at menarche among some controls, and some missing information on

covariates. Unlike some other endocrine disrupting chemicals that are estimated to have half-lives on the order of several years, peak rates of urinary excretion of phytoestrogens occur between 6 and 12 hours after ingestion (King & Bursill, 1998). Since phytoestrogens are excreted rather quickly, phytoestrogen exposure monitored through urinary excretion may under- or overestimate intermittent soy consumption and phytoestrogen exposure. Age at menarche was obtained through self-report on 'Growing and Changing' puberty questionnaires completed every year by parents and/or children, depending on age. There is some potential for misclassification of the outcome, such as the completion of the questionnaire by a parent unaware of the child's menarche status, or issues in the parent or child's recall of the month and year menstruation began.

It is also possible that the cases and controls selected were not representative of the cohort. When comparing female study participants who returned at least two 'Growing and Changing' questionnaires to those who did not return any questionnaires, non-respondents' parents tended to have somewhat lower educational attainment. Mothers of respondents were generally older at time of index birth compared to non-respondents. Finally, non-respondents were more likely to be of non-white race/ethnicity. This could have affected our findings since socioeconomic status is related to age at menarche (Braithwaite et al, 2008); however, whether socioeconomic status is related to phytoestrogen concentrations is unclear. Although race/ethnicity is also related to age at menarche (Biro et al, 2006; Wu et al, 2002; Freedman et al, 2002; Britton et al, 2004; McDonald et al, 2007) and was associated with maternal lignan concentrations in this study, we were not able to more closely examine the effect of race/ethnicity due to the small number of non-white girls. Last, due to a relatively modest sample size, this study

may have been underpowered to detect an association between *in utero* phytoestrogen exposure and age at menarche.

In summary, we compared exposure to phytoestrogens during pregnancy among mothers of girls who did and did not have earlier age at menarche in the ALSPAC cohort. Phytoestrogen concentrations of isoflavones and lignans varied by maternal characteristics. However, although *in utero O*-DMA was associated with early age at menarche, phytoestrogen exposure did not appear to be associated strongly with age at menarche in this cohort. This finding was not surprising in the context of previous studies of *in utero* exposure to endocrine disrupting chemicals and early life soy formula exposure, which have been equivocal.

CHAPTER III

Summary

In conclusion, we found evidence to suggest an association between *in utero* exposure to *O*-DMA and early menarche among British girls. There was little evidence to suggest an association between *in utero* exposure to other phytoestrogens (enterolactone, daidzein, genistein, enterodiol, and equol) and early menarche after adjusting for potential confounders.

Public Health Implications

While phytoestrogens have been lauded for their health benefits in regards to cardiovascular disease, cancer, osteoporosis, and menopausal symptoms, it is important to recognize their potential hazards as well. Because pregnancy is a critical time of organ development, including the brain, endocrine system, and reproductive tract, exposures during pregnancy can be relevant to future pubertal development. Since phytoestrogens are estrogen mimics, *in utero* exposure to phytoestrogens may have an impact on future pubertal outcomes, such as age at menarche. Given this biological plausibility and results that suggest there is an association between *in utero* exposure to *O*-DMA and early menarche, the public health implications of *in utero* exposure to phytoestrogens through maternal phytoestrogen consumption during pregnancy should be considered further. While there is insufficient evidence to indicate that pregnant women should lower their phytoestrogen consumption in order to improve their daughters' reproductive health, this is a growing area of research that merits further study.

Possible Future Directions

The results of this study present many opportunities for future research. This is the first study that has examined *in utero* exposure to phytoestrogens and early age at menarche, and thus the first to show an association between *in utero* exposure to *O*-DMA and early menarche. Therefore, given the lack of studies on the association between phytoestrogens and early age at menarche and the limited number of studies of *in utero* exposure to endocrine disrupting chemicals and early life soy exposure, there is a need for more studies in a variety of populations to further explore this association. In addition, future studies should aim to collect multiple maternal urine samples since phytoestrogen concentrations fluctuate depending on dietary intake, and phytoestrogens have short half-lives in the body. Lastly, future studies should examine associations between *in utero* exposure to phytoestrogens and other pubertal markers, and as well as explore potential mechanisms of action for *O*-DMA.

