Clinical Biochemistry 73 (2019) 82–89

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Continuous reference intervals for 38 biochemical markers in healthy children and adolescents: Comparisons to traditionally partitioned reference intervals

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ARTICLE INFO

Keywords:
Pediatric
Continuous reference intervals
Biochemical markers

ABSTRACT

Background: Reference intervals have traditionally been partitioned by age based on statistical significance and physiological relevance. However, analyte concentration does not change abruptly with age, but rather dynamically. In this study, we establish biochemical marker continuous reference intervals for a Canadian population using healthy pediatric reference individuals and compare these to partitioned reference intervals.

Methods: Continuous reference intervals spanning 1–18.5 years of age were established using data from healthy CALIPER children and adolescents aged 6 months- < 19 years. Continuous reference intervals (i.e. 2.5th and 97.5th quantiles) were generated by nonparametric quantile regression via penalized splines with non-crossing constraints. Abnormal flagging rates of established continuous reference intervals were compared to previously established age-partitioned CALIPER reference intervals for five biochemical markers using internal (CALIPER) and external (i.e. Canadian Health Measures Survey (CHMS)) datasets.

Results: Continuous reference intervals were determined for 38 biochemical markers, with 21 markers requiring sex-specific reference intervals. Despite similar total flagging rates to partitioned reference intervals, continuous reference intervals appeared to provide a more consistent and accurate estimation of reference limits for biomarkers with more complex age-related changes, including alkaline phosphatase and phosphate.

Conclusions: This is the first report of continuous biochemical marker reference intervals based on a healthy Canadian pediatric population. Reference limit point estimates based on continuous reference intervals are provided to aid clinical implementation. Continuous reference intervals offer a better estimation of dynamic changes in biochemical marker reference values with age, resulting in improved laboratory test result interpretation and clinical decision making in pediatrics.

1. Introduction

Appropriate laboratory test result interpretation depends on accurate health-associated benchmarks, including reference intervals and decision limits. Reference intervals are commonly defined as the central 95% of laboratory test results expected in a healthy reference population [1]. Reference interval establishment can be challenging for pediatrics due to recruitment difficulties, resource requirements, and vast physiological changes throughout childhood and adolescence [2]. To address this gap, The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) developed a database of pediatric age- and sex-specific reference intervals based on thousands of healthy

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase without pyridoxal phosphate; ALT ACT, alanine aminotransferase with pyridoxal phosphate; apoA1, apolipoprotein A1; apoB, apolipoprotein B; ASO, anti-streptolysin O; AST, aspartate aminotransferase without pyridoxal phosphate; AST ACT, aspartate aminotransferase with pyridoxal phosphate; C3, complement component 3; C4, complement component 4; CALIPER, Canadian Laboratory Initiative on Pediatric Reference Intervals; ChE, cholinesterase; CLSI, Clinical and Laboratory Standards Institute; CO_2, carbon dioxide; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; LDH, lactate dehydrogenase

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https://doi.org/10.1016/j.clinbiochem.2019.08.010
Received 16 July 2019; Received in revised form 11 August 2019; Accepted 21 August 2019
Available online 22 August 2019

Table 1
Comparison of flagging rates between partitioned and continuous reference intervals (CALIPER).

Analyte	Sex	Sample Size	Lower Reference Limit Flagging Rate (%)				Upper Reference Limit Flagging Rate (%)			
			Partitioned		Continuous		Partitioned		Continuous	
			Total	Mean Median Midpoint Range	Total	Mean Median Midpoint Range	Total	Mean Median Midpoint Range	Total	Mean Median Midpoint Range
ALP	F	459	0.87	0.66 0 **1.79** 0–3.57	**2.18**	**1.79** 0 3.45 0–6.90	2.18	2.15 0 5.56 0–11.11	2.18	**2.4** 0 **4.55** 0–9.09
	M	442	**2.26**	**2.3** **1.25** 4.35 0–8.7	2.04	1.95 0 **3.45** 0–6.90	1.81	**2** 0 5 0–10.00	1.81	1.66 0 **3.03** 0–6.06
Calcium	B	886	1.69	**1.49** 1.8 1.82 0–3.64	1.69	1.44 1.8 1.82 0–3.64	**2.37**	**2.72** **2.22** 5.17 0–10.34	1.69	1.68 0 **3.49** 0–6.98
Cholesterol	B	920	**2.39**	**2.39** **2.42** 3.97 0–7.94	1.74	1.69 0.66 **3.18** 0–6.35	**2.39**	**2.33** **2.58** **2.59** 0–5.17	2.07	1.93 1.79 2.83 0–5.66
Creatinine	F	516	**2.33**	**2.41** 0 5.77 0–11.54	2.13	1.96 **1.19** **3.85** 0–7.69	1.36	1.04 0 **2.94** 0–5.88	**2.13**	**2.17** 0 5.27 0–10.53
	M	473	**2.33**	**2.58** 0 6.25 0–12.50	1.9	1.91 0 **3.23** 0–6.45	**2.96**	**2.82** **2.68** **4.55** 0–9.09	1.9	1.79 0 7.15 0–14.29
Phosphate	F	458	**2.62**	**2.94** **2.66** 5.56 0–11.11	1.75	1.56 0 **3.57** 0–7.14	1.97	2.32 0 5.27 0–10.53	1.97	**2.41** 0 **4.17** 0–8.33
	M	444	1.58	1.59 0 5.56 0–11.11	**2.25**	**2.19** 0 5.56 0–11.11	**2.03**	**1.96** 0 5.27 0–10.53	1.35	1.14 0 **2.94** 0–5.88

ALP, alkaline phosphatase; B, both sexes; F, female; M, male.
Mean, median, midpoint, and range were calculated based on flagging rates obtained for each 1-year age interval (i.e. $n = 18$).
Bolded indicates values with a total, mean, median, or midpoint flagging rate closer to 2.5%, which indicate a more accurate reference interval estimation.

children and adolescents for over 170 biomarkers of pediatric disease [3].

