Continuous Reference Intervals for Pediatric Testosterone, Sex Hormone Binding Globulin and Free Testosterone using Quantile Regression

Daniel T. Holmes[a,b,*], J Grace van der Gugten[a,*], Benjamin Jung[f], Christopher R. McCudden[d,c,e]

[a] *St. Paul's Hospital Department of Pathology and Laboratory Medicine, 1081 Burrard St., Vancouver, BC V6Z 1Y6 Canada*
[b] *University of British Columbia Department of Pathology and Laboratory Medicine, 2211 Wesbrook Mall, Vancouver, BC V6T 1Z7 Canada*
[c] *Department of Pathology and Laboratory Medicine, Ottawa Hospital, General Campus, 501 Smyth Road, Ottawa, ON K1H 8L6 Canada*
[d] *Department of Pathology and Laboratory Medicine, University of Ottawa*
[e] *Eastern Ontario Regional Laboratory Association*
[f] *Hospital for Sick Children (SickKids), 555 University Ave., Department of Paediatric Laboratory Medicine, Toronto, ON, M5G 1X8*

Abstract

Testosterone (T), sex hormone binding globulin (SHBG), free testosterone (FT) and/or bioavailable testosterone (BAT) are common tests in pediatric endocrinology. As with most tests, these analytes required reference intervals for interpretation. Common methods to derive reference intervals require decisions about data shape and/or where to define partitions, which can result in sharp differences between age groups, particularly in children undergoing puberty. Partitioning also results in a form of data loss, where data from one age bin is completed disconnected from the next age bin. Non-parametric continuous reference intervals methods have previously been developed to avoid some of these drawbacks. These strategies use all the available data and smooth transitions between ages avoiding partitioning. However, the fitting process inolves selection and adjustment of many parameters and it can be difficult to sustain a reproducible approach. Here we provide non-parametric continuous reference intervals for T, FT, BAT, and SHBG. T measurements were determine by LC-MS/MS, FT and BAT was calculated, and SHBG measured on the Roche Cobas e601. The continuous interval methodology is described in detail with code examples in the statistical programming language R with and illustrations for reproducibililty.

*Corresponding Author
Email addresses: dtholmes@mail.ubc.ca (Daniel T. Holmes), gvandergugten@providencehealth.bc.ca (J Grace van der Gugten), benjamin.jung@sickkids.ca (Benjamin Jung), cmccudde@uottawa.ca (Christopher R. McCudden)

1. Introduction

Measurements of testosterone (T), sex hormone binding globulin (SHBG), free testosterone (FT) and/or bioavailable testosterone (BAT) are common in pediatric endocrinology for investigation of ambiguous genitalia, precocious puberty, and premature adrenarche. Age-dependent reference intervals for T as measured by liquid chromatography and tandem mass spectrometry (LC-MS/MS) have been previously investigated in a number of studies using a number of statistical procedures including, the Hoffmann method [1], various partitioning strategies [2–6], and continuous fitting procedures [7].

Statistical strategies for continuous fitting of age-dependent centiles became a matter of interest for the development of pediatric growth curves [8] but in time, these strategies have been applied to age-dependent biochemical and hematological parameters. There are numerous approaches to the problem, each addressing challenges of heteroscedasticity and non-normality in the raw data in different ways. Some of the more popular approaches include the those of Healy [8], Cole's lambda, mu and sigma (LMS) method [9], Royston's fractional polynomial method [10], the generalized additive models for location, scale and shape (GAMLSS) method [11] and quantile regression methods [12–14]

In this particular study we will demonstrate the application of the non-parametric quantile regression method deployed in the **quantregGrowth** package of the R programming language [15]. This methodology will be applied to T, SHBG, calculated FT and calculated BAT measured on a cohort of discarded anonymized samples from 421 children (195 F,226 M) aged 33 days to 19 years. We will illustrate the process of performing such an analysis using the **quantregGrowth** package.

2. Methods

2.1. Samples

Becton Dickenson and Greiner red top serum samples were obtained from British Columbia Children's and Women's Hospital after routine analysis for reactivity to allergens. After routine clinical analysis, specimens were sequestered, anonymized and decanted to 12 x 75 mm polystyrene tubes and frozen at -80 °C. No clinical exclusion criteria were applied to the cohort. These samples were then transferred to St. Paul's Hospital laboratory for the analysis of T and SHBG. SHBG was measured within 2 months of receipt while testosterone analysis was delayed for 3 years. Stability studies for SHBG have shown only modest changes at -25 °C for 25 years [16] while testosterone is similarly unaffected by years of storage at -80 °C when measured by LC-MS/MS [17].

2.2. Biochemical Analysis

T analysis was performed using a modification French's method [18] as previously described [19]. Briefly, liquid-liquid extraction was performed on 100 µL of sample/calibrator with 40 µL of internal standard (d3-testosterone at 11.7

nmol/L; 337 ng/dL) using 0.75 mL of hexane:ethyl acetate in a 96-well format using a Hamilton Starlet robotic liquid handler. After vortexing for 3 minutes, samples were centrifuged in plate for 10 min at 3000 rpm (948 g). Following centrifugation, 500 µL of the organic later was transferred to a second 96-well plate and evaporated under air warmed to 45 °C followed by reconstitution with 200 µL of 75:25 (0.1% formic acid in water):(0.1% formic acid, 2 mM ammonium acetate in 70:30 methanol:acetonitrile). LC-MS/MS analysis was performed using a SCIEX API 5000 triple quadrupole mass spectrometer using a Shimadzu 20AC liquid chromatography system and T was quantified using the following multiple reaction monitoring transitions: quantifier 289 → 97, qualifier 289 → 109, IS 292 → 97. The calibration range of the assay is 0.05–45.0 nmol/L and traceable to the National Institute of Standards (NIST) SRM 971 "Hormones in Frozen Human Serum" standard reference material. The assay total coefficient of variation (CV) ranges from 4.2–6.8% for concentrations of 0.14–21.76 nmol/L.

