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The estimated effect of season and vitamin D in the first trimester on pubertal timing in girls and boys: a cohort study and an instrumental variable analysis

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Abstract

Background: Season of birth has been associated with age at menarche. Maternal vitamin D levels in pregnancy may explain this effect. We investigated whether the season of first trimester or maternal 25-hydroxyvitamin D_3 [25(OH)D_3] levels were associated with pubertal timing in children.

Methods: We conducted a follow-up study of 15 819 children born in 2000–03 from the Puberty Cohort, nested in the Danish National Birth Cohort (DNBC). Mean differences in attaining numerous pubertal markers, including a combined estimate for the average age at attaining all pubertal markers, were estimated for low (November–April) relative to high (May–October) sunshine exposure season in the first trimester using multivariable interval-censored regression models. Moreover, we conducted a two-sample instrumental variable analysis using season as an instrument for maternal first-trimester 25(OH)D_3 plasma levels obtained from a non-overlapping subset ($n = 827$) in the DNBC.

Results: For the combined estimate, girls and boys of mothers who had their first trimester during November–April had earlier pubertal timing than girls and boys of mothers

whose first trimester occurred during May–October: –1.0 months (95% CI: –1.7 to –0.3) and –0.7 months (95% CI: –1.4 to –0.1), respectively. In the instrumental variable analysis, girls and boys also had earlier pubertal timing: respectively, –1.3 months (95% CI: –2.1 to –0.4) and –1.0 months (95% CI: –1.8 to –0.2) per SD (22 nmol/L) decrease in 25(OH)D_3.
Conclusions: Both first pregnancy trimester during November–April and lower 25(OH)D_3 were associated with earlier pubertal timing in girls and boys.

Key words: Seasonal effect, pregnancy season, vitamin D; 25-hydroxyvitamin D, prenatal exposure, delayed effects, maternal exposure, fetal programming, pubertal development, instrumental variable analysis

Key Messages

- Existing literature indicates that the season of birth is associated with markers of later reproductive health, including age at menarche.
- We found that the first pregnancy trimester during November through April, characterized by low exposure to sunshine and low endogenous synthesis of vitamin D_3 in the skin in Denmark, was associated with earlier pubertal timing in girls and boys.
- In an instrumental variable analysis using season at gestational Week 8 as an instrument for maternal 25-hydroxyvitamin D_3 [25(OH)D_3] levels, we found that lower maternal 25(OH)D_3 plasma levels in the first trimester were associated with earlier pubertal timing in girls and boys.
- Future studies should aim to elucidate the mechanism behind the observed association and to examine whether intervening on low maternal 25(OH)D_3 levels during seasons with low exposure to sunshine would be beneficial with regard to pubertal timing in the children.

Introduction

Season of birth, a marker of different environmental exposures at birth, has been associated with numerous different outcomes later in life, such as metabolic, neurologic and immunologic diseases.[1–5] Season of birth has also been associated with reproductive outcomes, including age at menarche (AAM),[1,6–8] fecundity in women,[9] fertility (offspring count and the risk of being childless) in men[10] and age at menopause.[11] Whether there is a seasonal effect on other markers of girls' pubertal development or with boys' pubertal development remains unexplored.

In studies investigating the association between season of birth and AAM from the Northern hemisphere, results suggest that birth during the summer or autumn may be associated with earlier AAM. However, the results and the directions of effect were not consistent.[1,6–8] At Northern latitudes, vitamin D_3 levels and levels of the metabolit 25-hydroxyvitamin D_3 [25(OH)D_3] fluctuate prominently across seasons[12] since the primary source hereof comes from the endogenous skin synthesis of vitamin D_3 following exposure to sunlight.[13] Interestingly, the season of birth has not been associated with AAM in areas with minor variations in sun exposure,[14,15] which highlights the potential importance of 25(OH)D_3 in the observed seasonal effects on AAM. However, other environmental exposures, such as infection rates, air pollution, temperature or melatonin, fluctuate by seasons and these factors may also affect the observed associations.[2,5,9,16,17]

Besides being a marker of different environmental exposures at birth, the season of birth is also a marker of *in utero* exposures during the prenatal period. Prenatal exposures, including vitamin D, may have a programming effect on the reproductive hormonal system, which develops and matures *in utero* and regulates pubertal development later in life.[18–21] In seeking to prevent earlier age at pubertal timing, which has been of public health concern due to observed associations with poor adult health,[22,23] identification of potential risk factors is warranted. We aimed to investigate whether the season of the first trimester, as a proxy for early 25(OH)D_3 exposure, was associated with pubertal timing in girls and boys using information on a range of pubertal markers collected longitudinally within a Danish population experiencing seasonal variations in sun exposure. Moreover, since maternal 25(OH)D_3 levels offer an opportunity for intervention in contrast to the season of first trimester, we aimed to use the season of first trimester as an instrumental variable for maternal 25(OH)D_3 levels to explore the potential causal effect of early *in utero* 25(OH)D_3 exposure on pubertal timing.

Methods

This population-based cohort study is based on the Danish National Birth Cohort (DNBC),[24] including its sub-cohorts, the Puberty Cohort[25] and the Fetal Programming of Semen Quality (FEPOS) Cohort.[26]

Study population

A large, population-based sample of pregnant Danish women was invited to the DNBC at the first antenatal visit to their general practitioners from 1996 to 2002.[24] Half of Danish general practitioners participated in the recruitment and ~92 000 women (participation rate 60%), corresponding to 30% of the source population of pregnant Danish women, were recruited. The women answered health behaviour and medical history questions during pregnancy and post-partum by using computer-assisted telephone interviews, had a blood sample drawn at around gestational Week 8 and follow-up questionnaires were provided when the children were 11 years old.

