Liquid Chromatography-Tandem Mass Spectrometry-Based Characterization of Steroid Hormone Profiles in Healthy 6 to 14-Year-Old Male Children

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To the Editor: Male steroid hormones are synthesized in the adrenal cortex and testis, and pediatric reference intervals for normal steroid hormone levels are essential for the diagnosis of adrenal and gonadal disorders. Importantly, steroid hormone profiles vary with age and pubertal development, and to date, only very limited data have been published concerning normal steroid hormone ranges in terms of age and pubertal development. The absence of such data has been primarily due to the lack of a sufficiently sensitive and specific method for determining appropriate reference intervals for steroid concentrations.

The immunoassay approach has been most commonly used to measure serum levels of steroid hormones to date. However, its specificity is rather low due to cross-reactivity and interference from other endogenous steroids and lipids.[1,2] The concentration values obtained by immunoassay tend to be higher than the actual values, particularly for steroid hormones present at low concentrations (e.g., 17α-hydroxyprogesterone), and thus, the reliability of the immunoassay is lower.[3-6] In addition, immunoassays can only target individual hormones, which limits the efficiency of this approach. Since radioimmunoassay, the most sensitive form of immunoassay, is even more susceptible to interference, different laboratories use their own self-developed reference ranges, which are not interchangeable. Therefore, different laboratories can produce very different results for the same sample. This is particularly problematic as diseases of the adrenal cortex and gonads usually require long periods of follow-ups, and samples collected over long intervals are more likely to be sent to different laboratories for analysis. Moreover, multicentric cohort studies on these conditions require that results obtained from different laboratories be comparable.

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. Use of this approach to measure steroid hormone levels has received considerable attention recently due to the high levels of sensitivity and specificity that can be achieved.[7,8] Moreover, LC-MS/MS can be applied to test multiple samples and analyze multiple steroid levels simultaneously, making this a very efficient approach relative to prior techniques.

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Although several studies have already used LC-MS/MS to measure steroid hormones' levels with age,[7-11] due to small sample sizes, few age- and puberty-associated reference ranges have been established. In the present study, we used the LC-MS/MS method to measure the serum levels of several steroid hormones and their precursors from different layers of the adrenal cortex in a large population of 6 to 14-year-old healthy male children in order to establish reference intervals for these hormones for male children at different ages and Tanner genital stages. Since we also wanted to provide reference intervals for free testosterone, we measured serum levels of this steroid by chemiluminescence immunoassay (CLIA) using an available kit. The steroid hormones analyzed included pregnenolone, 17α-hydroxyprogesterone, and corticosterone, which are produced in the zona glomerulosa (ZG) and zona fasciculate (ZF), as well as dehydroepiandrosterone (DHEA), androstenedione, and free testosterone of the zona reticularis (ZR) of the adrenal cortex.

Using the cluster sampling method, study participants were selected from middle and elementary schools in Shunyi, Beijing, where residents' financial conditions and urban versus rural populations are distributed evenly. The inclusion criteria were age between 6 and 14 years, normal weight based on a body mass index below the 85th percentile for boys in the same age group,[12] normal blood pressure and heart rate, and good health based on medical history. Participants with a history of any of the following conditions were excluded: hypogonadism, panhypopituitarism, diabetes, head trauma, renal failure, hemochromatosis, cirrhosis, hepatitis C infection, human immunodeficiency virus infection, and treatment with testosterone or oral steroids. In addition, participants with an

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active infection or recent surgery or hospitalization within 6 weeks prior to the study were excluded.

Puberty status was assessed by trained pediatricians according to the Tanner method, which includes five genital (G) stages ranging from stage G1, prepuberty to stage G5, and postpuberty. Testicular volume was determined with a Prader orchidometer as follows: <4 ml for stage G1, 4–8 ml for stage G2, >8–15 ml for stage G3, >15–20 ml for stage G4, and >20–25 ml for stage G5.

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.

Blood samples were acquired between 7:30 and 8:30 AM after an overnight fast of approximately 10 h. After preparation by centrifugation (3000 rpm, 15 min), serum samples were kept frozen at −80°C until use.

