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In utero exposure to atrazine analytes and early menarche in the Avon Longitudinal Study of Parents and Children Cohort

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Keywords

ALSPAC, endocrine disrupting compounds, puberty, menarche, atrazine

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Abstract word count 257/250

Abstract

Background: Evidence from experimental studies suggests that atrazine and its analytes alter the timing of puberty in laboratory animals. Such associations have not been investigated in humans.

Objective: To determine the association between *in utero* exposure to atrazine analytes and earlier menarche attainment in a nested case-control study of the population-based Avon Longitudinal Study of Parents and Children.

Methods: Cases were girls who reported menarche before 11.5 years while controls were girls who reported menarche at or after 11.5 years. Seven atrazine analyte concentrations were measured in maternal gestational urine samples (sample gestation week median (IQR): 12 (8-17)) for 174 cases and 195 controls using high performance liquid chromatography-tandem mass spectrometry. We evaluated the study association using multivariate logistic regression, adjusting for potential confounders.

Results: Diaminochlorotriazine (DACT) was the most frequently detected analyte (58% >limit of detection [LOD]) followed by desethyl atrazine (6%), desethyl atrazine mercapturate (3%), atrazine mercapturate (1%), hydroxyl atrazine (1%), atrazine (1%) and desisopropyl atrazine (0.5%). Because of low detection of other analytes, only DACT was included in the exposure–outcome analyses. The adjusted odds of early menarche (95% Confidence Interval) for girls with DACT exposures ≥median was 1.13 (0.82, 1.55) and exposure <median was 1.01 (0.73, 1.42) compared to girls with exposure <LOD (reference).

Conclusions: This study is the first to examine the association between timing of menarche and atrazine analytes. We found a weak, non-significant association between *in-utero* exposure to atrazine metabolite

DACT and early menarche. Further exploration of the role of these exposures in female reproduction in other cohorts is needed.

Main text word count: 3349

Introduction

Atrazine is used in more than 70 countries worldwide; most frequently in the United States (U.S.), Brazil, Argentina, Mexico and China to control broadleaf and grassy weeds in agricultural crops, mainly corn (LeBaron et al. 2008; EPA 2012). Human exposure to atrazine can occur through several routes. Agricultural workers and those living near farms can be exposed to atrazine dispersed in air through spraying (ATSDR 2003). Exposure can also occur through contact with contaminated soils or ingestion of contaminated agricultural products (ATSDR 2003). Water run-off from crops and lawn applications can get into ground and surface waters and contaminate drinking water wells (Munger et al. 1997). In water, atrazine breaks down to the following primary metabolites: desethyl atrazine (DEA), desisopropyl atrazine (DIA) and diaminochlorotriazine (DACT) (EPA 2012).

In areas where atrazine is used, the general population can be exposed to atrazine analytes (i.e., atrazine parent compound or any of its metabolites) through contaminated drinking water (Munger et al. 1997; Ocoa-Acuna et al. 1997; Villanueva et al. 2005). Developing fetuses and children can be exposed *in utero* or through breast milk (Balduini et al. 2003; Whyatt et al. 2003). Atrazine is broken down rapidly in the body and eliminated primarily in urine, within 24-48 hours (Catenacci et al. 1993). Although it does not bioaccumulate appreciably in humans, atrazine and its related compounds can persist in ground water, contaminating surface and drinking water sources (ATSDR 2003). This persistent contamination of drinking water can result in continuous exposure in humans, and therefore can be of concern (UNEP and WHO 2012).

In Great Britain, atrazine has mainly been used on maize and sweet corn crops (Fera Science Ltd 2016). During the years 1990 to 1992, about 42,000 kg of atrazine was applied annually in Great Britain, with about 17,000 kg applied to about 10,000 hectares annually in the South Western region of England

(Fera Science Ltd 2016). The European Union (E.U.) banned the use of atrazine in 2003 because of its persistent contamination of drinking and ground water above the E.U. recommended limit of 0.1 parts per billion (ppb) (Sass and Colangelo 2006). However, several countries still use this herbicide including the US where it is a restricted use pesticide (ATSDR 2003).

