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

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15 Abstract

16 Phytoestrogens are estrogenic compounds that occur naturally in plants. Phytoestrogens can
17 cross the placenta, and animal studies have found associations between *in utero* exposure to
18 phytoestrogens and markers of early puberty. We investigated the association between *in utero*
19 exposure to phytoestrogens and early menarche (defined as <11.5 years of age at onset) using
20 data from a nested case-control study within the Avon Longitudinal Study of Parents and
21 Children, a longitudinal study involving families living in the South West of England.
22 Concentrations of six phytoestrogens were measured in maternal urine samples collected during
23 pregnancy. Logistic regression was used to explore associations between tertiles of
24 phytoestrogen concentrations and menarche status, with adjustment for maternal age at
25 menarche, maternal education, pre-pregnancy body mass index (BMI), child birth order, and
26 duration of breastfeeding. Among 367 mother-daughter dyads, maternal median (interquartile
27 range) creatinine-corrected concentrations (in µg/g creatinine) were: daidzein 184.8 (88.8–
28 383.7), enterodiol 76.1 (39.1–135.8), enterolactone 911.7 (448.1–1558.0), equol 4.3 (2.8–9.0),
29 genistein 62.1 (27.1–160.9), and *O*-desmethylangolensin (*O*-DMA) 13.0 (4.4–34.5). In analyses
30 comparing those in the highest tertile relative to those in the lowest tertile of *in utero*
31 phytoestrogen exposure, higher enterodiol levels were inversely associated with early menarche
32 (OR=0.45; 95% CI: 0.25–0.81), while higher *O*-DMA levels were associated with early
33 menarche (odds ratio (OR) = 1.94; 95% confidence interval (CI): 1.07–3.51). These findings
34 suggest that *in utero* exposure to phytoestrogens may be associated with earlier age at menarche,
35 though the direction of association differs across phytoestrogens.

36 Keywords

37 ALSPAC, menarche, phytoestrogens, puberty, endocrine disruptors

38

39 Target journal: *Environment International*

40

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

43 **1. Introduction**

44 Puberty is a crucial period of growth and development. The timing and patterning of
45 pubertal events, such as age at menarche, can provide information on overall health and some
46 previous exposures, while potentially forecasting future health outcomes (Christensen et al,
47 2011; Biro et al, 2001; Golub et al, 2008).

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

60 EDCs are chemicals that may affect the body's endocrine system and cause adverse
61 developmental, reproductive, neurological, and immune effects in humans and animals. EDCs
62 can be natural or man-made, and research suggests that EDCs may have the greatest impact
63 during prenatal and early postnatal development when organ and neural systems are forming
64 (National Institute of Environmental Health Sciences, 2015). Most EDCs have estrogenic and/or
65 anti-androgenic actions (Daxenberger et al, 2001), which are thought to have puberty-inducing

66 effects in females (Mouritsen et al, 2010). Previous studies have examined the associations of *in*
67 *utero* exposure to various EDCs with pubertal development, particularly age at menarche, with
68 some conflicting results (Vasiliu et al, 2004; Blanck et al, 2000; Hatch et al, 2011; Christensen et
69 al, 2011). Most studies were limited by the use of retrospectively-collected age at menarche data.

70 One potential class of naturally-occurring EDCs of interest is phytoestrogens.
71 Phytoestrogens are estrogenic compounds that occur naturally in plants, with the most common
72 dietary source of phytoestrogens being soybean products (Kim & Park, 2012). Although
73 exposure to phytoestrogens is mostly dietary, phytoestrogens can cross the placental barrier in
74 humans (Foster et al, 2002). Phytoestrogen exposure may affect sexual development, including
75 altered pubertal timing (Kim & Park, 2012).

