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Marked Biological Variance in Endocrine and Biochemical Markers in Childhood: Establishment of Pediatric Reference Intervals Using Healthy Community Children from the CALIPER Cohort

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BACKGROUND: Reference intervals are indispensable in evaluating laboratory test results; however, appropriately partitioned pediatric reference values are not readily available. The Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) program is aimed at establishing the influence of age, sex, ethnicity, and body mass index on biochemical markers and developing a comprehensive database of pediatric reference intervals using an a posteriori approach.

METHODS: A total of 1482 samples were collected from ethnically diverse healthy children ages 2 days to 18 years and analyzed on the Abbott *ARCHITECT* i2000. Following the CLSI C28-A3 guidelines, age- and sex-specific partitioning was determined for each analyte. Nonparametric and robust methods were used to establish the 2.5th and 97.5th percentiles for the reference intervals as well as the 90% CIs.

RESULTS: New pediatric reference intervals were generated for 14 biomarkers, including α-fetoprotein, cobalamin (vitamin B_{12}), folate, homocysteine, ferritin, cortisol, troponin I, 25(OH)-vitamin D [25(OH)D], intact parathyroid hormone (iPTH), thyroid-stimulating hormone, total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), and free triiodothyronine. The influence of ethnicity on reference values was also examined, and statistically significant differences were found between ethnic groups for FT4, TT3, TT4, cobalamin, ferritin, iPTH, and 25(OH)D.

CONCLUSIONS: This study establishes comprehensive pediatric reference intervals for several common endocrine and immunochemical biomarkers obtained in a large cohort of healthy children. The new database will be of global benefit, ensuring appropriate interpretation of pediatric disease biomarkers, but will need further validation for specific immunoassay platforms and in local populations as recommended by the CLSI.

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Although the importance of reliable reference intervals (RIs)[4] is well understood, the establishment of RIs for new technologies or biomarkers has not always kept pace with technological advancements. This is especially true in the field of pediatrics, in which establishing RIs is particularly challenging given the continuous physiological changes that occur throughout childhood. To adequately characterize these changes, the development of continuous RIs with multiple age- and sex-specific partitions is required, which greatly increases the sample number necessary to develop reliable estimates. This issue is compounded by the small blood volume available in children and the ethical challenge of acquiring samples from healthy children. As a consequence of these difficulties, the majority of pediatric RIs currently available have applied statistical measures to hospital populations to estimate RIs (e.g., the Hoffman approach) *(1–3)*. However, the establishment of accurate pediatric RIs obtained from a healthy population is imperative for the correct clinical interpretation of laboratory results, particularly for immunochemical analytes.

The Canadian Laboratory Initiative for Pediatric Reference Intervals (CALIPER) program is a collabor-

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[4] Nonstandard abbreviations: RI, reference interval; CALIPER, Canadian Laboratory Initiative for Pediatric Reference Intervals; AFP, α-fetoprotein; tHcy, total homocysteine; TnI, troponin I; 25(OH)D, 25(OH)-vitamin D; iPTH, intact parathyroid hormone; TSH, thyroid stimulating hormone; TT3, total triiodothyronine; TT4, total thyroxine; FT4, free T4; FT3, free T3.

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ative study with the objective of addressing the critical gaps in pediatric RIs by determining the influence of key covariates, such as age, sex, and ethnicity, on pediatric RIs *(4)*. In this study, we report age- and sex-specific RIs for 14 immunochemical analytes measured on the Abbott *ARCHITECT* i2000 instrument system including: α-fetoprotein (AFP), cobalamin (vitamin B12), folate, total homocysteine (tHcy), ferritin, cortisol, troponin I (TnI), 25(OH)-vitamin D [25(OH)D], intact parathyroid hormone (iPTH), thyroid-stimulating hormone (TSH), total thyroxine (TT4), total triiodothyronine (TT3), free T4 (FT4), and free T3 (FT3). In addition, the influence of ethnicity was examined in children older than 1 year.

Materials and Methods

PARTICIPANT RECRUITMENT AND SAMPLE ACQUISITION

This study was approved by the institutional review board at the Hospital for Sick Children, Toronto, Canada. Healthy children, ages 2 days to 18 years, were recruited. Details regarding sample collection and other pertinent information have been described previously *(4)*. Briefly, participants were recruited through various community programs from December 2009 through February 2012. Exclusion criteria included history of chronic illness, metabolic disease, or recent acute illness. Serum samples were collected in a serum separator tube and were centrifuged, separated, and divided into aliquots within 4 h of collection, after which they were stored at −80 °C until analysis.

SAMPLE ANALYSIS

Serum samples for the aforementioned analytes were analyzed on the Abbott *ARCHITECT* i2000 system in batches over a 12-week period. Analytical methods were controlled according to the manufacturer's instructions as previously described *(4)*. Details regarding assay parameters can be found in Table 1 of the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue9.

STATISTICAL ANALYSIS AND DETERMINATION OF RIS

The data were analyzed in accordance with CLSI C28-A3 guidelines. Statistical analysis was performed using Excel and R software. Details regarding the statistical analysis of the data were described previously *(4)*. The geometric mean, which is the nth root of the product, was calculated to monitor the central tendency of analyte concentrations.

Dependence of analyte concentration on established covariates was assessed using a hierarchical linear modeling approach *(5)* that included age, ethnicity, sampling time (time of year and day), sex, and all relevant interaction variables. Before model building, the data were transformed to meet normality assumptions. The contribution of each variable to the linear model was assessed statistically (F test) and nonsignificant variables were removed, leaving only statistically significant main effects and/or interaction variables and their corresponding main effects in the model. A post-hoc pairwise comparison with Tukey correction was completed for 3 ethnic groups (whites, East Asians, and South Asians), 2 sexes, and 3 sampling times (morning, afternoon, and evening) to test for statistical differences among groups.

Results

Serum samples from 736 boys and 746 girls, ages 2 days to 18 years, were analyzed and the values used to calculate age- and sex-specific RIs, as provided in Table 1. The ethnic demographics of participants were previously reported *(4)*. Complete RI data and data following the International System of Units conversions can be found in the online CALIPER Raw Data Supplemental file and online Supplemental Table 2.

All analytes required age and/or sex partitioning. This was expected given the vast physiological changes that occur from infancy to adolescence. Similar to what was observed with many chemical analytes *(4)*, several immunochemical analytes required additional age partitioning within the first year of life, namely AFP, cortisol, ferritin, FT4, TnI, and 25(OH)D; insulin required both age and sex partitioning, but owing to uncertainty regarding the fasting status of the participants, RIs for insulin are not reported here.