Animal studies have associated exposure to atrazine with hormone-related tumors and with alterations in reproductive functions suggesting potential endocrine disrupting effects from these exposures. In rats, atrazine induced an earlier onset (Cooper et al. 2007; Wetzel et al. 1994) and increased incidence of mammary gland tumors by disrupting the ovarian function (Cooper et al 2007). Atrazine also caused a disruption in estrus cycle in rats by impairing the ovulatory surge of luteinizing hormone (Wetzel et al. 1994). Lactational exposure to atrazine or DACT (Laws et al. 2000, Laws et al. 2003) and prenatal exposure to atrazine (Davis et al. 2011) in female rats resulted in delayed puberty (vaginal opening) in the offspring. Further, DACT was demonstrated to be as potent as atrazine in delaying puberty (Laws et al. 2003). However, the doses associated with delayed puberty in these studies were high (50,000 – 200,000ppb daily).

Several studies have reported a secular trend towards earlier onset of puberty among girls in Europe and the United States (Aksglaede et al. 2008; Euling et al. 2008; McDowell et al. 2007; Parent et al. 2003; Semiz et al. 2008) with some reporting earlier age at onset of menarche. (Mc Dowell et al. 2007; Semiz et al. 2008) Early puberty is a risk factor for childhood risky behaviors (e.g., smoking, alcohol consumption and drug abuse) and adult-onset diseases (e.g., breast and ovarian cancer) (Gail et al. 1989; Moorman et al. 2009; vanJaarsveld et al. 2007; Walvoord 2010). Epidemiologic studies of other endocrine disrupting chemicals (EDCs) suggest that *in utero* exposure to the estrogenic effect of EDCs is associated with early onset of puberty (Blanck et al. 2000; Vasiliu et al. 2004). Evidence from experimental studies suggests that atrazine analytes alter the timing of puberty in laboratory animals, however, such associations have not been investigated in humans. This study uses a nested case-control

study design to measure atrazine analytes in maternal gestational urine as a proxy for *in utero* exposure of cases and controls to atrazine analytes, and examines the association with early menarche, as a marker of early puberty in girls.

Methods

Study population

Pregnant women living in the Bristol area, in the south west of England, United Kingdom, with an expected date of delivery from 1st April, 1991 to 31st December, 1992, were recruited to participate in the Avon Longitudinal Study of Parents and Children (ALSPAC) (Fraser et al. 2013). A total of 14,775 live births were included in the ALSPAC cohort (Boyd et al. 2013). Details about recruitment and the study participants have already been published (Fraser et al. 2013; Boyd et al. 2013). Beginning at the age of 8, a puberty questionnaire titled "Growing and Changing" was mailed to the participants each year until age 17 (except age 12) to collect information about the onset and progression of puberty in enrolled girls and boys (Rubin et al. 2009). For girls, onset of menarche information included the age and date of first menstrual period. The parents, the girls, or both completed the questionnaires (Rubin et al. 2009). The median age at onset of menarche (95% confidence interval [95% CI]) for girls who returned at least one puberty questionnaire (n= 3,938) was 12.87 years (10.82, 12.91) (Christensen et al. 2010). The questionnaire is available on the study website at http://www.bristol.ac.uk/media-library/sites/alspac/migrated/documents/ques-cb16a-mum-and-daughter-at-8.pdf. The website also contains detailed descriptions of available data accessible through a fully searchable online data dictionary (http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/).

The selection of cases and controls for the nested case-control study has been previously described (Christensen et al. 2011). In brief, 3,682 singleton girls returned at least 2 "Growing and Changing" questionnaires from ages 8 to 13 years. From this number 218 were identified as having

menarche before 11.5 years of age, and had one maternal gestational serum sample available for analysis. A subset of 174 cases also had one maternal gestational urine sample available for analysis. A random sample of 394 girls that had menarche at or after 11.5 years of age were selected as controls; 230 of these had one maternal gestational serum sample and 195 also had one maternal gestational urine sample available for analysis. We included only the 174 cases and 195 controls with analyzable maternal gestational urine samples in this study. The urine samples were collected from mothers as part of routine antenatal care at random times during pregnancy (between 1st April 1991 and 31st December 1992), (Fraser et al. 2013; Boyd et al. 2013) therefore, stage of gestation and timing of urine collection may vary among mothers' urine samples. Each urine sample was divided into small aliquots to maximize efficiency of the sample use, and banked at the University of Bristol (Boyd et al. 2013). The samples were stored at +4 or -20 degrees Celsius 0-6 days after initial collection then stored between -10 and -20 degrees Celsius until analysis. Only one aliquot per mother was used for this study. The ALSPAC Law and Ethics Committee, the Local Research Ethics Committees, and the Centers for Disease Control and Prevention (CDC) Institutional Review Board assessed and approved human subject protection (Christensen et al. 2011).