The Puberty Cohort comprised 22 439 children sampled from 56 641 eligible singletons born alive from 2000 to 2003 whose mothers had answered the first DNBC interview and had not withdrawn from the DNBC by May 2012.[27] In the Puberty Cohort, the children were invited to provide information on puberty half-yearly through web-based questionnaires from the age of 11.5 years of age until they reached 18 years of age or until reaching full maturity defined as Tanner Stage 5,[28,29] whichever came first. From the age of 11.5 years, 14 756 of the 22 439 invited children answered at least one of the half-yearly web-based questionnaires on pubertal timing. Moreover, 10 665 of the 22 439 invited children also answered similar questions on pubertal timing in the 11-year follow-up in the DNBC (Figure 1). When combining these data, 15 819 children provided information on pubertal timing (participation rate 70%).[25] In total, 98 195 questionnaires (median: 6 questionnaires, range 1–15) were completed and returned.

Exposure

The season of the first trimester was defined as the season at gestational Week 8, since this may be a critical time for the development and maturation of the reproductive system.[30,31] Further, the gestational blood samples from which 25(OH)D_3 was measured were obtained at around gestational Week 8, allowing us to use this same time in pregnancy for the instrumental variable analysis. We calculated the first day of gestational Week 8 based on the date of birth, which is embedded in the unique national personal identifier given to all newborns in Denmark,[32] and information on gestational age at delivery. Information on gestational age at delivery was obtained from the Danish Medical Birth Register or from self-reported data on expected due date from the first DNBC interview for those

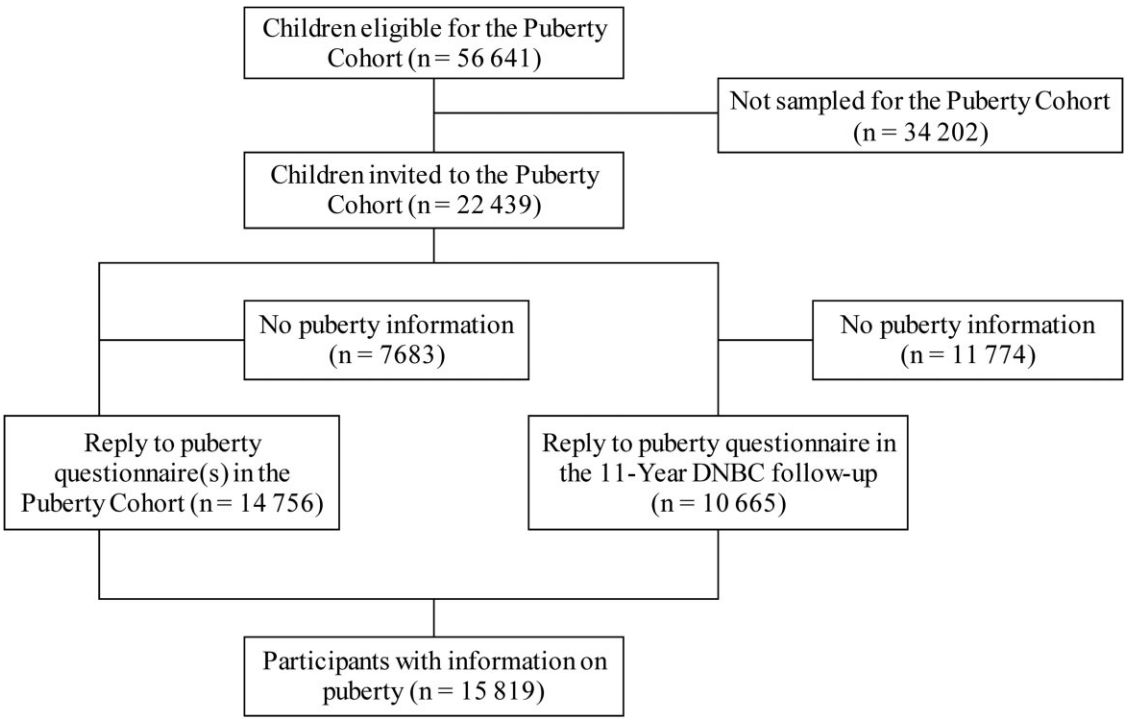

Figure 1 Flowchart of the inclusion of participants in the Puberty Cohort nested within the Danish National Birth Cohort, Denmark, 2000–21

with missing information on gestational age at delivery from the Danish Medical Birth Register ($n = 34$).

The endogenous vitamin D_3 production in the human skin varies by latitude.[33] In Denmark (latitude 54°–57° North), the endogenous production of vitamin D_3 occurs mainly from April to September.[12,34] As bioavailable 25(OH)D_3 reaches a steady state after ~6 weeks,[35,36] a low-exposure season was defined as November–April and a high-exposure season as May–October. This dichotomized variable was used in the primary analyses.

To refine the exposure, we further categorized the season at gestational Week 8 into calendar seasons (winter: December–February; spring: March–May; summer: June–August; autumn: September–November) and calendar months (January through December).

Maternal vitamin D levels

Information on maternal 25(OH)D_3 levels was obtained from a subset of the DNBC, whose sons participated in the FEPOS cohort.[26] This subset of pregnant women ($n = 1058$) was recruited to the DNBC from 1998 to 2000 and had no overlap with mothers of children recruited for the Puberty Cohort. All pregnant women from this subset had answered the two pregnancy interviews, had sons living in the area of Copenhagen or Aarhus and most pregnant women ($n = 827$) had plasma from the gestational blood samples obtained by the general practitioner at around gestational Week 8 stored at –80°C in the Danish National Biobank, Copenhagen, Denmark. According to the month in which the samples were taken, these measures were used to predict 25(OH)D_3 levels in the subset of women giving birth to a child participating in the Puberty Cohort. Plasma 25(OH)D_3 was measured from the stored gestational plasma samples. Quantitative analysis of 25(OH)D_3 was performed using 2D liquid chromatography tandem mass spectrometry (LC–MS/MS; QTRAP 6500+; AB Sciex, Framingham, MA, USA) at the Division of Occupational and Environmental Medicine, Lund University, as described in detail elsewhere.[37] No samples were below the limit of detection. Reference samples and QC samples from Chromsystems Instruments & Chemicals GmbH (MassCheck; Gräfelfing, Germany) were included in the analysis and met the target reference values.