SHBG was measured using the Roche Cobas e601 electrochemiluminescent assay according to the manufacturer's recommendations. Total CVs were observed to be 1.4–2.1% for concentrations ranging from 24.0–129 nmol/L. FT and BAT were calculated assuming an albumin concentration of 43 g/L using the Vermeulen equation [20].

2.3. Statistical Analysis

Continuous reference intervals were determined using non-parametric quantile regression. This method is resistant to outliers and makes no assumptions about symmetry, normality, linearity, and heteroscedasticity. The lower 2.5th and upper 97.5th centiles were modeled using the **quantregGrowth** [21,22], package using R version 4.1. Initial curve smoothing was done with penalized splines with 10-fold cross-validation as previously described [23].

To reproduce the analysis, the steps are to load the necessary R packages, import the data, split into male and female dataframes, and then call the function to determine the desired centiles (2.5, median, and 97.5) as shown below.

```r
# Load packages
library(openxlsx)
library(quantregGrowth)

# Import Data
testo <- read.xlsx("data/raw_data_w_ft_bat.xlsx")

# Split into Male and Female
male <- testo[testo$gender=='M',]
female <- testo[testo$gender=='F',]

# Create a variable with the appropriate centiles for use below:
tauss <- c(0.025,0.5,0.975)
```

```
#Call for cross-validation with a range of lambda smoothing values in Males:
mm <- gcrq(t_nmol_l ~ ps(age,
                 mon = 0,
                 lambda = seq(0, 100, l = 25)),
  tau = tauss,
  data = male,
  cv = T)
```

Breaking down the last lines of the above cross-validation call, we used the function for the continuous age-dependent centile curve, `gcrq`. The `gcrq` function, an acronym for `growth chart regression quantile`, is part of the **quantregGrowth** package and has a series required of arguments (also known as parameters). The `gcrq` function requirements are: 1) the formula containing the variables of interest (age and testosterone), 2) the centile or centiles (the variable we've called `tauss`, which has the lower 2.5th, median, and upper 97.5) and 3) the source dataframe with the variables, in this case `male`.

In R, formulae have a standard format of `dependent variable ~ predictor variable`. In our case, the dependent variable is testosterone (the column of data arbitrarily titled `t_nmol_l` in the dataframe). The predictor variable is age, but in this case it's embedded in another function, `ps()`, which is a spline. Splines will be familiar to most readers as a means to construct smooth curves. The smoothness and shape of the curve is determined by a series of piece-wise polynomials between fixed points or **knots**. There are as many options for the number of knots and polynomial order (linear, quadratic, cubic, etc.) as there are data points, which is not ideal for reproducible research. To address this, the `ps()` function uses a **penalized spline** with the option to perform cross-validation to automatically identify the **best** smoothness. In this case, **best** is the balance between fitting the line through each points and being too "wiggly" (overfit). The `ps()` function determines smoothness using a penalty term, λ, which is an error multiplier of how rough (wiggly) the curve is. The smaller the lambda, the smaller the penalty and the rougher the curve.

In figure 1, we illustrate the effect of different smoothing values for λ. Setting a λ smoothing term of zero shows overfitting, particularly >15 years old, whereas high λ values tend to underfit the data in a way won't accurately predict testosterone at older ages.