The changes in concentrations observed for each of the examined analytes were classified into 1 of 5 categories: (*a*) high variance and high concentration within the neonatal period that decreases abruptly shortly after birth; (*b*) high variance at birth that is significantly reduced around 1 year of age; (*c*) high variance and high concentration within the neonatal period that decreases gradually with age; (*d*) high variance at birth that decreases abruptly around 1 year of age and increases again in adolescence; and, (*e*) constant variance throughout life but variable concentration according to age.

ANALYTES WITH HIGH VARIANCE AND HIGH CONCENTRATIONS IN THE NEONATAL PERIOD

The analytes AFP, TnI, and ferritin all demonstrated high variance in analyte concentrations, with some exceptionally high values at birth, followed by significantly less variability and abruptly decreased concentrations shortly after birth, resulting in narrower RIs and significantly lower mean values throughout childhood and adolescence (Fig. 1). Consistent with previous reports *(1)*, AFP concentrations dropped almost

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Table 1. Age- and sex-stratified RIs for serum immunoassay analytes analyzed on the Abbott ARCHITECT i2000.[a]

		Male RIs						Female RIs					
Analyte	Age	No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI	No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI
AFP, ng/mL	3 to <6 months	72	49.13	4.15	274.7	2.74 to 5.76	186.37 to 342.58	72	49.13	4.15	274.7	2.74 to 5.76	186.37 to 342.58
	6 to <12 months	141	14.65	2.66	148.21	0.96 to 3.82	79.64 to 336.54	141	14.65	2.66	148.21	0.96 to 3.82	79.64 to 336.54
	1 to <3 years	62	6.51	2.88	20.94	2.54 to 3.34	15.83 to 25.22	62	6.51	2.88	20.94	2.54 to 3.34	15.83 to 25.22
	3 to <19 years	870	2.08	0.89	4.48	0.79 to 0.95	4.29 to 4.58	870	2.08	0.89	4.48	0.79 to 0.95	4.29 to 4.58
Cobalamin, pg/mL	5 days to <1 year	257	652	259	1576	181 to 274	1509 to 1620	257	652	259	1576	181 to 274	1509 to 1620
	1 to <9 years	267	744	283	1613	229 to 340	1420 to 1769	267	744	283	1613	229 to 340	1420 to 1769
	9 to <14 years	374	596	252	1125	234 to 291	1078 to 1207	374	596	252	1125	234 to 291	1079 to 1208
	14 to <17 years	217	493	244	888	214 to 261	848 to 926	217	493	244	888	214 to 262	848 to 926
	17 to <19 years	127	428	203	811	192 to 224	783 to 885	127	428	203	812	192 to 225	783 to 885
Cortisol, μg/dL	2 to <15 days	120	3.24	0.47	12.31	0.12 to 0.71	10.98 to 18.1	120	3.24	0.47	12.31	0.12 to 0.71	10.98 to 18.1
	15 days to <1 year	292	4.82	0.52	16.60	0.37 to 0.77	15.6 to 17.75	292	4.82	0.52	16.60	0.37 to 0.77	15.6 to 17.75
	1 to < 9 years	284	4.94	1.73	10.76	1.02 to 2.07	10.04 to 13.36	284	4.94	1.73	10.76	1.02 to 2.07	10.04 to 13.36
	9 to <14 years	359	5.99	2.19	12.66	1.67 to 2.41	12.13 to 13.66	359	5.99	2.19	12.66	1.67 to 2.41	12.13 to 13.66
	14 to <17 years	201	7.62	2.79	16.40	1.84 to 3.25	15.8 to 17.31	201	7.62	2.79	16.40	1.84 to 3.25	15.8 to 17.31
	17 to <19 years	125	9.46	3.52	18.33	3.08 to 4.65	16.44 to 19.24	125	9.46	3.52	18.33	3.08 to 4.65	16.44 to 19.24
Ferritin, ng/mL	4 to <15 days	127	288.4	99.6	717.0	63.1 to 122.9	648.7 to 741.2	127	288.4	99.6	717.0	63.1 to 122.9	648.7 to 741.2
	15 days to <6 months	172	109.2	14.0	647.2	9.8 to 22.1	521.5 to 734.2	172	109.2	14.0	647.2	9.8 to 22.1	521.5 to 734.2
	6 months to <1 year	140	41.7	8.4	181.9	4.6 to 8.9	167.1 to 250.4	140	41.7	8.4	181.9	4.6 to 8.9	167.1 to 250.4
	1 to <5 years	126	25.1	5.3	99.9	4.0 to 9.6	80.9 to 167.0	126	25.1	5.3	99.9	4.0 to 9.6	80.9 to 167.0
	5 to <14 years	497	34.3	13.7	78.8	12.9 to 15.4	73.0 to 86.6	497	34.3	13.7	78.8	12.9 to 15.4	73.0 to 86.6
	14 to <16 years	65	39.3	12.7	82.8	10.0 to 15.7	74.5 to 87.9						
	16 to <19 years	88	55.6	11.1	171.9	7.6 to 17.3	149.7 to 188.4						
	14 to <19 years							151	25.7	5.5	67.4	4.4 to 5.9	61.4 to 78.8
Folate (serum), ng/mL	5 days to <1 year	235	NA	10.6	NA	8 to 11.7	NA	235	NA	10.6	NA	8 to 11.7	NA
	1 to <3 years	50	NA	3.9	NA	0 to 6.4	NA	50	NA	3.9	NA	0 to 6.4	NA
	3 to <6 years	90	NA	11.9	NA	11.1 to 12.8	NA	90	NA	11.9	NA	11.1 to 12.8	NA
	6 to <8 years	70	NA	13.1	NA	10.2 to 14.4	NA	70	NA	13.1	NA	10.2 to 14.4	NA
	8 to <12 years	284	NA	11.4	NA	11.2 to 12.6	NA	284	NA	11.4	NA	11.2 to 12.6	NA
	12 to <14 years	144	NA	11.9	NA	10.8 to 12.3	NA	144	NA	11.9	NA	10.8 to 12.3	NA
	14 to <19 years	342	NA	7.9	NA	6.6 to 8.7	NA	342	NA	7.9	NA	6.6 to 8.7	NA

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Table 1. Age- and sex-stratified RIs for serum immunoassay analytes analyzed on the Abbott ARCHITECT i2000.[a] *(Continued from page 1395)*

