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Longitudinal Changes in Serum Concentrations of Adrenal Androgen Metabolites and their Ratios by LC-MS/MS in Healthy Boys and Girls

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ABSTRACT

Adrenarche is characterized by steadily rising levels of adrenal androgen metabolites from 4-6 years of age. We recently described marked gender-specific differences in circulating ratios between selected adrenal androgen metabolites in a cross-sectional study. This may suggest gender differences in steroidogenic enzyme activities.

We therefore aimed at verifying these findings in a prospective, longitudinal study of healthy boys and girls who were examined during pubertal transition.

A longitudinal study of 20 healthy children from the COPENHAGEN Puberty study, followed every 6 months for 5 years. Clinical examinations were conducted and serum concentrations of Androstenedione (Adione), 17-hydroxyprogesterone (17-OHP), testosterone (T), dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) were quantified by a newly developed LC-MS/MS method.

DHEA, DHEAS, Adione, 17-OHP and T increase with age. Boys had higher levels of DHEAS from 10.5 years of age, whereas girls had higher levels of Adione from 13 years of age compared to boys.

Interestingly, we observed significantly higher ratios of DHEAS/DHEA (sulfotransferase activity) in boys before and after pubertal onset compared to girls, whereas Adione/17-OHP (CYP 17 activity) appeared to increase more in pubertal girls compared to boys. This suggests that adrenal steroidogenic enzyme activities show developmental as well as gender-specific changes in healthy children.

1. Introduction

The adrenal cortex produces adrenal androgens and dehydroepiandrosterone (DHEA), and its abundant sulphated form DHEA-sulphate (DHEAS), as well as Δ^4 -Androstenedione (Adione). Large amounts are produced during fetal development, but the production of adrenal androgens decreases rapidly after birth, and the fetal adrenal zone (the source of fetal adrenal androgen production) regresses within the first three months of life after which zona glomerulosa, zona fasciculata and zona reticularis begin to develop. The production of the adrenal androgens is reactivated as the adrenal cortex matures, a process also called adrenarche [1;2]. The re-activation has been observed as early as three years of age and seems to be a gradual and ongoing process [3;4]. This process is reflected by an enlargement of the zona reticularis, resulting in an enzyme profile with low 3β -hydroxysteroid dehydrogenase type 2 expression (3β -HSD) and high cytochrome b5 (enhancer of 17,20-lyase activity of chromosome P450c17) and steroid sulfotransferase (SULT2A1) expression [5;6]. During puberty, DHEA is synthesized in high amounts, but the function of this steroid is discussed and the physiological role of adrenarche is still unknown [7;8]. The gradual increase in adrenal androgens precedes the appearance of androgen-dependent body hair: pubic hair (PH2+) and axillary hair by peripheral conversion of DHEAS to testosterone (T) and dihydrotestosterone (DHT) [9;10]. It is possible to quantify a variety of adrenal androgens and precursors, also at low concentrations using liquid chromatography tandem mass spectrometry (LC-MS/MS) [11;12]. This makes it possible to determine the gradual increase in circulating levels of adrenal androgen prior to development of pubic hair. We have recently evaluated the increase in adrenal androgens according to puberty in a cross-sectional study and found gender specific differences not only in concentrations but also in the interconversion of adrenal androgen metabolites (hormone ratios) [13], suggesting gender differences in either one or more of the following: hormone production, steroidogenic enzyme activities or hormone clearance, respectively. We therefore aimed at verifying these findings in a prospective, longitudinal study of healthy boys and girls

2. Materials and methods

2.1 Subjects

A sub-study of 20 children selected from a total of 208 children, who participated in the longitudinal part of the COPENHAGEN Puberty Study, were included [14;15]. The children were examined every 6th month for five years (2006-10). Only Caucasian children with at least seven examinations (three examinations prior to development of pubic hair and four examinations after development of pubic hair) were included in this study. One girl included had only three blood samples. The study was conducted at two schools, both belonging to the upper 20% of Danish schools characterized by higher parental income and higher socio-economic status according to a national investigation from 2011 [16]. Data regarding measurements of serum-T by LC-MS/MS in relation to radioimmunoassay (RIA) [17] and other aspects of this study have previously been published [18-24].

