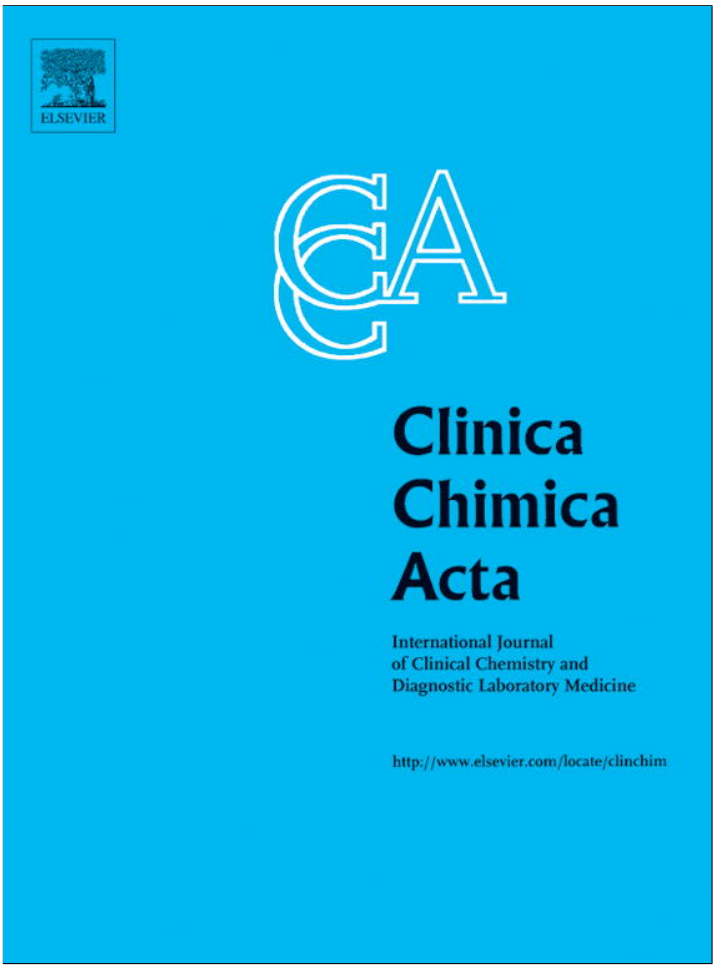

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Reference values for salivary testosterone in adolescent boys and girls determined using Isotope-Dilution Liquid-Chromatography Tandem Mass Spectrometry (ID-LC–MS/MS)

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ABSTRACT

The measurement of testosterone in saliva is an attractive alternative to serum analysis due to the simple and non-invasive sample collection. In children and adolescents salivary testosterone is mainly measured to investigate whether puberty has started or not. This study aimed to establish reference values for salivary testosterone during puberty in boys and girls. We measured salivary testosterone using ID-LC–MS/MS in a cohort of 131 girls and 123 boys of whom each had salivary testosterone measured at two time points during puberty. Salivary testosterone concentrations start to increase with the start of puberty around eight years and continuously increase up to adult concentrations in the following ten years. Reference values were calculated using the Lambda–Mu–Sigma (LMS)-curve fitting method and provided per year from 8 to 26 years of age in boys and girls. These reference ranges may help clinicians and researchers to interpret salivary testosterone results in both individual patients and study subjects.

1. Introduction

The measurement of steroid hormones in saliva is an attractive alternative to serum analysis due to the simple and non-invasive sample collection. Saliva samples are easily collected by patients or study subjects themselves by drooling their saliva in a simple polypropylene tube. This procedure needs no special training, is also suitable for older children and adolescents and can be performed at home or in field studies [1]. Salivary testosterone is one of the steroid hormones which is, in addition to cortisol, often requested for both clinical and research purposes. In children and adolescents testosterone is mainly measured to investigate whether puberty has started or not [2]. Reference values are available for children aged six to nine years old [3] as well as for adult males and females [5–7]. Reference ranges for children during puberty are still lacking.

Several issues in salivary testosterone testing need to be addressed in order to obtain reliable results. The first issue is the necessity of a reliable method to measure salivary testosterone. As testosterone levels in saliva are in the picomolar range, in both males and females, a very sensitive method is needed. In addition, as many steroid hormones are very similar to testosterone, which can cause cross reactivity in the assays, a very specific method is needed. Immunoassays are often used for steroid hormone measurement, as by Ostatníková et al. [4]. However, immunoassays may suffer from cross reactivity and high variation. Nowadays, Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS), mainly due to its superiority in specificity and lower variation, is the method of choice to measure testosterone [9,10]. The second issue is the saliva sample collection method. Several types of saliva collecting methods are available. However, many do not offer reliable results [8], suggesting that the most straightforward saliva collection method (drooling directly into a simple polypropylene tube) is the most reliable one.

