Reference intervals for steroid hormones in healthy 6- to 15-year-old girls based on liquid chromatography-tandem mass spectrometry in China

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To the Editor: Adrenal and gonadal function can be evaluated by analyzing steroid hormone levels in different age groups, especially adolescent girls. Liquid chromatography-tandem mass spectrometry (LC-MS/MS or high-performance LC [HPLC]-MS/MS) offers a combination of the physical separation capabilities of HPLC and the mass analysis capabilities of tandem MS, which improves sensitivity and specificity in measuring steroid hormone levels.[1,2] This method is more sensitive, effective, specific, and homogenous than other methods for analyzing children's steroid hormone levels in the clinic. Studies on the reference intervals of steroid sex hormones have more commonly performed with Caucasian subjects than with Chinese subjects.[3] LC-MS/MS-based reference intervals for steroid hormones in China, especially for healthy girls, have not yet been reported. With LC-MS/MS becoming increasingly common, the establishment of reference intervals is growing more urgent. Here, we define appropriate reference ranges for pregnenolone, 17α-hydroxyprogesterone, corticosterone, dehydroepiandrosterone, androstenedione, and free testosterone.

This study was approved by the Ethics Committee of Beijing Children's Hospital of Capital Medical University, and written informed consent was obtained from the participants' guardians.

A total of 981 girls (aged 6–15 years) were selected from middle and elementary schools in Shunyi, Beijing using the cluster sampling method. A total of 265 participants with severe disease, medication use or a body mass index at or above the 85th percentile for children of the same age and sex were excluded. A total of 716 healthy girls were included. No malnourished children were included. The girls were allocated to four age groups, namely, 6 to <9 years, 9 to <11 years, 11 to <13 years, and 13 to 15 years. Tanner stages of breast (B) and pubic hair (PH) development were assessed by trained pediatricians using on-the-spot interviews.

Serum samples were collected in a fasting state (8–12 h) between 7:30 and 8:30 AM. After preparation by centrifugation (3000 r/min, 15 min), serum samples were kept frozen at −80°C until use. Free testosterone in serum was measured with a chemiluminescence immunoassay (CLIA) using a Maglumi® 2000 automatic immunoassay analyzer (New Industries Biomedical Engineering Co., Ltd., Shenzhen, China). The intra- and inter-assay coefficients of variation (CVs) for free testosterone were <10%, and the lower limit of detection for free testosterone was 0.5 pg/mL. The serum concentrations of pregnenolone, 17α-hydroxyprogesterone, corticosterone, dehydroepiandrosterone, and androstenedione were measured by LC-MS/MS using an Agilent 1200 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) and an AB Sciex API5000 tandem mass spectrometer (AB Sciex Pte. Ltd, Foster City, CA, USA). The corresponding serum limit of quantification values and inter-assay CVs were determined and are listed in Supplementary Table 1, http://links.lww.com/CM9/A208.

All statistical analyses were carried out using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). Due to the non-normal distribution, the 2.5th, 25th, 50th, 75th, and 97.5th percentiles were calculated for each group. Interclass variance was analyzed with the Kruskal-Wallis test. Differences with *P* values <0.05 were considered statistically significant.

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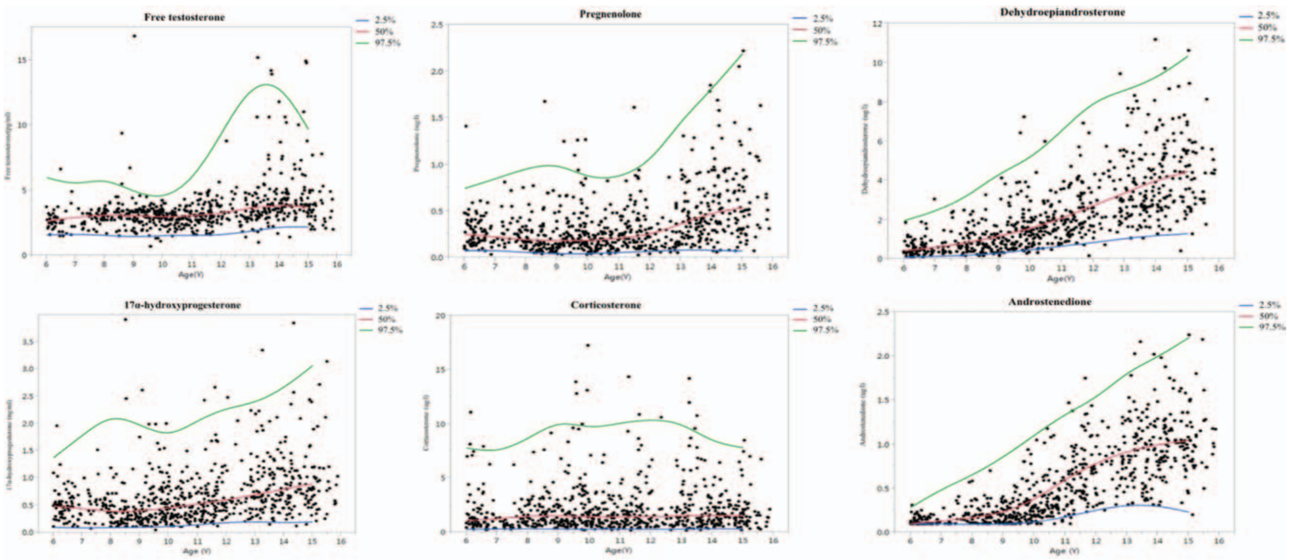