2.2 Clinical examination

Pubertal stages were evaluated by clinical examination according to Marshall and Tanner [25;26]. Breast stage and testicular volume (TV) were measured by palpation, TV to the nearest ml using Prader's orchidometer. In case of discrepancy between left and right side the largest measurement was used for classification. Pubertal onset was defined as breast stage \geq B2 in girls and TV > 3 ml in boys or pubic hair stage \geq PH2 (pubarche) in girls and boys. Gonadarche was defined as development of secondary sex characteristics due to pituitary-gonadal activation (B2 in girls and TV > 3 ml in boys). If breast tissue was palpated at an earlier examination but not present at the subsequent examination, the girl was graded as B1. Assessment of pubic hair staging was done by inspection.

All evaluations of puberty in the girls were done by one of two female paediatricians and all evaluations in boys by one of three male paediatricians. Age at onset of pubic hair (PH2+), breast tissue (B2+), genital stage 2 (G2+) or testis volume > 3 ml was assigned as the mean age between age at first examination in stage 2 and the latest examination in stage 1.

2.3 Hormone analyses

Blood samples were drawn between 8.00 AM and 10.00 AM. They were clotted, centrifuged and serum was stored at -20° C until hormone analyses were performed.

Concentrations of DHEA, DHEAS, 17 α -hydroxyprogesterone (17-OHP), Adione and T in serum were quantified using a newly developed and validated isotope diluted TurboFlow-LC-MS/MS method [27]. During the period of analysis of the current samples the relative standard deviations for quality control samples at low and high concentrations were: DHEA, 15.7 % and 8.6 %; DHEAS, 7.1 % and 8.3 %; 17-OHP, 10.9 % and 9.9 %; Adione, 11.0 % and 11.1 %; and T, 10.0 % and 5.7 %, respectively. Limits of quantification (LOQ) were determined according to the International Conference on Harmonisation guidelines (ICH, 2005) and were: DHEA, 0.88 nmol/l; DHEAS, 48 nmol/l; 17-OHP, 0.19 nmol/l, Adione, 0.18 nmol/l and T, 0.10 nmol/l.

3. Statistical analysis

Data are presented as medians and 25th and 75th percentiles. For hormone concentrations below the LOQ, the LOQ divided by the square root of 2 was used. To evaluate the longitudinal changes in hormone levels, we used a variance component model. The changes were evaluated as a function of age, where age was grouped into a categorical variable of six months interval (i.e. 8.75<9 years \leq 9.25; 9.25<9.5 years \leq 9.75 etc). The changes in hormone levels were also evaluated as a function of time to or from development of pubic hair (PH2), where time to PH2 were grouped into categorized variables (i.e. -0.75<-0.5 years \leq -0.25; -0.25<0 years \leq 0.25 etc). Longitudinal changes in hormone concentrations were evaluated by the Wilcoxon signed rank test. The Mann-Whitney U-test was used to compare hormone concentrations between genders. All statistical analyses were carried out using the SPSS software (Version 19; SPSS, Inc., Chicago, IL).

4. Ethical considerations

The Copenhagen Puberty Study was approved by the local ethical committee (# KF 01 282214 and # V200.1996/90). The study is registered in ClinicalTrials.gov (identifier NCT01411527).

5. Results

Serum concentrations of DHEAS were measurable (above LOQ) in all blood samples. The concentrations of DHEA, 17-OHP, Adione and T, respectively, were above LOQ in 175/177, 175/177, 175/177 and 168/177 samples, respectively. Serum levels of 17-OHP, DHEA, DHEAS and Adione, respectively, increased with age in both sexes, as illustrated in Figure 1.