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Tables

Table 1. Characteristics of the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (N=367 mother-daughter dyads).

	Menarche <11.5 years (N=172)		Menarche ≥11.5 years (N=195)		P-value for difference[b]
Characteristic[a]	N	%	N	%	
Child race					0.61
White	159	95.8	182	96.8	
Non-white	7	4.2	6	3.2	
Maternal education[d]					0.39
< O-level	36	21.7	32	16.8	
O-level	56	33.7	62	32.5	
>O-level	74	44.6	97	50.8	
Maternal age at menarche, years					0.0002
8-11	50	29.1	23	11.8	
≥12	100	58.1	142	72.8	
Missing	22	12.8	30	15.4	
Maternal pre-pregnancy BMI, kg/m^2					0.003
<18.5	4	2.6	11	6.1	
18.5-24.9	109	69.9	148	81.8	
25.0-29.9	28	17.9	15	8.3	
≥30.0	15	9.6	7	3.9	
Prenatal vegetarian diet					0.92
Yes	10	6.1	11	5.9	
No	154	93.9	177	94.1	
Prenatal smoking					0.47
Any	29	17.3	27	14.4	
None	139	82.7	161	85.6	
Maternal age at delivery, years					0.14[c]
<20	1	0.6	7	3.6	
20-24	31	18.1	28	14.4	
25-29	71	41.5	73	37.4	
≥30	68	39.8	87	44.6	
Child birth order					0.03
First born	101	61.6	97	51.6	
Second born	36	22.0	66	35.1	
Third born or later	27	16.5	25	13.3	
Child birth weight, g					1.00[c]
<2500	4	2.4	4	2.1	
≥2500	166	97.6	188	97.9	
Breastfeeding duration, months					0.04
Not breastfed	26	16.0	38	20.8	
<3	53	32.5	35	19.1	
3-5	26	16.0	34	18.6	
≥6	58	35.6	76	41.5	
Use of infant soy formula					0.43[c]
Any	4	2.4	2	1.1	
None	163	97.6	187	98.9	

Vegetarian diet during childhood					0.65
Yes	8	4.8	7	3.8	
No	158	95.2	176	96.2	
Childhood BMI Z-score					
<0	32	20.9	60	34.5	<0.0001
0-1	50	32.7	77	44.3	
≥1	71	46.4	37	21.3	
Age at menarche, years	Median	IQR	Median	IQR	
	11.0	10.7-11.3	12.8	12.3-13.4	

Abbreviations: N, number; CSE, Certificate of Secondary Education; g, grams; kg/m^2, kilograms per meter-squared; IQR, interquartile range

[a] Information was missing for some girls, including information on child ethnic background (n=13, 3.5%), maternal education (n=10, 2.7%), maternal age at menarche (n=52, 14.2%), maternal pre-pregnancy BMI (n=30, 8.2%), prenatal vegetarian diet (n=15, 4.1%), prenatal smoking (n=12, 3.3%), maternal age at delivery (n=1, 0.3%), child birth order (n=15, 4.1%), child birth weight (n=5, 1.4%), breastfeeding duration (n=21, 5.7%), use of infant soy formula (n=11, 3.0%), vegetarian diet during childhood (n=18, 4.9%), and childhood BMI Z score (n=40, 10.9%).

[b] Compared using chi-square tests unless otherwise noted

[c] Compared using Fisher's exact test

[d] <O-level=none, Certificate of Secondary Education, and vocational education, which are equivalent to no diploma or a GED in the United States. O-levels (ordinary levels) are required and completed at the age of 16. >O-level=A-levels (advanced levels) completed at 18, which are optional, but required to get into university; and a university degree.