		Male RIs						Female RIs					
Analyte	Age	No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI	No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI
FT3, pg/mL	4 days to <1 year	360	3.68	2.32	4.87	2.23 to 2.55	4.79 to 5.19	360	3.68	2.32	4.87	2.23 to 2.55	4.79 to 5.19
	1 to <12 years	528	3.54	2.79	4.42	2.74 to 2.85	4.3 to 4.49	528	3.54	2.79	4.42	2.74 to 2.85	4.3 to 4.49
	12 to <15 years	103	3.53	2.89	4.33	2.81 to 2.99	4.21 to 4.45	109	3.18	2.5	3.95	2.43 to 2.58	3.85 to 4.02
	15 to <19 years	133	3.19	2.25	3.85	2.1 to 2.66	3.8 to 3.97	124	2.96	2.31	3.71	2.01 to 2.45	3.59 to 3.92
FT4, ng/dL	5 to <15 days	66	2.07	1.05	3.21	0.87 to 1.31	3.04 to 3.35	66	2.07	1.05	3.21	0.87 to 1.31	3.04 to 3.35
	15 to <30 days	55	1.52	0.68	2.53	0.55 to 0.89	2.31 to 2.65	55	1.52	0.68	2.53	0.55 to 0.89	2.31 to 2.65
	30 days to <1 year	270	1.23	0.89	1.7	0.83 to 0.92	1.65 to 1.84	270	1.23	0.89	1.7	0.83 to 0.92	1.65 to 1.84
	1 to <19 years	952	1.11	0.89	1.37	0.86 to 0.91	1.36 to 1.39	952	1.11	0.89	1.37	0.86 to 0.91	1.36 to 1.39
tHcy, μg/mL	5 days to <1 year	266	0.73	0.39	1.35	0.31 to 0.41	1.31 to 1.48	266	0.73	0.39	1.35	0.31 to 0.41	1.31 to 1.48
	1 to <7 years	196	0.64	0.37	1.03	0.3 to 0.41	0.93 to 1.19	196	0.64	0.37	1.03	0.3 to 0.41	0.93 to 1.19
	7 to <12 years	279	0.76	0.46	1.14	0.44 to 0.52	1.09 to 1.24	279	0.76	0.46	1.14	0.44 to 0.52	1.09 to 1.24
	12 to <15 years	95	0.93	0.64	1.41	0.61 to 0.66	1.32 to 1.47	104	0.84	0.55	1.4	0.51 to 0.6	1.31 to 1.48
	15 to <19 years	113	1.19	0.74	1.81	0.7 to 0.78	1.75 to 1.87	116	0.96	0.67	1.61	0.63 to 0.71	1.52 to 1.69
iPTH, pg/mL	6 days to <1 year	172	26.79	6.42	88.58	3.96 to 8.58	74.81 to 146.04	172	26.79	6.42	88.58	3.96 to 8.58	74.81 to 146.04
	1 to <9 years	221	35.85	16.23	63.02	13.3 to 17.26	59.34 to 72.83	221	35.85	16.23	63.02	13.3 to 17.26	59.34 to 72.83
	9 to <17 years	534	43.3	21.89	87.55	20.57 to 22.64	80.38 to 92.64	534	43.3	21.89	87.55	20.57 to 22.64	80.38 to 92.64
	17 to <19 years	104	33.3	16.04	60.38	15 to 17.36	58.02 to 63.68	104	33.3	16.04	60.38	15 to 17.36	58.02 to 63.68
TnI, ng/L[c]	5 to <15 days	46	73.63	2.97	936.35	1.33 to 9.55	742.46 to 1083.83	46	73.63	2.97	936.35	1.33 to 9.55	742.46 to 1083.83
	15 days to <3 months	35	13.75	NA	NA	NA	NA	35	13.75	NA	NA	NA	NA
	3 months to <19 years	691	NA	NA	<9	NA	6.0 to 17.0	691	NA	NA	<9	NA	6.0 to 17.0
TSH, mIU/L	4 days to <6 months	139	2.31	0.73	4.77	0.367 to 0.98	4.27 to 5.54	139	2.31	0.73	4.77	0.367 to 0.98	4.27 to 5.54
	6 months to <14 years	640	1.98	0.7	4.17	0.61 to 0.82	4.04 to 4.43	640	1.98	0.7	4.17	0.61 to 0.82	4.04 to 4.43
	14 <19 years	259	1.51	0.47	3.41	0.25 to 0.57	3.15 to 3.45	259	1.51	0.47	3.41	0.25 to 0.57	3.15 to 3.45
TT3, ng/dL	4 days to <1 year	382	152.34	84.64	234.38	73.57 to 97.01	225.91 to 242.84	382	152.34	84.64	234.38	73.57 to 97.01	225.91 to 242.84
	1 to <12 years	513	151.69	113.28	189.45	106.77 to 114.58	185.55 to 194.66	513	151.69	113.28	189.45	106.77 to 114.58	185.55 to 194.66
	12 to <15 years	208	132.81	97.66	176.43	94.4 to 103.52	170.57 to 181.64	208	132.81	97.66	176.43	94.4 to 103.52	170.57 to 181.64
	15 to <17 years	64	122.40	93.75	156.25	89.84 to 98.96	150.39 to 159.51	59	113.93	92.45	141.93	89.84 to 97.01	135.42 to 143.23
	17 to <19 years	127	119.14	89.84	167.97	75.52 to 91.15	156.90 to 169.92	127	119.14	89.84	167.97	75.52 to 91.15	156.90 to 169.92

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Table 1. Age- and sex-stratified RIs for serum immunoassay analytes analyzed on the Abbott ARCHITECT i2000.[a] *(Continued from page 1396)*