The Clinical and Laboratory Standards Institute (CLSI) [1] recommends developing separate reference intervals for subgroups with different normative values. Statistical tests used to confirm physiologically relevant partitions include Harris and Boyd [4], Lahti [5], index of individuality [6], or cluster analysis [7]. However, age breakpoints arbitrarily simplify the complex physiological relationship between analyte concentration and pediatric age, particularly for biomarkers with extensive age-related changes (e.g. alkaline phosphatase (ALP) and creatinine) [8,9]. Result interpretation is further complicated when a patient's age classifies them for a new age-specific reference interval and the difference between adjacent reference intervals is large.

Continuous reference intervals can more accurately represent age-related biomarker concentration changes. Zierk and colleagues established continuous pediatric reference intervals for hematological and biochemical parameters through a retrospective analysis of clinical laboratory data [10,11], a similar approach to other studies [12–14]. Indeed, indirect methods provide a fast, more feasible and less costly approach for calculating reference intervals that uses data based on the actual preanalytical and analytical conditions used in routine clinical practice [15]. Direct sampling methods are preferred by some authors [16], which permit the use of defined inclusion and exclusion criteria, reduces variation due to preanalytical and analytical factors, and bypasses potentially inaccurate estimations when using a cohort comprised of healthy and unhealthy subjects. However, limited studies use direct sampling methods to establish pediatric continuous reference intervals [17–19]. The German Health Interview and Examination Survey for Children and Adolescents (KiGGS) has established continuous reference intervals for serum and urine laboratory biomarkers using healthy children and adolescents [19]. Another such study established continuous reference intervals based on a healthy Brazilian pediatric population but only for a limited number of transaminases [17]. Yet, no such study has been performed using a Canadian pediatric reference population.

The objectives of the present study were to [1] establish pediatric continuous reference intervals for 38 biochemical markers using CALIPER reference data, [2] calculate reference limit point estimates based on continuous reference intervals, and [3] compare flagging rates for newly established continuous reference intervals and previously established age-partitioned CALIPER reference intervals.

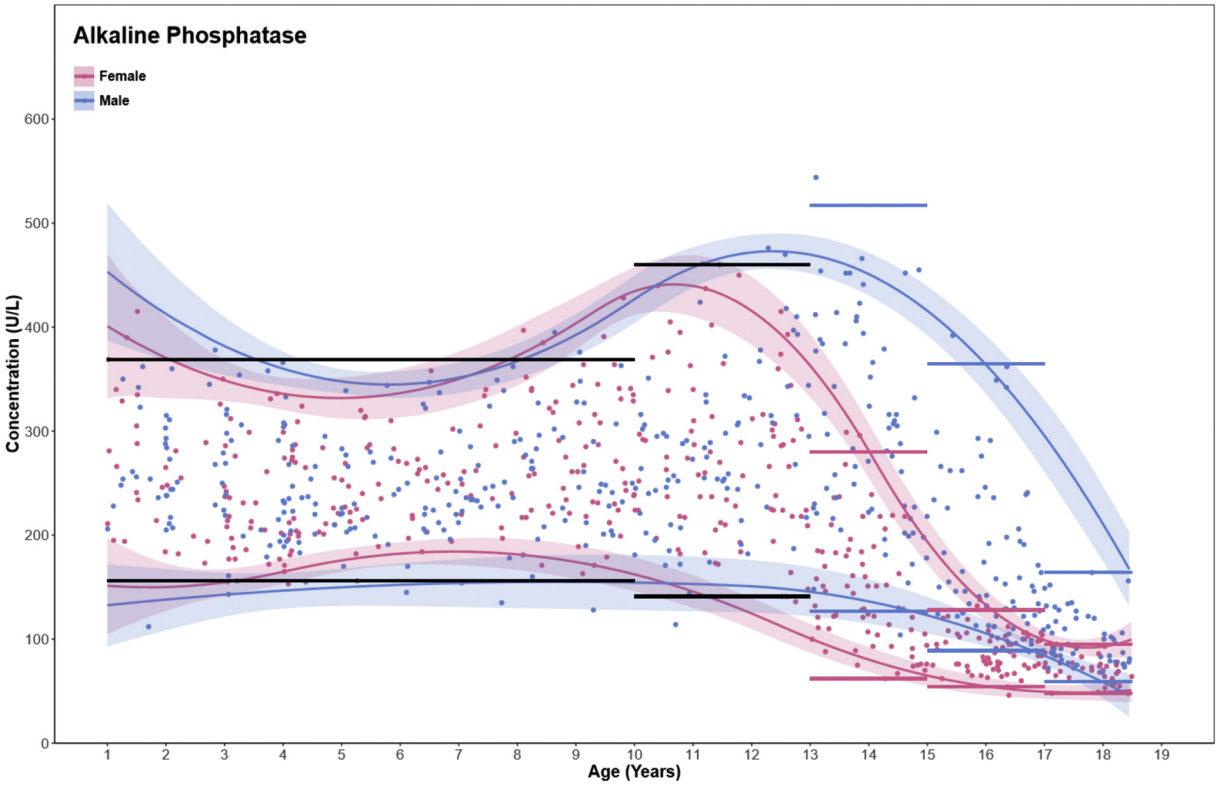

Fig. 1. Comparison of continuous reference intervals and partitioned reference intervals using CALIPER reference data for alkaline phosphatase (ALP) for children and adolescents 1–18.5 years of age. Female reference values, continuous reference intervals, and partitioned reference intervals are pink; male reference values, continuous reference intervals, and partitioned reference intervals are blue; partitioned reference intervals for both sexes are black. 95% confidence intervals for continuous reference intervals are indicated by the shaded area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