Free testosterone in serum was measured with 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. Serum concentrations of pregnenolone, 17α hydroxyprogesterone, corticosterone, DHEA, and androstenedione were measured by LC-MS method using an Agilent 1200 Series HPLC system for HPLC (Agilent Technologies Inc., California, USA) and an AB Sciex API5000 tandem mass spectrometer (AB Sciex Pte. Ltd, California, USA). The corresponding serum limit of quantification values and interassay CVs were determined and listed in Table 1.

All statistical analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Abnormally distributed data were presented as median and interquartile range. The reference intervals were determined using the 2.5 percentile as the lower limit and the 97.5 percentile as the upper limit. Interclass variance was analyzed with the Kruskal–Wallis test.

The study population included 820 boys from 6 to 14 years of age. The median participant age was 10.0 years with an interquartile range of 8.1–12.8 years. The sample sizes were small for the age of 12 years and stage G3, as children in these age groups were faced with stress related to their studies and refused to participate in the physical examination. Furthermore, we could not measure the hormone levels in all of the boys due to the limited volume of serum samples, and thus, the "n" values differ.

Table 2 provides the data for serum concentrations of steroid hormones by age, including the lower and upper limits for each hormone at every age from 6 to 14 years. The levels of 17α-hydroxyprogesterone and pregnenolone in the healthy boys did not vary significantly among the different adrenarche ages, from approximately 6–8 years old (before puberty initiation) to 9 years old. Beyond 10–11 years of age, these levels increased with age. By contrast, when we combined the age groups for 13 and 14 years together, the levels of corticosterone did not change significantly over the age range tested ($P = 0.34$). Although the difference among the age groups from 6 to 14 was significant, the binary fitting method also shown here revealed no correlation between age and corticosterone (estimated value was −0.07, $P = 0.06$). The concentrations of DHEA, androstenedione, and free testosterone increased with age starting from the age of 6 years [Table 2, Figure 1].

Table 1: Serum LOQ and interassay CV values for steroid hormones analyzed by LC-MS/MS

Steroid hormone	LOQ	Interassay CV
Pregnenolone	0.025 ng/ml	10.1% at 0.2 ng/ml
17α-hydroxyprogesterone	0.1 ng/ml	3.9% at 3 ng/ml
Corticosterone	0.1 ng/ml	8.3% at 3 ng/ml
DHEA	0.1 ng/ml	3.2% at 3 ng/ml
Androstenedione	0.1 ng/ml	4.2% at 3 ng/ml

DHEA: Dehydroepiandrosterone; LOQ: Limit of quantification; CV: Coefficient of variation; LC-MS/MS: Liquid chromatography-tandem mass spectrometry.

Table 3 displays the data for serum concentrations of steroid hormones by genital stage, including the lower and upper limits for each hormone from stage G1 to G5. The pregnenolone level increased significantly from stage G1 to G2 and remained steady from stage G3 onward. The level of 17α-hydroxyprogesterone increased obviously with the progression of puberty from stage G1 to G3, and then this increase slowed from stage G4. The corticosterone level did not change with the progression of puberty. DHEA and androstenedione levels increased significantly from stage G1 to G2 and then increased at a slower rate from stage G3. Free testosterone levels rose sharply during puberty from stage G1 to G3 and then slowly from stage G4 [Table 3, Figure 2].

In the present study, from adrenarche (6–8 years old) until 9 years of age, pregnenolone levels did not vary significantly nor did the levels of 17α-hydroxyprogesterone. This is because stimulation of the Δ4 pathway toward adrenocorticotropin in the ZR is not significant during adrenarche. In the Δ4-pathway in the ZF, cytochrome P-450 17 alpha-hydroxylase (CYP17) catalyzes the conversion of pregnenolone and progesterone to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively, so the levels of pregnenolone and 17α-hydroxyprogesterone do not vary significantly. In addition, the lower and upper limits for 17α-hydroxyprogesterone identified in our study were similar to previously published pediatric reference intervals.[11] No change in the level of corticosterone, the precursor of aldosterone, was observed from 6 to 14 years of age, and this result is consistent with a previous study showing that the aldosterone level is relatively constant from 1 to 15 years of age in boys and girls.[13] This is probably because the secretion of aldosterone was stable in relation to the constant size.[13] The above-mentioned results suggest that during adrenarche, hormone levels in the ZG and ZF do not change significantly. Beyond 10–11 years of age, however, pregnenolone and 17α-hydroxyprogesterone levels increase with age. Given that this timing coincides with the onset of puberty, we consider pubertal development to be the underlying cause for these increased hormone levels.

