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Prepubertal urinary estrogen excretion and its relationship with pubertal timing

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Shi L, Remer T, Buyken AE, Hartmann MF, Hoffmann P, Wudy SA. Prepubertal urinary estrogen excretion and its relationship with pubertal timing. *Am J Physiol Endocrinol Metab* 299: E990–E997, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00374.2010.—Whether prepubertal estrogen production impacts on the timing of puberty is not clear. We aimed to investigate prepubertal 24-h estrogen excretion levels and their association with early and late pubertal markers. Daily urinary excretion rates of estrogens of 132 healthy children, who provided 24-h urine samples 1 and 2 yr before the start of the pubertal growth spurt [age at takeoff (ATO)], were quantified by stable isotope dilution/GC-MS. E-sum3 (estrone + estradiol + estriol) was used as a marker for potentially bioactive estrogen metabolites and E-sum5 (E-sum3 + 16-epiestriol + 16-ketoestradiol) for total estrogen production. Pubertal outcomes were ATO, age at peak height velocity (APHV), duration of pubertal growth acceleration (APHV-ATO), age at Tanner stage 2 for pubic hair (PH2), genital (G2, boys) and breast (B2, girls) development, and age at menarche. Prepubertal urinary estrogen excretions (E-sum3 and E-sum5) were not associated with ATO, APHV, and age at PH2 but with duration of pubertal growth acceleration ($P < 0.01$) in both sexes. Girls with higher E-sum3 reached B2 0.9 yr ($P = 0.04$) and menarche 0.3 yr earlier ($P = 0.04$) than girls with lower E-sum3. E-sum3 was not associated with age at G2 in boys ($P = 0.6$). For most pubertal variables, the associations with E-sum3 were stronger than with E-sum5. In conclusion, prepubertal estrogens may not be critical for the onset of the pubertal growth spurt but are correlated with its duration in both boys and girls. Prepubertal estrogen levels may already predict the timing of girls' menstruation and breast development but do not appear to affect sexual maturation in boys.

sex differentiation; developmental biology; pediatrics; steroids

CHILDREN WITH EARLY PUBERTY are at risk for the development of hormone-related cancers [e.g., breast (4, 12) and testicular cancer (20)] later in life. Recent studies (10, 34) suggest that the increased prepubertal sexual hormone exposures may account for the earlier onset of puberty.

Sensitive gonadotropin assays have shown that the pulsatile nature of LH and FSH secretion, which traditionally characterizes the onset of puberty, is present already before the appearance of pubertal physical signs with a relative low-pulse amplitude (2, 14, 34). At the same time, a diurnal variation of circulating estradiol (E_2) in prepubertal children has also been suggested (34). Increasing evidence points at a physiological importance of even low-estrogen concentrations on somatic growth (9, 44) and sexual maturation (26, 46).

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Until now, most studies, which address directly (34) or indirectly (11, 26, 29) the association between prepubertal endogenous estrogen concentration and pubertal onset, are based exclusively on measurements of prepubertal serum concentrations of E_2. Although E_2 is the most potent estrogenic steroid, less potent estrogens estrone (E_1) (3) and estriol (E_3), as well as some of their metabolites (15, 53), also contribute to estrogenic activity. Additionally, an E_2 level measured in a single blood sample may not be representative of its 24-h secretion. Using stable isotope dilution/gas chromatography-mass spectrometry (ID/GC-MS), we could determine the urinary estrogen metabolites in 24-h urine samples of prepubertal children. Therefore, the first aim of the present study was to determine the daily estrogen metabolite excretion levels of healthy free-living boys and girls at a biologically comparable prepubertal stage, i.e., 1 and 2 yr before the onset of pubertal growth spurt (one of the earliest pubertal markers).

The second aim of this study was to investigate the association of prepubertal 24-h estrogen production with both somatic growth-related and sexual maturation-related pubertal markers in boys and girls, which had not yet been systematically investigated. In addition, the potential influences of birth characteristics (23), body mass (6, 45), and adrenarchal androgens (42) were also considered.

SUBJECTS AND METHODS

Study population. The children examined in the present study were a subsample of healthy participants in the Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) study, an ongoing, open-cohort study conducted at the Research Institute of Child Nutrition in Dortmund, Germany. Recruitment began in 1985, with about 40 infants entering the cohort each year at the age of 3 mo. The yearly assessments between infancy and early adulthood include 3-day weighed dietary records, anthropometric measurements, and collection of 24-h urine samples as well as interviews on lifestyle and medical assessments. The study is exclusively observational and noninvasive (until age 18) and has been approved by the Ethics Committee of the University of Bonn, Germany. All examinations and assessments were performed with parental and later on with the children's written consent. Further details of the DONALD study have been provided elsewhere (41).

The ages of the children who were initially recruited into the DONALD study when it began in 1985 were quite variable, and many current participants have not yet reached adolescence. Therefore, a plausible estimation of age at takeoff (ATO) was achieved in a subset of 376 participants with sufficient height measurements (for details, see Refs. 6 and 23). Of these, 132 children had not refused regular assessment of Tanner stages and had also collected 24-h urine samples at both time points (2 and 1 yr) before ATO. Complete information on birth characteristics was also available in all of these children. Hence,

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the subcohort analyzed herein included 132 prepubertal healthy children (69 boys).

Anthropometry. DONALD study participants were measured at each visit by trained nurses according to standard procedures. From the age of 2 yr onward, standing height is measured to the nearest 0.1 cm using a digital stadiometer. Weight was measured to the nearest 0.1 kg using an electronic scale (753 E; Seca, Hamburg, Germany). Body mass index (BMI) was calculated using the weight/height^2 (kg/m^2) formula. The Slaughter equations (49) for prepubertal children were used to estimate percent body fat. Information on birth weight, length, and gestational age was abstracted from a standardized assessment document provided by the obstetrician.