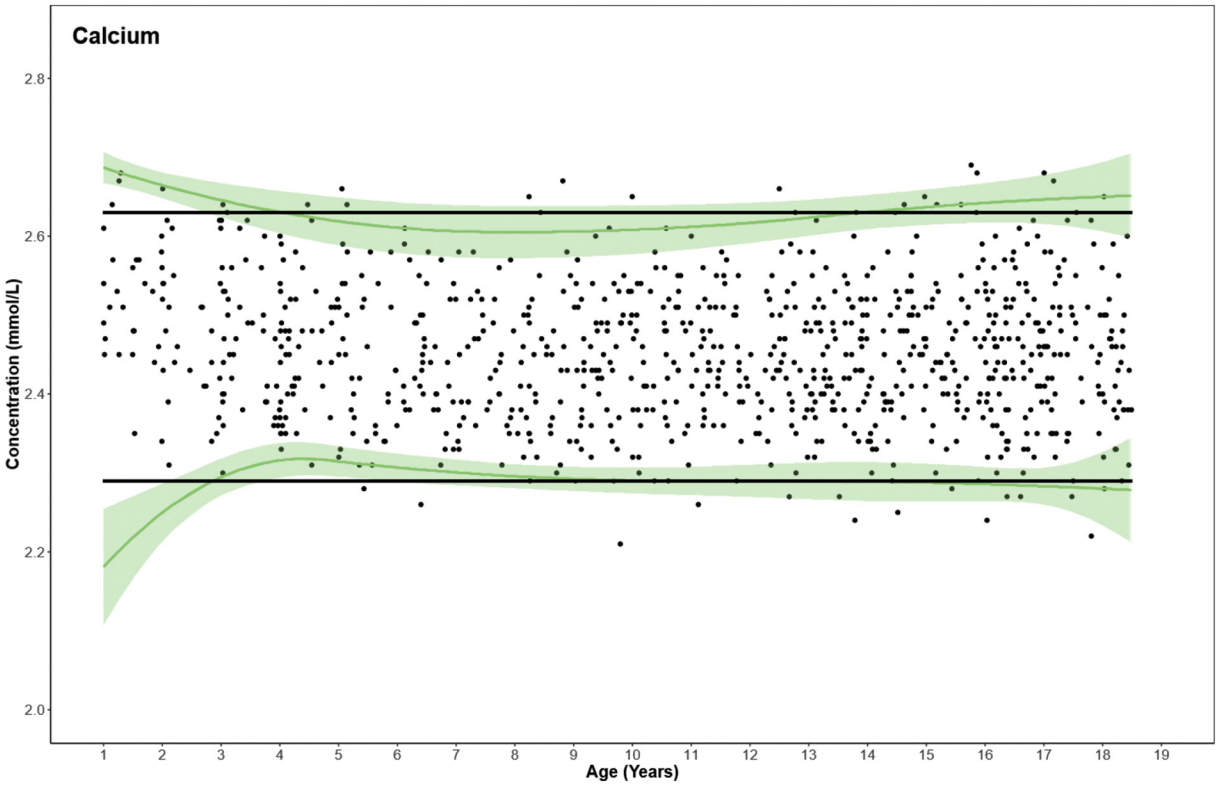

Fig. 2. Comparison of continuous reference intervals and partitioned reference intervals using CALIPER reference data for calcium for children and adolescents 1–18.5 years of age. Both sexes are shown. 95% confidence intervals for continuous reference intervals are indicated by the shaded area.

