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

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## Abstract

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 of age 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 and menarche status, with adjustment for maternal age at menarche, maternal education, pre-pregnancy body mass index (BMI), child birth order, and duration of breastfeeding. Among 367 mother-daughter dyads, maternal median (interquartile range) creatinine-corrected concentrations (in µg/g creatinine) were: daidzein 184.8 (88.8–383.7), enterodiol 76.1 (39.1–135.8), enterolactone 911.7 (448.1–1558.0), equol 4.3 (2.8–9.0), genistein 62.1 (27.1–160.9), and *O*-desmethylangolensin (*O*-DMA) 13.0 (4.4–34.5). In analyses comparing those in the highest tertile relative to those in the lowest tertile of *in utero* phytoestrogen exposure, higher enterodiol levels were inversely associated with early menarche (OR=0.45; 95% CI: 0.25–0.81), while higher *O*-DMA levels were associated with early menarche (odds ratio (OR) = 1.94; 95% confidence interval (CI): 1.07–3.51). These findings suggest that *in utero* exposure to phytoestrogens may be associated with earlier age at menarche, though the direction of association differs across phytoestrogens.

## Keywords

ALSPAC, menarche, phytoestrogens, puberty, endocrine disruptors

Target journal: *Environment International*

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

## 1. Introduction

Puberty is a crucial period of growth and development. The timing and patterning of pubertal events, such as age at menarche, can provide information on overall health and some previous exposures, while potentially forecasting future health outcomes (Christensen et al, 2011; Biro et al, 2001; Golub et al, 2008).

Age at menarche, on average, has decreased since the late 19$^{th}$ century (Wyshak & Frisch, 1982; Zacharias & Wurtman, 1969), and a secular trend towards earlier development of secondary sexual characteristics has been reported among girls in the Avon Longitudinal Study of Parents and Children (ALSPAC) based in the United Kingdom (Rubin et al, 2009). In the United States, recent estimates for average age at menarche (12.4 years) are almost a year younger than the average age at menarche of women born in the 1920s (13.3 years), and decreases in average age at menarche have been observed across all racial/ethnic groups (McDowell et al, 2007). While improvements in nutritional status since the 19$^{th}$ century and the increasing prevalence of childhood obesity may be in part responsible for this trend, exposure to endocrine disrupting chemicals (EDCs) may also lead to altered timing and patterning of pubertal development (Herman-Giddens et al, 1997; Buck Louis et al, 2008; Blanck et al, 2000; Biro et al, 2012; Christensen et al, 2011).

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 have the greatest impact 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 have examined the associations of *in utero* exposure to various EDCs with pubertal development, particularly age at menarche, with some conflicting results (Vasiliu et al, 2004; Blanck et al, 2000; Hatch et al, 2011; Christensen et al, 2011). Most studies were limited by the use of retrospectively-collected age at menarche data.

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). Although exposure to phytoestrogens is mostly dietary, phytoestrogens can cross the placental barrier in humans (Foster et al, 2002). Phytoestrogen exposure may affect sexual development, including altered pubertal timing (Kim & Park, 2012).

Animal studies have reported 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 have investigated the association between *in utero* phytoestrogen exposure and age at menarche. Our aim was to do so, using maternal gestational levels of phytoestrogen exposure and prospectively-collected age at menarche data in a population-based nested case-control study.

## 2. Study design and methods

### *2.1 Study population*

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 former 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 EDCs and age at menarche among the daughters. 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 parental- or self-report of menarche status, and, if it had occurred, month and year of occurrence so that age could be computed. 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 to satisfy sample size and power needed for the case-control study. 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 a 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 a maternal urine sample available. After evaluating the integrity of the maternal urine samples, 86.1% (n=174) of potential case and 81.3% (n=196) of potential control samples were analyzed. Two cases and one control were excluded due to missing creatinine concentrations, leaving a total sample size of 367 mother-daughter dyads.

*2.2 Laboratory analysis*

Maternal urine samples were stored at -20 degrees Celsius before being transferred under controlled conditions to the National Center for Environmental Health, Centers for Disease Control and Prevention (Atlanta, GA) for analysis using high-performance liquid chromatography–tandem mass spectrometry. The analytical methods are described elsewhere (Rybak et al, 2008). Phytoestrogens (enterolactone, daidzein, genistein, enterodiol, *O*-desmethylangolensin, and equol) were measured in maternal first morning void urine samples collected at a median gestational age of 12 weeks (interquartile range 8–17 weeks). Phytoestrogen concentrations were creatinine-corrected (CDC, 2012). Maternal urine concentrations were used as a proxy for fetal exposure (Green & Marsit, 2015; Ahmed et al, 2011).