The concentration of 17-OHP increased significantly with age in both girls and boys ($p < 0.005$). A significant increase within a six month period was observed in boys but not in girls. The significant increase in 17-OHP in boys was observed from 11.5 to 12.0 years (median increase 0.42 nmol/L; $p < 0.041$). DHEA increased significantly in boys from 9 to 13.5 years of age and in girls from 8.5 to 14 years of age. However a significant increase within 6 months was observed in boys but not in girls from 13.0 to 13.5 years of age (mean increase 1.58 nmol/L; $p = 0.029$) and from 14.0 to 14.5 years (mean increase 5.34 nmol/L; $p = 0.000$). The concentration of DHEAS increased significantly from 9 to 14.5 years in boys and from 8.5 to 14 years in girls ($p < 0.05$). Within 6 months the increase was only significant in boys from 14.0 to 14.5 years ($p = 0.015$). The level of DHEAS was higher in boys compared to girls from 10.5 years of age ($p = 0.007$). The concentration of Adione increased significantly in both sexes, however only significant within 6 months in the girls from 12 to 12.5 years ($p = 0.030$). The level of Adione was higher in girls from 13 years of age ($p = 0.009$). The concentration of T increased significantly in boys within 6 months from 12 to 12.5 years ($p = 0.043$). In girls the increases in T were small but significant within 6 months from 14 to 14.5 years ($p = 0.031$).

Levels of 17-OHP and DHEA increased in both sexes 6-12 months prior to onset of pubic hair development. 17OHP and DHEA increased significantly in boys from 12 months before until PH2+ ($p = 0.001$ and 0.006 , respectively) and DHEA increased significantly in girls from 12 months before development of pubic hair until PH2+ ($p = 0.046$). DHEAS increased significantly according to time to PH2+ in both sexes within 12 months, from 2 years before PH2+ until 2.5 years after in boys and from 1 years before PH2+ until 2.5 years after in girls (all $p < 0.05$). The level of Adione did also increase according to time to PH2+. A marked increase was observed 6 to 12 months before PH2+ and again 6 to 12 months after development of pubic

hair. The increase was significant within 12 months in both girls and boys, and from 6 months before PH2+ until PH2+ in boys. T increased significantly from 12 months prior to pubic hair development until PH2+ in boys and from 6 months prior to pubic hair development until 6 months after PH2 in girls.

The median concentrations of the hormones quantified by LC-MS/MS are summarized according to age groups in table 1 and according to time to first pubic hair development (PH2+) in table 2. Figure 2 illustrates developmental and gender differences in the ratios DHEAS to DHEA (reflecting the activity of the sulfotransferase), Adione to 17-OHP (reflecting the activity of the CYP17), Adione to DHEA (reflecting the activity of the 3β -HSD), and T to Adione (reflecting the activity of the 17β -Hydroxysteroid dehydrogenase [17β -HSD]), respectively, in healthy boys and girls according to time to pubarche. Higher ratios of DHEAS to DHEA were observed in boys and the levels did not change with pubertal onset in neither boys nor girls (780 vs 435 one year before PH2+, $p=0.023$ and 618 vs 293 one year after $p=0.000$). No gender differences were observed in the ratios of Adione to 17-OHP one year before PH2+ but a small but significant increased ratio was observed in girls one year after PH2+ (1.9 vs 1.2 $p=0.001$). The ratios of Adione to DHEAS did not differ between genders one year before and one year after PH2+, although an increase was observed in girls 2 years after PH2+.

6. Discussion

Longitudinal serum concentrations of adrenal androgens have previously been measured with various immunoassays in a few studies illustrating the increasing levels with increasing age and progressing puberty [28;29]. However, measurements of lower levels of the steroid metabolites by a more selective and sensitive LC-MS/MS method reveals that androgens increase at an earlier age and in prepubertal children. As T concentrations are measurable as low as at 0.1 nmol/L by our LS-MS/MS method compared to at 0.23 nmol/L by immunoassays, is it now possible to detect an increase in T in girls months prior to pubic hair development and in boys several years prior to pubic hair development, as earlier reported [17]. We observed no gender difference in DHEA-concentrations, but the DHEAS-concentrations were significantly higher in boys. The higher level of DHEAS, the sulphated form of DHEA, suggests a gender –specific difference in