Puberty outcome variables. For the growth-dependent puberty variable ATO, height measurements from age 5 yr onward for girls and from age 6 yr onward for boys were analyzed by using the parametric Preece and Baines model 1 (38), as recently described in detail (6, 23). This model also produces estimates of velocity at takeoff, age at peak height velocity (APHV), and peak height velocity (PHV). From these we have calculated the duration of the pubertal growth acceleration (APHV minus ATO), which represents an indicator for growth spurt duration or pubertal duration (1, 6). Tanner stages for pubic hair (PH) and either breast (girls) or genital (external and internal genitalia, boys) development were assessed by one of the DONALD study pediatricians from age 5 yr onward. Pubertal staging was performed according to the standardized criteria published by Marshall and Tanner (32) and testis volume determined by palpation using the Prader orchidometer. We used age at testis volume of $\geq$4 ml to define the onset of genital development in boys. Herein, we denoted Tanner stage 2 for breast and genital development in girls and boys, respectively, as B2 and G2. In addition, girls or their parents were asked whether menarche has occurred since the previous visit and, if so, in which month and year. This information was available for 51 of the 63 girls in this analysis.

Hormonal measurements and parameters. Prepubertal 24-h urine collections were performed at home under standardized conditions (41). Children and parents were carefully instructed for the collection of 24-h urine by written guidance and by a dietitian who visited the families at home to ascertain the children's compliance. All micturitions were stored immediately in preservative-free, Extran-cleaned, 1-l plastic containers at less than -20°C (41). For the purpose of this analysis, completeness of 24-h urine samples was checked via sex- and age-specific, body weight-related reference values of creatinine (i.e., samples with a daily creatinine excretion rate $<$0.1 mmol/kg body wt were not considered). Furthermore, samples that were reported to contain incomplete micturitions (according to the urine collection diary) were also excluded from analysis. The samples were thawed immediately before analysis.

Urinary estrogens and their metabolites were measured by stable ID/GS-MC (27). In our method (18), urine (5.0 ml) was spiked with a cocktail of internal standards (50 ng of [2,4-$^2H_2$]E_1, 25 ng of [2,4,16,16-$^2H_4$]E_2, 25 ng of [2,4,17-$^2H_3$]E_3, 25 ng of [1,4,16,16-$^2H_4$]2-methoxyestrone, 25 ng of [2,4,15,15,17-$^2H_5$]16-ketoestradiol, 50 ng of [2,4-$^2H_3$]16-epiestriol, and 6.25 ng of [1,4,16,16,17-$^2H_5$]2-hydroxyestradiol). Ascorbic acid (5 mg) was added to the sample to protect labile compounds. After equilibration (30 min at room temperature), extraction using SepPak C18 cartridges followed. The methanolic extract was dried and redissolved in sodium acetate buffer (pH = 4.5). Ascorbic acid and sulfatase from Helix pomatia Type H-1 (300 U) were added. After hydrolysis (3 h, 55°C) and reextraction, the methanolic extract was cleaned up by anion exchange chromatography (QAE Sephadex A-25 acetate form). Trimethylsilyl ethers were prepared as derivatives. The derivatized sample was dissolved in isooctane, and an aliquot of 2/200 μl was injected into the GC-MS. GC was performed using an Optima-1-MS fused silica capillary column (Macherey-Nagel, Dueren, Germany) housed in an Agilent 6890N series GC directly interfaced to an Agilent 5975 inert XL mass selective detector (MSD). The carrier gas was helium at a flow rate of 1 ml/min. The injector temperature was 270°C. The initial column temperature was set at 80°C, and after 2 min the temperature increased at a rate of 20°C/min to 190°C. Then, the column temperature was raised at a rate of 2.5°C/min to 240°C. Thereafter, it increased at a rate of 40°C/min up to 300°C, which was held for 6 min (postrun). For the determination of urinary estrogens, the MSD was operated in the selected ion-monitoring mode. Quantification was performed using the peak area ratio between analyte and internal standard. The limits of detection (LOD) were 0.025 ng/ml for E_1, E_2, 16-ketoestradiol, and 2-hydroxyestradiol, 0.25 ng/ml for E_3, 0.2 ng/ml for 16-epiestriol, and 0.05 ng/ml for 2-methoxyestrone. The interassay and intra-assay coefficients of variation (%) ranged between 0.85 and 2.21%.

In brief, GC-MS analysis of C19 and C21 urinary steroids proceeded as follows; free and conjugated urinary steroids were extracted from 5 ml of urine by solid-phase extraction (SepPak C18 cartridges), and conjugates were enzymatically hydrolyzed (helix pomatia type H-1, 300 U). After reextraction, internal standards (5α-androstane-3α,17α-diol, stigmasterol) were added to each extract before formation of methyloxime-trimethylsilyl ethers. The derivatized sample was dissolved in 500 μl of isooctane, and a 1-μl aliquot was injected for GC-MS analysis. GC was performed using an Optima-1-MS fused silica column (Macherey-Nagel) housed in an Agilent 6890 series GC directly interfaced to an Agilent 5973N MSD. Injections were made into an 80°C (2 min) oven with the temperature then increased by 20°C/min to 190°C (1 min). To separate steroids, the temperature was then increased by 2.5°C/min to 272°C. Interassay precision showed coefficients of variation in the range of 1.1% for α-C and 9.3% for dehydroepiandrosterone (DHEA). Intra-assay precision ranged between 1.7% for 5-androstene-3β,17β-diol and 6.2% for DHEA. Total adrenal androgen (AA) secretion was determined as the sum ($\sum$C19) of androsterone, etiocholanolone, 5-androstene-3β,17α-diol, 5-androstene-3β,17β-diol (androstenediol), DHEA, 16α-hydroxy-DHEA, and 5-androstene-3β,16α,17β-triol (39, 47), and total cortisol secretion was determined as the sum ($\sum$C21) of tetrahydrocortisone, tetrahydrocortisol, 5α-tetrahydrocortisol, α-cortolone, β-cortolone, α-cortol, and β-cortol, as reported previously (52).