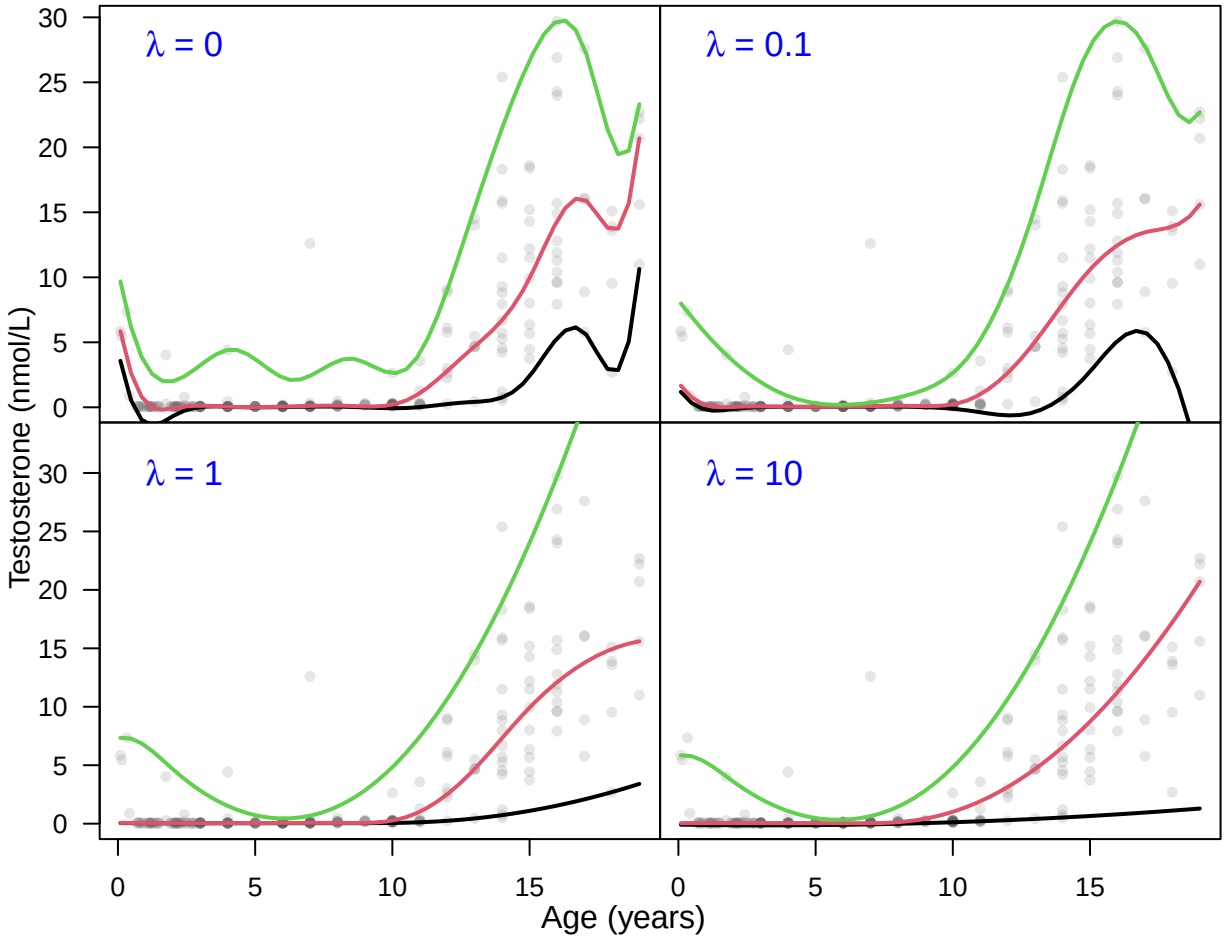

Figure 1: Comparison of different smoothing values (λ) for continuous reference interval curves for testosterone in males. Upper curve (green) is 97.5th, median (red), and lower 2.5th (black).

Instead of manually picking λ, in the `ps` function above, we provided a sequence of numbers, coded as: `lambda = seq(0,10,l=50)`, which is simply a sequence of numbers from 0 to 10 of length 50 (0, 0.2, 0.4, ..., 9.8, 10). By feeding `ps()` a sequence of numbers, the `gcrq` fitting function will calculate the testosterone vs. age curve for all those λ smoothing values and identify the optimum (smoothest curve that still passes closest to the points) using cross-validation. In the cross-validation, the curve is calculated using a subset of data points and the accuracy of the fit of the left out points is assessed iteratively. Using cross-validation, the optimum λ value for testosterone in males was 1.6.

While cross-validation selects an optimum, complete automation is seldom perfect and it is appropriate to visually inspect the results and use our knowledge of the expected data shape to hone the smoothing parameter. Specifically, we

know what the reference values are in adults and should use that knowledge to tune the fit if necessary. To improve the fit at the tails (age <1 and >18), the smoothing parameter (λ) was optimized manually by adjusting the initial values based on visual inspection of the curve. While this is subjective, it was determined with relative ease because we started with an optimal value of the cross-validation. Occasionally the optimal value will represent the data well and require no adjustment. In the present analysis, only SHBG in females required no additional tuning.

For completeness, the above description is one way to do the cross-validation. There are many other ways to do this using the **quantregGrowth** package, including defining a penalty matrix, weighting observations, selecting different types of spline (linear, quadratic), choosing the exact location of knots, forcing the curve to continuously increase or decrease, and forcing the curve shape to be concave-up or concave-down. The interested reader is referred to the package documentation [21,22].

Lastly, the confidence intervals (95%) of the fits were determined using the sandwich formula built into the **quantregGrowth** package [24] but bootstrapping can optionally be selected. Confidence intervals with shading are plotted using with the code: `plot(mm, conf.level = .95, shade = T)` Negative interval predictions were rounded to zero.

3. Results

Continuous intervals were determined for males and females between the ages of 6 months to 19 years for females (N=195) and 1 month to 19 years for males (N=226)—see tables 1 and 2 as well as figure 1. Intervals were calculated using non-parametric quantile regression for total T, SHBG, calculated free and bioavailable T. In both males and females, T values peaked in the age range of puberty, males being $\simeq 10\times$ higher. SHBG showed varying patterns with age, differing between males and females.

Confidence intervals were provided for the reference interval estimates, with higher variability around the tails of the intervals. Prediction outside the age interval (extrapolation) are not expected to be accurate given the absence of data.