Outcomes

Information on the age at attaining various pubertal markers was obtained from the Puberty Cohort. The markers included Tanner Stages 1–5 (breast and pubic hair development[28] in girls and genital and pubic hair development[29] in boys) with a short description and illustrations of each Tanner Stage; AAM (in years and months) in girls; the age at first ejaculation (in years and months) and voice break (yes, partly, no) in boys; and acne (yes, no) and axillary hair development (yes, no) in girls and boys. Questionnaires are available at https://www.dnbc.dk/data-available/puberty-follow-up.

Covariates

Potential confounding factors were identified using existing literature and directed acyclic graphs (Figure 2).[38] Information on maternal AAM, maternal pre-pregnancy body mass index (BMI), maternal first trimester smoking, couple fecundity, cohabitation of the parents and parental highest social class defined according to occupation and level of education derived from the Danish International Standard Class of Occupation and Education codes (ISCO-88 and ISCED) was obtained from the first interview in the DNBC. Information on maternal age at delivery was obtained from the Danish Medical Birth Register,[39] as was information on gestational age at delivery and birthweight, which was presented to describe the study population (Table 1). Since the DNBC primarily consists of Caucasian women and their children, ethnicity was controlled for by design. Pregnancy planning, season of conception and genetics involved in reproductive health were considered in the causal framework but not included in the statistical analyses (Figure 2). However, all backdoor paths related to these variables were closed by adjustment for the remaining covariates.

Statistical analysis

The data on pubertal timing were interval-censored, since the children provided current status of the pubertal markers half-yearly. The age at attaining the pubertal marker was left-censored if the child had already attained the given marker before completing the first questionnaire; it was interval-censored if the child attained the marker between two questionnaires; and it was right-censored if the child had not attained the marker when completing the last questionnaire. Data were therefore analysed using a multivariable regression model for censored, time-to-event data fitted by maximum-likelihood estimation (Stata's intreg package). Adjusted differences in age (months) with 95% CIs at attaining each of the pubertal markers according to the season when the mother was 8 weeks pregnant (low-exposure season relatively to high-exposure season) were estimated. In addition, all pubertal markers were combined into one model to obtain a combined estimate for the overall association between the exposure and all pubertal

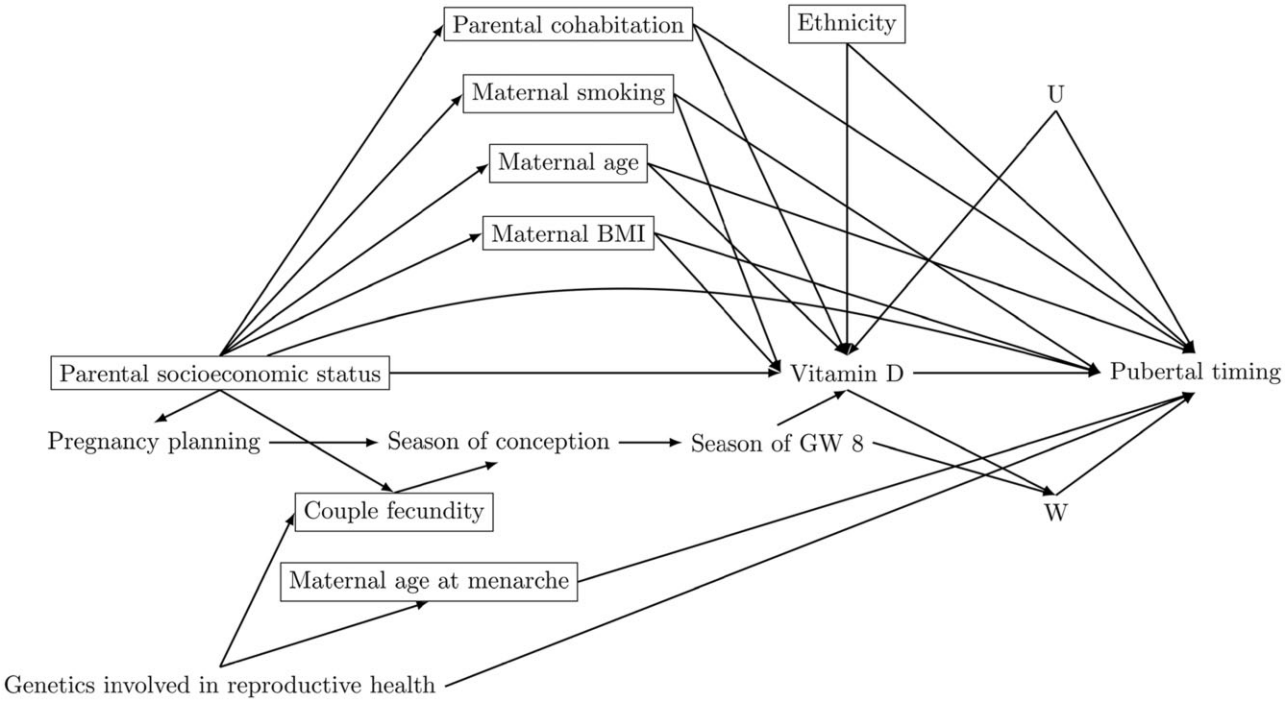

Figure 2 Directed acyclic graph illustrating the assumed causal framework of the study on the season at gestational Week 8 and pubertal timing, including the potential mediation by maternal vitamin D levels. Boxes indicate conditioning in the statistical analyses. BMI, body mass index; GW, gestational week. U represents diet, supplement use and outdoor activities. W represents air pollution, light intensity or melatonin, nutrient intake or diet quality, outdoor activities, physical activity, substance use, infections, rainfall, temperature, gestational diseases, sleep and sleep disturbances, age at menopause, autoimmune and immune disorders, cardiovascular diseases, cultural effects (educational attainment, sports performances), eye problems, metabolic effects, neurodevelopmental disorders, cancers, longevity