Statistical analysis. We performed all statistical analyses using SAS procedures (version 9.1; SAS Institute, Cary, NC). Percentiles of 24-h urinary estrogen metabolite excretion rates and the respective frequency of detection 1 and 2 yr before ATO were calculated. Two metabolites, 2-hydroxyestradiol and 2-methoxyestrone, with a frequency of detection $<$25% at both collection time points, were not considered further in the following statistical analyses. Subjects with missing values at both time points were excluded. In subjects with one measurable value either at 1 or 2 yr before ATO, the corresponding metabolite concentration of the respective other time point was set at the value equal to the LOD divided by the square root of 2 (7, 19). Paired *t*-test was used to examine the differences of estrogens and their metabolites between 1 and 2 yr before ATO.

To examine the association between prepubertal estrogen levels and pubertal timing, the sum of E_1, E_2, and E_3 (E-sum3) was used as a marker for potentially bioactive estrogen metabolites and the sum of E-sum3, 16-epiestriol, and 2-hydroxyestradiol (E-sum5) for total estrogen production. Preliminary analysis (Fig. 1) showed that 120 children of our original sample (n = 132) had applicable E-sum3 and E-sum5 data at 1 and 2 yr before ATO; i.e., at least one of these E-sums' components had measurable concentrations above LOD. Thus, the subsequent analyses were performed in these 120 children.

For description of the sample characteristics, means ± SD or median with interquartile range are given. To obtain more stable individual hormone values, means of 1 and 2 yr before ATO were calculated and used in the following analyses. Sex differences for sample characteristics were tested with unpaired *t*-test.

To illustrate the relationships between E-sum3, E-sum5, $\sum$C19, and BMI as well as their associations with chronological age, a preliminary Pearson's correlation was run. Nonnormally distributed E-sum3, E-sum5, and $\sum$C19 were (natural) log transformed.

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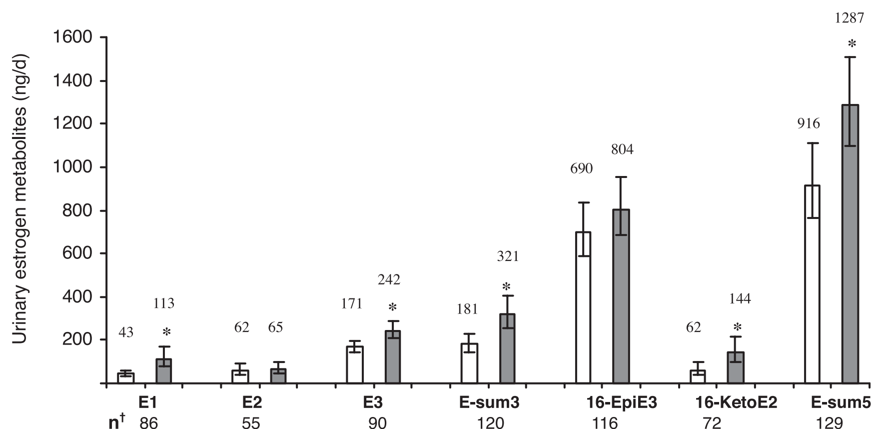

Fig. 1. Differences of estrogen metabolites between 1 (filled bars) and 2 yr (open bars) before age at takeoff (ATO). Data are geometric means (95% confidence interval) of respective estrogen metabolites. *Significant ($P < 0.05$) differences between 1 and 2 yr before ATO (tested with paired *t*-test). †No. of subjects with at least 1 measurable value 1 or 2 yr before ATO. For concentration below the limits of detection (LOD), a value equal to the LOD divided by the square root of 2 was used. E_1, estrone; E_2, estradiol; E_3, estriol; E-sum3, E_1 + E_2 + E_3; 16-EpiE3, 16-epiestriol; 16-ketoE$_2$, 16-ketoestradiol; E-sum5, E-sum3 + 16-EpiE3 + 16-ketoE$_2$.

Analyses of covariance were performed to test for sex-by-hormone interactions. No sex-by-E-sum3 or sex-by-E-sum5 interactions ($P >$ 0.1) were observed for any of the outcomes: ATO, APHV, duration of pubertal growth acceleration, age at Tanner stage B2/G2, or age at Tanner stage PH2. Accordingly, all of the subsequent analyses were first performed with boys and girls combined. Although somatic growth and PH development are physical signs occurring in both sexes, breast development, menarche, and external genitalia development are sex specific. Therefore, sex-stratified analyses were done with age at menarche and age at B2 as outcomes for girls and age at G2 as outcome for boys.