the activity of steroidogenic enzymes and/or co-enzymes participating in the conversion of DHEA to DHEAS. Furthermore, the concentrations of Adione were higher in girls, reflecting that a higher proportion of DHEA is converted to Adione in girls and to DHEAS in boys. A possible explanation could be a higher activity of sulfotransferase and/or a lower activity of 3β -HSD in boys and the inverse in girls. In the maturing adrenal the zona reticularis develops and is characterized by a higher level of the steroid sulfotransferase SULT2A1 and a lower level of the 3β -HSD, which resembles the observations in boys. This could indicate that the zona reticularis is developing earlier in boys despite development of pubic hair at later age. However, Orentreich *et al.* reported higher concentration of DHEAS in males in adulthood and a later peak in DHEAS in males than in females, as the DHEAS concentration peaked at age 20-24 years in men and at age 15-19 years in women [30]. Kushnir *et al.* reported higher concentration of DHEA in girls at six years of age compared to boys [12], and Sulcova *et al.* [31] observed higher concentration of DHEA in girls during puberty which is in contrast with our findings, although an increased DHEA/DHEAS ratio in girls compared to boys is in line with our observations. Furthermore, Hui *et al.* reported higher total thickness of the adrenal cortex, higher total thickness of zona reticularis and higher ratio of zona reticularis thickness of the adrenals in girls compared to boys, although not significant [32]. Higher concentrations of Adione in girls in puberty may be explained by ovarian contribution [33], and the higher level of T in boys are explained by a testicular contribution.

When comparing circulating hormone levels according to age with hormone levels according to onset of pubic hair development less variability in inter-individual concentrations of DHEA, Adione and T, respectively, at the time of PH₂₊ was observed. Conducting a variance component model to evaluate changes in hormone levels in six months intervals prior to pubic hair in this small sample size revealed significant changes in Adione levels 6 to 12 months prior to pubic hair development in both sexes and in levels of 17-OHP, T and DHEAS 6-12 months prior to pubic hair development in boys. These observations support that these steroid metabolites are associated with pubertal onset. Thus, DHEA or Adione may be the most important hormone in female pubic hair development and T in male pubic hair development as also reported by Shirtcliff *et al.* [34].

Boys enter puberty later than girls, and interestingly we observed opposing correlations between androgen levels at pubarche and age at PH2+ in girls compared to boys. The lower concentrations of 17-OHP and DHEA at pubarche in girls with later age may partly explain why these girls did not develop pubic hair as early as some of the other girls. However, the concentrations of Adione at pubarche were within a narrow range, which could indicate that a specific concentration of Adione is required to develop pubic hair.

The higher level of androgens at pubarche in boys with later pubarche could simply reflect a higher age, although it also seems as T increases at an older age in boys with later development of pubic hair (Figure 1).

It is a strength of the current study that we had accurate measurements both according to clinical examinations (every 6 months) and according to methodology (LC-MS/MS), but it is a limitation that only 20 children were included.

In conclusion, we assessed increasing circulating concentrations of adrenal steroids and their ratios during pubertal transition in girls and boys. Interestingly, we observed a higher ratio of DHEAS/DHEA in boys compared to girls during pubertal transition, a phenomenon that may reflect a sex difference in the enzymatic conversion of DHEA in the adrenals.

Acknowledgements

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Legend to Figures:

Figure 1. Serum concentrations of 17-OHP, DHEA, DHEAS, Adione and T according to age in girls (red lines) and boys (blue lines). Each line represents a child.

Figure 2. Activity of selected steroidogenic enzymes (sulfotransferase, CYP17, 3 β -HSD and 17 β -HSD) as illustrated by circulating ratios between androgen metabolites in 20 healthy children followed longitudinally. Red lines represent serum hormone ratios in individual girls (left panels) and blue lines represent boys (right panels) according to time to pubarche.