DHEA is mainly produced in the ZR of the adrenal cortex. Moreover, DHEA and DHEA sulfate (DHEAS) define adrenarche. Our results show that starting from 6 years of age, the level of DHEA increased with age, which is likely to be related to the adrenal morphology and alterations of steroidogenic enzyme expression. Anatomically, the adrenal gland differs before and after birth. The human fetal adrenal cortex is composed of a distinct fetal zone and neocortex. The fetal zone nearly vanishes within a few months after birth, and the neocortex develops into the adult adrenal cortex, with a distinct ZG and ZF. The ZR begins to grow at 3–5 years of age, becomes a continuous section by 7–8 years of age, and then continues to increase in size with age. Theoretically, the ZR of adults is morphologically the same as the fetal zone. DHEA and DHEAS production almost

Table 2: Reference intervals for serum steroid hormone concentrations in healthy 6 to 14-year-old male children

Age (years)	Pregnenolone (μg/L)			17α-hydroxyprogesterone (ng/ml)			Corticosterone (μg/L)		
	n	Median (IQR)	Interval*	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
6	97	0.07 (0.04–0.11)	0.03–0.22	109	0.18 (0.13–0.28)	0.10–0.82	115	1.50 (0.94–2.72)	0.32–10.60
7	66	0.05 (0.04–0.07)	0.03–0.16	74	0.21 (0.15–0.38)	0.10–1.68	76	1.55 (0.88–3.22)	0.42–17.25
8	90	0.08 (0.05–0.12)	0.03–0.33	104	0.21 (0.15–0.31)	0.10–0.90	104	1.21 (0.77–2.47)	0.36–13.91
9	88	0.12 (0.08–0.17)	0.03–0.51	95	0.21 (0.14–0.38)	0.10–0.85	93	1.64 (0.94–3.07)	0.22–12.40
10	57	0.09 (0.06–0.14)	0.03–0.32	85	0.33 (0.24–0.56)	0.12–1.16	84	1.41 (0.86–2.66)	0.34–12.90
11	60	0.14 (0.09–0.23)	0.03–0.46	81	0.44 (0.29–0.57)	0.10–1.21	81	1.43 (0.78–2.96)	0.22–10.21
12	43	0.20 (0.14–0.29)	0.04–0.76	49	0.45 (0.34–0.64)	0.15–1.36	46	1.35 (0.68–3.48)	0.26–12.14
13	70	0.15 (0.10–0.20)	0.03–0.42	80	0.54 (0.39–0.73)	0.19–1.23	80	0.92 (0.47–1.90)	0.15–6.70
14	103	0.18 (0.12–0.24)	0.05–0.43	108	0.66 (0.47–0.87)	0.24–1.35	109	1.67 (0.94–2.97)	0.30–7.93
P		<0.001			<0.001			0.01	

Age (years)	DHEA (μg/L)			Androstenedione (μg/L)			Free testosterone (pg/ml)		
	n	Median (IQR)	Interval	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
6	107	0.28 (0.14–0.50)	0.10–0.96	78	0.11 (0.10–0.14)	0.10–0.23	122	0.58 (0.50–0.89)	0.50–1.39
7	75	0.56 (0.27–0.91)	0.04–2.03	50	0.12 (0.10–0.18)	0.10–0.34	77	0.88 (0.62–1.22)	0.50–1.95
8	109	0.72 (0.42–1.03)	0.13–2.62	90	0.15 (0.11–0.20)	0.10–0.40	110	0.91 (0.60–1.25)	0.50–2.06
9	96	1.03 (0.66–1.74)	0.31–3.80	94	0.21 (0.16–0.29)	0.11–0.63	96	1.15 (0.81–1.53)	0.50–2.75
10	85	1.57 (1.08–2.48)	0.34–5.25	85	0.28 (0.19–0.42)	0.12–0.83	85	1.59 (1.27–2.02)	0.71–7.53
11	81	2.16 (1.60–3.07)	0.62–5.10	81	0.31 (0.23–0.42)	0.14–0.78	91	2.22 (1.73–3.68)	0.84–19.6
12	49	2.61 (1.83–3.68)	0.65–9.97	49	0.42 (0.27–0.57)	0.13–1.23	49	3.98 (2.30–9.82)	1.19–37.20
13	80	3.38 (2.39–4.29)	1.13–7.92	80	0.57 (0.41–0.76)	0.21–1.31	80	20.18 (9.35–34.55)	2.10–71.13
14	109	3.84 (2.72–5.07)	1.56–7.09	109	0.64 (0.48–0.81)	0.27–1.32	110	25.71 (16.70–40.40)	4.50–78.27
P		<0.001			<0.001			<0.001	