Least-squares regression analyses were used to analyze the associations of urinary estrogens with pubertal variables. The distribution of E-sum3 or E-sum5 was grouped into three categories: low (<25th percentile), medium (≥25th and ≤75th percentiles), and high (≥75th percentiles). The adjusted means were the least square means predicted by the model when the other variables were held at their mean values. In basic models (model 1), the respective pubertal variables were adjusted for sex difference only. Since BMI (6, 45) as well as AA (13, 24, 36, 42) may modulate pubertal timing, and both of them are also associated with estrogen levels, BMI-SDS and ∑C19-SDS (calculation based on Ref.39) were included separately as adjustment variables in two additional regression models. As suggested by previous studies (43), part of the variation of BMI is associated with the variation of adrenocortical activity, which itself is an important determinant of AA secretion (51). As a consequence, body mass

Table 1. *Percentiles (95% confidence interval) of detectable urinary estrogen metabolites (ng/day) 1 and 2 yr before ATO in 132 healthy children*

Variables	P10	P25	P50	P75	P90	FOD, %
E_1						
2 yr before ATO	<LOD	<LOD	<LOD	87 (19–202)	272 (217–485)	39
1 yr before ATO	<LOD	<LOD	<LOD	286 (194–462)	670 (599–1,287)	44
E_2						
2 yr before ATO	<LOD	<LOD	<LOD	70 (<LOD-131)	191 (148–284)	28
1 yr before ATO	<LOD	<LOD	<LOD	64 (<LOD-127)	171 (148–259)	27
E_3						
2 yr before ATO	<LOD	<LOD	<LOD	193 (154–294)	393 (334–569)	41
1 yr before ATO	<LOD	<LOD	133 (<LOD-188)	261 (322–408)	424 (559–670)	51
E-sum3						
2 yr before ATO	<LOD	<LOD (<LOD-11)	155 (90–205)	318 (449–536)	569 (701–813)	68
1 yr before ATO	<LOD	77 (<LOD-171)	324 (258–454)	711 (566–886)	1,159 (909–2,069)	77
16-Epiestriol						
2 yr before ATO	<LOD	71 (<LOD-638)	879 (768–1,004)	1,183 (1,085–1,293)	1,459 (1,331–1,665)	75
1 yr before ATO	<LOD	485 (<LOD-753)	972 (882–1,067)	1,263 (1,185–1,481)	1,655 (1,516–1,831)	77
16-Ketoestradiol						
2 yr before ATO	<LOD	<LOD	<LOD	139 (<LOD-244)	337 (256–1,207)	27
1 yr before ATO	<LOD	<LOD	<LOD	247 (190–287)	540 (356–1,327)	39
E-sum5						
2 yr before ATO	<LOD (<LOD-169)	612 (222–276)	1,152 (979–1,287)	1,678 (1,470–1909)	2307 (2,378–3,298)	89
1 yr before ATO	282 (<LOD-550)	969 (612–1,159)	1,512 (1,309–1,649)	2,187 (1,920–2,568)	2903 (2,628–3,749)	92
2-Hydroxyestradilol						
2 yr before ATO	<LOD	<LOD	<LOD	<LOD	183 (107–485)	17
1 yr before ATO	<LOD	<LOD	<LOD	<LOD (<LOD-109)	168 (137–281)	20
2-Methoxyestrone						
2 yr before ATO	<LOD	<LOD	<LOD	<LOD	62 (<LOD-212)	11
1 yr before ATO	<LOD	<LOD	<LOD	<LOD	<LOD (<LOD-171)	9
E-total						
2 yr before ATO	<LOD (<LOD-191)	612 (232–841)	1,196 (1,041–1,375)	1,828 (1,628–2,149)	2496 (2,307–3,667)	89
1 yr before ATO	301 (<LOD-607)	1,071 (775–1,233)	1,574 (1,385–1,724)	2,251 (1,964–2,635)	3153 (2,820–3,855)	93

ATO, age at takeoff; E_1, estrone; E_2, estradiol; E_3, estriol; FOD, frequency of detection; LOD, limit of detection; E-sum3 sum of E_1, E_2, and E_3; E-sum5, sum of E-sum3, 16-epiestriol, and 16-ketoestradiol; E-total, sum of E-sum5, 2-hydroxyestradilol, and 2-methoxyestrone. LOD for E_1, E_2, 2-hydroxyestradilol, and 16-ketoestradiol is 0.025 ng/ml, for E_3 is 0.25 ng/ml, for 16-epiestriol is 0.2 ng/ml, and for 2-methoxyestrone is 0.05 ng/ml.

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Table 2. *Characteristics of the study sample for examination of association between daily urinary estrogen excretion and pubertal timing (n = 120)*

	Boys (n = 64)	Girls (n = 56)	P for Difference[a]
Age, yr[b]	8.8 ± 0.9	7.3 ± 0.7	<0.0001
Anthropometric[b]			
Weight, kg	31.8 ± 6.2	26.2 ± 5.5	<0.0001
Height, cm	137.0 ± 6.9	126.9 ± 6.3	<0.0001
%Body fat	14.4 (12.1, 18.9)	15.3 (13.2, 19.7)	0.4
BMI, kg/mb	16.8 ± 2.2	16.1 ± 2.1	0.1
BMI-SDS[c]	−0.09 ± 1.13	−0.13 ± 1.1	0.3
Urinary[b]			
ΣC19, μg/day[d]	451 (330, 624)	249 (187, 398)	<0.0001
ΣC19-SDS[c]	−0.06 ± 1.15	−0.10 ± 0.88	0.8
ΣC21, μg/day[e]	4,246 (3,669, 4,760)	3,453 (3,092, 3,956)	<0.0001
ΣC21-SDS[e]	0.33 ± 0.90	0.75 ± 0.87	0.01
E-sum3, ng/day[f]	389 (244, 644)	346 (186, 525)	0.4
E-sum5, ng/day[g]	1,566 (1,028, 1,964)	1,424 (931, 1,712)	0.3
Pubertal			
ATO, yr	10.4 ± 0.8	8.9 ± 0.6	<0.0001
APHV, yr	13.5 ± 0.9	11.7 ± 0.7	<0.0001
Duration of pubertal growth acceleration, yr[h]	3.1 ± 0.3	2.9 ± 0.4	0.0002
Age at B2/G2, yr	10.8 ± 1.1	10.5 ± 1.0	0.1
Age at PH2, yr	11.5 ± 1.2	10.7 ± 1.1	0.0004
Age at menarche, yr[i]		13.0 ± 0.8	
Early-life related			
Birth weight, g	3,609 ± 448	3,427 ± 352	0.02
Gestational age, wk	40 (40, 41)	40 (39, 40)	0.7