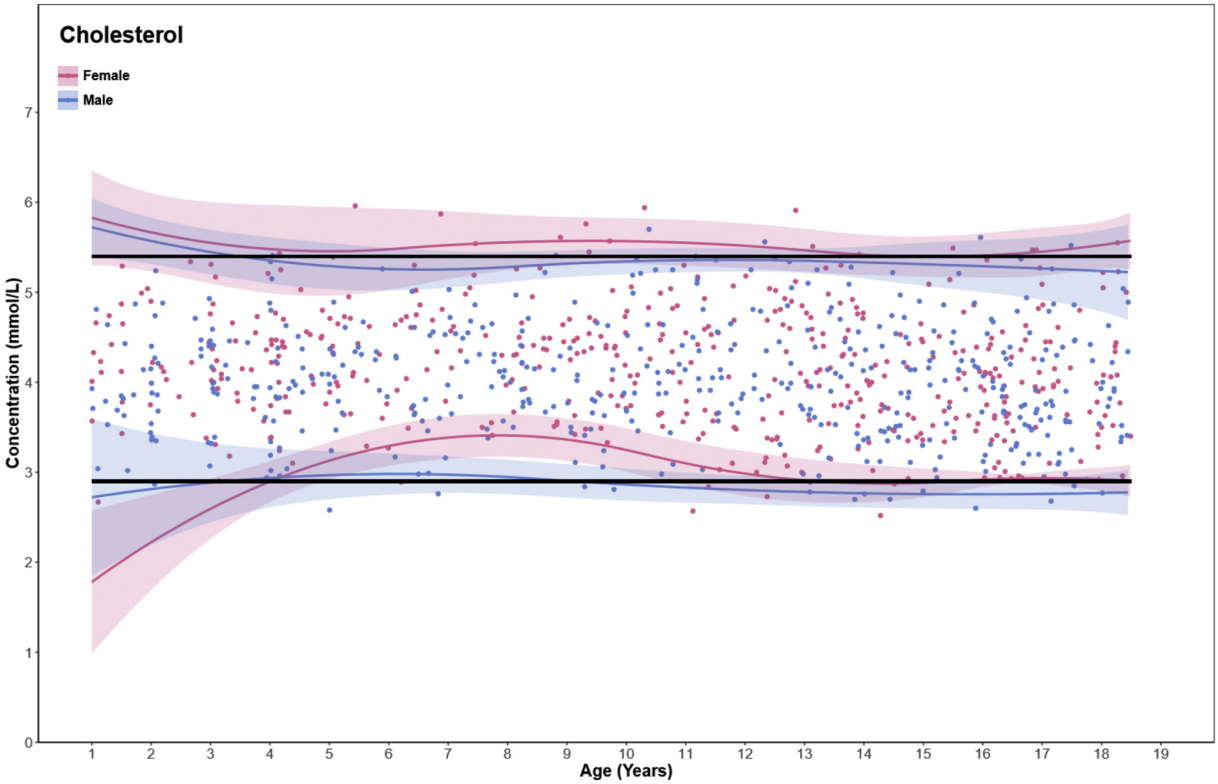

Fig. 3. Comparison of continuous reference intervals and partitioned reference intervals using CALIPER reference data for cholesterol for children and adolescents 1–18.5 years of age. Female reference values, continuous reference intervals, and partitioned reference intervals are pink; male reference values, continuous reference intervals, and partitioned reference intervals are blue; partitioned reference intervals for both sexes are black. 95% confidence intervals for continuous reference intervals are indicated by the shaded area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. Participant recruitment, sample acquisition, and sample analysis

This study was approved by the Research Ethics Board at The Hospital for Sick Children (Toronto, ON). Community children and adolescents were recruited as part of the CALIPER study as previously described [8]. Study participation included written informed consent, completing a short questionnaire, and donation of a blood sample. The ethnic composition was based on the 2006 Canadian census for Ontario. Data from participants 6 months- < 19 years (n = 788–1072) were analyzed. Subjects < 6 months of age exhibited high variability in reference values, often greater than that found during puberty. The rapid change in concentration and variability resulted in an inaccurate continuous reference interval estimation and therefore subjects < 6 months of age were removed from analysis. Samples were collected in serum separator tubes (SST; BD), centrifuged, separated within 4 h of collection, and stored at −80 °C until testing. Results for 38 biochemical markers were obtained using the Abbott ARCHITECT c8000 system. Analytical and calibration/traceability information has been previously described [8].

2.2. Continuous reference interval determination

Statistical analysis was performed using R software version 3.5.1 [20,21]. While several methods can determine continuous reference intervals, nonparametric quantile regression is robust to outliers, influential observations, and deviations from symmetry, normality, linearity, and homogeneity of variance. Preliminary continuous reference intervals were established using various nonparametric quantile regression methods, including local polynomial quantile regression and quantile regression with smoothing splines including restricted cubic, natural, and B-splines. Ultimately, nonparametric quantile regression via penalized splines with non-crossing constraints was used to establish continuous reference intervals (i.e. 2.5th and 97.5th quantiles). Bootstrap replicates (case resampling) were performed to quantify uncertainty in the estimates and 95% pointwise confidence intervals for the fitted quantiles are shown. This method was chosen because it is flexible enough to account for the nonlinear effect of age and avoids issues related to the knot selection process [21]. "K-fold" cross-validation determined the optimal smoothing parameter (i.e. λ) value and the model was fitted using the selected smoothing parameter [21]. When the lower confidence interval limit was negative, it was changed to 0. If sex differences for a biochemical marker were previously determined to be statistically significant for at least one age partition [8], continuous reference intervals were established separately for each sex in the current study. Otherwise, sexes were combined to establish one continuous reference interval for both sexes.

Data from subjects 6 months- < 19 years were used to determine the quantile regression line, while data from 1 to 18.5 years (n = 721–991) are depicted on the scatterplots. This ensured data beyond the upper and lower end of the age range were considered and the continuous upper and lower reference limits reflect the true age trends around 1 year and 18.5 years. Including data beyond the lowest (i.e. 1 year) and highest (i.e. 18.5 years) age narrowed the confidence intervals at these extremes of age, increasing the accuracy of the estimation. Therefore, only the regression line from 1 to 18.5 years can be reported with confidence. Exceptions to this approach were transferrin and GGT, in which it was arbitrarily decided to not include data from subjects 6 months- < 1 year due to high variability in reference values < 1 year that led to inaccurate estimations by the nonparametric quantile regression model. Lower and upper reference limit point estimates for every 6 months of age were calculated.