Exposure Assessment

Banked urine samples from the University of Bristol were transferred to CDC's Division of Laboratory Sciences within the National Center for Environmental Health in 2008, and analyzed during the same year. The following atrazine analytes were measured using on-line solid phase extraction-isotope dilution-high performance liquid chromatography-tandem mass spectrometry: (Panuwet et al. 2008, Panuwet et al. 2010) Atrazine, DACT, DEA, DIA, atrazine mercapturate, desethyl atrazine mercapturate, and hydroxyl atrazine. The limits of detection (LODs) were calculated as three times the standard deviation of the noise at zero concentration (Panuwet et al. 2008, Panuwet et al. 2010). An estimate of the noise was based on the variation in precision at concentrations close to the LODs

(Panuwet et al. 2008, Panuwet et al. 2010). Creatinine was measured for each urine sample on a Roche Hitachi Modular P Chemistry Analyzer (Hitachi, Pleasanton, CA, USA) using the Creatinine Plus Assay, as described in Roche's Creatinine Plus Product Application # 04903773003.

We calculated the median, and the 25th and 75th percentile only for analytes detected in more than 50% of sample measurements. Survival analyses methods are recommended when estimating distributions using data with values <LOD instead of methods that replace values <LOD with LOD/2 or LOD/√2 (Gillespie et al. 2010). Therefore, to calculate the median and percentiles, we used non-parametric survival analysis methods using the LIFETEST procedure in SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA) to account for the left censored values (<LOD) after transforming the data for left censoring.

Exposure and early menarche analyses

We classified exposure to analytes detected in more than 50% of sample measurements into the following 3 categories: <LOD, LOD to <median, and ≥median. We determined the median using the distribution of controls ≥LOD. We also dichotomized exposure as "Yes" (≥LOD) or "No" (<LOD).

We selected the following covariates *a priori* from the literature, and evaluated them for their potential to confound the outcome–exposure association: Mother's age at delivery (<20, 20–24, 25–29, 30–39, or ≥40 years) (Adair 2001), mother's pre-natal body mass index (BMI) (<18.5 [underweight], 18.5–24.9 [normal], 25–29.9 [over-weight], or ≥30 [obese]) (Rubin et al. 2009), mother's age at menarche (8–11, 12–14, or ≥15 years) (Rubin et al. 2009), mother's education (Certificate of secondary education (CSE)/None, Vocational, O-level, A-level, or Degree) (Parent et al. 2009; Adair 2001), child's birth weight (<2500g or ≥2500g) (Adair 2001; Ruder et al. 2010), child's birth order (first born, second born, or third born or later) (Rubin et al. 2009), child's BMI at age 7 (Rubin et al. 2009; Biro et al. 2010), child's duration of breast feeding (never, < 6 months , or ≥6 months) (Balduini et al. 2003),

trimester when gestational urine sample was collected (<12 weeks, 13–≤28 weeks, or ≥29 weeks) and month of urine sample collection (December–February, March–May, June–August, or September–November), or (March–August or September–February) (Whyatt et al. 2003; Winchester et al. 2008).

We used logistic regression methods in SAS 9.3 to evaluate the covariate–exposure and the covariate–outcome association. We considered covariates with a p-value (p) <0.25 (Mickey and Greenland 1989) for both associations to be potential confounders.

We used multiple imputation methods in SAS 9.3 to impute values for missing covariate data (Liu and De 2015), and used these datasets with imputed covariate values to estimate the outcome–exposure association. We used logistic regression to analyze the association between early menarche and *in utero* exposure to atrazine analytes, and then used the MIANALYZE procedure to combine the results from imputed datasets. We adjusted for urine creatinine by including it as a covariate in the multivariate model.