Analyte	Age	Male RIs					Female RIs				
		No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI	No. of samples	Geometric mean	Lower limit	Upper limit	Lower limit CI	Upper limit CI
TT4, μg/dL	7 days to <1 year	301	9.12	5.87	13.67	4.62 to 6.27	13.44 to 14.55	301	9.12	5.87	13.67	4.62 to 6.27	13.44 to 14.55
	1 to <9 years	239	7.86	6.16	10.32	5.18 to 6.24	10.04 to 11.15	239	7.86	6.16	10.32	5.18 to 6.24	10.04 to 11.15
	9 to <12 years	177	7.1	5.48	9.31	4.9 to 5.67	8.9 to 9.91	177	7.1	5.48	9.31	4.9 to 5.67	8.9 to 9.91
	12 to <14 years	54	6.37	5.01	8.28	4.85 to 5.12	8.02 to 8.48	62	6.47	5.08	8.34	4.83 to 5.31	8.26 to 8.73
	14 to <19 years	145	6.45	4.68	8.62	4.00 to 5.04	8.45 to 9.12	131	7.18	5.46	12.99	5.28 to 5.63	11.31 to 14.03
25 (OH)D, ng/mL	5 to <15 days	100	10.27	1.7	33.99	0.99 to 3.41	28.56 to 39.3	100	10.27	1.7	33.99	0.99 to 3.41	28.56 to 39.3
	15 days to <3 months	52	19.19	6.16	40.48	4.25 to 8.21	35.63 to 43.72	52	19.19	6.16	40.48	4.25 to 8.21	35.63 to 43.72
	3 months to <1 year	111	23.73	6.94	47.28	5.5 to 9.04	44.15 to 49.91	111	23.73	6.94	47.28	5.5 to 9.04	44.15 to 49.91
	1 to <9 years	244	29.67	13.24	54.88	9.04 to 15.2	51.88 to 61.76	244	29.67	13.24	54.88	9.04 to 15.2	51.88 to 61.76
	9 to <14 years	281	24.83	12.68	46.52	9.52 to 13.68	43.76 to 50.2	281	24.83	12.68	46.52	9.52 to 13.68	43.76 to 50.2
	14 to <19 years	270	19.69	4.8	42.32	3.76 to 5.56	40.68 to 45.68	270	19.69	4.8	42.32	3.76 to 5.56	40.68 to 45.68

[a] Bold and underlined values indicate sex-specific differences within age partitions.
[b] NA, not applicable.
[c] Corresponding 99th percentiles for TnI obtained by linear interpolation: 968 ng/L (5 days to <15 days); 59 ng/L (15 days to <3 months); 21 ng/L (3 months to <19 years).

8-fold shortly after birth [geometric mean 397 ng/mL (0 to <3 months) to 49 ng/mL (3 to <6 months)]. After this abrupt decrease, concentrations gradually fell until reaching adult concentrations at around 3 years of age (RI 3 to <19 years, 0.89–4.48 ng/mL; geometric mean 2.08 ng/mL). Similarly, TnI concentrations dropped from 74 ng/L (5 to <15 days) to 14 ng/L (15 days to <3 months) and reached adult concentrations after 3 months of age. Ferritin concentrations decreased more gradually, from 288 ng/mL between 4 and <15 days, to 109 ng/mL between 15 days and <6 months, and 41.7 ng/mL between 6 and 12 months. Although both AFP and TnI decreased to nearly undetectable concentrations, the decrease in ferritin concentrations was less drastic and concentrations rose again after 14 years of age, particularly in boys.

ANALYTES WITH HIGH VARIANCE AT BIRTH THAT DECREASED SIGNIFICANTLY AROUND 1 YEAR OF AGE

All the thyroid hormones, FT3, FT4, TT3, and TT4, presented with higher variance at birth that decreased significantly around 1 year of age and subsequently remained constant (Fig. 2). The absolute concentrations gradually decreased with age, with the exception of TT4, for which the concentration decreased during childhood and then increased in girls between 14 and 18 years of age. As a consequence, the RIs were characterized by multiple age and sex partitions (Table 1). Modest sex differences were noted in the concentrations of FT3, TT3, and TT4.

ANALYTES WITH HIGH VARIANCE AND HIGH CONCENTRATIONS WITHIN THE NEONATAL PERIOD THAT DECREASED GRADUALLY WITH AGE

Both TSH and cobalamin demonstrated higher variance at birth, with a gradual reduction in both variance and analyte concentrations according to age (Fig. 3). In accord with recent publications *(6)*, the geometric mean TSH concentration decreased with age, from 1.98 mIU/L (6 months to <14 years) to 1.51 mIU/L (14–18 years). No TSH values were recorded for infants younger than 4 days.

ANALYTES WITH HIGH VARIANCE AT BIRTH THAT DECREASED ABRUPTLY AROUND 1 YEAR OF AGE AND INCREASED AGAIN IN ADOLESCENCE

The majority of analytes examined followed this distribution pattern, which can be further subdivided into 3 subclasses. In subclass A (tHcy and cortisol), the mean concentration increased with age; in subclass B (iPTH), the mean concentration remained constant; and, in subclass C (folate), the mean concentration decreased with age (Fig. 4).

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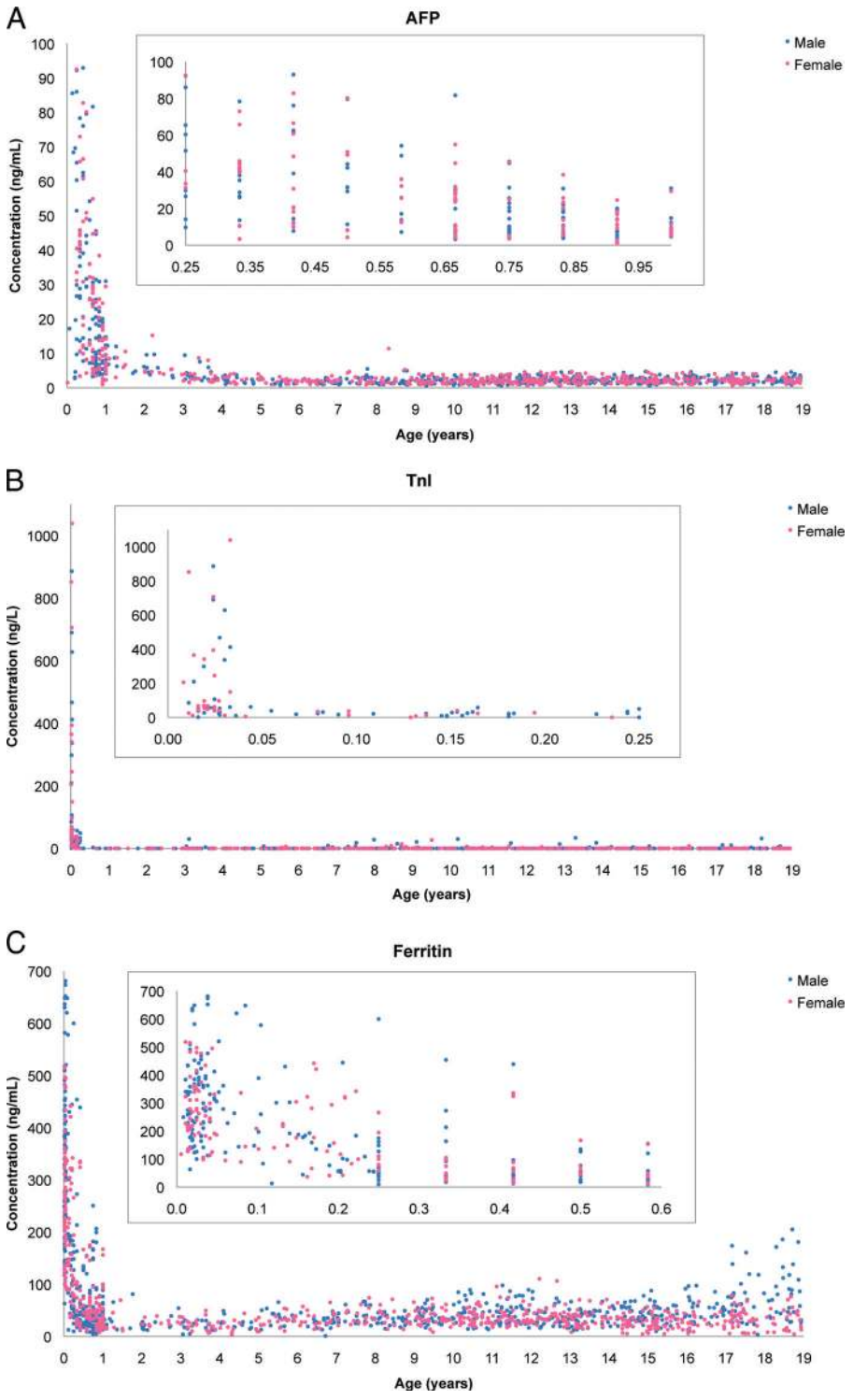