markers for girls and boys separately using Huber–White robust variance estimation. This provides a combined estimate for the average age difference in pubertal timing between exposure groups while accounting for the risk of type 1 errors due to multiple testing of correlated outcomes.[40,41]

For the combined estimate only, we also estimated associations between calendar season (winter, spring, summer and fall) and calendar month at gestational Week 8 (January through December). The associations were conducted with summer and July as references, respectively, since these represent the high-exposure calendar season and month.

All interval-censored regression analyses were conducted assuming normally distributed residuals. To assess this assumption, we compared the non-parametric cumulative incidence function based on the Turnbull estimator with the normal distribution[42,43] in R (x64 3.3.1). Due to local regulations [General Data Protection Regulation, Regulation (EU), 2016/679 of 25 May 2018], these plots cannot be published. However, the data were compatible with the assumption. For the earliest pubertal markers, only the right part of the distribution could be visualized due to late entry in the Puberty Cohort. The assumption of normality of age at attaining the earliest pubertal markers is supported by other studies on pubertal timing from similar study populations.[17,44,45]

Instrumental variable analysis for the potential effect of vitamin D on pubertal timing

To explore the potential causal effect of vitamin D in the first trimester on pubertal timing, we performed an instrumental variable analysis using season (low-exposure season and high-exposure season) at gestational Week 8 as an instrument for maternal plasma $25(OH)D_3$ levels. Associations were estimated using a two-sample two-stage least squares regression approach under the core assumptions of relevance (the instrument being associated with the exposure), exchangeability (no confounding of the association between the instrument and the outcome) and exclusion restriction criteria (the instrument affecting the outcome only through the exposure).[46,47]

In the first-stage regression, we used the FEPOS sample to fit an ordinary least squares regression model of maternal $25(OH)D_3$ plasma levels obtained in gestational Week 8 on the season at gestational Week 8 (the dichotomized low- and high-exposure season) and a priori chosen covariates. First-stage statistics (F-statistics and r^2) were obtained.[46] Based on this first-stage regression model, we

Table 1 Distribution of covariates according to high- or low-exposure season at gestational Week 8 in 15 819 girls and boys from the Puberty Cohort, Denmark, 2000–21

Baseline characteristics	Season					
	High-exposure season		Low-exposure season		Missing	
	No.	%	No.	%	No.	%
	8 365	53	7 454	47		
Predicted mean vitamin D in nmol/L[a]	64		47		0	0
Couple fecundity[b]					44	0
Unplanned pregnancy	1374	16	1116	15		
0–5 month TTP	4504	54	4037	54		
6–12 month TTP	1074	13	964	13		
> 12 month TTP + MAR	1389	17	1317	18		
Maternal age at delivery (years)[c]	30.5 (4.4)		30.5 (4.3)		6	0
Maternal age at menarche					123	1
Earlier than peers	2117	25	1894	25		
Same as peers	4753	57	4235	57		
Later than peers	1434	17	1263	17		
Maternal BMI (kg/m^2)					217	1
<18.5	578	7	478	6		
18.5 to <24.9	5093	61	4563	61		
25 to <29.9	1729	21	1576	21		
>30	850	10	735	10		
Parental cohabitation					>7[d]	>0
Yes	8198	98	<7286[d]	<98		
No	160	2	168	2		
Parental highest social class					31	0
High-grade professional	1975	24	1713	23		
Low-grade professional	2722	33	2473	33		
Skilled worker	2281	27	2072	28		
Unskilled worker	1161	14	988	13		
Student	156	2	156	2		
Economically inactive	49	0.5	42	0.5		
Smoking during first trimester (cigarettes/day)					53	0
0	5984	72	5363	72		
1–10	1839	22	1673	22		
>10	513	6	394	5		
Birthweight (grams)[c]	3533 (603)		3520 (600)		45	0
Gestational age at delivery (days)[c]	279 (17)		279 (18)		34	0

Due to rounding of percentages, numbers may not add up to 100%. TTP, time to pregnancy; MAR, medically assisted reproduction; BMI, body mass index.

[a]Predicted in mothers of sons enrolled in the Fetal Programming of Semen Quality (FEPOS) cohort based on being in gestational Week 8 + 0 in the low-exposure season (November–April) or high-exposure season (May–October).

[b]Including unplanned pregnancy, time to pregnancy and medically assisted reproduction.

[c]Mean (SD).

[d]Due to local data regulations, it is not permitted to report smaller numbers than five, including missing data, which is why the numbers in the table have been changed to mask the numbers smaller than five.

predicted maternal 25(OH)D_3 plasma levels in the Puberty Cohort sample. In the second-stage regression, we applied the predicted maternal 25(OH)D_3 plasma levels as the exposure in the interval-censored regression models of pubertal timing while adjusting for the same covariates as in the first-stage regression model. Standard errors in all analyses were bootstrapped using 1000 repetitions stratified on the two subsets to obtain valid 95% CIs. To ease interpretation and clinical relevance, we calculated estimates as mean differences in age at attaining the pubertal markers in months per SD (22 nmol/L) decrease in vitamin D.