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

Table 3: Reference intervals for serum steroid hormone concentrations during different Tanner stages among healthy male children

Tanner genital stage	Pregnenolone (μg/L)			17α-hydroxyprogesterone (ng/ml)			Corticosterone (μg/L)		
	n	Median (IQR)	Interval*	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
G1	383	0.08 (0.05–0.13)	0.03–0.31	448	0.22 (0.15–0.38)	0.10–1.05	455	1.45 (0.87–2.92)	0.36–12.52
G2	97	0.13 (0.09–0.21)	0.04–0.33	122	0.37 (0.26–0.51)	0.13–1.16	114	1.36 (0.80–2.98)	0.21–12.94
G3	67	0.16 (0.11–0.25)	0.05–0.57	78	0.53 (0.36–0.75)	0.16–2.74	70	1.38 (0.63–2.54)	0.23–6.13
G4	94	0.18 (0.13–0.23)	0.06–0.49	101	0.65 (0.47–0.86)	0.27–1.57	100	1.31 (0.75–2.25)	0.16–7.22
G5	39	0.16 (0.12–0.21)	0.03–0.34	43	0.68 (0.49–0.94)	0.26–1.33	43	1.58 (0.74–2.93)	0.29–7.68
P		<0.001			<0.001			0.32	

Tanner genital stage	DHEA (μg/L)			Androstenedione (μg/L)			Free testosterone (pg/ml)		
	n	Median (IQR)	Interval	*n*	Median (IQR)	Interval	*n*	Median (IQR)	Interval
G1	452	0.70 (0.37–1.34)	0.13–3.82	378	0.18 (0.13–0.25)	0.10–0.63	470	0.97 (0.59–1.36)	0.50–2.49
G2	123	2.12 (1.56–3.16)	0.53–5.32	122	0.32 (0.23–0.44)	0.12–0.93	123	2.24 (1.69–3.49)	0.62–18.15
G3	78	3.10 (2.21–4.24)	1.07–10.86	78	0.48 (0.34–0.65)	0.18–1.31	78	11.52 (5.66–23.35)	1.89–63.82
G4	101	3.65 (2.64–4.59)	1.43–6.70	101	0.63 (0.48–0.77)	0.20–1.14	102	26.39 (17.12–37.36)	6.50–64.85
G5	43	4.36 (2.83–5.49)	1.49–8.60	43	0.71 (0.57–0.90)	0.28–1.40	43	34.16 (24.98–47.07)	8.22–88.67
P		<0.001			<0.001			<0.001	

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

ceases at birth and does not resume until the age of 6 years.[14] Continued increase in DHEA production with age corresponds to morphological development of the adrenal cortex. The Δ5 pathway in the ZR is dominant during adrenarche, CYP17 catalyzes the 17α-hydroxylation of pregnenolone and the 17, 20-lyase reaction of its 17-hydroxy derivative, 17α-hydroxypregnenolone, and then 17α-hydroxypregnenolone is converted into DHEA. This is probably another cause for the increase of DHEA at adrenarche age.

Recent studies have shown that the hybrid parenchymal cells of the ZF and ZR co-express 3β-hydroxysteroid dehydrogenase (3βHSD) and cytochrome b5 (Cyt-b5), affecting the production of androstenedione.[15,16] Moreover, Cyt-b5 enhances the activity of 3βHSD by reinforcing the affinity of 3βHSD and its cofactor nicotinamide adenine dinucleotide NAD(+). The dynamic pattern