Values are means ± SD or medians (25th, 75th percentiles). BMI, body mass index; ΣC19, marker of urinary adrenal androgen secretion; APHV, age at peak height velocity; PH2, Tanner stage 2 for pubic hair; B2/G2, Tanner stage 2 for breast (girls) and for external genitalia (boys) development. [a]Sex differences were tested with unpaired *t*-test for continuous variables. [b]Values were derived from each individual's arithmetic mean from 1 and 2 yr before ATO; i.e., data represent average values ~1.5 yr before ATO. [c]SDS, SD score was calculated based on the data of children with C19 (39) and C21 (52) steroid gas chromatography-mass spectrometry reference values. [d]Sum of C19 metabolites (androsterone, etiocholanolone, 5-androstene-3β,17α-diol, 5-androstene-3β,17β-diol, dehydroepiandrosterone (DHEA), 16α-hydroxy-DHEA, and 5-androstene-3β,16α,17β-triol), reflecting total adrenal androgen secretion (39). [e]Sum of C21 metabolites (tetrahydrocortisone, tetrahydrocortisol, 5α-tetrahydrocortisol, α-cortolone, β-cortolone, α-cortol, and β-cortol), reflecting total cortisol secretion (52). [f]Sum of E_1, E_2, and E_3. [g]Sum of E-sum3, 16-epiestriol, and 16-ketoestradiol. [h]Duration between APHV and ATO. [i]No information on age at menarche for 5 girls.

variation (via covariation with adrenocortical activity) partly reflects the potential C19 influence on the puberty outcomes (i.e., the part of C19 variation caused by adrenocortical activity). Therefore, we removed the influence of adrenocortical activity on BMI by preadjustment of BMI-SDS with total cortisol secretion (∑C21, as an indicator for adrenocortical activity) before entering BMI-SDS in the regression models. This way, we should be able to better identify the association of AA and their converted products, estrogens with pubertal timing. Trend testing was performed with E-sum3 or E-sum5 as continuous independent variables. In addition, gestational age and birth weight were also considered. Since they did not modify the association of E-sum3 or E-sum5 with the respective pubertal outcomes, they were not retained in the models.

RESULTS

Urinary estrogen metabolites: measurement assessment. Percentiles of daily urinary estrogen metabolite excretion rates and the respective frequency of detection 1 and 2 yr before ATO are presented in Table 1. The detection frequencies of three major estrogens, E_1, E_2, and E_3, ranged between 30 and 50%. Among all of the estrogen metabolites, 16-epiestriol had the highest detection frequency (~75%), and quantitatively, it was also the most predominant estrogen metabolite, which made up >50% of total urinary estrogen excretion in healthy children before ATO. For most of the single estrogen metabolites as well as E-sum3 and E-sum5, values of 1 yr before ATO were significantly higher than 2 yr before ATO (Fig. 1).

Association between estrogens and pubertal timing. Sample characteristics for investigating the associations of estrogens with early and late pubertal markers are presented in Table 2. Boys and girls at a biologically comparable prepubertal stage (on average 1.5 yr before ATO) showed ~1.5 yr difference in chronological age but no difference for E-sum3 and E-sum5. Boys showed a significantly longer "duration of pubertal growth acceleration" than girls. ATO, APHV, age at B2/G2, and age at PH2 occurred earlier in girls than in boys.

BMI, ∑C19, E-sum3, and E-sum5 were significantly positively correlated with each other (Table 3). BMI correlated stronger with ∑C19 ($r = 0.54$) than with E-sum3 ($r = 0.33$) or E-sum5 ($r = 0.22$). E-sum3 and E-sum5 were not significantly correlated with chronological age in the present sample.

Associations of E-sum3 or E-sum5 with ATO, APHV, age at B2/G2, and age at PH2 are presented in Table 4. There were no associations between E-sum3 and E-sum5 with ATO or APHV. Children with higher E-sum3 tended to reach B2/G2 earlier ($P = 0.07$) and had an earlier age at PH2 ($P = 0.02$)

Table 3. *Cross-correlation between age, BMI, urinary adrenal androgen, and estrogen secretion in 120 prepubertal healthy children*

	Age, yr	BMI	ΣC19	E-sum3
BMI, kg/m²	0.26*			
ΣC19, log e	0.55***	0.54***		
E-sum3, log e	0.12	0.33**	0.46***	
E-sum5, log e	0.10	0.22*	0.26*	0.51***

*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

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independent of BMI. After adjusting for ∑C19, these associations were attenuated, and significance was no longer discernible. However, even after the adjustments for both BMI and ∑C19, E-sum3 was still associated with duration of pubertal growth acceleration ($P = 0.002$). Children with higher E-sum3 (before ATO) had a 0.3 yr shorter duration of pubertal growth acceleration. For most of the pubertal variables (but not for APHV), the associations with E-sum3 (marker of potentially bioactive estrogen metabolites) were stronger than those with E-sum5. The latter was only significantly associated with duration of pubertal growth acceleration.