Figure 1: Changes in the concentrations of six hormones with age in healthy girls.

Table 1: Reference intervals for the concentrations of six steroid hormones in each age group.

	Free testosterone (pg/mL)			Pregnenolone (μg/L)			Dehydroepiandrosterone (μg/L)		
Age (years)	*n*	Median (IQR)	Interval*	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
6 to <9	136	3.00 (2.50–3.40)	1.54–6.13	183	0.20 (0.13–0.34)	0.05–0.76	183	0.61 (0.36–1.05)	0.13–2.45
9 to <11	170	2.90 (2.50–3.40)	1.36–4.27	181	0.19 (0.12–0.33)	0.04–1.01	180	1.41 (0.79–2.01)	0.30–4.27
11 to <13	124	3.10 (2.63–3.90)	1.60–5.18	128	0.23 (0.14–0.39)	0.05–0.88	127	2.23 (1.63–3.53)	0.73–6.29
13–15	193	3.80 (3.20–4.80)	1.89–13.90	197	0.46 (0.30–0.75)	0.08–1.70	197	3.80 (2.67–5.38)	1.27–8.81
P value		<0.001			<0.001			<0.001	

	17α-hydroxyprogesterone (ng/mL)			Corticosterone (μg/L)			Androstenedione (μg/L)		
Age (years)	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
6 to <9	184	0.42 (0.25–0.57)	0.10–1.60	184	1.27 (0.74–2.42)	0.29–7.73	184	0.14 (0.10–0.20)	0.10–0.41
9 to <11	184	0.44 (0.25–0.72)	0.10–1.77	184	1.38 (0.77–2.69)	0.26–11.05	183	0.28 (0.17–0.39)	0.10–1.03
11 to <13	128	0.56 (0.38–0.85)	0.16–2.39	128	1.50 (0.82–2.45)	0.37–10.32	128	0.68 (0.45–0.89)	0.19–1.43
13–15	197	0.79 (0.55–1.17)	0.21–2.58	197	1.58 (0.88–2.76)	0.31–8.72	197	0.97 (0.74–1.21)	0.33–2.02
P value		<0.001			0.22			<0.001	

*Intervals: The 2.5 percentile value is the lower limit and the 97.5 percentile value is the upper limit. IQR: Interquartile range.

Two outliers were eliminated according to CLSI C28-A3, and the median age of 714 healthy girls was 10.6 years with an interquartile range of 8.8 to 13.2 years.

The percentages for Tanner stages of B and PH development in each age group of healthy girls are shown in Supplementary Figure 1, http://links.lww.com/CM9/A208. The proportion of B2 and above was 6.84% from age 6 to <9 years, 64.10% from age 9 to <11 years, 93.75% from age 11 to <13 years, and 100% from age 13 to 15 years. The proportion of PH2 and above was 0 from age 6 to <9 years, 10.26% from age 9 to <11 years, 64.06% from age 11 to <13 years, and 100% from age 13 to 15 years.