To explore whether a violation of the exclusion restriction criteria could explain or reverse the observed association, we conducted multidimensional bias analyses proposed by Baiocchi *et al.*[46]. We conducted two different

bias analyses in which we considered that the pregnancy season may affect pubertal timing through either air pollution or maternal infections during pregnancy (see Supplementary text, available as Supplementary data at *IJE* online).

All interval-censored regression models and all instrumental variable analyses were adjusted for the a priori selected covariates and were fitted with inverse probability of sampling and selection weights. Maternal age was modelled as a second-order polynomial to allow for non-linearity. Inverse probability of sampling weights, corresponding to the inverse probability of being sampled to the Puberty Cohort, were applied to create a pseudo-population representative of the source population.[27] Inverse probability selection weights, corresponding to the inverse probability of participation, were used to account for the potential risk of selection bias due to non-participation.[48] Selection weights were estimated by a logistic regression model based on the month of gestational Week 8 and the identified potential confounders as explanatory variables for participation. Robust standard errors were used to account for the inverse probability weights and the clustering of siblings. Data management and statistical analyses were conducted in Stata 17.0 (StataCorp LLC, College Station, TX).

Results

In total, 8365 (53%) mothers were 8 weeks pregnant during the high-exposure season (May–October) and 7454 (47%) were 8 weeks pregnant during the low-exposure season (November–April). The distributions of covariates were similar in the two seasons (Table 1).

Children of mothers who were 8 weeks pregnant during November–April had, on average, earlier age at attaining all pubertal markers than children of mothers who were 8 weeks pregnant during May–October (Table 2). For the combined estimate, girls and boys of mothers who were 8 weeks pregnant during the low-exposure season had –1.0 months (95% CI: –1.7 to –0.3) and –0.7 months (95% CI: –1.4 to –0.1) earlier pubertal timing than girls and boys of mothers who were 8 weeks pregnant during the high-exposure season.

Calendar season (4 categories) and calendar month (12 categories) of gestational Week 8 were also associated with pubertal timing in girls and boys. For the combined estimate, girls and boys of mothers who were 8 weeks pregnant during the winter (December–February) had –1.5 months (95% CI: –2.4 to –0.6) and –1.0 months (95% CI: –1.9 to –0.1) earlier pubertal timing than girls and boys of mothers who were 8 weeks pregnant during the summer (June–August) (Table 3). The same tendency was observed when examining calendar months, where girls and boys whose mothers were 8 weeks pregnant around winter months had earlier pubertal timing than their peers whose mothers were 8 weeks pregnant in July (Supplementary Table S1, available as Supplementary data at *IJE* online).

Instrumental variable analyses

Maternal 25(OH)D_3 plasma levels fluctuated throughout the seasons in a sinusoidal pattern with the highest concentrations from May through October (Figure 3).[37] Mean 25(OH)D_3 plasma levels from the FEPOS cohort were 64 nmol/L in the high-exposure season (May–October) and 47 nmol/L in the low-exposure season (November–April) (Table 1). First-stage F-statistics was 138 and r^2 was 14%. Lower predicted vitamin D levels were associated with earlier pubertal timing in girls and boys for all pubertal markers (Table 4). For the combined estimate girls and boys had –1.3 months (95% CI: –2.1 to –0.4) and –1.0 months (95% CI: –1.8 to –0.2) earlier pubertal timing per SD decrease in vitamin D levels, respectively. The results from the multidimensional bias analyses indicated that this finding was likely not explained by violation of the exclusion restriction criteria by seasonal effects on air pollution or infections, given that the bias parameters were correctly specified (Supplementary Tables S2 and S3, available as Supplementary data at *IJE* online).

Discussion

Key results

In this longitudinal cohort study, we investigated pubertal timing in a population-based sample of girls and boys using the unique data from the Puberty Cohort in which the current status of a range of pubertal markers was collected throughout pubertal development. Children of mothers who were 8 weeks pregnant during the winter months, characterized by low sun exposure, had earlier pubertal timing than children of mothers who were in gestational Week 8 during the summer months, characterized by high sun exposure. In the instrumental variable analysis using season at gestational Week 8 as an instrument for maternal 25(OH)D_3 plasma levels, we found that lower levels of predicted maternal 25(OH)D_3 in gestational Week 8 were associated with earlier pubertal timing. This was not likely explained by a potential association between season and pubertal timing through increased risk of infections or air pollution during the winter months.

Strengths and limitations

The risk of selection bias is considered low. The participation rate in the Puberty Cohort was high (70%) and we

Table 2 Crude and adjusted[a] age difference in months with 95% CIs in pubertal timing according to high- and low-exposure seasons[b] in gestational Week 8. Low-exposure season relative to high-exposure season