76 Animal studies have reported the effects of phytoestrogens to be quite different according
77 to time, dosage and route. Studies in rodents found that exposure to high doses of phytoestrogens
78 (isoflavones) *in utero* and through diet in early life accelerated pubertal onset in female animals
79 (Casanova et al, 1999; Takashima-Sasaki et al, 2006). In humans, the effect of soy-based infant
80 formula on pubertal development has been studied to some extent, though this has yielded mixed
81 results regarding an association with age at menarche (Adgent et al, 2011; Strom et al, 2001).
82 However, there have been no human studies published to date that have investigated the
83 association between *in utero* phytoestrogen exposure and age at menarche. Our aim was to do so,
84 using maternal gestational levels of phytoestrogen exposure and prospectively-collected age at
85 menarche data in a population-based nested case-control study.

86 **2. Study design and methods**

87 *2.1 Study population*

88 The Avon Longitudinal Study of Parents and Children (ALSPAC) is an ongoing
89 prospective birth cohort of 14,541 pregnancies. ALSPAC enrolled pregnant women with an
90 expected delivery date between April 1st, 1991 and December 31st, 1992 from three health
91 districts in the former county of Avon, Great Britain. Information has been collected on these
92 parents and children through interviews, mailed questionnaires, and clinic visits. Details on
93 ALSPAC recruitment and study methods have been described elsewhere (Boyd et al, 2013).

94 A nested case-control study was conducted within the ALSPAC cohort to explore
95 associations of prenatal maternal concentrations of various EDCs and age at menarche among
96 the daughters. A ‘Growing and Changing’ questionnaire was developed to collect information on
97 the offspring’s pubertal development and distributed to participants annually between the ages of
98 8–17 years (1999–2008), with the exception of age 12 (2003). Menarche was determined through
99 parental- or self-report of menarche status, and, if it had occurred, month and year of occurrence
100 so that age could be computed. From the original base population of 14,062 live births, case and
101 control series were selected from singleton (n=11,820) female subjects (n=5,756) who had
102 completed at least two puberty staging questionnaires between the ages of 8 and 13 (5 possible
103 questionnaires returned; n=3,682). Girls meeting eligibility criteria were ordered according to
104 reported age at menarche when the 13-year old data became available. A cut-off of 11.5 years
105 was established as defining ‘early’ menarche to satisfy sample size and power needed for the
106 case-control study. Eligible cases could complete any two questionnaires in the series, provided
107 that one was completed after menarche, while controls had to complete the 13-year old
108 questionnaire in order to ascertain that menarche had not occurred by the cut-off of 11.5 years.
109 Of the girls who reported menarche before the age of 11.5 (n=338), 59.8% (n=202) had a
110 prenatal maternal urine sample available, and were considered potential cases. Among girls who

111 reported menarche at or after the age of 11.5, a random sample of 394 was chosen as potential
112 controls, and of these, 61.2% (n=241) had a maternal urine sample available. After evaluating the
113 integrity of the maternal urine samples, 86.1% (n=174) of potential case and 81.3% (n=196) of
114 potential control samples were analyzed. Two cases and one control were excluded due to
115 missing creatinine concentrations, leaving a total sample size of 367 mother-daughter dyads.

116 *2.2 Laboratory analysis*

117 Maternal urine samples were stored at -20 degrees Celsius before being transferred under
118 controlled conditions to the National Center for Environmental Health, Centers for Disease
119 Control and Prevention (Atlanta, GA) for analysis using high-performance liquid
120 chromatography–tandem mass spectrometry. The analytical methods are described elsewhere
121 (Rybak et al, 2008). Phytoestrogens (enterolactone, daidzein, genistein, enterodiol, *O*-
122 desmethylangolensin, and equol) were measured in maternal first morning void urine samples
123 collected at a median gestational age of 12 weeks (interquartile range 8–17 weeks).
124 Phytoestrogen concentrations were creatinine-corrected (CDC, 2012). Maternal urine
125 concentrations were used as a proxy for fetal exposure (Green & Marsit, 2015; Ahmed et al,
126 2011).