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Figure 1

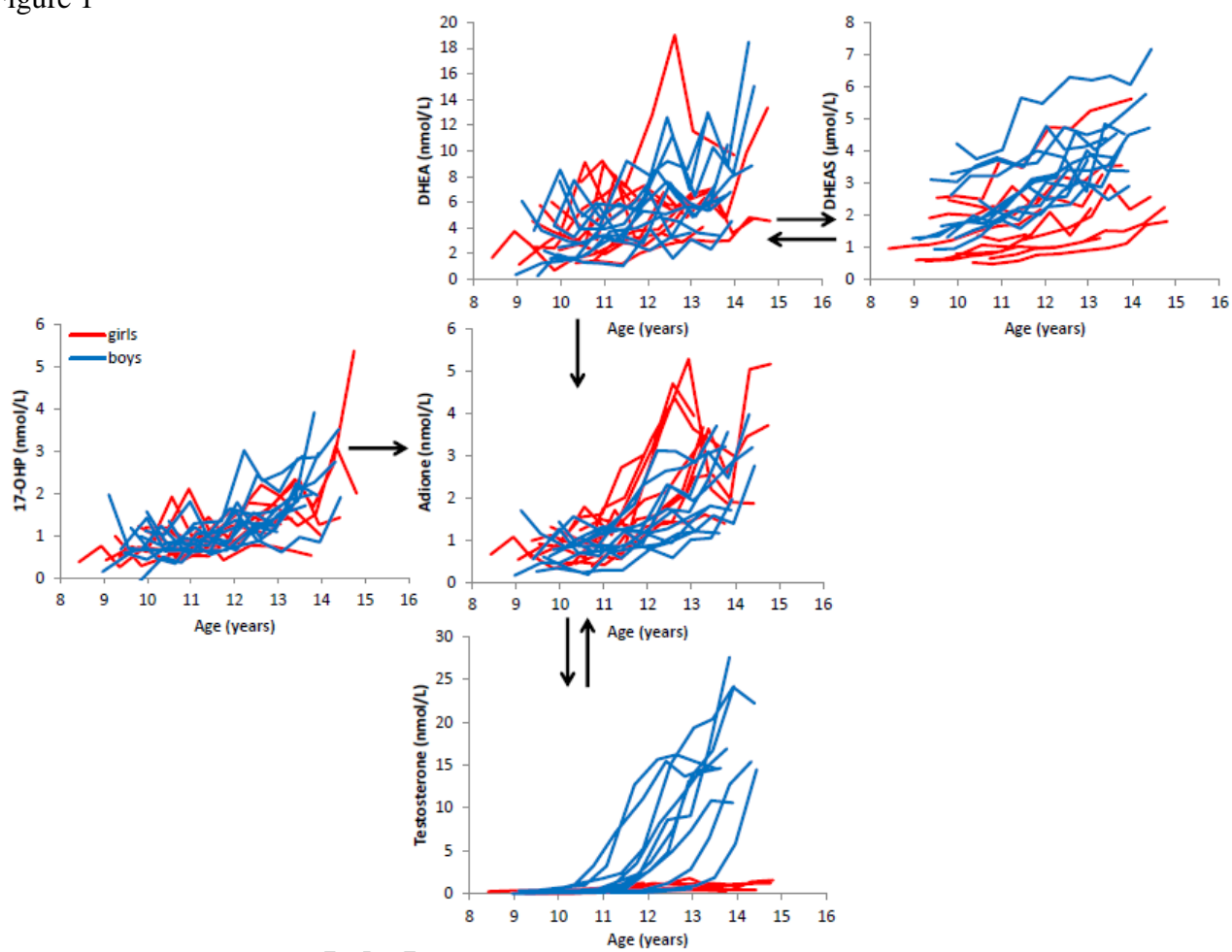


Figure 2

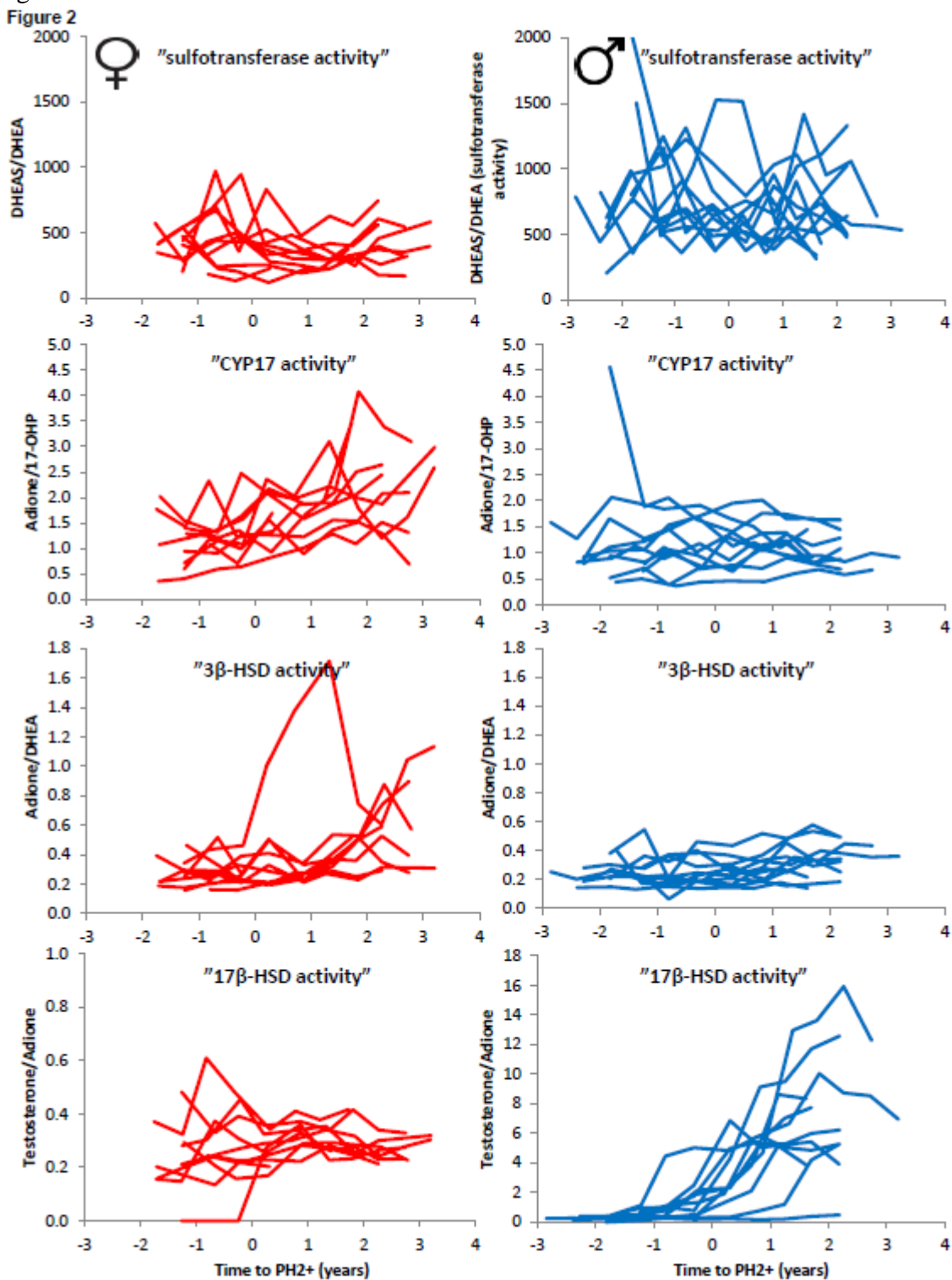


Table 1

Median serum concentrations of 17-OHP, DHEA, DHEAS, Adione and testosterone in girls and boys according to age groups