*2.3 Statistical analysis*

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 time points. We considered the following as covariates: child race (white/non-white); maternal education (ordinally classified as <O-level (ordinary level: required, completed at age 16), O-level, or >O-level); maternal age at menarche (8–11 years / 12–15 years); maternal pre-pregnancy self-reported body mass index (BMI) ($kg/m^2$); prenatal vegetarian diet (yes/no);

prenatal smoking (any/none); maternal age at delivery (years); child birth order (first born, second born, or third born or later); child birth weight (<2500 g / ≥2500 g); 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 objectively measured childhood BMI Z-score at age 8 (if missing for age 8, used age 7, 9, or 10).

All data analysis was performed using SAS 9.3 (Cary, NC). Descriptive statistics were calculated for the sample comprised of 367 mother-daughter dyads; chi square and Fisher's exact tests were used to compare groups by menarche status. Medians and interquartile ranges 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.

Prior to modeling, phytoestrogen concentrations were log transformed. Phytoestrogen concentrations were also divided into tertiles by using cut points based on the distribution among the controls. To investigate the association between maternal phytoestrogen concentration and earlier age at menarche, unconditional logistic regression models were used. Using the set of potential confounding variables selected *a priori* for consideration in multivariable regression models, the final model was achieved through hierarchical backwards elimination of insignificant variables (Kleinbaum et al, 1982). Maternal education, maternal age at menarche, maternal vegetarian diet, and childhood BMI were considered as potential effect modifiers. Given that 15% (n=56) of observations were missing data for covariates included in the model, multiple imputation using the fully conditional specification method was performed to address missing covariate data (Liu & De, 2015).

Please note that the ALSPAC study website contains details of all the data that are available through a fully searchable data dictionary: http://www.bristol.ac.uk/

157 alspac/researchers/access/ (University of Bristol, 2015). Ethical approval for the study was
158 obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics
159 Committees. The U.S. Centers for Disease Control and Prevention (CDC) Institutional Review
160 Board assessed and approved human subjects protection. Mothers provided informed consent at
161 time of enrollment.

162 **3. Results**

163 In the ALSPAC cohort, girls were predominantly born to white mothers, most of whom
164 had ordinary levels of education or higher (Table 1). Cases were more likely to have mothers
165 who had an earlier age at menarche; one-third of case mothers reported menarche between 8 and
166 11 years of age, compared to less than 14% of control mothers. Mothers of cases were more than
167 twice as likely to have an overweight or obese pre-pregnancy BMI, and cases were more than
168 twice as likely to have a childhood BMI that was more than one standard deviation above the
169 mean. Cases were more likely to be the first born child (61.6% versus 51.6%) and more likely to
170 have never been breastfed or breastfed for less than 3 months (48.5% versus 39.9%). The median
171 age of menarche among cases was 11.0 years, while the median age of menarche among controls
172 was almost two years later at 12.8 years.

173 **Table 1**. Characteristics of the Avon Longitudinal Study of Parents and Children (ALSPAC)
174 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[a] |
|---|---|---|---|---|---|
| Characteristic | N | % | N | % | |
| Child race | | | | | 0.61 |
| White | 159 | 95.8 | 182 | 96.8 | |
| Non-white | 7 | 4.2 | 6 | 3.2 | |
| Maternal education[c] | | | | | 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 | | | | | <0.0001 |
| 8–11 | 50 | 33.3 | 23 | 13.9 | |

| | | | | | |
|---|---|---|---|---|---|
| ≥12 | 100 | 66.7 | 142 | 86.1 | |
| Maternal pre-pregnancy BMI, kg/m$^2$ | | | | | 0.0004 |
| <25 (under/normal weight) | 113 | 72.4 | 159 | 87.8 | |
| ≥25 (overweight/obese) | 43 | 27.6 | 22 | 12.2 | |
| 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 | 160 | 85.6 | |
| Maternal age at delivery, years | | | | | 0.63 |
| <25 | 32 | 18.7 | 35 | 17.9 | |
| 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 [d] | | | | | 1.00[b] |
| <2500 | <5 | -- | <5 | -- | |
| ≥2500 | 166 | -- | 188 | -- | |
| 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 [d] | | | | | 0.43[b] |
| Any | <5 | -- | <5 | -- | |
| None | 163 | -- | 187 | -- | |
| Vegetarian diet during childhood | | | | | 0.65 |
| Yes | 8 | 4.8 | 7 | 3.8 | |
| No | 158 | 95.2 | 176 | 96.2 | |
| Childhood BMI Z-score | | | | | <0.0001 |
| <0 | 32 | 20.9 | 60 | 34.5 | |
| 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: g, grams; kg/m$^2$, kilograms per meter-squared; IQR, interquartile range

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

[b] Compared using Fisher's exact 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.