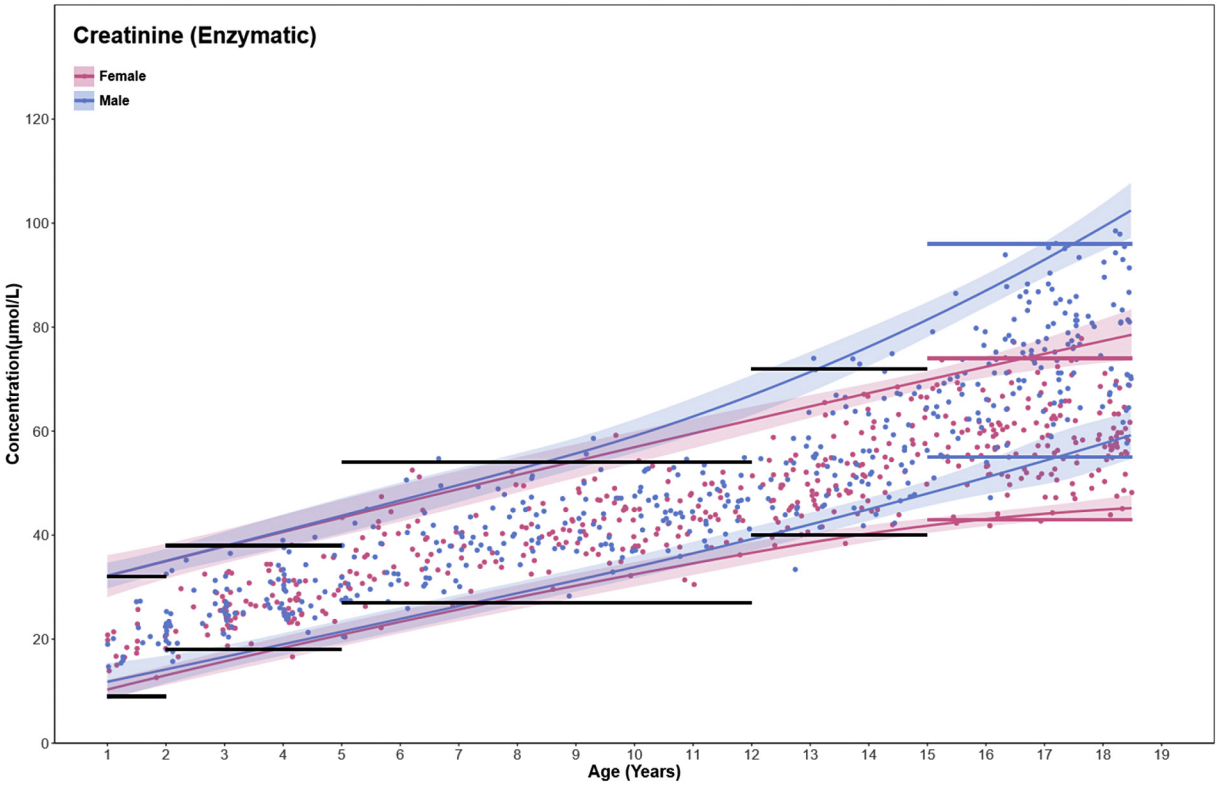

Fig. 4. Comparison of continuous reference intervals and partitioned reference intervals using CALIPER reference data for creatinine (enzymatic) for children and adolescents 1–18.5 years of age. Female reference values, continuous reference intervals, and partitioned reference intervals are pink; male reference values, continuous reference intervals, and partitioned reference intervals are blue; partitioned reference intervals for both sexes are black. 95% confidence intervals for continuous reference intervals are indicated by the shaded area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Comparison of discrete and continuous reference intervals

Continuous reference intervals were compared to age-partitioned reference intervals previously established using the same CALIPER dataset [8] for ALP, calcium, cholesterol, creatinine, and phosphate, which represent different concentration distributions. Continuous and partitioned reference intervals were compared by lower and upper limit flagging rates. The total flagging rate (1–18.5 years) and the flagging rate for each 1-year age interval were calculated using CALIPER reference data. For the 1-year age interval flagging rates, the mean, median, midpoint, and range were obtained to compare the consistency in flagging rates across age. A flagging rate close to 2.5% for both the upper and lower reference limits for each 1-year of age indicates that the reference interval accurately represents the relationship between age and analyte concentration. Flagging rates for continuous and partitioned reference intervals were also determined using the Canadian Health Measures Survey (CHMS) data (ages 3–18.5 years) obtained using the Ortho Vitros 5600 FS analyzer for the same five analytes [9] to validate their accuracy using an external dataset. While individual data points were used to establish CHMS reference intervals, data points used for scatterplots and flagging rates represented the mean of ≥11 closely associated results, due to confidentiality protocols of Statistics Canada [9].

3. Results

3.1. Continuous reference interval estimation

Continuous reference intervals for children and adolescents aged 1–18.5 years were determined for 38 biochemical markers (Supplemental Figs. 1–38). Sex-specific continuous reference intervals were calculated for 21 biochemical markers, due to sex-specific differences [8], and sexes were combined for the remaining 17 biochemical markers. Six-month point estimates for lower and upper reference limits and corresponding confidence intervals are provided for 6 months- < 19 years, although point estimates at age 6 months and 19 years should be interpreted with caution, due to wide confidence intervals (Supplemental Tables 1–38).

To ensure continuous reference intervals were accurately estimated, data from participants aged 6 months- < 1 year and 18.5- < 19 years were included for analysis (Supplemental Figs. 39–76). Exceptions included transferrin and GGT, where including data from subjects < 1 year lead to an overestimation of the GGT upper reference limit for 1- < 5 years (Supplemental Fig. 77) and an underestimation of the transferrin lower reference limit for 1- < 3 years (Supplemental Fig. 78). Therefore, only data from 1- < 19 years were used (Supplemental Figs. 60 and 73), while the final plots show continuous reference intervals for 1–18.5 years (Supplemental Fig. 22 and 35).