Main results	Season at gestational Week 8	
	Low-exposure season	
	Crude difference	Adjusted difference in months (95% CI)
Girls $n = 7870$[c]		
Tanner Breast Stage 2[d]	–1.3	–1.6 (–3.0; 0.3)
Tanner Breast Stage 3[d]	–1.1	–1.2 (–2.0; –0.3)
Tanner Breast Stage 4[d]	–1.1	–1.0 (–1.9; –0.1)
Tanner Breast Stage 5[d]	–1.0	–1.5 (–3.2; 0.2)
Tanner Pubic Hair Stage 2[d]	–0.8	–0.7 (–1.5; 0.0)
Tanner Pubic Hair Stage 3[d]	–1.0	–0.8 (–1.5; –0.1)
Tanner Pubic Hair Stage 4[d]	–1.1	–0.8 (–1.7; 0.2)
Tanner Pubic Hair Stage 5[d]	–0.7	–0.9 (–2.4; 0.5)
Axillary hair	–0.8	–0.7 (–1.7; 0.3)
Acne	–1.6	–1.7 (–2.9; –0.6)
Menarche	–0.6	–0.5 (–1.2; 0.2)
Combined estimate	–1.0	–1.0 (–1.7; –0.3)
Boys $n = 7256$[c]		
Tanner Genital Stage 2[d]	–0.8	–0.7 (–1.7; 0.3)
Tanner Genital Stage 3[d]	–0.8	–0.6 (–1.6; 0.3)
Tanner Genital Stage 4[d]	–0.8	–0.7 (–1.6; 0.3)
Tanner Genital Stage 5[d]	–1.2	–1.2 (–2.7; 0.4)
Tanner Pubic Hair Stage 2[d]	–0.5	–0.7 (–1.7; 0.2)
Tanner Pubic Hair Stage 3[d]	–0.5	–0.3 (–1.2; 0.5)
Tanner Pubic Hair Stage 4[d]	–0.9	–0.6 (–1.4; 0.2)
Tanner Pubic Hair Stage 5[d]	–0.9	–0.5 (–1.6; 0.6)
Axillary hair	–0.3	–0.6 (–1.6; 0.5)
Acne	–1.2	–0.8 (–1.8; 0.1)
Voice break	–0.9	–1.1 (–2.1; –0.2)
First ejaculation	–1.3	–1.0 (–1.9; –0.1)
Combined estimate	–0.8	–0.7 (–1.4; –0.1)

[a]Adjusted for maternal age at delivery (modelled as a second-order polynomial to allow for non-linearity), maternal age at menarche, maternal body mass index, parental highest social class, parental cohabitation, first-trimester smoking and couple fecundity.

[b]High-exposure season defined as May–October and low-exposure season defined as November–April.

[c]*n* refers to the number of boys and girls that gave information on all of the pubertal markers.

[d]Tanner Stages defined based on the work by Marshall and Tanner.[28,29]

Table 3 Crude and adjusted[a] age difference in months with 95% CIs in the combined estimate for pubertal timing according to calendar season at gestational Week 8. Calendar seasons relative to summer

Results for calendar seasons	Girls[b]		Boys[b]	
	Crude difference	Adjusted difference (95% CI)	Crude difference	Adjusted difference (95% CI)
Winter	–1.3	–1.5 (–2.4; –0.6)	–0.8	–1.0 (–1.9; –0.1)
Spring	–1.5	–1.2 (–2.1; –0.3)	–0.2	–0.3 (–1.2; 0.7)
Summer	Ref.	Ref.	Ref.	Ref.
Fall	–0.7	–0.9 (–1.8; 0.1)	–0.4	–0.2 (–1.1; 0.8)

[a]Adjusted for maternal age at delivery (modelled as a second-order polynomial to allow for non-linearity), maternal age at menarche, maternal body mass index, parental highest social class, parental cohabitation, first-trimester smoking and couple fecundity.

[b]$n = 7870$ for girls and $n = 7256$ for boys.

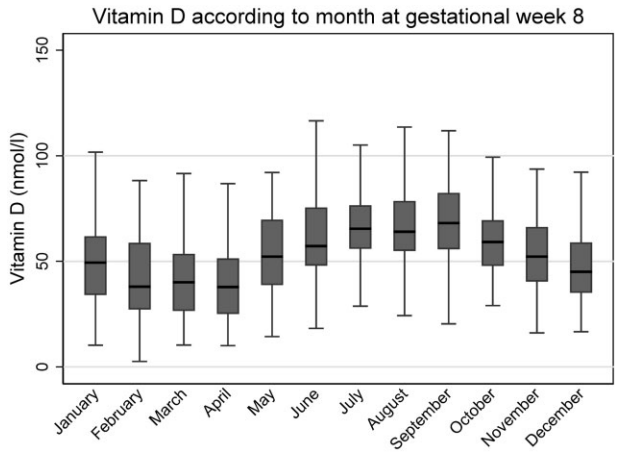

Figure 3 Vitamin D [25(OH)D_3] levels in nmol/L according to calendar month at blood sampling in gestational Week 8 from the Fetal Programming of Semen Quality (FEPOS) cohort, nested within the Danish National Birth Cohort, Denmark, 1998–2019. Previously published in[37] and republished with permission from Springer Nature

applied selection weights to all regression models. Moreover, participation in the Puberty Cohort was not associated with a marker of pubertal timing [the height difference in standard deviations (HD: SDS)] obtained from external registers.[49] Participation was not associated with the season at gestational Week 8 [equally many mothers of non-participants were 8 weeks pregnant during high (53%) and low (47%) exposure seasons].

Information on pubertal timing was obtained by self-report, which limits the risk of selection bias due to non-participation but induces the risk of bias due to misclassification. However, the children gave information on current pubertal development throughout puberty, thereby limiting the risk of misclassifications. The self-assessment of the Tanner Stages in the Puberty Cohort was investigated in a validation study on 197 girls and boys 13–16 years of age.[50] In girls, the agreement for Tanner Breast and Pubic Hair Stages comparing a clinical examination by a trained clinical investigator and the self-reported evaluation by the participant was fair (52% and 54%, respectively). In boys, the agreement between Tanner Genital and Pubic Hair Stages was moderate to fair (33% and 55%, respectively).[50] Due to their age at the clinical examination, most children had attained Tanner Stage 4 to 5 and it was impossible to assess the validity of the earlier pubertal markers in the validation study. However other studies have also found moderate to good agreement between self-assessment and an expert evaluation of the pubertal stages in populations similar to ours, also for the earlier stages.[51] In addition, in a recent meta-analysis, Campisi *et al.* found moderate to substantial agreement between self-reported Tanner Stage and the assessment done by a trained clinician, especially regarding onset of puberty (Tanner Stage 2) and full maturation (Tanner Stage 5).[52] Reassuringly, in the validation study, we found no evidence of any bias in either direction and self-assessment of the Tanner Stages was independent of parental education. We also expect any misclassification to be non-differential according to pregnancy season. AAM in girls and age at first ejaculation in boys are instantaneous events and most girls (81%) and boys (63%) provided the exact month and year of attaining these pubertal markers.[53] Accordingly, this information is expected to be valid.