Table 2. Gestational urinary phytoestrogen concentrations among mothers of girls with and without earlier age at menarche in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (N=367 mother-daughter dyads).

	Total	Menarche ≥11.5 years (N=172)	Menarche <11.5 years (N=195)	p-value[b]
Analyte[a]	Geom. Mean (95% CI)	Geom. Mean (95% CI)	Geom. Mean (95% CI)	
Enterolactone	754.7 (673.5 – 845.7)	776.3 (668.5 – 901.5)	736.2 (621.1 – 872.5)	0.89
Daidzein	183.8 (162.3 – 208.3)	188.7 (159.3 – 223.5)	179.7 (149.6 – 215.7)	0.79
Genistein	63.5 (54.9 - 73.4)	60.6 (49.2 - 74.5)	66.2 (53.9 - 81.2)	0.58
Enterodiol	71.5 (64.6 - 79.1)	63.5 (54.7 - 73.7)	79.3 (69.2 - 91.0)	0.02
O-DMA	11.2 (9.34 - 13.5)	13.7 (10.5 - 17.7)	9.4 (7.3 - 12.3)	0.06
Equol	5.7 (4.9 - 6.5)	5.5 (4.5 - 6.7)	5.8 (4.8 - 7.0)	0.74

Abbreviations: N, number; Geom. Mean, geometric mean; *O*-DMA, *O*-Desmethylangolensin

[a] Creatinine corrected concentrations in µg/g creatinine

[b] p-value for difference between cases and controls using the Wilcoxon Rank Sum Test

[c] There are missing concentrations for genistein (n=1 where menarche <11.5 years), *O*-DMA (n=2 where menarche <11.5 years), and equol (n=1 where menarche <11.5 years and n=1 where menarche ≥11.5 years)

Table 3. Median and interquartile range of maternal isoflavone (genistein and daidzein) and lignan (enterodiol and enterolactone) concentrations by selected maternal and child characteristics in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (N=367 mother-daughter dyads).

		Isoflavone[a]			Lignan[a]		
Group	N	Median	IQR	p-value[b]	Median	IQR	p-value[b]
Child ethnic background				0.58			<0.0001
White	341	249	(122 - 547)		1035	(573 - 1758)	
Non-white	13	261	(71 - 519)		382	(181 - 526)	
Maternal education[c]				0.29			0.005
<O-level	68	268	(126 - 538)		718	(478 - 1204)	
O-level	118	291	(132 - 564)		1018	(452 - 1805)	
>O-level	171	236	(112 - 530)		1098	(646 - 1915)	
Maternal age at menarche, years				0.14			0.09
8-11	73	281	(192 - 533)		813	(395 - 1370)	
≥12	242	238	(106 - 521)		1046	(571 - 1682)	
Missing	52	314	(151 - 672)		1013	(610 – 1773)	
Maternal pre-pregnancy BMI, kg/m^2				0.24			0.003
<18.5	15	303	(114 - 447)		1056	(824 - 2594)	
18.5-24.9	257	260	(128 - 590)		1082	(596 - 1783)	
25.0-29.9	43	201	(82 - 427)		924	(384 - 1363)	
≥30.0	22	322	(101 - 662)		584	(334 - 988)	
Prenatal vegetarian diet				0.73			0.82
Yes	21	197	(114 - 611)		1177	(688 - 1444)	
No	330	254	(124 - 530)		991	(526 - 1682)	
Prenatal smoking				0.49			0.20
Yes	56	297	(156 - 564)		999	(407 - 1588)	
No	299	245	(118 - 533)		1007	(562 - 1783)	
Maternal age at delivery, years				0.03			0.003
<20	8	627	(428 - 1079)		457	(312 - 1748)	
20-24	59	331	(197 - 718)		724	(389 - 1574)	
25-29	144	260	(121 - 521)		932	(518 - 1369)	
≥30	155	215	(105 - 517)		1215	(669 - 1983)	