3.2. Comparing continuous and partitioned reference intervals using CALIPER reference data

Flagging rates for continuous and partitioned reference intervals using CALIPER data for ALP, calcium, creatinine, cholesterol, and phosphate are shown in Table 1 and Figs. 1–5. For ALP (Fig. 1; Table 1), continuous and partitioned upper reference limit total flagging rates were the same (i.e. 2.18% (females); 1.81% (males)), while the continuous lower reference limits for females and partitioned lower reference limits for males had a flagging rate closer to 2.5%. For ALP 1-year age interval flagging rates, the midpoint was closer to 2.5% for upper continuous than partitioned reference limits, suggesting a more accurate estimation of the 97.5th percentile. However, this advantage

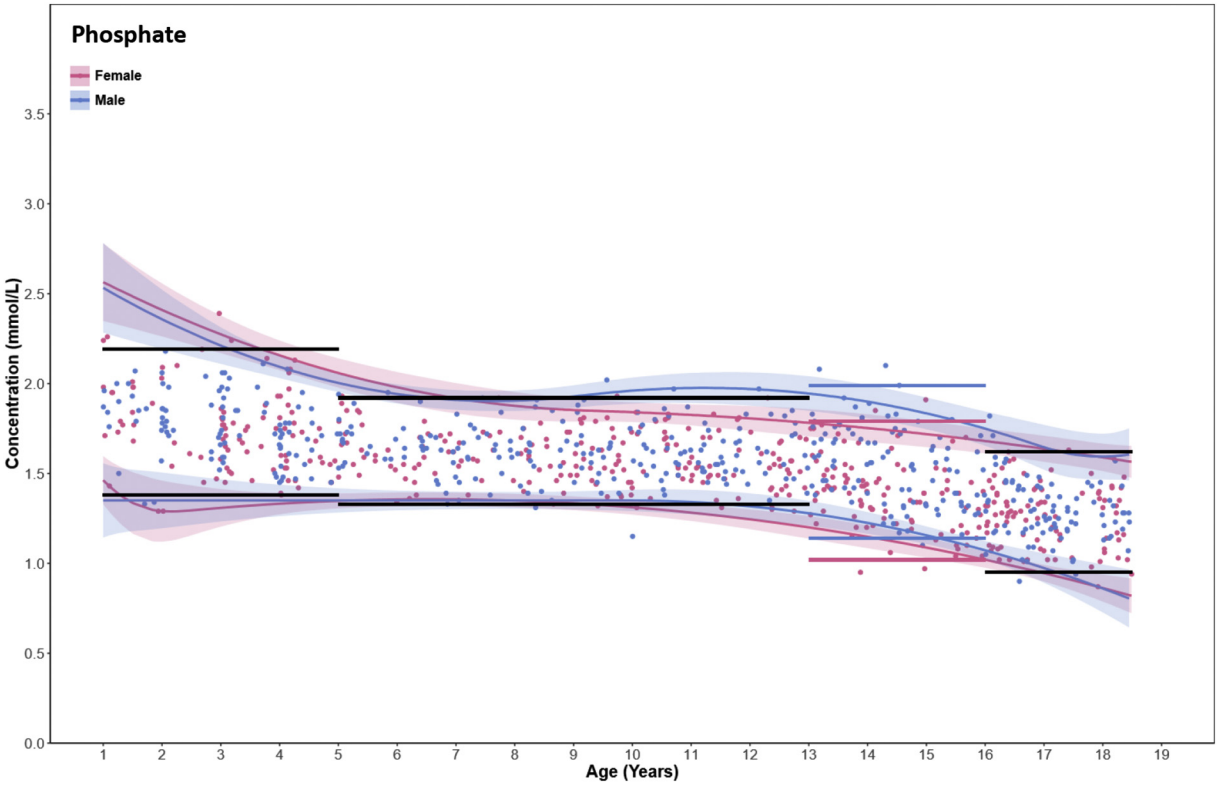

Fig. 5. Comparison of continuous reference intervals and partitioned reference intervals using CALIPER reference data for phosphate for children and adolescents 1–18.5 years of age. Female reference values, continuous reference intervals, and partitioned reference intervals are pink; male reference values, continuous reference intervals, and partitioned reference intervals are blue; partitioned reference intervals for both sexes are black. 95% confidence intervals for continuous reference intervals are indicated by the shaded area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was less clear for the lower reference limits. Phosphate exhibits a similar age-related trend as ALP (Fig. 5). Total phosphate flagging rates were the same for continuous and partitioned upper female reference limits (i.e. 1.97%), although partitioned lower reference limits exhibited a flagging rate closer to 2.5% for both sexes. Nevertheless, continuous upper reference limit flagging rates for 1-year age intervals had a narrower range and midpoint closer to 2.5%. A less clear advantage of continuous reference intervals was evident for the lower reference limit. Creatinine concentration gradually increased with age and was higher in adolescent males compared to females (Fig. 4). However, neither partitioned nor continuous reference intervals offered a clear advantage, likely due to the more gradual change in creatinine concentration with age. Furthermore, as numerous (i.e. 6) age partitions were previously established by CALIPER, age-partitioned reference intervals in this case offer reference limits of similar accuracy to continuous reference intervals.