Fig. 1. Age-dependent scatter plots by sex of analytes with high variance and high concentrations in the neonatal period.

(A), AFP; (B), TnI; (C), ferritin.

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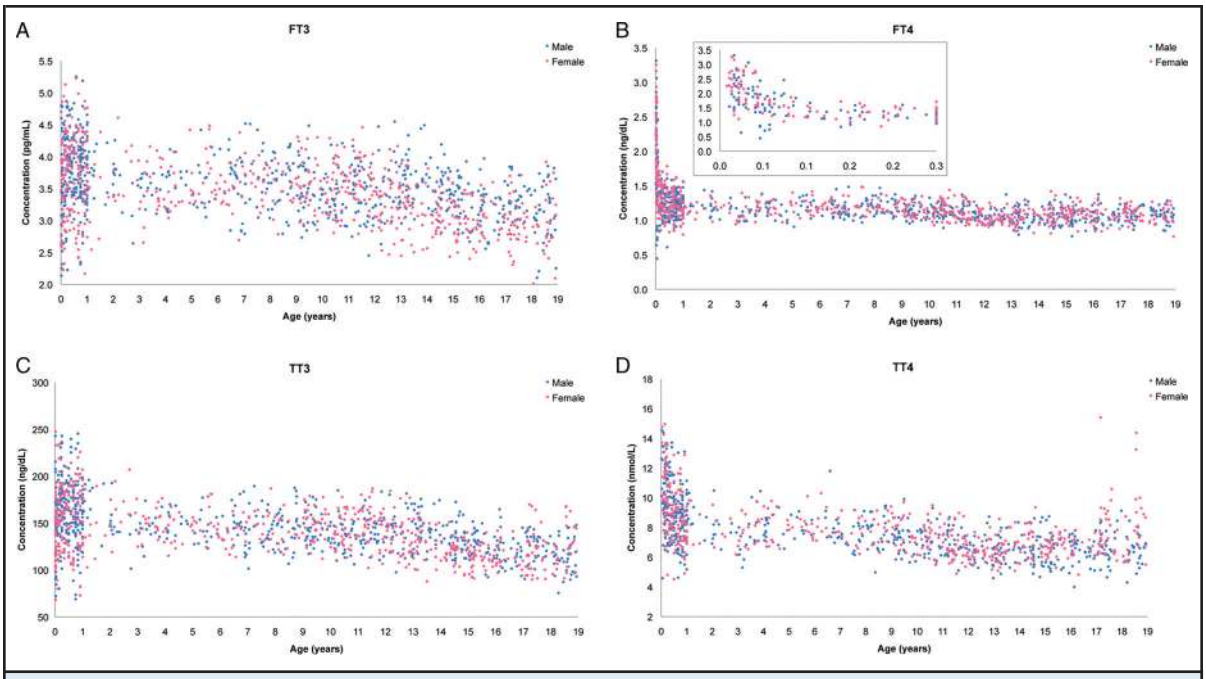

Fig. 2. Age-dependent scatter plots by sex of analytes with high variance at birth that decreases significantly around 1 year of age.

(A), FT3; (B), FT4; (C), TT3; (D) TT4.

Subclass A. After the first year of life, tHcy was observed to increase as a function of age and sex, with higher tHcy concentrations in boys vs girls >12 years of age.

A significant age-effect was noted for cortisol, whereby after infancy, increased cortisol concentration as well as increased variance were associated with increasing age, particularly for individuals >17 years of age. Cortisol concentrations were additionally examined for the effect of sampling time (morning 9:00–10:59; afternoon 11:00–14:59; and evening 15:00–22:00). Using linear modeling, a modest increase in concentration of approximately 10% was noted for samples obtained in the morning as compared to the evening ($P = 0.03$).

Subclass B. After infancy, the geometric mean iPTH concentration remained largely constant throughout childhood, with a peak in concentration occurring between 9 and <17 years (Table 1). The variance, however, increased substantially and abruptly around 9 years of age, thereby widening the RI, and subsequently decreased gradually from 15 to 19 years.

Subclass C. Consistent with the hypothesis that folate is efficiently transferred from maternal to fetal circulation and through breast milk, high serum folate concentrations were observed in infants less than 1 year of age (Table 1). This was followed by a significant drop in concentrations occurring between 1 and 3 years of age, an increase in children 3 to 6 years of age, and a gradual decrease in children older than 6 years of age until adolescence.