The potential bias due to late entry in the Puberty Cohort, i.e. potential bias due to left-censoring of the early markers of pubertal timing, has previously been found to be limited under plausible violations to the normal distribution.[53] Further, age at attaining the earliest pubertal markers follows a normal distribution in populations similar to ours.[17,44,45]

We adjusted for multiple potential confounders to limit the risk of introducing a false association between the season at gestational Week 8 and pubertal timing due to potential genetic confounding, arising if highly fecund couples conceive around the time of pregnancy planning, which peaks during the late summer in Denmark.[54] Unfortunately, we did not have information on the timing of pregnancy planning. However, we saw no differences in the proportion of unplanned and planned pregnancies or couple fecundity across seasons (Table 1).

Interpretation

No studies have reported associations between the season of first trimester and pubertal timing. Still, previous studies have reported associations between season of birth and AAM, though with differences in the direction and magnitude of associations.[1,6–8] In general, the studies are difficult to compare due to differences in analyses and reporting of associations; most do not present adjusted results and some studies were not designed to investigate the association between season of birth and AAM. Importantly, the relevant exposure timing is likely not around the season of birth, but rather in fetal life, where the reproductive system develops and is particularly vulnerable to interferences.[55]

Best comparable to our study was the study by Day *et al.* that found associations between season of birth and AAM after adjustment for a composite measure of socioeconomic position and lifestyle factors in a sample of 238 014 participants from UK Biobank.[1] Girls born during the autumn had earlier AAM and girls born during the summer had later AAM compared with all other birth seasons combined. Magnitudes of associations were ~1 month earlier and later AAM, comparable to the magnitude of

Table 4 Crude and adjusted[a] age difference in months with 95% CIs in pubertal timing according to vitamin D levels predicted by high- and low-exposure seasons[b] in gestational Week 8. Estimates presented per SD[c] decrease in maternal 25-hydroxyvitamin D_3 [25(OH)D_3] levels

Instrumental variable analysis	Vitamin D	
	Per SD decrease in maternal 25(OH)D_3 levels predicted by season in gestational Week 8	
	Crude difference	Adjusted difference (95% CI)
Girls $n = 7870$[d]		
Tanner Breast Stage 2[e]	–1.5	–2.2 (–3.9; –0.4)
Tanner Breast Stage 3[e]	–1.4	–1.6 (–2.7; –0.5)
Tanner Breast Stage 4[e]	–1.5	–1.3 (–2.6; 0.0)
Tanner Breast Stage 5[e]	–1.2	–1.9 (–4.2; –0.4)
Tanner Pubic Hair Stage 2[e]	–1.1	–1.0 (–2.0; 0.0)
Tanner Pubic Hair Stage 3[e]	–1.3	–1.1 (–2.1; –0.1)
Tanner Pubic Hair Stage 4[e]	–1.4	–1.0 (–2.2; 0.3)
Tanner Pubic Hair Stage 5[e]	–1.1	–1.2 (–3.1; 0.7)
Axillary hair	–1.1	–0.9 (–2.2; 0.4)
Acne	–2.2	–2.3 (–3.8; –0.7)
Menarche	–0.8	–0.7 (–1.7; 0.3)
Combined estimate	–1.4	–1.3 (–2.1; –0.5)
Boys $n = 7256$[d]		
Tanner Genital Stage 2[e]	–1.0	–0.9 (–2.3; 0.5)
Tanner Genital Stage 3[e]	–1.0	–0.8 (–2.0; 0.3)
Tanner Genital Stage 4[e]	–0.9	–0.8 (–2.1; 0.4)
Tanner Genital Stage 5[e]	–1.3	–1.6 (–3.7; 0.5)
Tanner Pubic Hair Stage 2[e]	–0.7	–1.1 (–2.4; 0.2)
Tanner Pubic Hair Stage 3[e]	–0.6	–0.5 (–1.6; 0.6)
Tanner Pubic Hair Stage 4[e]	–1.1	–0.8 (–1.9; 0.3)
Tanner Pubic Hair Stage 5[e]	–1.0	–0.6 (–2.1; 0.8)
Axillary hair	–0.5	–0.9 (–2.2; 0.4)
Acne	–1.6	–1.1 (–2.4; 0.2)
Voice break	–1.2	–1.5 (–2.8; –0.2)
First ejaculation	–1.4	–1.3 (–2.6; –0.1)
Combined estimate	–0.9	–1.0 (–1.8; –0.2)

[a]Adjusted for maternal age at delivery (modelled as a second-order polynomial to allow for non-linearity), maternal age at menarche, maternal body mass index, parental highest social class, parental cohabitation, first-trimester smoking and couple fecundity.

[b]High-exposure season defined as May–October and low-exposure season defined as November–April.

[c]SD = 22 nmol/L 25(OH)D_3.

[d]*n* refers to the number of boys and girls that gave information on all of the pubertal markers.

[e]Tanner Stages defined based on the work by Marshall and Tanner.[28,29]

associations in our study with earlier pubertal timing in children of mothers who were in gestational Week 8 during the low-exposure season, which may, in turn, translate into an effect of being born during the summer or autumn. Kliś *et al.* found that girls born during the summer had earlier AAM of ∼2–4 months compared with girls born in all other seasons in a cross-sectional sample of Polish students.[6] This may also reflect an association between mothers being in the first trimester during a low-exposure season and thereby having children with earlier pubertal timing as found in our study.