The present study aimed to establish reference values for salivary testosterone during puberty in boys and girls. Saliva samples were collected by drooling into a polypropylene tube and salivary testosterone was measured using a sensitive and accurate LC–MS/MS method.

2. Materials and methods

The current study was part of a large longitudinal study, referred to as Braintime, conducted at Leiden University, the Netherlands [11]. All participants or their legal representatives gave informed consent. For

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Table 1
LMS-parameters and percentiles for salivary testosterone per year in 123 boys, measured twice with 2 years between the two measurements. The 2.5th and 97.5th percentiles form the boundaries of the reference range.

Boys									
Age (years)	LMS parameter			Percentiles [testosterone] (pmol/L)					
	L	M	S	2.5%	15.9%	50.0%	84.1%	97.5%	n
				−1.96 SD	−1.00 SD	Median	+1.00 SD	+1.96 SD	
8	0.1	1.6	1.2	0.1	0.4	1.6	4.9	12.4	4
9	0.1	3.4	1.2	0.2	1.0	3.4	10.1	24.9	8
10	0.1	5.6	1.1	0.4	1.7	5.6	15.6	37.2	15
11	0.2	15.6	1.1	1.2	4.8	15.6	41.0	91.0	22
12	0.3	42.5	1.0	2.8	13.5	42.6	102.5	203.7	24
13	0.4	84.5	0.9	4.2	27.6	84.5	184.4	328.9	31
14	0.5	134.9	0.8	6.8	48.3	134.9	268.0	441.3	25
15	0.5	186.7	0.7	18.8	78.8	186.7	341.9	536.1	29
16	0.5	234.7	0.6	44.3	117.2	234.7	397.4	598.4	22
17	0.4	276.5	0.5	79.8	157.9	276.5	438.1	638.4	22
18	0.3	311.9	0.4	115.5	194.6	311.9	472.7	675.9	9
19	0.2	341.5	0.4	143.2	223.4	341.5	504.9	715.6	10
20	0.2	366.1	0.4	161.5	244.7	366.1	533.5	749.3	2
21	0.2	386.2	0.4	172.5	260.7	386.2	554.2	764.3	2
22	0.3	402.2	0.4	178.5	273.3	402.2	566.0	759.3	7
23	0.5	415.1	0.3	181.8	284.4	415.1	569.6	740.8	2
24	0.7	425.8	0.3	182.5	294.6	425.9	569.5	717.6	7
25	1.0	435.5	0.3	178.0	303.2	435.5	569.3	698.8	2
26	1.2	444.8	0.3	166.4	310.2	444.9	570.6	685.7	3

the determination of reference values in the present study, 131 girls and 123 boys of whom each had salivary testosterone measured at two time points during puberty from this larger cohort were included. Their salivary testosterone data were described earlier in a study concerning longitudinal changes in adolescent risk-taking [3]. At the first time point (T1), median age was 13.60 years (age range 8.01–24.55 years) for boys and mean age was 12.83 years (age range 8.20–22.79 years) for girls. Approximately two years later (median time-difference 2.00 years; range time-difference 1.01–2.86 years) all participants provided a second saliva sample. At the second time point (T2), median age was 15.57 years (age range 9.92–26.22 years) for boys and median age was 14.84 years (age range 10.26–24.83 years) for girls.

Saliva samples were collected by passive drool, immediately after waking up in the morning, and before eating or brushing teeth. Saliva was directly drooled in a polypropylene tube. Samples were sent to the laboratory and frozen at −20 °C until analysis. Postmenarcheal females collected saliva on the seventh day of their menstrual cycle. Females using contraceptives interfering with the hypothalamic–pituitary-gonadal axis, such as oral contraceptives or hormonal intrauterine devices, were excluded.

Testosterone concentrations in all saliva samples were analysed in duplicate at the Endocrine Laboratory, Department of Clinical Chemistry of the VU University Medical Center Amsterdam. Measurements of samples of which the duplicate measurement had a difference > 15% were repeated. Salivary testosterone was determined by isotope dilution-liquid chromatography–tandem mass spectrometry (ID-LC–MS/MS). This method was described in detail earlier and was calibrated on an LC–MS/MS method for serum testosterone which was found to be concordant with a reference method [5,12]. In short, after thawing and centrifugation (30 min at 1900*g*), 200 μL saliva was pipetted in duplicate.