Sex-stratified analyses (after adjustment for BMI) showed that girls with higher E-sum3 reached Tanner stage B2 0.9 yr earlier ($P = 0.04$) than girls with lower E-sum3 (Fig. 2*A*). After adjusting for ∑C19-SDS, significance no longer existed ($P = 0.2$). Girls experienced theirs menarche 0.3 yr earlier ($P = 0.04$) than girls with a lower E-sum3 (Fig. 2*B*) independent of BMI and ∑C19. However, E-sum3 was not associated with age at G2 for boys ($P = 0.6$; Fig. 2*C*).

DISCUSSION

This study is the first to determine the estrogen excretion levels in 24-h urine samples of healthy free-living boys and girls using ID/GC-MS at a biologically comparable prepubertal stage, i.e., 1 and 2 yr before the onset of one of the earliest pubertal markers (ATO). Despite the relatively low detection frequencies for some metabolites, we could identify significant increases for most of the estrogenic steroids from a younger biological age (2 yr before ATO) to an older one (1 yr before ATO), which confirmed the biological validity of our measurements. On the basis of these measurements, we found that higher prepubertal urinary estrogen excretions (indicated by E-sum3 and E-sum5) were not associated with earlier ATO and APHV but with a shorter duration between them, i.e., a shorter duration of pubertal growth acceleration. Girls with higher E-sum3 showed a 0.9-yr earlier onset of breast development and a 0.3-yr earlier onset of menstruation than girls with lower E-sum3. Prepubertal estrogens did not appear to be related to the onset of genital development of boys or pubic hair development in both boys and girls.

Most of the previous studies on prepubertal estrogens (3, 34, 44) have examined blood samples. To our knowledge, only one study (33) has measured estrogen profiles in overnight urines of girls (8–14 yr; $n = 20$), and the estimated 24-h total urinary estrogen excretions were 2.85–10.68 μg. In agreement with these data, 63 girls in our study at 1 yr before ATO (7–9 yr), i.e., at a clearly younger average age, had correspondingly also a lower total estrogen excretion (E-sum5) ranging from 0.3 to 3.2 μg/day. The total estrogen excretion level of our 69 boys (1.6 μg/day) was nearly the same as that of girls (1.4 μg/day) of the same biological age. Chronologically, boys were 1.5 yr older than girls. Therefore, although we did not measure the estrogen excretion levels of boys and girls at the same chronological age, we can conclude that prepubertal girls have a higher estrogen production (on a chronological age basis) than boys. This is in line with the suggestions from the published blood data (10, 25, 33).

When comparing the 24-h excretion pattern of urinary estrogen metabolites with that of healthy premenopausal women, the relative amounts of E_2 of prepubertal children appeared to be somewhat lower [also illustrated by Maskarinec et al. (33)], whereas 16-EpiE_3 was obviously much higher (ratio of 16-

Table 4. *Association of 24-h urinary estrogen excretions with early and late pubertal markers in healthy prepubertal children*[a]

	Categories of E-sum3[a]				Categories of E-sum5[a]			
	Low	Normal	High	*P* for trend[b]	Low	Normal	High	*P* for trend[b]
ATO ($n = 120$)								
Model 1 (M1)[c]	9.5 (9.2, 9.8)[d]	9.6 (9.5, 9.8)	9.6 (9.4, 9.9)	0.4	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.7 (9.4, 9.9)	0.7
M1+ BMI-SDS[e]	9.5 (9.2, 9.8)	9.7 (9.5, 9.9)	9.6 (9.3, 9.9)	0.6	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.6 (9.4, 9.9)	0.8
M1+ ΣC19-SDS	9.5 (9.2, 9.8)	9.6 (9.5, 9.8)	9.6 (9.3, 9.9)	0.5	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.6 (9.4, 9.9)	0.8
APHV ($n = 118$)								
Model 1 (M1)[c]	12.8 (12.5, 13.1)	12.6 (12.4, 12.8)	12.5 (12.2, 12.8)	0.1	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.5 (12.2, 12.8)	0.09
M1+ BMI-SDS[e]	12.8 (12.5, 13.1)	12.6 (12.4, 12.8)	12.5 (12.2, 12.8)	0.2	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.6 (12.4, 12.9)	0.1
M1+ ΣC19-SDS	12.7 (12.4, 13.0)	12.6 (12.4, 12.8)	12.6 (12.2, 12.9)	0.5	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.6 (12.3, 13.0)	0.2
Duration of pubertal growth acceleration ($n = 118$)								
Model 1 (M1)[c]	3.3 (3.1, 3.4)	3.0 (2.9, 3.1)	2.8 (2.7, 3.0)	<0.0001	3.2 (3.0, 3.3)	3.0 (2.9, 3.1)	2.9 (2.7, 3.0)	<0.0001
M1+BMI-SDS[e]	3.2 (3.1, 3.3)	2.9 (2.9, 3.0)	2.9 (2.8, 3.0)	<0.0001	3.1 (3.0, 3.3)	3.0 (2.9, 3.1)	2.9 (2.8, 3.0)	0.0001
M1+ΣC19-SDS	3.2 (3.1, 3.3)	3.0 (2.9, 3.0)	2.9 (2.8, 3.1)	0.0008	3.1 (3.0, 3.2)	3.0 (2.9, 3.1)	3.0 (2.9, 3.1)	0.001
Age at B2/G2 ($n = 117$)								
Model 1 (M1)[c]	10.9 (10.6, 11.3)	10.7 (10.4, 11.0)	10.2 (9.8, 10.6)	0.02	10.7 (10.4,11.1)	10.7 (10.5,11.0)	10.3 (9.9,10.7)	0.1
M1+ BMI-SDS[e]	10.9 (10.5, 11.3)	10.7 (10.4, 10.9)	10.3 (9.9, 10.7)	0.07	10.7 (10.4,11.1)	10.7 (10.5,11.0)	10.4 (10.0,10.7)	0.2
M1+ ΣC19-SDS	10.8 (10.4, 11.2)	10.7 (10.4, 11.0)	10.3 (9.9, 10.7)	0.3	10.7 (10.3,11.0)	10.7 (10.4,11.0)	10.5 (10.1,10.9)	0.4
Age at PH2 ($n = 116$)								
Model 1 (M1)[c]	11.5 (11.1, 12.0)	11.1 (10.8, 11.4)	10.7 (10.3, 11.2)	0.02	11.2 (10.8,11.6)	11.2 (10.9,11.5)	10.7 (10.3,11.1)	0.3
M1+ BMI-SDS[e]	11.5 (11.1, 11.9)	11.0 (10.7, 11.3)	10.9 (10.5, 11.3)	0.06	11.2 (10.8,11.6)	11.2 (10.9,11.5)	10.8 (10.4,11.7)	0.4
M1+ ΣC19-SDS	11.3 (10.9, 11.7)	11.0 (10.8, 11.3)	11.1 (10.6, 11.4)	0.6	11.1 (10.7,11.5)	11.2 (10.9,11.4)	11.0 (10.6,11.4)	0.9