[d] Counts and percents suppressed due to small cell sizes

Median enterodiol concentrations were almost 15 µg/g creatinine lower among cases than among controls, while median *O*-desmethylangolensin (*O*-DMA) concentrations were more than

2 µg/g creatinine higher among cases than among controls (Table 2). There were no other differences evident.

**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] | Median | IQR | Median | IQR | Median | IQR | |
| Enterolactone | 911.7 | 448.1–1558.0 | 901.1 | 494.8–1478.2 | 923.8 | 440.6–1650.9 | 0.89 |
| Daidzein | 184.8 | 88.8–383.7 | 187.8 | 91.9–369.7 | 183.0 | 88.8–423.8 | 0.79 |
| Genistein | 62.1 | 27.1–160.9 | 60.2 | 26.6–157.6 | 65.1 | 27.2–162.7 | 0.58 |
| Enterodiol | 76.1 | 39.1–135.8 | 69.6 | 33.6–123.6 | 84.3 | 49.4–144.4 | 0.02 |
| *O*-DMA | 13.0 | 4.4–34.5 | 14.0 | 5.7–43.8 | 11.7 | 3.4–31.5 | 0.06 |
| Equol | 4.3 | 2.8–9.0 | 4.4 | 2.8–7.4 | 4.2 | 2.6–9.6 | 0.74 |

Abbreviations: IQR, interquartile range; *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)

In the unadjusted model, decreased odds of early menarche were observed for those in the second and third tertiles of *in utero* enterodiol exposure compared to those in the lowest tertile (odds ratio $(OR)_{second}$=0.60; 95% confidence interval $(CI)_{second}$: 0.36–0.98; p-$value_{second}$=0.04; $OR_{third}$=0.58; 95% $CI_{third}$: 0.35–0.96; p-$value_{third}$=0.03; p-trend: 0.03) (Table 3). Increased odds of early menarche were observed for those in the second and third tertiles of *in utero O*-DMA exposure compared to those in the lowest tertile ($OR_{second}$=1.91; 95% $CI_{second}$: 1.12–3.27; p-$value_{second}$: 0.02; $OR_{third}$=1.97; 95% $CI_{third}$: 1.16–3.35; p-$value_{third}$: 0.02; p-trend: 0.02).

The results of the analyses adjusting for maternal age at menarche, maternal education, pre-pregnancy BMI, child birth order, and duration of breastfeeding were similar to those from the unadjusted analyses (Table 3). In the adjusted model, the odds of early menarche for each

unit increase of logged mothers' enterodiol concentration were OR=0.75 (95% CI: 0.59–0.96; p-trend: 0.02). When enterodiol was treated categorically, decreased odds of early menarche were observed for those in the second and third tertiles of *in utero* enterodiol exposure compared to those in the lowest tertile ($OR_{second}$=0.44; 95% $CI_{second}$: 0.25–0.77; p-$value_{second}$=0.005; $OR_{third}$=0.45; 95% $CI_{third}$: 0.25–0.81; p-$value_{third}$=0.008; p-trend: 0.007). The odds of early menarche for each unit increase of logged mothers' *O*-DMA concentration were OR=1.14 (95% CI: 1.00–1.29; p-trend: 0.05). When comparing those in the third tertile of *O*-DMA concentration to those in the lowest tertile, a 94% increase in the odds of early menarche was observed ($OR_{third}$=1.94; 95% $CI_{third}$: 1.07–3.51; p-$value_{third}$=0.03; p-trend: 0.03). No other significant associations were observed between phytoestrogen concentration and early menarche. There was no evidence of effect modification by maternal education, maternal age at menarche, maternal vegetarian diet, or childhood BMI.