Table 2
LMS-parameters and percentiles for salivary testosterone per year in 131 girls measured twice with 2 years between the two measurements. The 2.5th and 97.5th percentiles form the boundaries of the reference range.

Girls									
Age (years)	LMS parameter			Percentiles [testosterone] (pmol/L)					
	L	M	S	2.5%	15.9%	50.0%	84.1%	97.5%	n
				−1.96 SD	−1.00 SD	Median	+1.00 SD	+1.96 SD	
8	0.1	2.9	0.6	0.7	1.5	2.9	5.4	9.3	9
9	0.2	5.0	0.7	1.2	2.5	5.0	9.4	16.2	13
10	0.2	7.8	0.7	1.8	3.9	7.8	14.4	24.8	26
11	0.2	10.5	0.7	2.4	5.3	10.5	19.6	33.4	25
12	0.2	13.3	0.7	3.0	6.6	13.3	24.5	41.5	33
13	0.2	15.8	0.6	3.5	7.9	15.8	28.9	48.0	27
14	0.3	18.1	0.6	4.0	9.0	18.1	32.4	52.5	27
15	0.3	19.9	0.6	4.3	10.0	19.9	35.0	55.3	22
16	0.4	21.6	0.6	4.7	11.0	21.6	37.0	57.2	19
17	0.4	23.0	0.6	5.3	12.0	23.0	38.7	58.8	16
18	0.4	24.3	0.6	6.2	13.2	24.3	40.2	60.4	9
19	0.3	25.6	0.5	7.2	14.4	25.6	41.6	62.1	15
20	0.3	27.0	0.5	8.5	15.7	27.0	43.0	63.8	5
21	0.2	28.4	0.5	9.8	17.1	28.4	44.5	65.6	7
22	0.2	29.7	0.5	11.3	18.5	29.7	45.8	67.3	7
23	0.1	31.1	0.4	12.8	20.0	31.1	47.1	68.7	0
24	0.1	32.4	0.4	14.4	21.6	32.4	48.2	69.7	2

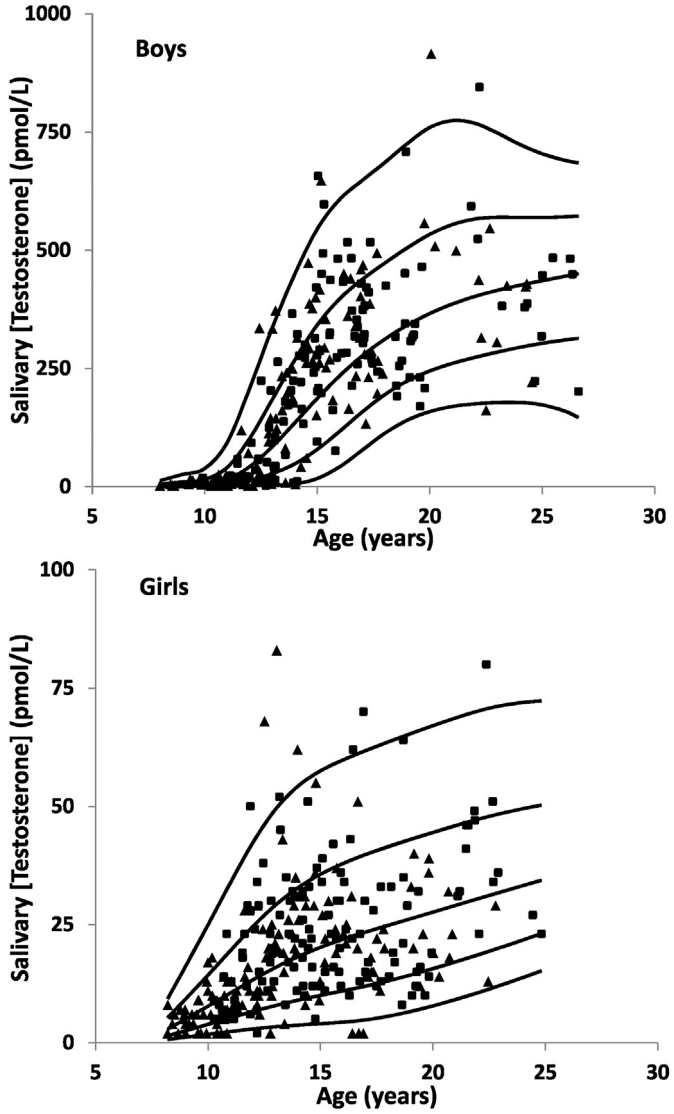

Fig. 1. Individual points and fitted percentile curves (2.5; 15.9; 50.0; 84.1; 97.5 percentiles) for salivary testosterone in boys (n = 123) and girls (n = 131). First measurement is indicated by ▲; the second by ■.