Analytes with constant variance but variable concentration according to age

Unique to the other analytes, the variance associated with 25(OH)D remained relatively constant throughout the pediatric population, with significant changes in the geometric mean concentration occurring with age (Fig. 5). Importantly, 25(OH)D concentrations increased significantly from birth to approximately 6 years, and then declined steadily. This relationship was consistent even when results were adjusted for seasonality (see online Supplemental Fig. 1). However, within each age partition, 25(OH)D concentrations were significantly higher in samples collected in the summer months (June, July, and August) compared to non-summer months [$P < 0.008$, ANOVA with the Tukey-HSD (honestly significant difference) posttest].

Influence of ethnicity on pediatric biomarker concentrations

In addition to identifying the dependence of analyte concentrations on age and sex, a modeling approach, as described in Methods, was used to identify the influ-

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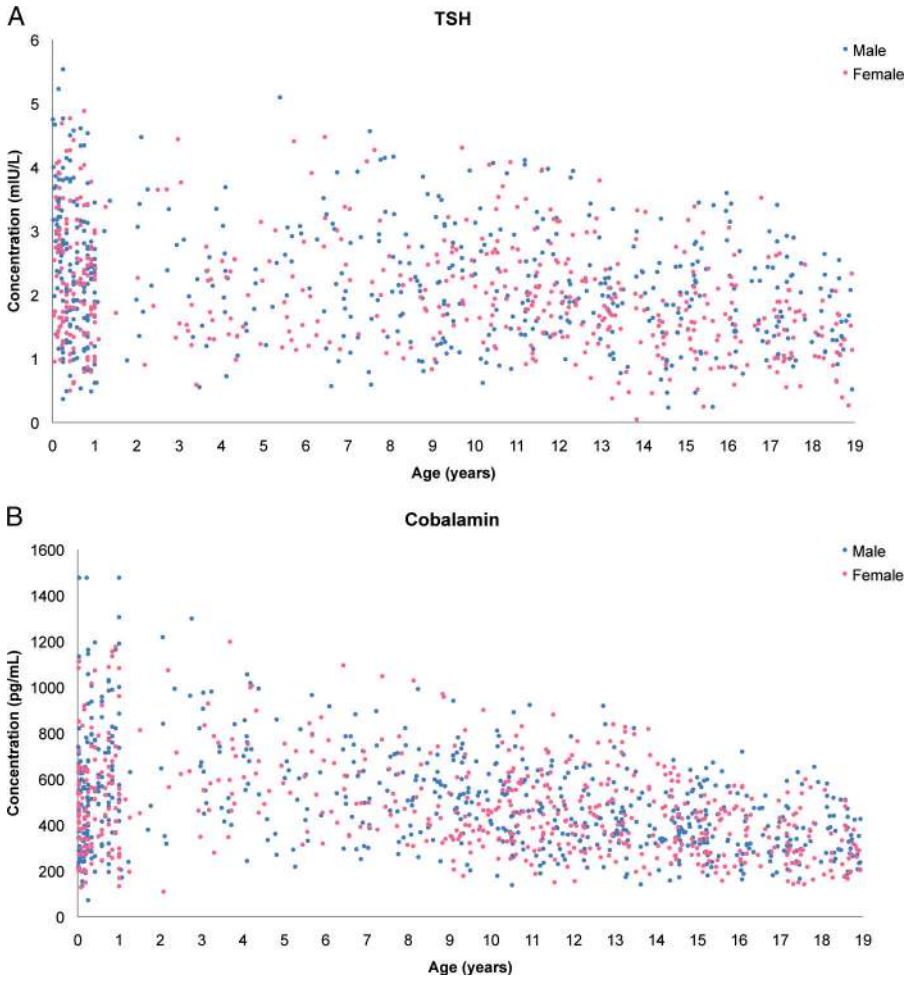

Fig. 3. Age-dependent scatter plots by sex of analytes with high variance and high concentration within the neonatal period that decreases gradually with age.

(A), TSH; (B), cobalamin.

ence of ethnicity. Statistically significant differences were observed in the concentrations of FT4, TT3, TT4, cobalamin, ferritin, iPTH, and 25(OH)D across ethnic groups (see online Supplemental Table 3). Although modest effect sizes were noted for TT3 and FT4, both TT4 and ferritin were substantially higher in East Asians and lower in South Asians compared to whites. Cobalamin was substantially higher in East Asians, iPTH was substantially higher in South Asians, and 25(OH)D was substantially lower in East Asians.

Discussion

Objective data from the clinical laboratory are critical to the majority of medical decisions, including initial diagnosis and monitoring of treatment outcome. Laboratory errors, including inaccurate RIs, can lead to inappropriate diagnosis, unnecessary test repetition, and inappropriate follow-up investigations *(7)*. Excessively wide RIs may mask subclinical disorders, and the use of incorrect RIs may confuse the clinical picture. Therefore, relevant RIs are essential for correct test result interpretation and reducing the risk of false negatives and false positives. Failure to provide proper RIs in the pediatric population carries obvious consequences for immunochemical analytes such as TSH *(8)* and ferritin *(9)*.

The current investigation established RIs in a large, multiethnic pediatric cohort. However, the study was not without its limitations. As it was a cross-sectional investigation, the long-term health of the participating children was not verified. Additionally, the RIs provided are method- and population-specific,