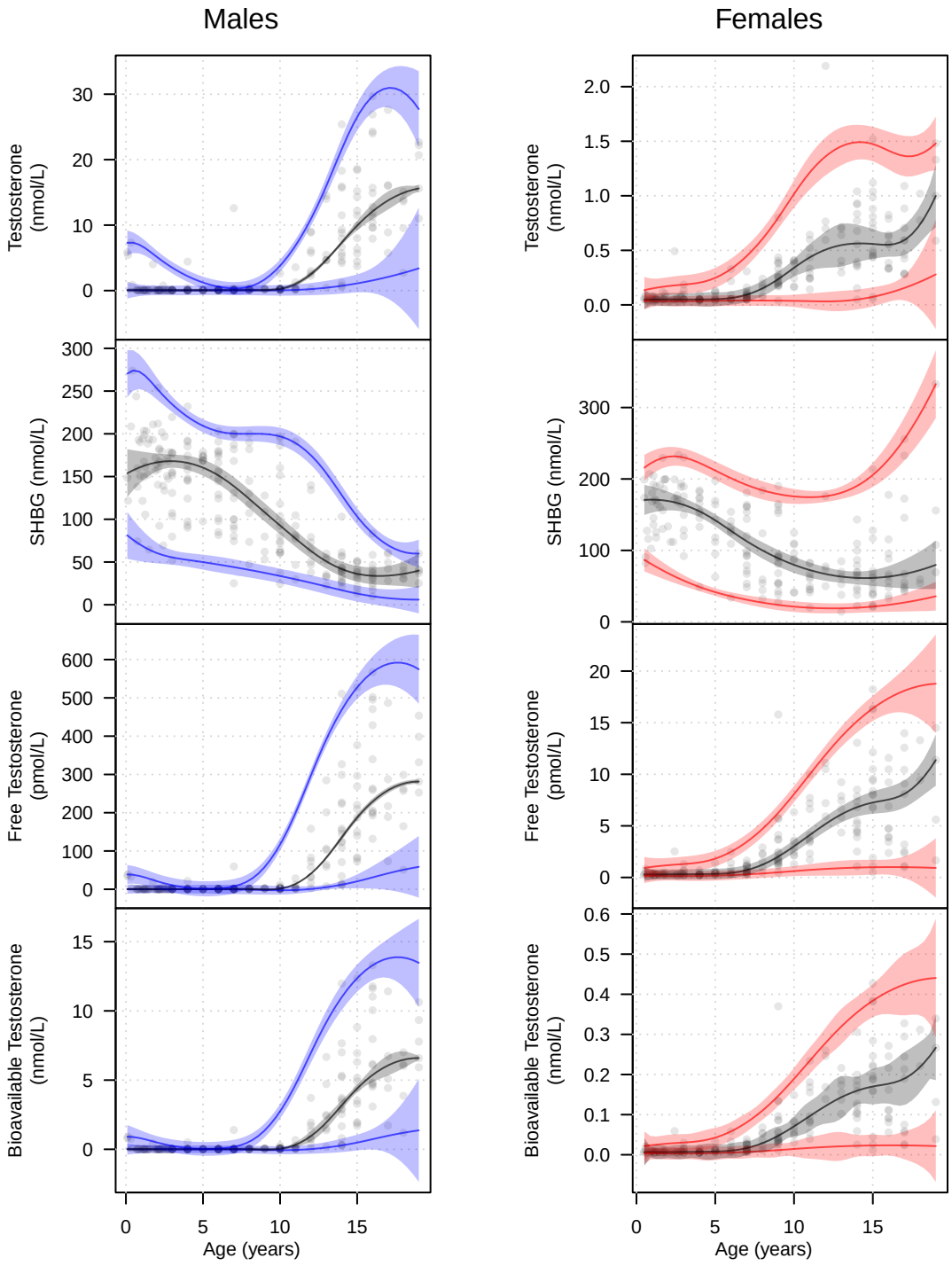

Figure 2: Non-parametric continuous pediatric reference intervals for testosterone, SHBG, BAT and FT in males (left) and females (right) showing confidence intervals calculated using the sandwich formula [15].

Table 1: Male centile estimates by age in years. Point estimates of the reference intervals are selected at the mid-point of each respective age-bin.

	T (nmol/L)			SHBG (nmol/L)			FT (pmol/L)			BAT (nmol/L)			
Age(y)	2.5th	50th	97.5th	2.5th	50th	97.5th	2.5th	50th	97.5th	2.5th	50th	97.5th	N
0–<1	0.05	0.06	7.31	76	158	274	0.23	0.41	37.73	0.01	0.01	0.88	11
1–<2	0.04	0.06	6.13	65	164	264	0.16	0.31	28.08	0.00	0.01	0.66	17
2–<3	0.03	0.05	4.39	58	168	245	0.17	0.26	16.12	0.00	0.01	0.38	21
3–<4	0.03	0.05	2.93	54	168	228	0.20	0.26	7.47	0.00	0.01	0.18	12
4–<5	0.03	0.05	1.79	51	164	215	0.22	0.31	2.49	0.00	0.01	0.06	14
5–<6	0.04	0.06	0.97	49	158	207	0.17	0.42	1.17	0.00	0.01	0.03	9
6–<7	0.05	0.07	0.47	46	147	202	0.00	0.57	3.50	0.00	0.01	0.08	17
7–<8	0.05	0.08	0.34	43	134	200	0.00	0.69	10.97	0.00	0.02	0.26	15
8–<9	0.04	0.08	0.81	40	119	200	0.00	0.31	30.82	0.00	0.01	0.72	9
9–<10	0.03	0.09	2.15	36	104	199	0.00	0.00	70.97	0.00	0.00	1.66	5
10–<11	0.05	0.37	4.42	33	89	196	0.00	3.24	133.85	0.00	0.08	3.14	17
11–<12	0.11	1.25	7.63	29	74	187	0.00	18.14	218.80	0.00	0.43	5.13	8
12–<13	0.26	2.85	11.77	25	61	172	1.38	47.17	315.17	0.03	1.11	7.39	8
13–<14	0.48	5.08	16.85	21	49	150	6.25	90.28	404.93	0.15	2.12	9.49	7
14–<15	0.77	7.67	22.30	16	41	125	13.02	142.45	477.60	0.31	3.34	11.19	16
15–<16	1.14	10.18	26.94	13	36	101	21.71	192.42	532.51	0.51	4.51	12.48	12
16–<17	1.59	12.24	29.87	10	34	82	31.85	231.87	569.68	0.75	5.43	13.35	13
17–<18	2.12	13.84	30.97	8	34	69	42.07	259.83	589.09	0.99	6.09	13.81	5
18–<19	2.72	14.96	30.26	6	36	62	51.08	276.29	590.75	1.20	6.47	13.84	5
19–<20	3.40	15.60	27.72	6	40	60	58.64	281.25	574.67	1.37	6.59	13.47	5