After addition of internal standard ([$^2H_5$]-testosterone) and derivatization using methoxylamine hydrochloride, the samples were injected into a Symbiosis online solid phase extraction (SPE) and liquid chromatography system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA). Intra-assay coefficient of variation (CV) was 11%, 4%, and 2% at 10, 140, and 900 pmol/L, respectively and inter-assay CV was 5% at 200 and 2000 pmol/L, respectively. Mean recovery was 93% (SD 7%). Testosterone levels from 23 participants on T1 and 3 participants on T2 fell below the detection limit of 4 pmol/L. For the calculation of the reference ranges these samples were assigned a testosterone concentration of 2 pmol/L. Reference ranges for boys and girls (n = 123 and n = 131 for boys and girls, respectively: all subjects were sampled twice with a 2 years between the measurements) were calculated using the LMS method (lmsChartMaker) [13]. This is a curve fitting method using Lambda((L) skewness), Mu ((M) median) and Sigma ((S) coefficient of variation) as variables to calculate percentile curves.

3. Results

The estimated percentiles (2.5%, 15.9% 50%, 84.1% and 97.5%) as well as the variables L (skewness), M (median) and S (coefficient of variation) are given per year in Table 1 for boys and in Table 2 for girls. SD-scores can be calculated using the following formula: SD-score = $(\text{Measurement}/M)^L - 1] / [L \times S]$. Fig. 1 shows the individual data points as well as the fitted percentiles for boys and girls.

4. Discussion

We established salivary testosterone reference values for boys and girls during puberty in a cohort of healthy children. We demonstrated that salivary testosterone concentrations gradually increase during puberty. It is known that in most children serum as well as saliva testosterone concentrations start to increase with the start of puberty between seven and nine years and continuously increase up to adult concentrations in the following ten years [14–17]. This is in line with our findings. Ostatníková et al. found prepubertal salivary testosterone levels below 93 pmol/L and below 60 pmol/L in boys and girls aged six to nine years, respectively [3]. These levels are higher than those found in the current study in children aged eight and nine years (testosterone levels were below 25 pmol/L). This difference might be due to differences between the assays used to asses testosterone, a radioimmunoassay by Ostatníková et al. and LC–MS/MS in the current study. This underlines the importance of method specific reference values.

The upper cut-off value found in young males and females above 19 years in the current study were clearly higher, than those reported in adults. Adult reference ranges for salivary testosterone reported earlier were 135–400 pmol/L [4], 73–343 pmol/L [5] and 64–356 pmol/L [6] for adult males and <39 pmol/L for adult females [6]. One of the reasons for this difference is that due to the lower number of data points above 19 years in the current study, the reference ranges show increased variation. This might be due to a peak in testosterone concentrations occurring in the late teens and early twenties [15]. An earlier study on salivary testosterone concentrations in males between 18 and 30 years showed a reference range 190–680 pmol/L [7]. The difference between these values and those reported in the current study might be caused by assay differences as mentioned earlier and is probably also caused by the younger age of the subjects in the current study.

Our study has several strengths. First, samples for this study were collected in the morning which is optimal, as testosterone shows a circadian rhythm [18]. Secondly, saliva samples were collected and handled adequately [8]. Finally, we used an accurate method [4]. A limitation of this study is that these subjects were sampled twice. This seems to increase the number of observations, but might have influenced the final reference ranges since the same children have been analysed twice, rather than using samples from different children. However, as a relatively large and heterogeneous group of healthy children was used in this study, it is unlikely that the analysis of more children using single collections would have severely altered the reference ranges found. In addition, we did not analyse testosterone levels in relation to pubertal stage. Although this might be interesting information for clinicians, we believe that conclusions about the start of puberty can also be drawn based upon these age-based reference values. In the present study, most measurements were between 10 and 17 years of age. At younger as well as older age the number of measurements is limited. This causes an increased variation in the reference values found in these ages. Further research, including more subjects aged below 10 and above 17 years of age, needs to be performed, in order to confirm the reference ranges presented in our study.