Results

Child's birth order, child's BMI at age 7, mother's prenatal BMI and mother's age at menarche significantly (p<0.05) differed between cases and controls (Table 1). Cases were more likely than controls to be first born children (61.5% vs. 52.1%), have a mother who was overweight (BMI 25–29.9) or obese (BMI>30) (17.7% and 10.4%, respectively vs. 8.3% and 3.9% of controls), or have a mother with onset of menarche between the ages 8–11 years (32.9% vs. 13.4% of controls) (Table 1). Cases had significantly higher BMIs at age 7 compared to controls (p <0.0001). Cases were also more likely to have a mother with either no education or a CSE (15.5% vs. 9.4% of controls) although this difference was not statistically significant. The median age at menarche for cases was 11 years compared to 12.8 years for controls (Table 1).

Atrazine was detected in only 0.8% of the study participants. DACT was the only analyte detected above the LOD in over 50% of the participants (58.3%) (Table 2). Other less frequently detected analytes were DEA (6.2%), desethyl atrazine mercapturate (3.3%), atrazine mercapturate (1.4 %), hydroxyl atrazine (1%) and DIA (0.5%).

Because of low detection of the other atrazine analytes, only DACT was included in the exposure–outcome analyses. Mothers prenatal BMI, child's BMI at age 7, and child's duration of breastfeeding were identified as potential confounders of the *in utero* exposure to DACT and early menarche association. Fifty two cases and 53 controls did not have the covariate data required for the adjusted analysis of DACT exposure and early menarche. Therefore, we used multiple imputation for the exposure–outcome association analyses.

For the association between early menarche and dichotomized DACT exposure, the unadjusted odds ratio (95% CI) was 1.18 (0.78, 1.78), and the adjusted odds ratio (95% CI) was 1.12 (0.90, 1.40) (Table 3). For the association between early menarche and DACT exposure categories, the unadjusted odds ratio (95% CI) was 1.01 (0.61, 1.67) for the <median category and 1.34 (0.83, 2.18) for the ≥median category compared to the reference (<LOD) (Table 3). The adjusted odds ratio (95% CI) was 1.03 (0.74, 1.45) for the <median category and 1.13 (0.82, 1.55) for the ≥median category compared to the reference (Table 3). Even though the odds ratios were >1, none of the associations were statistically significant (the 95% CI included the null).

When we excluded the cases and controls without covariate data from the analyses, the odds ratio for dichotomous exposure increased though the association remained non-significant (see supplemental material; see Table S1). However, for the categorical exposure, the unadjusted odds ratio (95% CI) was 1.11 (0.60, 2.06) for the <median category and 2.06 (1.16, 3.64) for the ≥median category compared to the reference (see supplemental material; see Table S1). The adjusted odds of early

menarche (95% CI) for girls with DACT exposures <median was 1.26 (0.65, 2.42) and for the ≥median was 1.86 (1.03, 3.38) compared to the reference (see supplemental material; see Table S1). The association was significant for both unadjusted and adjusted *in utero* exposure to DACT ≥median compared to the reference.

Discussion

To our knowledge, this study is the first to analyze the association between exposure to atrazine analytes and timing of menarche in humans. Our results suggest that earlier menarche is associated with *in utero* exposure to DACT, though the association is weak, the confidence intervals wide and include the null.

The distribution profile of the analytes detected in the present study population suggests environmental exposure to atrazine as opposed to occupational exposures. Assessments of streams and ground water in the US showed that as atrazine persists in ground water, it breaks down to its degradates (e.g., DEA, DACT and DIA) such that the degradates are detected as frequently as, (Barbash et al. 1999) and at higher concentrations than the parent compound. (Gilliom et al. 2006) Barr et al. (2007), also reported that DACT is the analyte predominantly detected (77%) in populations exposed environmentally to atrazine (e.g., through contaminated water or food), followed distantly by DEA (15%), DIA (6%) and then atrazine mercapturate (2%). In our study, we detected DACT at 58%, followed by DEA (6%). We detected atrazine mercapturate and DIA in 1.4% and 0.5% of our study participants respectively. The environmental exposure profile we observed in ALSPAC participants is consistent with exposure to atrazine analytes through drinking water or food (Barr et al. 2007). The Barr et al. (2007) study had a small number of participants (N = 24), and even though DACT was the predominant atrazine analyte detected in the different exposure categories (environmental, occupational

and acute), the inter-person variations were about 30%. Further evaluation in larger studies is needed (Barr et al. 2007).