Whether these associations are a result of fluctuations in 25(OH)D_3 levels during pregnancy has not been established. We have previously found that lower maternal intake of vitamin D supplements in mid-pregnancy was associated with later pubertal timing in boys in a dose-dependent manner. In contrast, there was no consistent association with pubertal timing in girls (in review). However, as maternal intake may not reflect the actual bioavailability of vitamin D, the study may not compare directly to our current study.

In the instrumental variable analysis, we found that lower predicted 25(OH)D_3 levels in gestational Week 8 were associated with earlier pubertal timing in both girls and boys. Whether this reflects causality depends on the core assumptions underlying the analysis.[46] The relevance

assumption that season at gestational Week 8 is associated with maternal 25(OH)D_3 levels can be tested and was considered fulfilled due to the high variability in 25(OH)D_3 levels that was explained by season at gestational Week 8 (first-stage F-statistics was 138 and r^2 was 14%); an F-statistics above 10 is regarded as sufficient to avoid weak instrument bias.[46,56] The exchangeability assumption is not testable. However, season at gestational Week 8 was independent of all measured potential confounders available in the DNBC (Table 1) supporting our assumption that season at gestational Week 8 was likely also independent of unmeasured potential confounders and, hence, that the association between season at gestational Week 8 and pubertal timing was unconfounded. Moreover, we adjusted for parental couple fecundity and maternal AAM to close the potential backdoor from season at gestational Week 8 to season of conception and pregnancy planning to genetics involved in reproductive health (Figure 2).

The exclusion restriction criteria are also an untestable assumption stating that season at gestational Week 8 only affects pubertal timing through maternal plasma 25(OH)D_3 levels.[46] However, the external environment changes by season, suggesting that exposures other than 25(OH)D_3 levels may fluctuate over the year and potentially affect pubertal timing. If season at gestational Week 8 affects another factor (e.g. risk of infections or high level of air pollution) during the low-exposure season that may be causally related to pubertal timing, then the exclusion restriction assumption is violated, which would induce bias of the estimated causal effect of 25(OH)D_3 on pubertal timing (Supplementary Figure S1, available as Supplementary data at *IJE* online). However, the bias analyses that assessed the risk of bias due to violation of the exclusion restriction criteria suggested that the observed effect of 25(OH)D_3 on pubertal timing was not likely to be introduced by a potential violation of the exclusion restriction criteria for most realistic scenarios. The results suggested that lower 25(OH)D_3 most likely may have accelerated pubertal timing even more, given that our suggested bias parameters were correct (see Supplementary text, available as Supplementary data at *IJE* online).

In order to quantify and interpret a point estimate as a causal association, a fourth assumption—the monotonicity or homogeneity assumption—must also be considered.[56,57] For example, the monotonicity assumption entails that all pregnant women should have the same direction of effect of low-exposure or high-exposure season on their 25(OH)D_3 levels, although the magnitude of the effect does not need to be identical for all individuals.[56] Some pregnant women may travel to southern destinations during the low-exposure season and hence be exposed to more sunlight in the low-exposure than the high-exposure season. However, only very few will likely be overall exposed to more sunlight during the low-exposure than the high-exposure season and any bias introduced by this violation will likely be negligible. Furthermore, some women may stay indoors during the high-exposure season, although they are probably unlikely to be exposed to more sunlight during the low-exposure season anyway. Therefore, this would likely not cause considerable bias either.

In conclusion, we found associations between season of first trimester and pubertal timing in girls and boys in this Danish population with seasonal variations in endogenous vitamin D_3 synthesis. Since the results from this study depend on geographic location, our results may be generalizable to other populations at around the same latitude as in Denmark. Children of mothers who were 8 weeks pregnant during the winter months characterized by limited sun exposure had earlier pubertal timing than children of mothers who were 8 weeks pregnant during the summer months. Moreover, lower predicted 25(OH)D_3 was associated with earlier pubertal timing in girls and boys. Future studies should aim to elucidate the mechanism underlying the observed association and to examine whether intervening in low maternal 25(OH)D_3 levels during seasons with low exposure to sunshine would be beneficial with regard to pubertal timing in children.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki. The data collection in the DNBC and the Puberty Cohort was approved by the Committee for Biomedical Research Ethics in Denmark (KF 01–471/94). This study was approved by the Danish Data Protection Agency (2012–41-0379 and 2015–57-0002) and the steering committee of the DNBC (2012–04, 2015–47 and 2018–09). The data were de-identified of personal identifiers, hence no protocol approval from an Institutional Review Board was required. All pregnant women provided informed consent including also their children's participation.

Data availability

The data set analysed in the study is not publicly available due to national data security legislation on sensitive personal data. Researchers may apply for access to data from the DNBC. Please see https://www.dnbc.dk/data-available or write to dnbc-research@ssi.dk for additional information.

Supplementary data

Supplementary data are available at *IJE* online.

Author contributions

C.H.R.-H. planned and obtained funding for the data collection in the Puberty Cohort. A.G.-S. planned this study in collaboration with O.A.A. and C.H.R.-H. A.G.-S. performed data management with N.B., L.L.H.L. and A.E. The analytic strategy was designed by A.G.-S., N.B., O.A.A. and C.H.R.-H. A.G.-S. performed the statistical analyses. The instrumental variable analysis and bias analysis were supervised by N.B. and O.A.A. A.G.-S. wrote the first draft. All authors supported the discussion, interpreted the data, revised the manuscript critically and approved the final manuscript.

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Conflict of interest

None declared.

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