Calcium lower reference limit total flagging rates were the same for continuous and partitioned (i.e. 1.69%), but were closer to 2.5% when using partitioned upper reference limits (Fig. 2; Table 1). For 1-year age interval flagging rates, the range and midpoint were the same for calcium lower reference limits, but the range was narrower and midpoint closer to 2.5% for continuous upper reference limits. A less clear advantage for continuous reference intervals is likely due to minimal variation in calcium concentration with age. Similarly, cholesterol changes minimally with age (Fig. 3), resulting in a less clear advantage of continuous over partitioned reference limits. Total flagging rates were closer to 2.5% using partitioned upper and lower reference limits (Table 1). However, when assessing the flagging rate for 1-year age intervals, the range was narrower and the midpoint closer to 2.5% for continuous, rather than partitioned, upper reference limits, with the opposite true for the lower reference limits.

3.3. Comparing continuous and partitioned reference intervals using an external dataset (CHMS)

Flagging rates for continuous and partitioned reference intervals were also calculated using an external dataset (i.e. CHMS) (Supplemental Table 39; Supplemental Figs. 79–83). For ALP, the total female upper reference limit flagging rate was the same for partitioned and continuous (i.e. 6.16%), while for males, continuous reference limits provided a more accurate estimation. Again, this advantage was less clear for the total lower reference limit flagging rate. When assessing 1-year flagging rates, continuous and partitioned reference intervals exhibited less variation in lower and upper reference limit flagging rates, respectively. Both phosphate and creatinine concentrations from the CHMS study were higher than CALIPER reference values, supported by little to no flagged values below the lower reference limit (continuous or partitioned). Creatinine and phosphate upper continuous reference intervals had a total flagging rate closer to 2.5% compared to partitioned reference intervals. For 1-year interval flagging rates, creatinine upper continuous reference limits exhibited less variation, with a mean and median closer to 2.5%.

Similar to creatinine and phosphate, calcium concentration measured in the CHMS study was higher than CALIPER, again resulting in no values flagged as below the lower reference limits. Partitioned reference intervals exhibited a total upper reference limit flagging rate closer to 2.5%, in addition to the mean and median flagging rates when assessing 1-year flagging intervals. Lastly, cholesterol continuous lower reference limit flagging rates were closer to 2.5%, while the opposite was true for upper reference limits. However, when assessing 1-year interval flagging rates, continuous and partitioned reference intervals performed equally well.

4. Discussion

Due to the profound changes in biochemical marker concentration throughout pediatric age [2], particularly during puberty, age-specific reference interval partitions are often developed based on both statistical (i.e. Harris and Boyd [4]) and physiological relevance. However, discrete age-partitioned reference intervals inherently reduce the complexity of the relationship between analyte concentration and age and inaccurately represent the onset and degree of concentration change. Partitioning reference intervals also reduces the sample size for any given partition and does not consider reference values in surrounding age partitions. Therefore, partitioning lessens the accuracy of reference interval estimations and can lead to an abrupt change in reference limits between two adjacent age partitions. Indeed, if there were infinite age partitions, age-partitioned reference intervals would provide an accurate estimation of the normative range. A continuous statistical model is therefore needed to estimate reference intervals when dynamic concentration changes with age are evident.

Continuous reference intervals reported here are similar to those established by others, including for ALT, AST, and GGT [10,17,18]. Serum transaminase measurement in pediatrics can be used to assess non-alcoholic fatty liver disease (NAFLD) in patients with the metabolic syndrome [22]. Hypertransaminasemia can also present in children with genetic/metabolic conditions, accounting for 20–30% of pediatric liver disease [23,24]. Bussler et al. established continuous reference intervals for serum transaminases using a healthy pediatric population (11 months – 16 years) by an LMS (i.e. lambda, mu, sigma) method [17]. We observed similar concentration patterns for ALT, with peak concentration for both sexes in infancy. Females exhibit a decline in ALT levels upon pubertal onset, while males maintain their ALT levels until later in puberty. While Zierk et al. [10] used an indirect sampling approach, similar age and sex trends in ALT levels were observed. The early decline in ALT levels was also observed by England et al., who developed continuous reference intervals for the first 5 years of life [18]. Our AST estimation was also similar to previously established continuous reference intervals using direct [17] and indirect [10] sampling methods, where levels gradually decline throughout the pediatric age, with a steeper decline in females. This sex difference may be due to changes in skeletal muscle during puberty, also highlighted by the steady increase in creatinine observed during puberty, with a greater rise in males. While Bussler et al. reported a sex difference for GGT, there was no sex difference in our cohort [8]. Nevertheless, the trend in GGT concentration with age mirrored an intermediate trend between those previously reported for males and females [10,17,19], with GGT increasing with age.

Continuous reference intervals are commonly established using the LMS method, which relies on Box-Cox transformation to achieve normality [17,18]. Nonparametric quantile regression via penalized splines with non-crossing constraints is more practical and flexible for constructing continuous reference intervals. This approach estimates the conditional quantile as a smooth function of age regardless of its distribution, making it valuable in the absence of a suitable transformation method to achieve normality. Importantly, cross-validation to determine the optimal smoothing parameter also improves its reproducibility. To further improve the accuracy of our estimation, data from subjects below and above the age range were included for quantile regression determination. The World Health Organization (WHO) growth reference curves for children and adolescents aged 5–19 years [25] used a similar approach in which data from subjects aged 18 months-24 years was used to ensure a smooth transition with previously established curves for children < 5 years [26] and adult BMI cut-offs [27]. While our cohort was limited to the amount of additional data that could be included while still providing estimates for a sufficiently wide pediatric age range, we included data for an additional 6 months below and above the age range to ensure our estimations considered adjacent trends in biochemical marker concentration.