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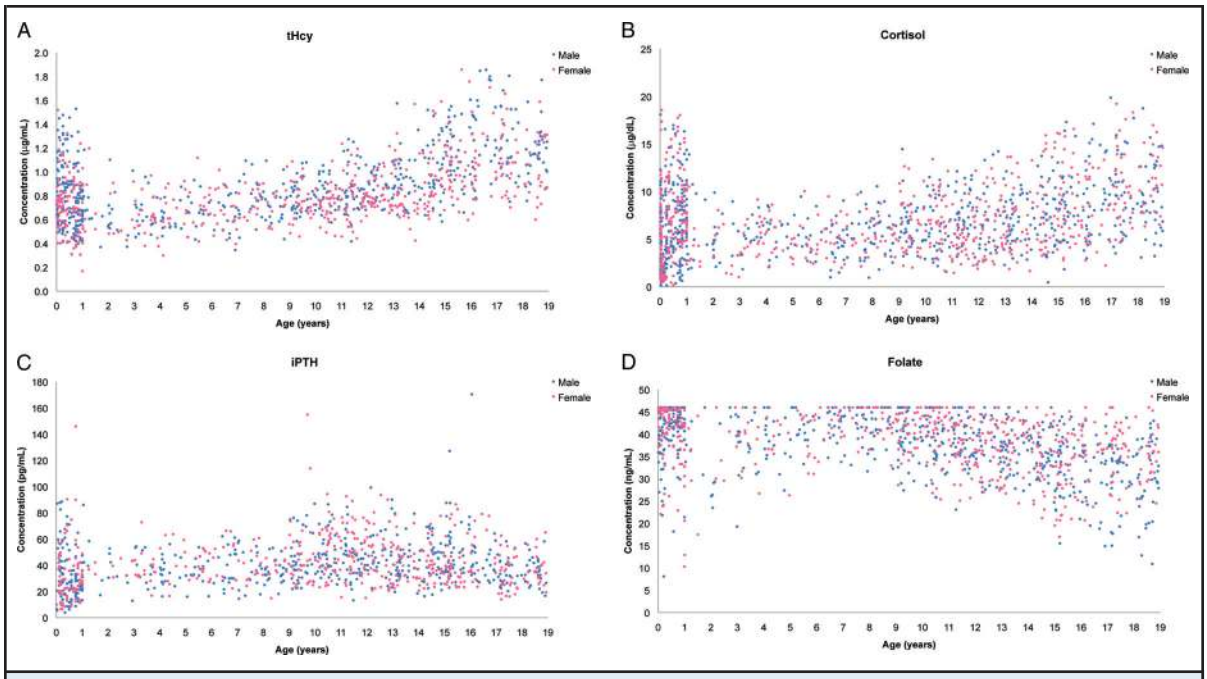

Fig. 4. Age-dependent scatter plots by sex of analytes with high variance at birth that decreases abruptly around 1 year of age and increases again in adolescence.

(A), tHcy; (B), cortisol; (C), iPTH; (D), folate.

meaning that the RI data must be appropriately transferred and validated based on CLSI guidelines if they are to be used with alternate platforms and/or populations. Finally, because fasting was not mandated, the preprandial/postprandial status varied across participants, thereby clouding the interpretation of some analytes, such as insulin. However, a recent study of 27 children revealed minimal influence of fasting on most biochemical markers *(10)*.

We were able to categorize each of the immunochemical analytes into 1 of 5 classes based on the dynamic changes observed. In the first group, which in-

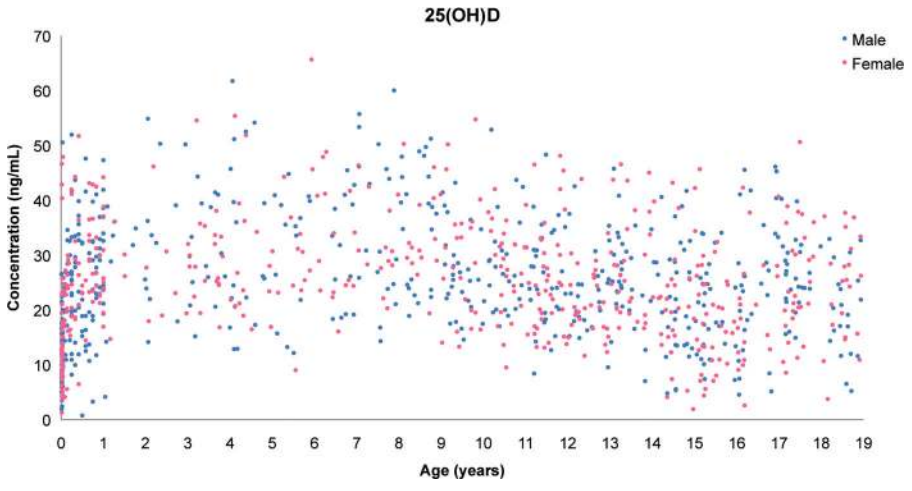

Fig. 5. Age-dependent scatter plots by sex of 25(OH)D with constant variance but variable concentration according to age.

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cluded AFP, TnI, and ferritin, a high concentration was observed in the neonatal period, with subsequent substantial decreases shortly after birth. This pattern of analyte change can be explained by several mechanisms. For example, hepatic gene expression, which is high for AFP in the fetus, switches predominantly to albumin expression shortly after birth *(11)*, and reexpression of AFP occurs only with disease. For TnI, the process of birth itself is believed to impart stress on the fetal heart and consequently increase TnI expression and/or release, with concentrations returning to adult levels by 3 months of age. We observed TnI increases above 300 ng/L, the manufacturer's recommended diagnostic cutoff, in 28% of neonates under 15 days of age, which may have been caused by several factors including functional hypoxemia of the fetus *(12)*, perinatal asphyxia, cardiovascular stress during delivery *(12)*, and cardiac remodeling. Additional studies are necessary to determine what factors contribute to troponin increases in neonates.

For ferritin, the high concentrations observed during the first 6 months of life reflect iron stores accumulated during the last trimester of gestation *(13)*. Additionally, the increased concentrations observed immediately postnatally may be a consequence of erythrocyte turnover *(14)*. Ferritin concentrations were additionally increased in boys during adolescence (ages 14 to 19 years) (Fig. 1) (geometric mean 55.6 ng/mL in boys vs 25.7 ng/mL in girls). Previous studies have similarly identified a significant increase in ferritin concentrations in male adolescents and have attributed this effect to higher dietary iron intake *(15)*.

In the second group, comprising various thyroid hormones, the RI narrowed significantly during childhood, likely reflecting immature homeostatic mechanisms in infancy. These observations are consistent with some *(6)* but not all *(16, 17)* previous publications. Additionally, FT3 concentrations were lower in 12- to 18-year-old girls and TT4 concentrations were higher in 14- to 18-year-old girls as compared to boys, whereas no significant sex differences were seen for TT3 or FT4, consistent with some reports *(2, 6)*, but not others *(17)*. Interestingly, a subgroup of young women ages 17 to 18 years had increased TT4 concentrations (Fig. 2), consistent with the effects of exogenous estrogen, which is known to increase the concentration of thyroxine-binding globulin and consequently TT4 *(18)*. Indeed, all of these young women were identified as using oral contraceptives on the basis of completed questionnaires.