Table 2: Female centile estimates by age in years. Point estimates of the reference intervals are selected at the mid-point of each respective age-bin.

	T (nmol/L)			SHBG (nmol/L)			FT (pmol/L)			BAT (nmol/L)			
Age(y)	2.5th	50th	97.5th	2.5th	50th	97.5th	2.5th	50th	97.5th	2.5th	50th	97.5th	N
0–<1	0.04	0.05	0.14	87	171	216	0.18	0.30	0.93	0.00	0.01	0.02	8
1–<2	0.03	0.05	0.16	74	171	228	0.15	0.30	1.11	0.00	0.01	0.03	21
2–<3	0.03	0.05	0.18	63	167	232	0.13	0.30	1.24	0.00	0.01	0.03	12
3–<4	0.03	0.05	0.19	54	160	227	0.13	0.31	1.35	0.00	0.01	0.03	7
4–<5	0.04	0.05	0.22	46	150	218	0.15	0.33	1.57	0.00	0.01	0.04	10
5–<6	0.04	0.05	0.27	39	137	208	0.19	0.36	2.03	0.00	0.01	0.05	8
6–<7	0.04	0.07	0.37	34	122	198	0.25	0.50	2.78	0.01	0.01	0.07	7
7–<8	0.04	0.11	0.49	30	109	189	0.32	0.85	3.81	0.01	0.02	0.09	16
8–<9	0.04	0.17	0.66	26	97	182	0.42	1.44	5.13	0.01	0.03	0.12	6
9–<10	0.04	0.26	0.86	23	86	178	0.53	2.27	6.72	0.01	0.05	0.16	18
10–<11	0.04	0.36	1.07	21	78	175	0.64	3.27	8.53	0.02	0.08	0.20	8
11–<12	0.03	0.45	1.25	19	71	174	0.75	4.35	10.40	0.02	0.10	0.24	10
12–<13	0.03	0.52	1.38	19	66	176	0.84	5.37	12.23	0.02	0.13	0.29	4
13–<14	0.04	0.55	1.46	19	63	182	0.91	6.24	13.89	0.02	0.15	0.33	6
14–<15	0.05	0.56	1.49	20	61	192	0.96	6.89	15.32	0.02	0.16	0.36	13
15–<16	0.08	0.56	1.47	22	62	208	0.99	7.29	16.50	0.02	0.17	0.39	15
16–<17	0.11	0.55	1.41	24	64	229	1.00	7.57	17.44	0.02	0.18	0.41	10
17–<18	0.16	0.60	1.36	27	67	257	1.00	8.14	18.13	0.02	0.19	0.43	10
18–<19	0.21	0.74	1.38	31	73	292	0.97	9.38	18.58	0.02	0.22	0.44	2
19–<20	0.28	1.00	1.48	36	80	333	0.92	11.38	18.78	0.02	0.27	0.44	4

4. Discussion

We have determined continuous age-dependent reference intervals for T, SHBG, FT and calculated BAT for male and female children under 20 years. Only a few studies have investigated total T reference intervals using LC-MS/MS in pediatric populations [1,4–7]. A graphical comparison of these is shown in figure 3 showing reasonable agreement among the studies bearing in mind that the statistical analyses are performed differently and samples are differentially binned into age-categories. However, figure 3 also starkly illustrates the problem with binned reference interval studies, namely, that there are large jumps in the reference intervals seen when transitioning from one age-bin to the next. Obviously this does not reflect physiology and would induce misclassification with increasing frequency as bin sizes grow. For example in both the studies of Soldin [1] and Bae [7], a large jump of $\simeq 17 - 25$ nmol/L is seen in the upper limit of normal for boys after the age of 10 y and a similar phenomenon is seen in Kushnir's study [5], attenuated somewhat by the narrower bin sizes.

Of note, concern has been raised about incorrect application of the Hoffmann indirect method leading to inappropriately narrow reference intervals [25], which would affect the results from Soldin et al, but the material effect of this error is hard to appreciate in figure 3.