	Age groups									(each child +/- 3 months of age in same age group)
	9.5	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	
	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	Median (25th – 75th percentiles)	
Girls										
17-OHP nmol/L	0.6 (0.5-0.7)	0.7 (0.6-1.2)	0.7 (0.4-1.2)	0.7 (0.6-0.9)	1.0 (0.8-1.1)	0.9 (0.8-1.3)	1.4 (1.2-1.8)	1.5 (1.1-1.8)	1.6 (1.2-2.3)	
DHEA nmol/L	2.9 (2.8-4.5)	3.6 (2.3-4.0)	2.7 (1.6-5.5)	3.9 (2.0-6.5)	6.2 (3.0-7.2)	3.9 (2.7-6.2)	5.4 (3.6-6.8)	5.9 (4.0-6.8)	6.1 (4.1-6.8)	
DHEAS μ mol/L	1.1 (0.6-1.9)	1.2* (0.7-2.5)	1.1 (0.7-1.5)	1.1 (0.8-2.2)	1.4 (1.0-2.0)	2.1 (1.0-2.3)	1.6 (1.0-3.2)	2.1 (1.2-2.7)	2.2* (1.3-3.3)	
Adione nmol/L	0.9 (0.7-0.9)	0.8 (0.4-1.1)	0.6 (0.4-1.2)	1.0 (0.8-1.5)	1.4* (0.7-2.0)	1.9 (1.3-3.0)	2.1* [#] (1.4-4.4)	2.6 (2.1-3.8)	3.0 (2.2-3.6)	
Testosterone nmol/L	0.2 (0.2-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.3* (0.2-0.3)	0.3* (0.2-0.7)	0.6 (0.3-0.9)	0.6* (0.4-1.1)	0.7 (0.6-1.0)	0.8 (0.7-1.0)	
Boys										
17-OHP nmol/L	0.7 (0.6-0.9)	0.7 (0.6-1.1)	0.7 (0.6-1.0)	0.8 (0.7-1.0)	0.9 (0.6-1.1)	1.3* (0.8-1.6)	1.2 (1.0-1.5)	1.5 (1.2-1.6)	1.9* (1.8-2.2)	
DHEA nmol/L	3.0 (1.4-3.8)	2.7 (1.9-4.2)	3.0 (2.0-4.9)	4.0 (3.5-4.8)	4.0 (3.1-5.9)	5.4 (4.8-6.8)	7.0 (4.5-9.2)	5.6 (4.9-6.5)	5.9* [#] (5.3-10.3)	
DHEAS μ mol/L	1.5 (1.2-2.4)	1.8 (1.3-3.0)	2.2 (1.7-3.4)	2.3 (1.9-3.7)	3.0 (2.1-3.5)	3.2 (2.3-4.0)	3.7 (2.9-4.6)	3.9 (3.0-4.1)	4.1 (3.4-4.7)	
Adione nmol/L	0.6 (0.4-0.9)	0.8 (0.6-1.1)	0.9 (0.7-1.0)	0.9 (0.7-1.2)	1.2 (0.8-1.3)	1.2 (0.9-1.3)	1.7* [#] (1.2-2.3)	1.6 (1.2-2.0)	2.1* (1.6-3.0)	
Testosterone nmol/L	0.2 (0.1-0.3)	0.3 (0.2-0.3)	0.3 (0.2-1.0)	0.6 (0.3-1.8)	1.0* (0.6-3.5)	3.2* (2.3-8.2)	8.1* [#] (4.8-15.1)	13.4* (7.4-14.1)	14.6* (10.8-16.6)	

*Significant difference in median concentration compared to the concentration six months earlier (Wilcoxon signed rank test, $p \leq 0.05$).

Significant difference in median concentration compared to the concentration six months earlier (Variance component model, $p \leq 0.05$).

Table 2

Median serum concentrations of 17-OHP, DHEA, DHEAS, Adione and testosterone in girls and boys according to time to PH2+ (each child +/- 3 months of age in same time to PH2+ group).