Continuous reference intervals can represent detailed physiological changes that may be masked by discrete age partitions [13]. We directly compared the accuracy of continuous and partitioned reference intervals established using the same dataset. Both continuous and partitioned reference limits represent 2.5th and 97.5th percentiles and thus their accuracy can be assessed by their ability to flag 2.5% of CALIPER reference data as high and 2.5% as low. Indeed, accurate reference intervals should exhibit 1–4% of results outlying the upper and lower reference limits, with the most ideal value being 2.5% [4]. In theory, continuous reference intervals would perform consistently with age, while age-partitioned reference intervals would perform best near the centre of the partition, with greater under and/or over-flagging rates near the extremes of the age partition. Therefore, even if the total flagging rates are similar between reference intervals established with two different statistical procedures, there is an important difference in which individuals are flagged as abnormal. ALP, creatinine, and phosphate continuous and partitioned reference limits exhibited comparable ability to estimate the 2.5th and 97.5th percentile based on total flagging rates. However, ALP and phosphate continuous reference limits provided a more consistent accurate estimation of upper reference limits, likely due to the profound variation in ALP and phosphate concentration with age Conversely, neither partitioned nor continuous reference intervals offered a clear advantage for estimating calcium and cholesterol reference limits, potentially due to their minimal fluctuation with age. Overall, continuous reference intervals appear advantageous for estimating reference limits for biochemical markers with dynamic age-related changes. Conversely, discrete reference intervals are suitable for biochemical markers that remain relatively constant with age. These findings were further confirmed using data from an external dataset (i.e. CHMS). However, the necessity of using data points from CHMS that represent the mean of ≥11 closely associated results introduces a limitation that may either overstate or diminish the flagging rates compared to using individual data points. The aggregation of datapoints is unfortunately a requirement imposed by the Government for privacy that could not be avoided.

One strength of this study is the cohort of healthy children and adolescents recruited from the community. Hospital laboratory data are often used to establish continuous reference intervals, particularly for pediatrics [10,11] or invasive sampling [28], given their easy access and low cost. However, these datasets exhibit an intrinsic limitation of containing both healthy and pathological subjects. Furthermore, we developed continuous reference intervals for a wide array of routinely used biochemical markers, in contrast to studies commonly assessing one [11,28] or a few [17] analytes. Lastly, we were able to directly compare continuous reference intervals to partitioned reference intervals established using the same dataset. Despite the ability of continuous reference intervals to accurately reflect dynamic trends in biochemical marker concentration throughout pediatric age, obstacles remain for their clinical implementation. Laboratory information systems (LIS) cannot accommodate mathematical functions to allow clinical laboratory reports to include continuous reference intervals. It is important for these obstacles to be overcome in the near future by developers of LIS software to ensure accurate interpretation of laboratory test results and optimal patient care. Therefore, providing reference limit point estimates based on continuous reference intervals are still required for clinical implementation. Therefore, we provide 6-month point estimates, including sex-specific estimates when required, to aid laboratories in implementing pediatric continuous reference intervals. Nevertheless, point estimates still reduce the complexity of true age-related trends in biochemical marker concentration.

Establishing accurate reference intervals for neonatal/infantile age, which exhibits extensive age-related changes, remains a challenge and was not addressed in the current publication due to limited available data and the drastic change in analyte concentration < 1 year. The CALIPER program is beginning initiatives to fill this gap through recruitment of healthy neonates and infants and establishment of

continuous reference intervals. Furthermore, both sex- and age-related changes are exaggerated, and in the case of sex, only become evident, during puberty. For analytes that were previously determined to exhibit a sex difference for at least one age partition, continuous reference intervals were established separately for each sex across the entire age range. This is because the age at which a sex difference becomes evident would again have to be arbitrarily chosen and reduce the complex relationship between analyte concentration and age. Therefore, with the exception of IgM, which exhibits higher levels in females during the entire age range, analytes with sex-specific continuous reference intervals only exhibit a statistically significant sex difference at the onset of puberty. While adolescents at all stages of puberty were included in this study, the statistical method and sample size used in the current study are limited in their ability to provide a robust estimation of continuous reference intervals during this period of profound change. It would therefore be of interest to perform a focussed study on continuous reference curves during pre-pubertal, pubertal, and post-pubertal stages with a larger sample size and the use of various statistical methods to determine which is most optimal to establish estimates of very dynamic trends. However, it is important to note that the need to incorporate Tanner Stage may pose challenges for implementation and interpretation. Lastly, it is important to note that total CO_2 reference values may be falsely lowered due to measurement following freeze-thaw. Indeed, the total CO_2 reference intervals reported in the present study appear slightly lower than those reported in the Abbott package insert (child: 20–28 mmol/L; adult: 22–29 mmol/L) and those recently reported for adults (23–20 mmol/L) [29].

In conclusion, this is the first comprehensive report of continuous pediatric reference intervals for the Canadian population, based on healthy reference individuals. Comparing continuous and partitioned reference intervals further supported the improved accuracy of continuous reference intervals, especially for analytes with profound age-related concentration changes. To aid clinical implementation, sex-specific (when required) 6-month reference limit point estimates are provided. However, reference intervals should be verified for each clinical laboratory's specific analytical platform and local population, as per CLSI guidelines [30], prior to implementation.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Acknowledgements

This study would not have been possible without the generous participation of children from the community. We thank all participants, their families and the numerous CALIPER volunteers whose dedication and time commitment made this study possible. This study was supported by a Foundation Grant from the Canadian Institutes of Health Research (CIHR). VH was supported by a CIHR Doctoral award.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.08.010.

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