127 *2.3 Statistical analysis*

128 Potential confounders to be considered in the analyses were identified *a priori* based on
129 previously published literature and biological plausibility. Covariates were collected at various
130 time points. We considered the following as covariates: child race (white/non-white); maternal
131 education (ordinally classified as <O-level (ordinary level: required, completed at age 16), O-
132 level, or >O-level); maternal age at menarche (8–11 years / 12–15 years); maternal pre-
133 pregnancy self-reported body mass index (BMI) (kg/m²); prenatal vegetarian diet (yes/no);

134 prenatal smoking (any/none); maternal age at delivery (years); child birth order (first born,
135 second born, or third born or later); child birth weight (<2500 g / ≥2500 g); breastfeeding
136 duration (ordinally classified as not breastfed, <3 months, 3–5 months, ≥6 months); use of infant
137 soy formula (any/none); vegetarian diet during childhood (yes/no); and objectively measured
138 childhood BMI Z-score at age 8 (if missing for age 8, used age 7, 9, or 10).

139 All data analysis was performed using SAS 9.3 (Cary, NC). Descriptive statistics were
140 calculated for the sample comprised of 367 mother-daughter dyads; chi square and Fisher's exact
141 tests were used to compare groups by menarche status. Medians and interquartile ranges were
142 calculated for each phytoestrogen for the total sample and by menarche status, and the Wilcoxon
143 rank sum test was used to compare groups by menarche status.

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

155 Please note that the ALSPAC study website contains details of all the data that are
156 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).

Characteristic	Menarche <11.5 years (N=172)		Menarche ≥11.5 years (N=195)		P-value for difference ^a
	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	

175 Abbreviations: g, grams; kg/m², kilograms per meter-squared; IQR, interquartile range

176 ^a Compared using chi-square tests unless otherwise noted

177 ^b Compared using Fisher's exact test

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

182 ^d Counts and percents suppressed due to small cell sizes

183 Median enterodiol concentrations were almost 15 µg/g creatinine lower among cases than

184 among controls, while median *O*-desmethylangolensin (*O*-DMA) concentrations were more than

185 2 µg/g creatinine higher among cases than among controls (Table 2). There were no other
186 differences evident.

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

Analyte ^a	Total		Menarche <11.5 years (N=172)		Menarche ≥11.5 years (N=195)		p- value ^b
	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

190 Abbreviations: IQR, interquartile range; O-DMA, O-Desmethylangolensin

191 ^a Creatinine-corrected concentrations in µg/g creatinine

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

193 ^c There are missing concentrations for genistein (n=1 where menarche <11.5 years), O-DMA (n=2 where
194 menarche <11.5 years), and equol (n=1 where menarche <11.5 years and n=1 where menarche ≥11.5
195 years)

196 In the unadjusted model, decreased odds of early menarche were observed for those in
197 the second and third tertiles of *in utero* enterodiol exposure compared to those in the lowest
198 tertile (odds ratio (OR)_{second}=0.60; 95% confidence interval (CI)_{second}: 0.36–0.98; p-
199 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).
200 Increased odds of early menarche were observed for those in the second and third tertiles of *in*
201 *utero* O-DMA exposure compared to those in the lowest tertile (OR_{second}=1.91; 95% CI_{second}:
202 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:
203 0.02).

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

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

219 **Table 3.** Associations of maternal urinary phytoestrogen concentrations with earlier age at
 220 menarche in the Avon Longitudinal Study of Parents and Children (ALSPAC) nested case-
 221 control study population (N=367 mother-daughter dyads).