In our study, a single sample of urine collected during pregnancy from each mother was used. Since atrazine has a short half-life of 8 hours with elimination from the body occurring in 24-48 hours, (Catenacci et al. 1993) atrazine analytes measured in the urine sample would most likely only describe exposure in the past 24–48 hours, and may not be an accurate representation of exposure throughout the pregnancy. However, the use of atrazine, e.g., agricultural applications, can lead to contaminated surface and ground water, which can result in persistent contamination of drinking water supplies (ATSDR 2003; Munger 1997). If exposure to atrazine in this population occurred regularly through drinking water as suggested by the environmental exposure profile, the measurement of atrazine analytes in this study may be an adequate approximation of long-term exposure. This assumption does not take into account that some of the mothers may have other sources of drinking water, e.g., bottled water. Another factor that may not be taken into consideration is the water filtration systems at public drinking water supply systems or in the home. Although the use of only one urine sample may introduce error to the classification of exposure, this classification error is likely to be non-differential for cases and controls.

The results of our study show that participants were exposed to several atrazine analytes. A recent study in France measured exposure to 8 atrazine analytes in urine samples from 579 pregnant women in the PELAGIE cohort, and examined the association with several adverse birth outcomes including fetal growth restriction and small head circumference (Chevrier 2011). DACT was detected in 7% of the participants, DEA and DIA in 10%, and 4% of the participants respectively, and atrazine mercapturate in 4% (Chevrier 2011). In previous human exposure studies in the U.S., the analyte mainly measured has been atrazine mercapturate (Arcury 2007; Bakke 2009). As atrazine is still in use in several countries including U.S., other metabolites including DACT, DEA and DIA need to be measured to better assess exposure. However, Barr et. al. (2007) state that "measuring atrazine compound or

atrazine mercapturate in urine is the unequivocal indication that a person was exposed to atrazine and not an environment degradate".

Our study results conflict with animal studies suggesting that atrazine and DACT may delay the onset of puberty by altering the hypothalamic-pituitary activity. In most of these studies the doses used to induce the observed outcomes are typically very high (50,000-200,000ppb), and do not compare to environmental levels that human populations are usually exposed to (median concentrations for DACT in our study are 0.27ng/mL (ppb) for cases and 0.24ppb for controls). Epidemiologic studies suggest that *in utero* exposure to the estrogenic effect of some EDCs is associated with early puberty (Blanck 2000; Vasiliu 2004). Animal studies suggest that atrazine does not bind to the estrogen receptor, and that the hypothalamic function is the main target of atrazine exposure (Cooper et al. 2007). A suggested endocrine disrupting mechanism of action associated with atrazine exposure is aromatase induction, which results in an increase in estrogen (Hayes et al. 2002; Sanderson et al. 2001). This increase may contribute to some estrogen-mediated toxicities observed in animal studies (e.g., induction and earlier onset of the incidence of mammary gland tumors, and lengthening of estrus cycle in rats) (Sanderson et al. 2001). More experimental studies conducted using doses of atrazine analytes at background levels, i.e., doses that populations are more likely to be exposed to, are needed to determine health outcomes, including onset of puberty, and mechanisms of action.

A strength of this study is the ability to link to several demographic and health-related characteristics of the child and mother. A disadvantage of this study is use of only one urine sample during the pregnancy period. A second disadvantage is that age at menarche was self-reported by children or their parents, which could be subject to recall bias. Lastly, this study analyzed the association between *in utero* exposure to DACT and early menarche, not onset of puberty, which is marked by Tanner stage 2 breast development.

Conclusion

Our results show a non-significant association between early menarche and *in utero* exposure to the atrazine analyte DACT in the ALSPAC cohort. Further exploration of the role of exposure to atrazine analytes in female reproduction in other large independent cohorts is needed. To comprehensively assess atrazine exposure in human populations, several analytes including DACT, DEA, and DIA, may need to be measured along with atrazine parent compound and atrazine mercapturate.