[a]Categories of E-sum3 or E-sum5 were defined as follows: low (E-sum3 <25th percentile), normal (E-sum3 ≥25th and ≤75th percentile), and high (E-sum3 > 75th percentile). [b]From linear regression models with E-sum3 (log e) or E-sum5 (log e) as continuous variables. [c]Model 1 was adjusted for sex difference. [d]All such values are least-squares means (95% confidence interval). [e]Adjusted for cortisol secretion (ΣC21-SDS).

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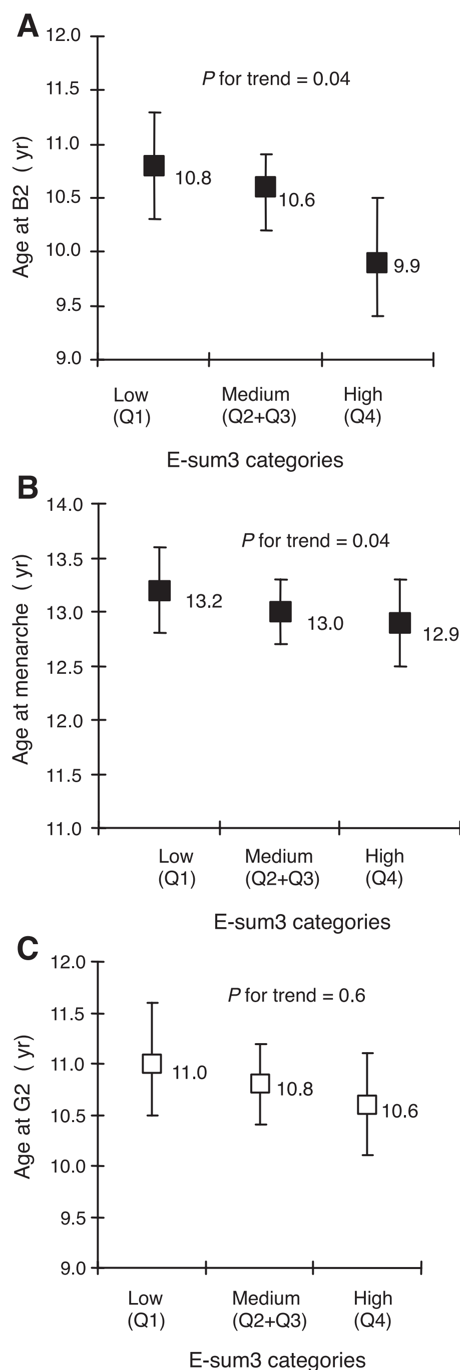

Fig. 2. *A*: age at B2 (age at Tanner stage 2 for breast development; 55 girls). *B*: age at menarche (51 girls). *C*: age at G2 (age at Tanner stage 2 for genital development; 62 boys) by categories of E-sum3 (mean of 1 and 2 yr before ATO) in healthy children. Data are means (95% confidence interval) adjusted for sex and body mass index-SDS. *P* for trend refers to the *P* value obtained in linear regression models with E-sum3 as continuous variable. Q, quartile. Medians of E-sum3 in low, medium, and high groups were 143, 371, and 750 ng/day, respectively.

$EpiE_3$ to E_2 was 10:1 in children vs. 1:2 in adults). The reason could be that, in children, the bioactive estrogens like E_2 may be preferentially metabolized to less active compounds, and the metabolic pathway to 16-EpiE3 appears to dominate in children.

Although the role of metabolites such as 16α-hydroxylated estrogens with potentially breast carcinogenic effects (15, 53) on pubertal timing would have been particularly interesting, the low detection frequency limited the examination of the effects of these single metabolites. However, we could use the sum of three major estrogens, E_1, E_2, and E_3 (E-sum3), to estimate daily excretion of the potentially bioactive estrogen fraction and the sum of the five major estrogen metabolites (E-sum5) as a surrogate for 24-h total estrogen production in a relatively large number of healthy children ($n = 120$). This allowed us to investigate the potential roles of childhood estrogen production at the onset and progression of pubertal growth spurt as well as on the early and late sexual maturation markers.