Analyte ^c	Unadjusted ^a			Adjusted ^{ab}		
	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
<i>O</i>-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

222 ^a Unconditional logistic regression

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

225 ^c Creatinine-corrected concentrations in µg/g creatinine

226 ^d Continuous represents natural log transformed values of phytoestrogen concentration

227 ^e Tertiles represent the comparison of the higher tertiles, tertiles 2 or 3, to the lowest tertile of
228 phytoestrogen concentration

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

231

232 4. Discussion

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

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

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

246 less structurally similar to 17 β -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,

269 2006; Casanova et al, 1999; Kouki et al, 2003; Lewis et al, 2003; Bateman & Patisaul, 2008; Lee
270 et al, 2009; Nagao et al, 2001).

271 To our knowledge, no previous studies have investigated *in utero* phytoestrogen
272 exposure; therefore, we looked to previous studies on the effect of *in utero* exposure to other
273 potential EDCs, which produced mixed results, as have previous studies on early life
274 phytoestrogen exposure to soy infant formula. A cohort study (n=151) assessing *in utero*
275 exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE) with
276 age at menarche found that increased exposure to DDE was associated with an earlier age at
277 menarche, while exposure to PCBs was not associated (Vasiliu et al, 2004). A nested case-
278 control study (n=448) found no association with *in utero* exposure to polyfluoroalkyl chemicals
279 (PFCs) and age at menarche (Christensen et al, 2011). Studies on the association between soy-
280 based infant formula and age at menarche are also inconclusive (the phytoestrogens found in soy
281 are daidzein and genistein; *O*-DMA and equol are metabolites of daidzein). A retrospective
282 cohort study (n=811) found no association between soy-based formula and self-reported recalled
283 age at menarche (Strom et al, 2001). However, a prospective cohort study (n=2,028) in British
284 girls of the ALSPAC study found a 53% increased risk of early menarche among those fed soy-
285 based formula, when compared to cows' milk-based formula (Adgent et al, 2011); it should be
286 noted that this paper examined the ALSPAC cohort as a whole, as opposed to the nested case-
287 control study used in this paper. While it is difficult to compare across classes of EDCs and at
288 different times of exposure, previous studies have yet to suggest a clear association between *in*
289 *utero* and early life exposure to EDCs and age at menarche.

290 Strengths of this study are the inclusion of multiple phytoestrogen biomarkers, substantial
291 covariate data available on mothers and children from multiple time points over gestation and

292 childhood, and outcome data generally collected in the year that the outcome occurred.
293 Limitations of this study include a single spot urine measurement of phytoestrogen exposure
294 which was not collected at a uniform time of gestation, the absence of a ‘Growing and Changing’
295 questionnaire at age 12, missing information on age at menarche among some controls,
296 incomplete information on some covariates, and no assessment of phytoestrogen exposure or
297 early childhood soy consumption (excluding soy formula) in the daughters, which might
298 influence timing of menarche. Unlike some other EDCs that are estimated to have half-lives on
299 the order of several years, peak rates of urinary excretion of phytoestrogens occur between 6 and
300 12 hours after ingestion (King & Bursill, 1998). Since phytoestrogens are excreted rather
301 quickly, phytoestrogen exposure assessed through urinary excretion at a single time point may
302 under- or overestimate intermittent phytoestrogen exposure. Age at menarche was obtained
303 through self-report on ‘Growing and Changing’ puberty questionnaires completed every year by
304 parents and/or children, depending on age. There is some potential for misclassification of the
305 outcome, such as the completion of the questionnaire by a parent unaware of the child’s
306 menarche status, or issues in the parent or child’s recall of the month and year menstruation
307 began.

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

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

330 5. Conclusions

331 In summary, we compared exposure to phytoestrogens during pregnancy among mothers
332 of girls who did and did not have earlier age at menarche in the ALSPAC cohort. We found an
333 association between *O*-DMA and increased odds of earlier age at menarche, while decreased
334 odds of earlier age at menarche were observed for enterodiol. As demonstrated in our study by
335 the conflicting effects of phytoestrogens, plus the general lack of human studies on the
336 associations between phytoestrogens and pubertal outcomes, there is a need for additional studies

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

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