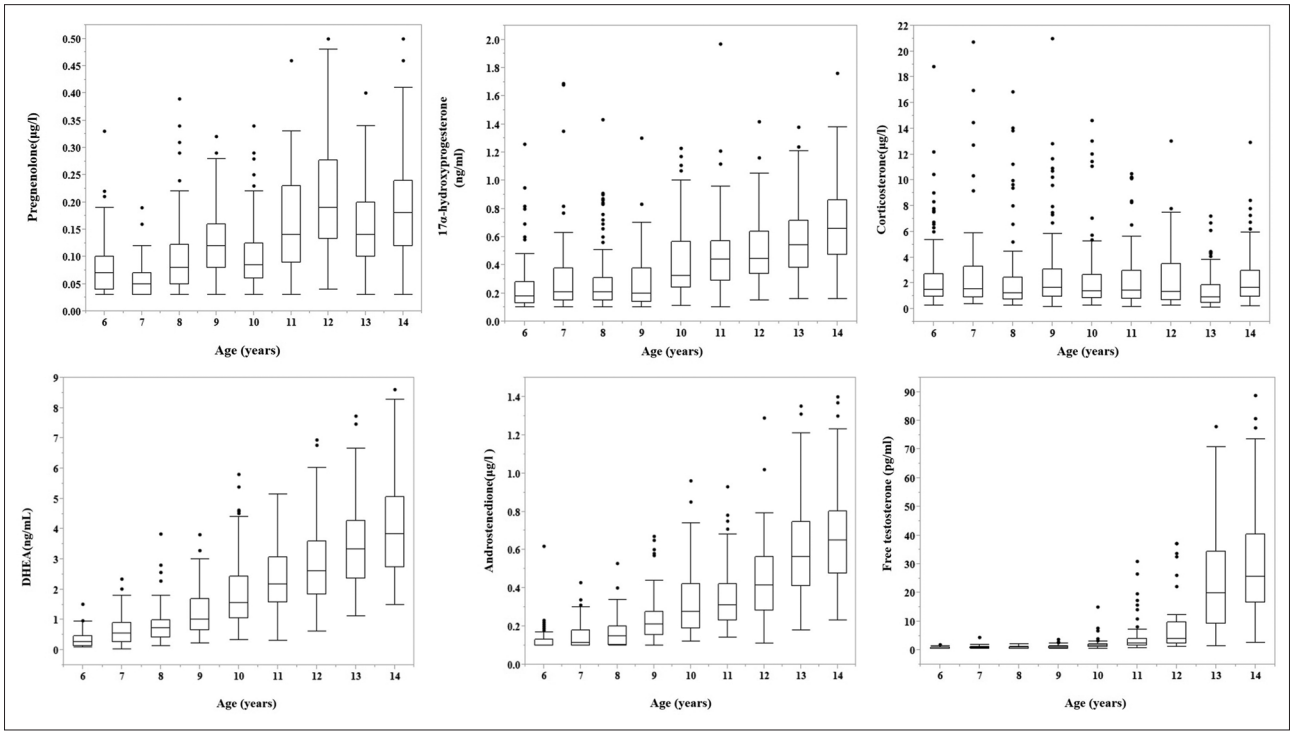

Figure 1: Median serum concentrations of steroid hormones of 6 to 14-year-old healthy male children.