Tables

Table 1 Study population characteristics

Characteristic	Cases (N=174) N	%	Controls (N=195) N	%	p-value[a]
Mother's ethnicity					0.88
White	167	98.2	189	98.4	
Other Ethnicity	3	1.8	3	1.6	
Missing	4		3		
Mother's education[b]					0.45
CSE/None	26	15.5	18	9.4	
Vocational	12	7.1	14	7.3	
O-Level	56	33.3	61	31.9	
A-Level	48	28.6	64	33.5	
Degree	26	15.5	34	17.8	
Missing	6		14		
Mother's age at delivery (yrs)					0.36
<20	1	0.6	6	3.1	
20-24	32	18.5	28	14.4	
25-29	72	41.6	74	38	
30-39	65	37.6	84	43.1	
>40	3	1.7	3	1.5	
Missing	1				
Mother's prenatal BMI					0.004
<18.5	5	3.3	11	6.2	
18.5-24.9	109	69.0	148	81.8	
25-29.9	28	17.7	15	8.3	
>30	16	10.4	7	3.9	
Missing	16		14		
Mother's age at menarche (yrs)					0.0003
8 – 11	50	32.9	22	13.4	
12 – 14	92	60.5	126	76.8	
≥ 15	10	6.6	16	9.8	
Missing	22		31		
Child's birth order					0.03
First born	102	61.5	98	52.1	
Second born	36	21.7	65	34.6	
Third born or later	28	16.9	25	13.3	
Missing	8		7		
	Median	Quartile	Median	Quartile	<0.0001
Child's BMI at age 7	17	15.7–18.9	15.9	15.2–16.8	
Child's age at menarche (yrs)	11	10.7–11.3	12.8	12.3–13.3	

Urine Sample gestation week	12	8– 17	13	9 – 18	0.23

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

Table 2 Gestational urine concentrations (ng/mL)

Analyte	Total N (%) > LOD[a]	Cases[c] N (%) > LOD	Median (quartiles)	Controls[c] N (%) > LOD	Median (quartiles)	p-value
Diaminochlorotriazine (DACT)	369 (58.3)	174 (60.3)	0.27 (0.16–0.44)	195 (56.4)	0.24 (0.16–0.41)	0.31[b]
Desethyl atrazine (DEA)	369 (6.2)	174 (4.6)		195 (7.7)		
Desethyl atrazine mercapturate	369 (3.3)	174 (2.3)		195 (4.1)		
Atrazine mercapturate	369 (1.4)	174 (0.6)		195 (2.1)		
Hydroxyl atrazine	369 (1.1)	174 (0.6)		195 (1.5)		
Atrazine	369 (0.8)	174 (0.6)		195 (1)		
Desisopropyl atrazine (DIA)	369 (0.5)	174 (0.6)		195 (0.5)		

[a] The LODs were: atrazine, 0.05ng/mL; DACT, 0.19ng/mL; DEA, 0.10ng/mL; DIA, 0.12ng/mL; atrazine mercapturate, 0.08ng/mL; desethyl atrazine mercapturate, 0.09ng/mL; and hydroxyl atrazine, 0.14ng/mL
[b] Comparison between cases and controls using Wilcoxon Rank Sum test
[c] Urine creatinine median (IQR) µg/g: Cases 81 (48-134) µg/g; Controls 80 (52-118) µg/g

Table 3 Association between DACT gestational urine concentrations (ng/mL) and early menarche

DACT	n cases/controls	Unadjusted OR (95%CI)	Adjusted OR (95% CI)
Dichotomous exposure			
<LOD	69/ 85	1 Reference	1 Reference
≥LOD	105/ 110	1.18 (0.78 – 1.78)[a]	1.12 (0.90 – 1.40)[a, c]
Categorical exposure			
< LOD[d]	69/ 85	1 Reference	1 Reference
LOD – < Median[d]	45/ 55	1.01 (0.61–1.67) [b]	1.03 (0.74–1.45) [b, c]
≥ Median[d]	60/ 55	1.34 (0.83–2.18) [b]	1.13 (0.82–1.55) [b, c]

[a] Odds of early menarche for DACT exposure ≥LOD compared to exposure <LOD
[b] Odds of early menarche by DACT exposure category compared to reference (<LOD)
[c] Adjusted for Mother's pre-natal BMI, childhood BMI at age 7, duration of breastfeeding, and urine sample creatinine
[d] DACT LOD = 0.19 ng/mL and Median of controls ≥LOD = 0.4 ng/mL

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