In the third group, which included TSH and cobalamin, the RI was observed to narrow gradually with age. For TSH, this may reflect gradual fine-tuning of homeostatic mechanisms, whereas for cobalamin, it likely reflects gradual depletion of cobalamin stores that were generated in utero. Because the first TSH measurement was performed on a 4-day-old infant, we were unable to address the TSH surge that occurs in the first 4 days of life. However, our findings are not consistent with the suggestion of increased TSH concentrations between 2 and 20 weeks of age *(18)*. Consistent with recent reports *(2)*, log-transformed TSH values did not correlate with FT4 concentrations. However, this may be a result of the narrow RI for TSH and FT4 in conjunction with the wide biological variability between TSH and respective FT4 concentration in a healthy population, rather than any assay performance issues *(19)*. There has been substantial controversy regarding the upper limit of the TSH RI. Although the National Association of Clinical Biochemistry has advocated lowering this limit *(19)*, others have challenged this recommendation *(20)*. The results herein support reducing the upper limit for TSH in adolescence to 3.5 mIU/L, and are in accord with recent studies in adult populations *(21)*. This reduction in the upper limit of the RI for TSH may be a result of the immunoassays used in these studies. It should also be appreciated that TSH increases are commonly observed in obese children *(22)*, and therefore reducing the upper limit of the RI may misclassify an unacceptable number of children in overweight or obese populations.

In contrast with previously published data *(23)*, both the variance and the geometric mean of cobalamin were observed to decrease with age until 14 years, after which mean concentrations remained constant (Table 1). This result differs from those in previous reports indicating low (approximately 270 pg/mL) cobalamin concentrations until 1 year of life, a peak concentration occurring between 5 and 7 years of age, and a subsequent decline to approximately 540 pg/mL into adolescence *(25)*. The reason for this discrepancy is unclear but is likely related to dietary factors as well as cobalamin supplementation during pregnancy and fetal transfer *(24)*. For both cobalmin and folate, significantly higher RIs were reported across all ages compared with previous studies *(25)*, likely reflecting maternal supplementation and folate fortification programs.

In the fourth group, which included the majority of analytes, wider RIs were observed during both the neonatal period and adolescence, likely reflecting the influence of growth and development on analyte concentrations. Consistent with previous reports, a sex effect was noted for tHcy. However, the age at which the sex difference was apparent differs slightly from previous studies, which have revealed sex partitions occurring anywhere from 8 years *(26)* to 15 years of age *(27)*. This difference is likely population specific, resulting from developmental changes central to puberty and

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differences in nutritional status. Although previous reports have indicated that tHcy concentrations vary according to ethnicity *(27)*, we did not observe a relationship with ethnic origin.

Our observations for cortisol contrasted markedly with previous findings using either hospitalized patients *(3)* or healthy children *(28)*, in which either the opposite effect or no relationship with age was noted. Additionally, no substantial diurnal variation was observed for cortisol concentrations. Numerous factors may account for this observation. The stress of phlebotomy may have proportionally increased cortisol concentrations to a greater extent in samples obtained in the afternoon compared to the morning, thereby minimizing the difference between morning and afternoon/evening samples. Physiologically, children may have less pronounced cortisol circadian rhythms so that their interindividual variation masked the intraindividual diurnal variations. Additionally, the sampling time may have been ineffective to capture the diurnal variation. Infants are known to lack circadian rhythm in cortisol concentrations *(29)* and not to develop marked decreases in afternoon cortisol concentrations (9:00–11:00 vs 15:00–17:00 h) until approximately 6–8 years of age *(30)*. Given the study sampling time (9:00–22:00 h) and the intra- and interindividual variations for cortisol, which are estimated for adults to be 20.9% and 45.6%, respectively *(31)*, it is likely that the effect of interindividual variation surpassed that of circadian rhythm. In brief, when applying RIs for cortisol to a pediatric population, daytime sampling time does not appear to importantly affect interpretation.

For PTH, our results were consistent with previous reports *(1)*. The increased variation in iPTH concentrations between 9 and 19 years of age likely reflected biological responses to interindividual variations in linear growth, bone turnover, and calcium and vitamin D requirements *(32)*.

The RIs for serum folate in our cohort were significantly higher than prior estimates *(33)*, likely resulting from folic acid supplementation that is mandated in Canada. Indeed, with serum folate deficiency being defined by NHANES (the National Health and Nutrition Examination Survey) as <6.8 nmol/L [<3 ng/mL *(34)*], our minimum concentration of 3.6 ng/mL was well above this cutoff. The concurrent decrease in both cobalamin and folate and increase in tHcy observed during adolescence may reflect the abstraction of these nutrients from serum to support growth and consequent accumulation of tHcy when folate and/or cobalamin concentrations are insufficient to support its remethylation *(23)*.

In the fifth group, which consisted of 25(OH)D, the width of the RI, but not the mean concentration, was maintained throughout childhood and adolescence. Across the age partitions, 74%, 27%, 14%, 5%, 10%, and 29% of children manifested serum 25(OH)D concentrations <16 ng/mL, a concentration threshold noted to correspond with increased iPTH concentrations *(35)*. Alarmingly, 88%, 52%, 26%, 14%, 27%, and 43% of children from each respective partition had 25(OH)D concentrations <20 ng/mL, which is the deficiency concentration recommended by the Institute of Medicine *(36)*. Contributing to this were the effects of ethnicity and season, wherein concentrations of 25(OH)D from children of East Asian descent (Table 2) and samples collected in nonsummer months (see online Supplemental Fig. 1) were observed to be significantly lower compared to those obtained from white children and/or collected in the summer.

In terms of the influence of ethnicity on analyte concentrations, significant differences were noted for FT4, TT3, TT4, cobalamin, ferritin, iPTH, and 25(OH)D. Previous studies conducted in adult populations have similarly noted an effect of ethnicity on these analytes *(37–40)*, resulting from a combination of hereditary factors [e.g., transcobalamin concentration is significantly increased in blacks *(39)*] as well as in response to acquired causes [e.g., dietary factors relating to iron intake *(37)*]. Together, these findings highlight the importance of considering ethnicity when interpreting data in relation to a RI.

Conclusions

This study is a part of the CALIPER initiative to establish a comprehensive database of pediatric RIs from healthy children to benefit pediatric centers worldwide. The data contained herein highlight the dynamic changes that occur in immunochemical analytes throughout childhood. For all analytes examined, at least 2 age and sex partitions were required, and in some cases, as many as 8 were necessary to adequately capture the changes observed in both the geometric mean and the variance about this mean. The complete database used to calculate the RIs is available as an online supplemental file to allow each laboratory to analyze the data and determine which partitions are appropriate for their service. Future studies will be necessary to transfer and validate these RIs to other platforms to allow wider application of the database.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting*

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or revising the article for intellectual content; and (c) final approval of the published article.

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