Tables 1 and 2 provide the data for the serum concentrations of six steroid hormones in each age group and B stage. We could not measure the hormone levels in all of the girls due to the limited volume of serum samples, and thus, the "*n*" values differ. The differences in pregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, and free testosterone among the age groups from 6 to 15 years were significant, but there was no significant difference in corticosterone among the age groups ($\chi^2 = 3$, $P = 0.22$). The interval of corticosterone from 6 to 15 years is 0.30 to 8.99 μg/L, and the median (interquartile range) is 2.41 (0.80–2.55) μg/L. The age-associated changes in the levels of the six hormones in healthy girls are shown in Figure 1. The concentrations of pregnenolone and 17α-hydroxyprogesterone remained stable with age among girls younger than 9 years and started to increase thereafter. The levels of dehydroepiandrosterone, androstenedione and free testosterone continuously increased from 6 to 15 years old, while that of corticosterone did not change significantly with age.

Table 2: Reference intervals for the concentrations of six steroid hormones during different Tanner stages of breast development.

Tanner stages of breast	Free testosterone (pg/mL)			Pregnenolone (μg/L)			Dehydroepiandrosterone (μg/L)		
	n	Median (IQR)	Interval*	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
I	198	2.90 (2.48–3.30)	1.50–4.92	243	0.22 (0.14–0.35)	0.05–0.82	243	0.72 (0.40–1.25)	0.17–3.30
II	159	3.10 (2.60–3.50)	1.60–4.80	171	0.19 (0.12–0.34)	0.04–0.91	169	1.74 (1.16–2.52)	0.29–6.35
III	169	3.50 (2.80–4.40)	1.80–10.60	172	0.32 (0.18–0.52)	0.05–1.36	172	2.72 (1.96–3.71)	0.87–7.67
IV–V	98	3.90 (3.20–4.85)	1.54–14.10	103	0.48 (0.30–0.78)	0.07–1.93	103	4.54 (3.08–5.66)	1.18–10.09
P value		<0.01			<0.01			<0.01	

Tanner stages of breast	17α-hydroxyprogesterone (ng/mL)			Corticosterone (μg/L)			Androstenedione (μg/L)		
	n	Median (IQR)	Interval	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
I	246	0.42 (0.25–0.62)	0.10–1.98	246	1.23 (0.73–2.43)	0.28–9.02	245	0.15 (0.10–0.22)	0.10–0.50
II	172	0.47 (0.26–0.73)	0.10–1.74	172	1.57 (0.89–2.65)	0.43–10.52	172	0.35 (0.22–0.56)	0.10–1.22
III	172	0.65 (0.46–0.93)	0.19–2.24	172	1.30 (0.65–2.48)	0.24–8.44	172	0.86 (0.61–1.06)	0.34–1.73
IV–V	103	0.88 (0.57–1.2)	0.22–2.97	103	1.63 (1.01–2.96)	0.35–8.58	103	1.01 (0.8–1.26)	0.32–2.09
P value		<0.01			0.015			<0.01	

*Intervals: The 2.5 percentile value is the lower limit and the 97.5 percentile value is the upper limit. IQR: Interquartile range.

In this study, the changes in the six steroid sex hormones with age and puberty in healthy girls from 6 to 15 years old were consistent with the development of the adrenal glands and gonads. The serum levels of pregnenolone and 17α-hydroxyprogesterone showed no significant changes from adrenarche (6−8 years old) to 9 years old, but after 10 years of age, they increased with age as a result of the adrenal gland response to adrenocorticotropic hormone (ACTH). This is because the Δ5 pathway predominates, while the Δ4 pathway does not respond to ACTH well at adrenarche. At the same time, 3β-hydroxysteroid dehydrogenase (3β-HSD) showed very low activity. The level of dehydroepiandrosterone, as the main marker of adrenarche, increased with age beginning at 6 years and continued to increase throughout puberty. This is the result of the development and differentiation of the adrenal gland, as well as changes in steroid hormone syntheses.[4] The serum levels of androstenedione and free testosterone increase with age because the number of 3β-HSD and Cyt-b5 double-positive hybrid cells increases with the growth of the adrenal cortex,[5] and the production of androstenedione in the ovary also increases with the initiation of puberty. The serum level of free testosterone increases with age as a result of the increase in the substrate level and 17β-HSD activity. The level of corticosterone, as a precursor of aldosterone, did not change with age.

We provide reference intervals for steroid sex hormones in 6- to 15-year-old healthy female children based on the LC-MS/MS method. The levels of certain hormones change with age and pubertal status. These references are valuable for the diagnosis and treatment of pediatric adrenal and gonadal diseases, as well as for future research on adrenarche and gonadal initiation and progression.

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Conflicts of interest

None.

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