**Table 3**. 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=367 mother-daughter dyads).

| | Unadjusted[a] | | | Adjusted[ab] | | |
|---|---|---|---|---|---|---|
| Analyte[c] | OR (95% CI) | p value[f] | p for trend[f] | OR (95% CI) | p value[f] | p for trend[f] |
| **Enterolactone** | | | | | | |
| Continuous[d] | 1.04 (0.87–1.26) | | 0.65 | 1.23 (0.98–1.55) | | 0.07 |
| Tertile 2[e] (551.49–1314.96) | 1.39 (0.85–2.28) | 0.19 | | 1.78 (1.02–3.10) | 0.04 | |
| Tertile 3[e] (1314.96–8718.04) | 0.98 (0.58–1.65) | 0.93 | 0.94 | 1.65 (0.90–3.02) | 0.10 | 0.10 |
| **Daidzein** | | | | | | |
| Continuous | 1.03 (0.87–1.22) | | 0.70 | 1.02 (0.84–1.24) | | 0.83 |
| Tertile 2 (110.09–319.03) | 1.37 (0.83–2.25) | 0.21 | | 1.14 (0.66–1.99) | 0.64 | |
| Tertile 3 (319.03–21880.45) | 1.00 (0.59–1.68) | 0.99 | 0.99 | 0.85 (0.47–1.51) | 0.57 | 0.58 |
| **Genistein** | | | | | | |
| Continuous | 0.96 (0.83–1.11) | | 0.55 | 0.92 (0.78–1.08) | | 0.30 |
| Tertile 2 (38.86–118.38) | 1.02 (0.62–1.67) | 0.94 | | 0.90 (0.52–1.56) | 0.70 | |
| Tertile 3 (118.38–17916.60) | 0.87 (0.52–1.44) | 0.58 | 0.59 | 0.76 (0.43–1.35) | 0.35 | 0.31 |
| **Enterodiol** | | | | | | |
| Continuous | 0.79 (0.64–0.98) | | 0.03 | 0.75 (0.59–0.96) | | 0.02 |
| Tertile 2 (56.49–117.99) | 0.60 (0.36–0.98) | 0.04 | | 0.44 (0.25–0.77) | 0.005 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tertile 3 (117.99–1188.20) | 0.58 (0.35–0.96) | 0.03 | 0.03 | 0.45 (0.25–0.81) | 0.008 | 0.007 |
| ***O*-DMA** | | | | | | |
| Continuous | 1.12 (1.00–1.26) | | 0.05 | 1.14 (1.00–1.29) | | 0.05 |
| Tertile 2 (4.77–21.04) | 1.91 (1.12–3.27) | 0.02 | | 1.54 (0.85–2.77) | 0.15 | |
| Tertile 3 (21.04–1631.58) | 1.97 (1.16–3.35) | 0.02 | 0.02 | 1.94 (1.07–3.51) | 0.03 | 0.03 |
| **Equol** | | | | | | |
| Continuous | 0.97 (0.83–1.13) | | 0.70 | 0.98 (0.82–1.15) | | 0.78 |
| Tertile 2 (3.19–6.93) | 1.22 (0.74–1.99) | 0.44 | | 1.10 (0.63–1.91) | 0.74 | |
| Tertile 3 (6.93–9005.85) | 0.86 (0.51–1.44) | 0.55 | 0.57 | 0.90 (0.51–1.60) | 0.72 | 0.73 |

[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
[f] The p-value is for the comparison of tertile 2 or 3 to the lowest tertile of phytoestrogen concentration; the p for trend is for the trend across all three tertiles

## 4. Discussion

In this study, we observed strong associations between enterodiol and decreased odds of earlier age at menarche, and some evidence of an association between *O*-DMA and increased odds of earlier age at menarche.

Studies have suggested that lignans such as enterodiol and enterolactone exhibit biphasic effects (estrogenic and antiestrogenic effects), which are dependent on exposure level (Tang et al, 2015; Mousavi and Adlercreutz, 1992; Mueller et al, 2004; Pettersson and Gustafsson, 2001; Waters and Knowler, 1982; Welshons et al, 1987; Adlercreutz, 2007; Wang, 2002). At relatively low doses, some lignan exposures demonstrate estrogenic activity, stimulating cell growth, while at higher doses appear to behave as antiestrogenic agents, suppressing cell growth (Wang, 2002). The biphasic effects of lignans could potentially provide an explanation for the negative association observed between enterodiol and early menarche status.

*O*-DMA is an intestinal bacterial metabolite of daidzein, and about 90% of individuals harbor bacteria capable of metabolizing daidzein to *O*-DMA (Frankenfield, 2011). *O*-DMA is