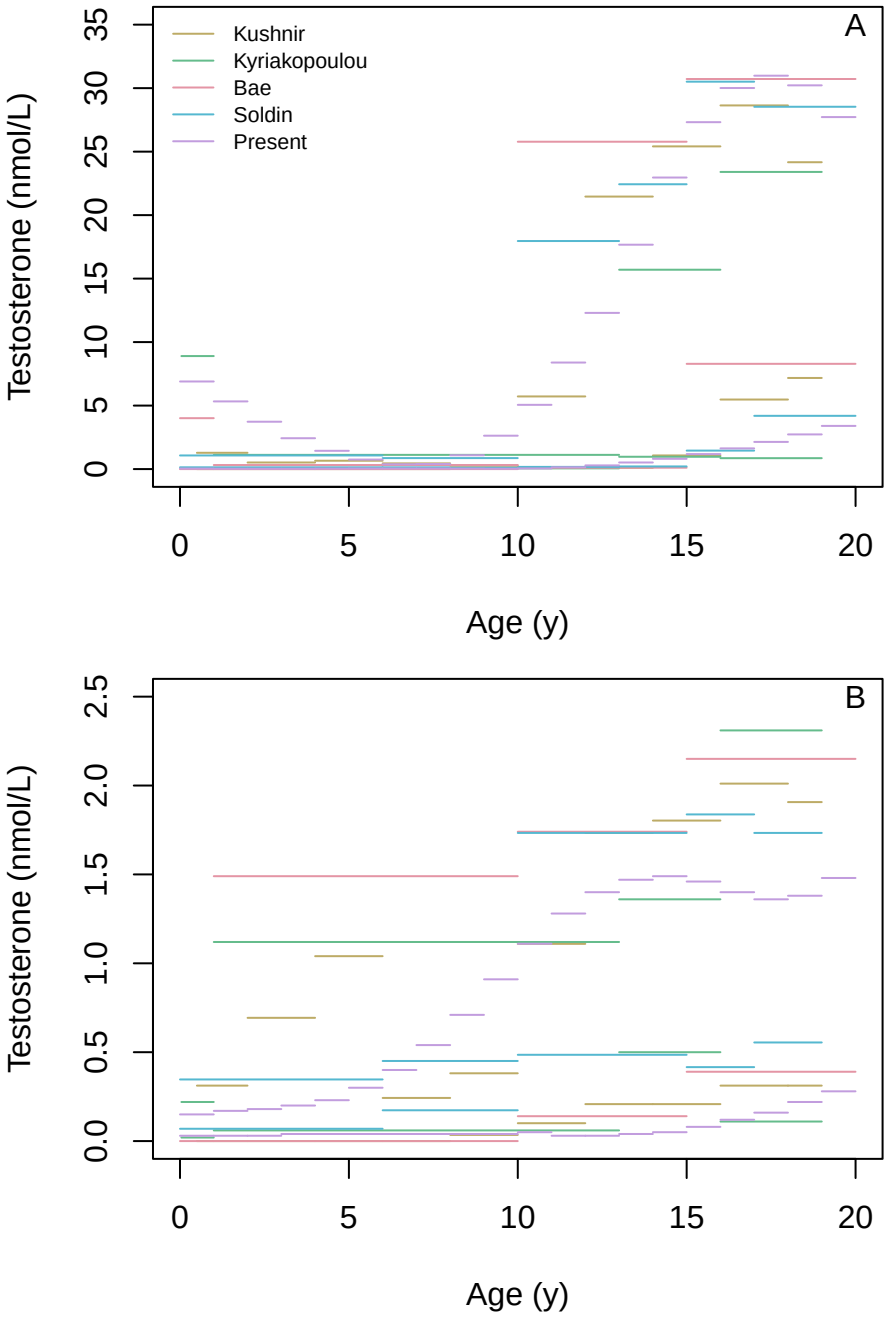

Figure 3: A graphical comparison of reference intervals for total tesotsterone by LC-MS/MS from various studies for male (A) and female (B) children. Lower and upper reference intervals for a given study are shown as horizontal lines.

A weakness of the present study in comparison to others is the relative dearth of subjects in the age-category of 0–12 months (N=8 F and N=11 M) which means that subjects demonstrating the phenomenon of mini-puberty [26] are few. While we do see elevated T for males in this age-category, we do not see them in our female data, similar to Kyriakopoulou et al. However, Bae et al do have a large number of female subjects less than 1 year of age showing increased T levels as does a subsequent study from the same group as Kyriakopoulou et al [27] using Roche Cobas 8000 for both T and SHBG. The phenomenon of mini-puberty is a potential hazard in continuous fitting algorithms because the age window where mini-puberty occurs is so narrow and the androgen concentrations so relatively high that the modeled centile functions may have a shallower slope than is required to accurately reflect physiology. In this sense a dedicated (i.e. binned) analysis of results for patients less than 1 year of age may be warranted.

Another weakness of this study is that androgen and SHBG concentration is dependent on Tanner Stage for pubescent males and females and are also affected by phase of the menstrual cycle and/or the use of oral contraceptives. Given the anonymity of the specimens, this information was not available.

This study has also not taken into account the fact that median albumin concentrations by age are not fixed at 43 g/L throughout life but show age-dependence, being lower in early childhood–especially in the neonatal period and in children under 4 years [28]. While we acknowledge this, attempts to address it would not have practical impact. The Vermeulen equation itself is really a *metric* of free testosterone calculated by means of mass-action using estimates of the binding coefficients of albumin and SHBG from studies now quite old [29] and estimates of FT and BAT are not strongly affected by albumin concentration. In this sense, we make the assumption that if the reference intervals are determined with the same albumin estimates as the patient, then results will be interpretable, though not applicable to other analytical methodologies.