	Time to PH2+ (years)								
	-1.5	-1.0	-0.5	0.0	0.5	1.0	1.5	2.0	2.5
	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)	Median (25th – 75th percentile s)
Girls									
17-OHP nmol/L	0.7 (0.6-1.0)	0.7 (0.5-0.7)	0.6 (0.4-1.1)	0.9 (0.6-1.3)	1.0 (0.8-1.4)	1.4 (1.1-1.7)	1.2 (0.9-1.7)	1.4 (1.1-1.7)	1.6 [#] (1.3-3.1)
DHEA nmol/L	2.9 (1.4-3.8)	2.9 (2.0-3.2)	3.2 (2.4-4.5)	4.7 (2.6-6.2)	6.1 (4.2-6.8)	6.2 (4.8-6.8)	5.8 (4.9-6.4)	5.1 (3.3-7.8)	5.9 (4.7-9.7)
DHEAS µmol/L	0.8 (0.6-2.0)	1.0 (0.6-2.0)	1.2 (0.7-2.3)	1.4 (1.0-2.0)	1.6 (1.0-2.6)	1.7 (1.4-2.4)	2.2 (1.5-2.9)	2.0 (1.3-3.4)	2.2 [#] (1.7-2.6)
Adione nmol/L	0.7 (0.5-1.1)	0.8 (0.6-0.9)	0.8 (0.6-1.5)	1.4 (1.3-1.8)	1.6 (1.4-2.0)	2.5 [#] (2.1-3.4)	2.6 (2.2-3.0)	2.8 (1.9-3.5)	3.7 (3.0-3.9)
Testosterone nmol/L	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.2-0.4)	0.3 (0.3-0.6)	0.5* (0.4-0.7)	0.7 [#] (0.6-1.2)	0.7 (0.7-1.1)	0.7 [#] (0.4-1.1)	1.0 (0.9-1.2)
Boys									
17-OHP nmol/L	0.7 (0.6-0.9)	0.6 (0.5-0.7)	1.0* (0.8-1.2)	1.3 (1.0-1.5)	1.3 (0.9-1.8)	1.6 (1.2-2.3)	2.0 [#] (1.5-2.5)	1.9 (1.8-2.8)	2.9 [#] (2.9-2.9)
DHEA nmol/L	3.5 (1.9-5.4)	3.5 (2.8-4.3)	4.1 (3.1-6.6)	6.4 (4.0-7.6)	5.5 (3.9-8.5)	6.1 (5.3-8.6)	5.5 (4.9-10.5)	6.5 (3.4-7.5)	6.3 (4.5-8.0)
DHEAS µmol/L	2.2 (1.8-3.5)	1.9 (1.7-3.6)	2.3 (1.9-4.0)	3.1 [#] (2.2-4.5)	3.0 (2.7-4.0)	4.1 [#] (3.4-4.8)	4.0 (3.4-4.5)	3.8 [#] (3.4-4.5)	3.7 (2.9-4.5)
Adione nmol/L	0.8 (0.7-1.0)	0.8 (0.6-1.0)	1.3 [#] (0.7-1.3)	1.5 (1.1-1.9)	1.2 (0.9-2.6)	1.9 (1.4-2.5)	1.9 (1.4-3.0)	2.3 (1.2-3.2)	2.4 (2.0-2.8)
Testosterone nmol/L	0.3 (0.2-0.6)	0.3 (0.2-1.0)	1.1* (0.6-2.3)	2.8* (2.4-8.6)	5.9* (4.8-9.1)	12.9 [#] (7.5-15.1)	14.7* (13.8-15.4)	14.6 (14.5-16.9)	24.1 [#] (24.1-24.2)

*Significant difference in median concentration compared to the concentration six months earlier (Wilcoxon signed rank test, $p \leq 0.05$).

[#] Significant difference in median concentration compared to the concentration six months earlier (Variance component model, $p \leq 0.05$).

HIGHLIGHTS

Adrenal androgens increase prior to pubic hair development

Boys higher levels of DHEAS from 10.5 years of age

Girls higher levels of Adione from 13 years of age

No sex difference in levels of 17-OHP and DHEA

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