E_2 has been shown to be essential for the increase in growth velocity and epiphyseal fusion during pubertal growth in both sexes (17, 30). Nevertheless, it is not clear whether the elevation of estrogen levels in childhood may already contribute to or predict the increase of growth velocity and the subsequent earlier onset of pubertal growth spurt. In the present study, we did not find a significant association between prepubertal urinary estrogen excretions and ATO, which was not determined by adrenal androgen secretion either (42). Therefore, it seems possible that the start of the pubertal growth spurt may not be determined primarily by total endogenous sex hormone production. Whether a specific sex steroid metabolite, e.g., androstenediol (31, 35, 40), which can act as an estrogen or an androgen receptor agonist, or certain neuropeptide like kisspeptin (37) may play a role in the initiation of the pubertal growth spurt needs to be examined further.

In this study, both E-sum3 and E-sum5 showed modest associations with a later growth spurt marker, APHV; however, after adrenal androgens and body mass were considered, these associations vanished. These results suggest that, at that early prepubertal stage, the variations of adrenal androgen levels and body mass (6) may play a more important role in predicting APHV. Nevertheless, in our view, it does not mean that estrogen is not necessary for pubertal growth. On the contrary, adequate reserve of adrenal androgen and body fat at the early prepubertal stage may predict a higher estrogen level at a later prepubertal stage and during puberty, since adrenal androgens are important substrates converted to estrogens by aromatase, i.e., by the enzyme expressed largely in adipose tissues (16). The good correlation between $\sum$C19 and E-sum3 ($r = 0.46$) also suggests that adrenal androgens could be the major sources of estrogens in the prepubertal stage.

Clinical studies (5, 8) have shown that males with syndromes of estrogen deficiency experienced normal prepubertal growth and were able to sustain linear growth but had no epiphyseal fusion. Therefore, it appears that one important physiological contribution of estrogens is not attributed to their stimulative but repressive function on linear growth due to their role in the closure of the epiphyseal plate (30). A shorter duration of growth spurt acceleration may indicate an earlier maturation of the epiphysis. Thus, based on our findings (children with higher estrogen excretions before ATO had a shorter duration of growth spurt acceleration), we speculate that prepubertal estrogen levels may already predict the timing of epiphyseal maturation in puberty.

E-sum3 showed a significant association with age at PH2; however, after BMI and especially $\sum$C19 were included, the

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association was no longer significant. It is noteworthy that the present findings of prepubertal sex steroid effects on pubarche (i.e., the preferential androgen effects) are based on urinary analysis (42) and have been for the first time verified by a longitudinal examination in healthy children. This provides novel evidence compared with the previous clinical results in patients with metabolic disorders (50) or cross-sectional observations (28) based on blood measurements. Although somatic growth and pubic hair development are physical signs occurring in both sexes, breast development, menarche, and external genitalia development are sex specific. In boys, prepubertal urinary estrogen excretions did not appear to affect onset of their genital development. In girls, E-sum3 but not E-sum5 (reflecting total estrogen production) was associated with age at B2 and menarche. A reason could be that the quantitatively most dominant component of E-sum5 (i.e., 16-epiE3) may be a peripherally catabolized product without prior action in the breast and other estrogen-dependent tissues. Therefore, we propose that the estrogen effect on breast development and menstruation of girls might be better identified by using E-sum3 than by using E-sum5. Estrogen effects (at least in the early prepubertal stage) on breast development appear to be attributed partly to the local conversion from adrenal androgens, since after inclusion of adrenal androgens in the model, E-sum3 was no longer significant. However, the predictive effect of E-sum3 on the timing of menarche was independent of adrenal androgens, which did not show any association with age at menarche (27).

Nevertheless, the observed estrogen effects were weaker than we had expected. One reason could be the sample size (n = 51–56 healthy girls), which is rather large compared with previous studies (33, 34) but may be not large enough to clearly differentiate the effects between two relatively closely related variables such as adrenal androgens and estrogens. Another reason could be that the time window that we investigated, i.e., 1.5 yr before ATO, is relatively far away from the onset of breast development (≈3 yr) and especially menarche (≈5.5 yr). So it would be interesting to know whether the estrogen effect is more pronounced in a later prepubertal stage, i.e., directly before the start of breast development or menstruation. In this early prepubertal stage, even our very sensitive isotope GC-MS method could not detect all metabolites; therefore, the frequency of undetectable hormone values may have introduced a correlation bias and also limited our ability to investigate the physiological role of single metabolites. However, the mean levels of two 24-h urine measurements (i.e., 1 and 2 yr before ATO) provided a more stable assessment of individual estrogen levels than that obtained from a single urine or blood sample. Another strength of the present study is that both somatic growth-related (ATO and APHV) and sexual maturation-related pubertal markers [age at Tanner stage II (boys, girls), menarche (girls)] could be determined. Their values are in accordance with values reported from other European studies (1, 21, 22, 48).

In conclusion, we found that daily estrogen production of boys is comparable with that of girls at the same biological developmental stages, i.e., 1 and 2 yr before the onset of pubertal growth spurt. Higher prepubertal estrogen production may not be critical for the initiation of the pubertal spurt, but it may increase the rate of growth spurt progression in both boys and girls. Prepubertal estrogen levels may already predict the age at menarche. However, the effect of estrogens on the onset of breast development might be attributed partly to the conversion from adrenal androgens. Prepubertal estrogens do not appear to influence sexual maturation in boys.

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DISCLOSURES

The authors have nothing to disclose.

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