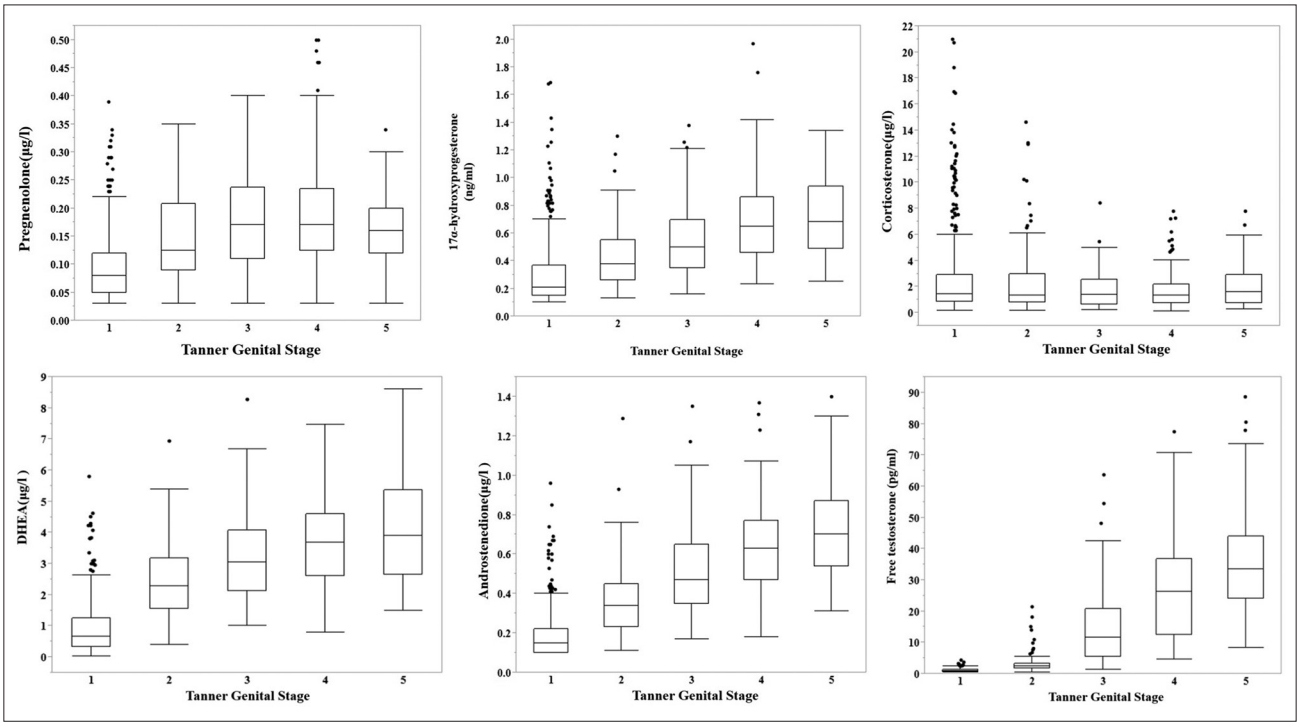

Figure 2: Median serum concentrations of steroid hormones during different Tanner genital stages in healthy male children.

of androstenedione with age could be explained by the morphology of the adrenal cortex and changes in the ratios of hybrid cell types with age. 3βHSD and Cyt-b5 double-positive cells are present in the highest ratio in 13–20 year olds compared with levels in 4 to 6- and 7 to 12-year-old groups.[17] Although not every age group has been studied yet, current data indicate that 3βHSD and Cyt-b5 double-positive cells increase with age, and adrenal androstenedione also increases with age. Our results showing an increase in the androstenedione level with age are consistent with this hypothesis.

The level of adrenal testosterone is rather low before puberty, existing mostly in the form of DHEA. By examining free testosterone levels, our results demonstrate that testosterone can be measured at adrenarche. Furthermore, the free testosterone levels were shown to increase from the ages of 6 to 10 years. Testosterone

predominantly originates from androstenedione through the catalysis of 17βHSD, and therefore, we speculate that the activity of 17βHSD increases during adrenarche.

In most boys, the onset of puberty occurs after the age of 10. In addition to the adrenal glands, gonads also produce sex hormones, but adrenal initiation and gonad initiation are two independent processes. The Leydig cells can synthesize and secrete multiple androgen hormones, for example, testosterone, androstenedione, and DHEA, through the same processes as in the adrenal cortex. After pubertal onset, androgen hormone production by the gonads increases with age, and our results confirmed that the concentrations of pregnenolone, 17α-hydroxyprogesterone, DHEA, androstenedione, and free testosterone increased with the progression of puberty. The pregnenolone level showed a significant increase from stage G1 to G2 and remained steady from G3 onward. 17α–hydroxyprogesterone, DHEA, androstenedione, and free testosterone increased significantly during the early stage of puberty and then remained steady from mid puberty. These results imply that the increase in enzymatic activities is more significant with the initiation of the hypothalamus–pituitary–gonadal axis, and with maturity, the enzyme activity reaches a plateau.

The present study provides the profiles of steroid hormones from adrenarche through the onset and progression of puberty in healthy male children using LC-MS/MS for sensitive and specific detection of hormone concentrations. The changes observed in the levels of certain hormones correlated with the known age-related changes in adrenal morphology and steroidogenic enzyme expression. The reference intervals established in this work could be valuable in the diagnosis of adrenal and gonad diseases as well as future research of adrenarche and gonadal initiation and progression.

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Conflicts of interest

There are no conflicts of interest.

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