Child birth order				0.34			0.36
First born	198	237	(114 - 525)		1005	(550 - 1593)	
Second born	102	281	(119 - 652)		1040	(646 - 1840)	
Third born or later	52	269	(167 - 598)		825	(449 - 1542)	
Child birth weight, g				0.36			0.37
<2500	8	360	(197 - 608)		554	(320 - 1772)	
≥2500	354	250	(120 - 533)		1005	(550 - 1661)	

Abbreviations: N, number; kg/m^2, kilograms per meter-squared; g, grams; CSE, Certificate of Secondary Education; IQR, interquartile range

[a] Creatinine corrected concentrations in µg/g creatinine

[b] p value for difference using the Wilcoxon Rank Sum Test or the Kruskal-Wallis Test

[c] <O-level=none, Certificate of Secondary Education, and vocational education, which are equivalent to no diploma or a GED in the United States. O-levels (ordinary levels) are required and completed at the age of 16. >O-level=A-levels (advanced levels) completed at 18, which are optional, but required to get into university; and a university degree.

Table 4. Associations of maternal urinary phytoestrogen concentrations with earlier age at menarche in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (N=309 mother-daughter dyads).

	Unadjusted[a]		Adjusted[ab]	
Analyte[c]	OR (95% CI)	p for trend	OR (95% CI)	p for trend
Enterolactone				
Continuous[d]	1.04 (0.87 – 1.26)	0.65	1.22 (0.96 – 1.55)	0.10
Tertile 2[e] (551.49 – 1314.96)	1.39 (0.85 – 2.28)		1.64 (0.91 – 2.97)	
Tertile 3[e] (1314.96 – 8718.04)	0.98 (0.58 – 1.65)	0.94	1.37 (0.72 – 2.61)	0.34
Daidzein				
Continuous	1.03 (0.87 – 1.22)	0.70	1.10 (0.89 – 1.34)	0.38
Tertile 2 (110.09 – 319.03)	1.37 (0.83 – 2.25)		1.43 (0.80 – 2.56)	
Tertile 3 (319.03 – 21880.45)	1.00 (0.59 – 1.68)	0.99	1.11 (0.60 – 2.07)	0.74
Genistein				
Continuous	0.96 (0.83 – 1.11)	0.55	0.96 (0.81 – 1.15)	0.69
Tertile 2 (38.86 – 118.38)	1.02 (0.62 – 1.67)		1.04 (0.58 – 1.87)	
Tertile 3 (118.38 – 17916.60)	0.87 (0.52 – 1.44)	0.59	0.97 (0.54 – 1.77)	0.93
Enterodiol				
Continuous	0.79 (0.64 – 0.98)	0.03	0.85 (0.66 – 1.09)	0.19
Tertile 2 (56.49 – 117.99)	0.60 (0.36 – 0.98)		0.42 (0.23 – 0.78)	
Tertile 3 (117.99 – 1188.20)	0.58 (0.35 – 0.96)	0.03	0.61 (0.34 – 1.11)	0.09
***O*-DMA**				
Continuous	1.12 (1.00 – 1.26)	0.05	1.19 (1.03 – 1.36)	0.01
Tertile 2 (4.77 – 21.04)	1.91 (1.12 – 3.27)		1.62 (0.87 – 3.02)	
Tertile 3 (21.04 – 1631.58)	1.97 (1.16 – 3.35)	0.02	2.36 (1.26 – 4.43)	0.008
Equol				
Continuous	0.97 (0.83 – 1.13)	0.70	0.97 (0.82 – 1.16)	0.77
Tertile 2 (3.19 – 6.93)	1.22 (0.74 – 1.99)		1.12 (0.63 – 2.00)	
Tertile 3 (6.93 – 9005.85)	0.86 (0.51 – 1.44)	0.57	0.87 (0.48 – 1.61)	0.68