In conclusion, we determined reference values testosterone in properly collected saliva specimens from boys and girls aged 8–26 years using a sensitive and accurate LC–MS/MS method. These reference ranges may help clinicians and researchers to interpret salivary testosterone results in both individual patients and study subjects.

References

[1] J. Durdiakova, H. Fabryova, I. Koborova, D. Ostatnikova, P. Celec, The effects of saliva collection, handling and storage on salivary testosterone measurement, Steroids 78 (2013) 1325–1331.

[2] G.E. Butler, R.F. Walker, R.V. Walker, P. Teague, D. Riad-Fahmy, S.G. Ratcliffe, Salivary testosterone levels and the progress of puberty in the normal boy, Clin. Endocrinol. 30 (1989) 587–596.

[3] D. Ostatnikova, K. Pastor, Z. Putz, M. Dohnanyiova, A. Mat'aseje, R. Hampl, Salivary testosterone levels in preadolescent children, BMC Pediatr. 2 (2002) 5.

[4] H.N. Bui, S.E.E. Schagen, D.T. Klink, H. Delemarre-van de Waal, M.A. Blankenstein, A.C. Heijboer, Salivary testosterone in female-to-male transgender adolescents during treatment with intra-muscular injectable testosterone esters, Steroids 78 (2013) 91–95.

[5] P.R. Macdonald, L.J. Owen, F.C. Wu, W. Macdowall, B.G. Keevil, A liquid chromatography–tandem mass spectrometry method for salivary testosterone with adult male reference interval determination, Clin. Chem. (2011) 774–781.

[6] U. Turpeinen, E. Hamalainen, M. Haanpaa, L. Dunkel, Determination of salivary testosterone and androstendione by liquid chromatography–tandem mass spectrometry, Clin. Chim. Acta 413 (2012) 594–599.

[7] V. Gonzalez-Sanchez, O. Moreno-Perez, L. Garcia de Guadiana, et al., Reference ranges for serum and salivary testosterone in young men of Mediterranean region, Endocrinol. Nutr. 62 (2015) 4–10.

[8] M. Gröschl, H. Kohler, H.G. Topf, T. Rupprecht, M. Rauh, Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs, J. Pharm. Biomed. Anal. 47 (2008) 478–486.

[9] D.J. Handelsman, L. Wartofsky, Requirement for mass spectrometry sex steroid assays in the journal of clinical endocrinology and metabolism, J. Clin. Endocrinol. Metab. 98 (2013) 3971–3973.

[10] R.M. Büttler, F. Martens, F. Fanelli, et al., Comparison of 7 published LC–MS/MS methods for the simultaneous measurement of testosterone, androstenedione, and dehydroepiandrosterone in serum, Clin. Chem. 61 (2015) 1475–1483.

[11] B.R. Braams, A.C.K. van Duijvenvoorde, J.S. Peper, E.A. Crone, Longitudinal changes in adolescent risk-taking: a comprehensive study of neural responses to rewards, pubertal development, and risk-taking behavior, J. Neurosci. 35 (2015) 7226–7238.

[12] H.N. Bui, P.M. Sluss, S. Blincko, D.L. Knol, M.A. Blankenstein, A.C. Heijboer, Dynamics of serum testosterone during the menstrual cycle evaluated by daily measurements with an ID-LC–MS/MS method and a 2nd generation automated immunoassay, Steroids 78 (2013) 96–101.

[13] T.J. Cole, P.J. Green, Smoothing reference centile curves: the LMS method and penalized likelihood, Stat. Med. 11 (1992) 1305–1319.

[14] A. Khairullah, L.C. Klein, S.M. Ingle, et al., Testosterone trajectories and reference ranges in a large longitudinal sample of male adolescents, PLoS One 9 (2014), e108838.

[15] M.M. Kushnir, T. Blamires, A.L. Rockwood, et al., Liquid chromatography–tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals, Clin. Chem. 56 (2010) 1138–1147.

[16] A.E. Kulle, F.G. Riepe, D. Melchior, O. Hiort, P.M. Holterhus, A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age- and sex-specific reference data, J. Clin. Endocrinol. Metab. 95 (2010) 2399–2409.

[17] J.K. Rilling, C.M. Worthman, B.C. Campbell, J.F. Stallings, M. Mbizva, Ratios of plasma and salivary testosterone throughout puberty: production versus bioavailability, Steroids 61 (1996) 374–378.

[18] J.M.J. Dabbs, Salivary testosterone measurements: reliability across hours, days, and weeks, Physiol. Behav. 48 (1990) 83–86.