246 less structurally similar to 17β-estradiol than its parent compound and therefore may exhibit
247 different biological actions than daidzein. The underlying bacteria that metabolize daidzein to *O*-
248 DMA may have a distinct physiological role; urinary excretion of *O*-DMA is a marker of
249 harboring intestinal bacteria capable of C-ring cleavage, and therefore it is suspected that the role
250 of the phenotype may extend beyond daidzein metabolism (Frankenfield, 2011).
251 To our knowledge, this is the first published study of associations of *in utero*
252 phytoestrogen exposure with age at menarche. Although there were few significant associations
253 found between phytoestrogen levels and age at menarche, there is biological plausibility for such
254 an association. Exposures during pregnancy are extremely relevant to pubertal development,
255 since this represents the period of initial organ development, including the brain, endocrine
256 system, and reproductive tract. Furthermore, the fetus is more susceptible to such exposures due
257 to smaller size, lack of a complete blood-brain barrier, and absence of metabolizing enzymes
258 (Todaka et al, 2005). Studies have found that phytoestrogens can cross the placental barrier in
259 humans, and one study (n=53) of Californian women undergoing amniocentesis found that 96%
260 of second trimester amniotic fluid samples contained quantifiable amounts of dietary
261 phytoestrogens (Foster et al, 2002). Based on evidence from animal studies, the main mechanism
262 of action of phytoestrogens—the binding of phytoestrogens to estrogen receptors—may be
263 particularly relevant for *in utero* exposure to phytoestrogens because of the timing of
264 differentiation and development (Takagi et al, 2004; Takashima-Sasaki et al, 2006; Casanova et
265 al, 1999). Studies in rodents have found that isoflavones administered through diet or
266 subcutaneous injection during gestation or early life can lead to early vaginal opening (akin to
267 early menarche in humans), irregular estrous cyclicity, and decreased GnRH activation (GnRH
268 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).

To our knowledge, no previous studies have investigated *in utero* phytoestrogen exposure; therefore, we looked to previous studies on the effect of *in utero* exposure to other potential EDCs, which produced mixed results, as have previous studies on early life phytoestrogen exposure to soy infant formula. 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 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). Studies on the association between soy-based infant formula and age at menarche are also inconclusive (the phytoestrogens found in soy are daidzein and genistein; *O*-DMA and equol are metabolites of daidzein). A retrospective cohort study (n=811) found no association between soy-based formula and self-reported recalled age at menarche (Strom et al, 2001). However, a prospective cohort study (n=2,028) in British girls of the ALSPAC study found a 53% increased risk of early menarche among those fed soy-based formula, when compared to cows' milk-based formula (Adgent et al, 2011); it should be noted that this paper examined the ALSPAC cohort as a whole, as opposed to the nested case-control study used in this paper. While it is difficult to compare across classes of EDCs and at different times of exposure, previous studies have yet to suggest a clear association between *in utero* and early life exposure to EDCs 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, the absence of a 'Growing and Changing' questionnaire at age 12, missing information on age at menarche among some controls, incomplete information on some covariates, and no assessment of phytoestrogen exposure or early childhood soy consumption (excluding soy formula) in the daughters, which might influence timing of menarche. Unlike some other EDCs 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 assessed through urinary excretion at a single time point may under- or overestimate intermittent 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.

Urinary phytoestrogen concentrations during 1991–1992 among mothers of girls participating in the ALSPAC cohort were roughly two to three times higher for all phytoestrogens except equol (which was half as high) when compared to 2003–2006 National Health and Nutrition Examination Survey (NHANES) data for white women between 20 and 39 years old (CDC, 2012) (data not shown). It should be noted though that these samples were taken more than a decade apart.

It is also possible that the girls selected for this nested case-control study were not representative of the base cohort. When comparing ALSPAC girls who returned at least two 'Growing and Changing' questionnaires to those who did not return any questionnaires, non-respondents' parents were more likely to have lower educational attainment. Compared to non-respondents, mothers of respondents were generally older at time of index birth. Further, non-respondents were more likely to be of non-white race. This could have affected our findings since socioeconomic status is related to age at menarche (Braithwaite et al, 2009); however, whether socioeconomic status is related to phytoestrogen concentrations is unclear. Although race is related to age at menarche (Biro et al, 2006; Biro et al, 2001; Wu et al, 2002; Freedman et al, 2002; Britton et al, 2004; McDowell et al, 2007) and was associated with maternal lignan concentrations in this study, we were not able to examine the effect of race due to the small number of non-white girls enrolled in ALSPAC. Furthermore, it has been suggested that several genes in Caucasians code for early menarche (Dvornyk and Waqar-ul-Haq, 2012), and since we did not include genetic data in this study, we do not know if our results could be affected. Last, due to a modest sample size, this study may have been underpowered to detect additional associations between *in utero* phytoestrogen exposure and age at menarche.

## 5. Conclusions

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. We found an association between *O*-DMA and increased odds of earlier age at menarche, while decreased odds of earlier age at menarche were observed for enterodiol. As demonstrated in our study by the conflicting effects of phytoestrogens, plus the general lack of human studies on the associations between phytoestrogens and pubertal outcomes, there is a need for additional studies

to explore these associations in a variety of populations and to describe potential mechanisms of action for *O*-DMA and enterodiol.

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