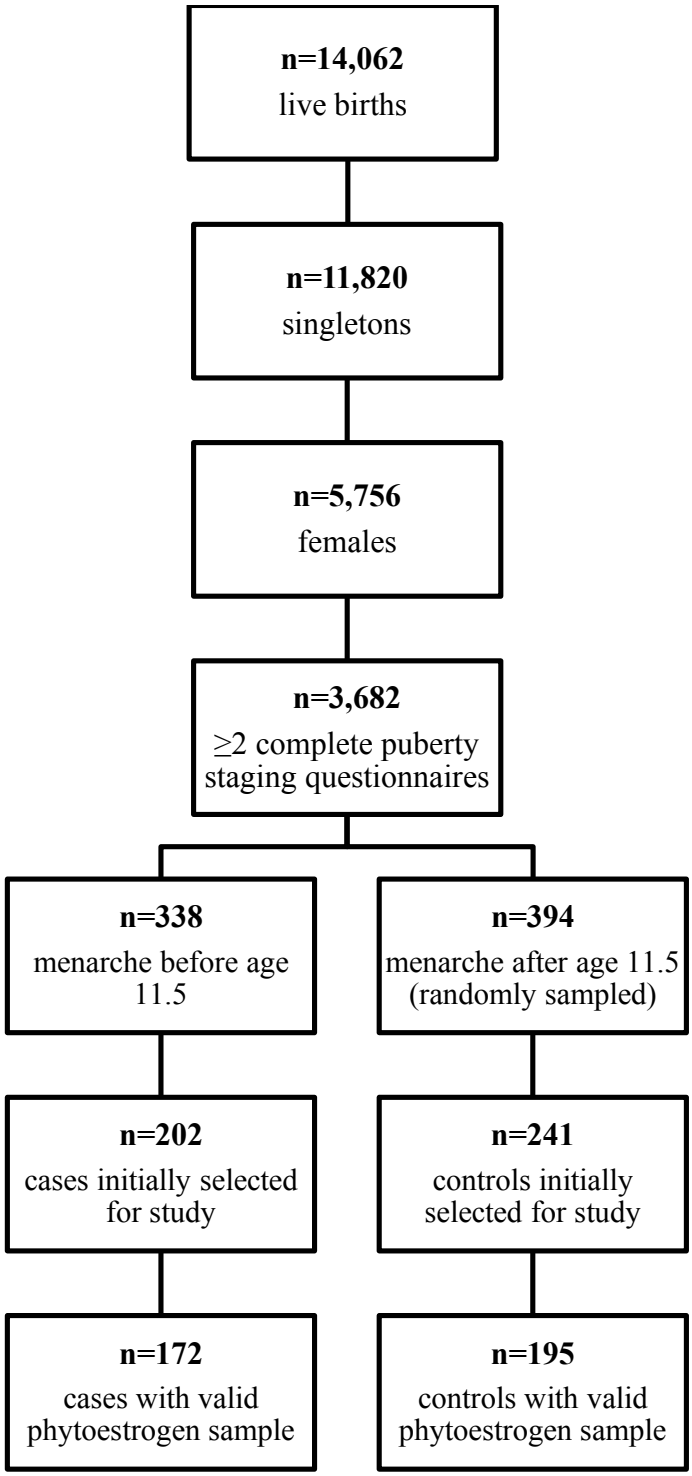
[a] Unconditional logistic regression

[b] Adjusted for maternal age at menarche, maternal education, pre-pregnancy BMI, child birth order, and duration of breastfeeding

[c] Creatinine corrected concentrations in µg/g creatinine
[d] Continuous represents natural log transformed values of phytoestrogen concentration
[e] Tertiles represent the comparison of the higher tertiles, tertiles 2 or 3, to the lowest tertile of phytoestrogen concentration; the p for trend is for the trend across all three tertiles

Figures

Figure 1. Flowchart of eligibility and exclusions.