Pediatric reference intervals for SHBG using the Roche Cobas e411 and e601 methods have been reported previously [27,30] though our results can only be superficially compared because of differences in biochemical and statistical analysis. Zec et al only provide reference interval results for children aged 1–10 y and obtain results of 22.7–166.5 nmol/L for females and 21.5–186.2 nmol/L for boys. While these do not seem incompatible with the present study, detailed comparison is not possible. The more recent study from the CALIPER group [27] is also difficult to compare because samples > 200 nmol/L were not diluted and re-run so as to obtain a numerical result–all results > 200 are replaced with 200.

We are not aware of free or bioavailable testosterone pediatric reference interval studies using LC-MS/MS for the T measurement though results for the Roche Cobas e411 [30] and Abbott Architect ci4100 are reported [31].

The strength of this study, and continuous reference intervals in general, is that we did not need to make any assumptions or arbitrary age partitions. Non-parametric continuous intervals use all the data, are robust to outliers, and avoid large jumps between intervals at puberty. Another advantage of continuous intervals is the ability to calculate point estimates for any age using the curve

model. For example, the lower, median, and upper limits for testosterone in a female aged 14.5, is determine with the code: `round(predict(fm, newdata = data.frame(age=14.5)),2)`, yielding: 0.06, 0.56, 1.49; the model is `fm`, we add our age of interest as a `data.frame` and surround the call with `round` to get 2 decimal places (as opposed to the default 8). This can be helpful in challenging cases and part of the discussion with a clinician. Such models may also be shared as web applications (e.g. RShiny [32]) to allow users to generate point estimates themselves. Though it not possible to implement fully continuous age-dependent reference intervals into a laboratory information system, point estimates naturally permit reference interval estimates that are as granular as month-by-month or year-by-year.

5. Conclusion

In this study, we established continuous reference intervals for T, SHBG, and calculated free and biovailable testosterone in males and females under the age of 20. Reference intervals are an essential tool against which to evaluate results of individual patients for clinical decision-making. Continuous reference intervals are a superior method for determining intervals where values vary with age. Continuous intervals avoid problems with arbitrary age partitions, sharp differences between age group, and data sparsity. In, particular non-parametric methods have advantages in using all of the data (rather than age partitions), does not require arbitrary removal of outliers, and is resistant to asymmetric, non-normality, and heteroscedastic (different variance with age) data [22]. We attempted to describe the process of generating such intervals in a reproducible way such that readers could derive their own continuous intervals for this or other analytes. The source code for this paper can be downloaded at https://github.com/drdanholmes/jmsacl_continuous_reference_interval.

6. Conflicts of Interest

The authors have no relevant conflicts of interest to disclose.

References

[1] O.P. Soldin, H. Sharma, L. Husted, S.J. Soldin, Pediatric reference intervals for aldosterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry, Clinical Biochemistry. 42 (2009) 823–827.

[2] D. Konforte, J.L. Shea, L. Kyriakopoulou, D. Colantonio, A.H. Cohen, J. Shaw, D. Bailey, M.K. Chan, D. Armbruster, K. Adeli, Complex biological pattern of fertility hormones in children and adolescents: A study of healthy children from the CALIPER cohort and establishment of pediatric reference intervals, Clinical Chemistry. 59 (2013) 1215–1227.

[3] R.F. Greaves, J. Pitkin, C.S. Ho, J. Baglin, R.W. Hunt, M.R. Zacharin, Hormone modeling in preterm neonates: Establishment of pituitary and steroid hormone reference intervals, The Journal of Clinical Endocrinology & Metabolism. 100 (2015) 1097–1103.

[4] A. Kulle, F.G. Riepe, D. Melchior, O. Hiort, P. 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, The Journal of Clinical Endocrinology & Metabolism. 95 (2010) 2399–2409.

[5] M.M. Kushnir, T. Blamires, A.L. Rockwood, W.L. Roberts, B. Yue, E. Erdogan, A.M. Bunker, A.W. Meikle, Liquid chromatography–tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals, Clinical Chemistry. 56 (2010) 1138–1147.

[6] L. Kyriakopoulou, M. Yazdanpanah, D. Colantonio, M. Chan, C. Daly, K. Adeli, A sensitive and rapid mass spectrometric method for the simultaneous measurement of eight steroid hormones and CALIPER pediatric reference intervals, Clinical Biochemistry. 46 (2013) 642–651.

[7] Y.J. Bae, R. Zeidler, R. Baber, M. Vogel, K. Wirkner, M. Loeffler, U. Ceglarek, W. Kiess, A. Körner, J. Thiery, others, Reference intervals of nine steroid hormones over the life-span analyzed by LC-MS/MS: Effect of age, gender, puberty, and oral contraceptives, The Journal of Steroid Biochemistry and Molecular Biology. 193 (2019) 105409.

[8] M. Healy, Notes on the statistics of growth standards, Annals of Human Biology. 1 (1974) 41–46.

[9] T.J. Cole, P.J. Green, Smoothing reference centile curves: The LMS method and penalized likelihood, Statistics in Medicine. 11 (1992) 1305–1319.

[10] P. Royston, D.G. Altman, Regression using fractional polynomials of continuous covariates: Parsimonious parametric modelling, Journal of the Royal Statistical Society: Series C (Applied Statistics). 43 (1994) 429–453.