Appendix

Supplementary Table 1. Adjusted associations of maternal urinary phytoestrogen concentrations with earlier age at menarche in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (N=309 mother-daughter dyads).

Analyte[a]	OR (95% CI)[bc]	p for trend
Enterolactone		
Continuous[d]	1.15 (0.92 – 1.45)	0.22
Tertile 2[e] (551.49 – 1314.96)	1.46 (0.82 – 2.58)	
Tertile 3[e] (1314.96 – 8718.04)	1.16 (0.62 – 2.14)	0.65
Daidzein		
Continuous	1.11 (0.91 – 1.35)	0.31
Tertile 2 (110.09 – 319.03)	1.58 (0.89 – 2.78)	
Tertile 3 (319.03 – 21880.45)	1.16 (0.63 – 2.12)	0.64
Genistein		
Continuous	0.97 (0.82 – 1.16)	0.75
Tertile 2 (38.86 – 118.38)	1.06 (0.60 – 1.87)	
Tertile 3 (118.38 – 17916.60)	0.99 (0.56 – 1.77)	0.97
Enterodiol		
Continuous	0.85 (0.66 – 1.09)	0.34
Tertile 2 (56.49 – 117.99)	0.51 (0.29 – 0.91)	
Tertile 3 (117.99 – 1188.20)	0.71 (0.40 – 1.27)	0.21
***O*-DMA**		
Continuous	1.19 (1.03 – 1.36)	0.02
Tertile 2 (4.77 – 21.04)	1.72 (0.93 – 3.18)	
Tertile 3 (21.04 – 1631.58)	2.27 (1.22 – 4.20)	0.01
Equol		
Continuous	0.97 (0.82 – 1.16)	0.72
Tertile 2 (3.19 – 6.93)	1.22 (0.70 – 2.13)	
Tertile 3 (6.93 – 9005.85)	0.85 (0.47 – 1.54)	0.61

[a] Creatinine corrected concentrations in µg/g creatinine

[b] From unconditional logistic regression

[c] Adjusted for maternal education, pre-pregnancy BMI, child birth order, and duration of breastfeeding

[d] Continuous represents natural log transformed values of phytoestrogen concentration

[e] Tertiles represent the comparison of the higher tertiles, tertiles 2 or 3, to the lowest tertile of phytoestrogen concentration; the p for trend is for the trend across all three tertiles

Supplementary Table 2. Gestational urinary phytoestrogen concentrations among mothers of girls in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-control study population (1991-1992) (N=367) versus urinary phytoestrogen concentrations among white women aged 20 to 39 years old in the 2003-2006 National Health and Nutrition Examination Survey (NHANES) (N=277).

Analyte[ab]	ALSPAC	NHANES
Enterolactone	755 (674 – 846)	296 (236 – 373)
Daidzein	184 (162 – 208)	57.9 (46.6 – 71.9)
Genistein	63.5 (54.9 – 73.4)	26.5 (20.8 – 33.7)
Enterodiol	71.5 (64.6 – 79.1)	42.2 (33.9 – 52.5)
O-DMA	11.2 (9.34 – 13.5)	5.12 (3.67 – 7.15)
Equol	5.65 (4.91 – 6.49)	10.3 (8.07 – 13.1)

Abbreviations: N, number; Geom. Mean, geometric mean; *O*-DMA, *O*-Desmethylangolensin

[a] Creatinine corrected concentrations in µg/g creatinine

[b] In NHANES, the reportable range of results were 0.1 – 3,300 ng/mL for enterolactone, 0.4 – 1,600 ng/mL for daidzein, 1 – 730 ng/mL for genistein, 0.04 – 320 ng/mL for enterodiol, 0.2 – 300 ng/mL for *O*-DMA, and 0.06 – 100 ng/mL for equol