[11] R.A. Rigby, D.M. Stasinopoulos, Generalized additive models for location, scale and shape, Journal of the Royal Statistical Society: Series C (Applied Statistics). 54 (2005) 507–554.

[12] R. Koenker, Quantile regression, Cambridge University Press, 2005. https://doi.org/10.1017/CBO9780511754098.

[13] V.M. Muggeo, M. Sciandra, A. Tomasello, S. Calvo, Estimating growth charts via nonparametric quantile regression: A practical framework with application in ecology, Environmental and Ecological Statistics. 20 (2013) 519–531.

[14] V.M. Muggeo, F. Torretta, P.H. Eilers, M. Sciandra, M. Attanasio, Multiple smoothing parameters selection in additive regression quantiles, Statistical Modelling. (2020) 1471082X20929802.

[15] V.M. Muggeo, quantregGrowth: Growth charts via smooth regression quantiles with automatic smoothness estimation and additive terms, (2021 (accessed July 7, 2021)).

[16] R.E. Gislefoss, T.K. Grimsrud, L. Mørkrid, Stability of selected serum proteins after long-term storage in the janus serum bank, Clinical Chemistry and Laboratory Medicine. 47 (2009) 596–603.

[17] D. Handelsman, R. Desai, M. Seibel, D. Le Couteur, R. Cumming, Circulating sex steroid measurements of men by mass spectrometry are highly reproducible after prolonged frozen storage, The Journal of Steroid Biochemistry and Molecular Biology. 197 (2020) 105528.

[18] D. French, Development and validation of a serum total testosterone liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay calibrated to NIST SRM 971, Clinica Chimica Acta. 415 (2013) 109–117.

[19] D. French, J. Drees, J.A. Stone, D.T. Holmes, J.G. van der Gugten, Comparison of four clinically validated testosterone LC-MS/MS assays: Harmonization is an attainable goal, Clinical Mass Spectrometry. 11 (2019) 12–20.

[20] W. De Ronde, Y.T. Van Der Schouw, H.A. Pols, L.J. Gooren, M. Muller, D.E. Grobbee, F.H. De Jong, Calculation of bioavailable and free testosterone in men: A comparison of 5 published algorithms, Clinical Chemistry. 52 (2006) 1777–1784.

[21] V.M.R. Muggeo, F. Torretta, P.H.C. Eilers, M. Sciandra, M. Attanasio, Multiple smoothing parameters selection in additive regression quantiles, Statistical Modelling. 0 1471082X20929802. https://doi.org/10.1177/1471082X20929802.

[22] V.M. Muggeo, M. Sciandra, A. Tomasello, S. Calvo, Estimating growth charts via nonparametric quantile regression: A practical framework with application in ecology, Environmental and Ecological Statistics. 20 (2013) 519–531.

[23] S. Asgari, V. Higgins, C. McCudden, K. Adeli, Continuous reference intervals for 38 biochemical markers in healthy children and adolescents: Comparisons to traditionally partitioned reference intervals, Clinical Biochemistry. 73 (2019) 82–89.

[24] J.W. Hardin, The sandwich estimate of variance, in: Maximum Likelihood Estimation of Misspecified Models: Twenty Years Later, Emerald Group Publishing Limited, 2003.

[25] D.T. Holmes, K.A. Buhr, Widespread incorrect implementation of the hoffmann method, the correct approach, and modern alternatives, American Journal of Clinical Pathology. 151 (2019) 328–336.

[26] M.G. Forest, A.M. Cathiard, J.A. Bertrand, Evidence of testicular activity in early infancy, The Journal of Clinical Endocrinology & Metabolism. 37 (1973) 148–151.

[27] M.K. Bohn, V. Higgins, S. Asgari, F. Leung, B. Hoffman, J. Macri, K. Adeli, Paediatric reference intervals for 17 roche cobas 8000 e602 immunoassays in the CALIPER cohort of healthy children and adolescents, Clinical Chemistry and Laboratory Medicine (CCLM). 57 (2019) 1968–1979.

[28] G. Lockitch, A. Halstead, S. Albersheim, C. MacCallum, G. Quigley, Age- and sex-specific pediatric reference intervals for biochemistry analytes as measured with the ektachem-700 analyzer., Clinical Chemistry. 34 (1988) 1622–1625.

[29] A. Vermeulen, T. Stoica, L. Verdonck, The apparent free testosterone concentration, an index of androgenicity, The Journal of Clinical Endocrinology & Metabolism. 33 (1971) 759–767.

[30] I. Zec, I. Kučak, I. Begčević, A.-M. Šimundić, D. Tišlarić-Medenjak, Ž.B. Megla, N. Vrkić, Reference intervals for reproductive hormones in prepubertal children on the automated roche cobas e 411 analyzer, Clinical Biochemistry. 45 (2012) 1206–1212.

[31] J.E. Raizman, F. Quinn, D.A. Armbruster, K. Adeli, Pediatric reference intervals for calculated free testosterone, bioavailable testosterone and free androgen index in the CALIPER cohort, Clinical Chemistry and Laboratory Medicine (CCLM). 53 (2015) e239–e243.

[32] W. Chang, J. Cheng, J. Allaire, C. Sievert, B. Schloerke, Y. Xie, J. Allen, J. McPherson, A. Dipert, B. Borges, Shiny: Web application framework for R, (2021). https://CRAN.R-project.org/package=shiny.