Pediatric Population Reference Value Distributions for Cancer Biomarkers and Covariate-Stratified Reference Intervals in the CALIPER Cohort

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BACKGROUND: Cancer biomarkers are commonly used in pediatrics to monitor cancer progression, recurrence, and prognosis, but pediatric reference value distributions have not been well established for these markers. The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) sought to develop a pediatric database of covariate-stratified reference value distributions for 11 key circulating tumor markers, including those used in assessment of patients with childhood or adult cancers.

METHODS: Healthy community children from birth to 18 years of age were recruited to participate in the CALIPER project with informed parental consent. We analyzed serum samples from 400–700 children (depending on the analyte in question) on the Abbott Architect ci4100 and established reference intervals for α-fetoprotein (AFP), antithyroglobulin (anti-Tg), human epididymis protein 4 (HE4), cancer antigen 125 (CA125), CA15-3, CA19-9, progastrin-releasing peptide (proGRP), carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC), and total and free prostate specific antigen (PSA) according to CLSI C28-A3 statistical guidelines.

RESULTS: We observed significant fluctuations in biomarker concentrations by age and/or sex in 10 of 11 biomarkers investigated. Age partitioning was required for CA153, CA125, CA19-9, CEA, SCC, proGRP, total and free PSA, HE4, and AFP, whereas sex partitioning was also required for CA125, CA19-9, and total and free PSA.

CONCLUSIONS: This CALIPER study established a database of childhood reference intervals for 11 tumor biomarkers and revealed dramatic fluctuations in tumor marker concentrations between boys and girls throughout childhood. In addition, important differences between the adult and pediatric population were observed, further highlighting the need for pediatric-specific reference intervals.

Increasingly, cancer biomarkers have become an important tool in the fight against the growing rates of childhood and adult cancers. Although there may be limitations to how and when cancer biomarkers can be used in patient diagnosis, there is great potential for these cancer biomarkers to aid in monitoring and follow-up assessments of cancer patients. However, information on normal concentrations of these markers in the pediatric population is sorely lacking. Table 1 provides a summary of the current clinical applications for 11 key cancer biomarkers in both pediatric and adult cancers. Table 1 also provides a summary of the pediatric reference intervals currently available for these 11 key cancer biomarkers. The gaps are evident, with little to no information available for 7 of the 11 markers of interest.

To harness the full potential of tumor biomarkers in predicting patient outcomes and monitoring treatment, it is important to have age- and sex-stratified reference intervals for comparison. Indeed, the importance of age- and sex-stratified reference intervals for tumor biomarkers has been previously noted in the adult population (1). It is reasonable to assume that this may also apply to the pediatric population, where pivotal developmental changes such as puberty are likely to affect the concentrations of these markers. In addition to the clinical importance of reference intervals, understanding how the concentrations of key biomarkers fluctuate in the healthy population is also important from a research perspective.

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Several studies have noted and illustrated the ways in which gaps in pediatric reference values hinder our ability to assess potential applications of specific cancer biomarkers (2, 3).

With the majority of the literature on tumor biomarkers focusing on the adult population, it is important to note the reasons for establishing reference values in a pediatric population. First, tumor biomarkers are not simply byproducts of tumor metabolism. Rather, many of these markers have been shown to play an important role in cancer progression (4, 5). It is reasonable, therefore, to speculate that these markers may also play a role in the progression of pediatric cancers. Second, disease incidence can change over time; thus, cancers that have, in the past, typically arisen in the adult population can begin to arise in the pediatric population. For example, the incidence of melanoma in the pediatric population has been steadily increasing since 1973 (6).

To ensure proper interpretation of laboratory test results, it is crucial that appropriately established reference intervals be available (8). The establishment of reference

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**Table 1. Clinical applications and available pediatric reference intervals for 11 key circulating tumor biomarkers.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pediatric cancer</th>
<th>Adult cancer*</th>
<th>Available pediatric reference intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>Monitoring of sacrococcygeal teratomas (3); prognosis of germ cell tumors (2)</td>
<td>Diagnosis and prognosis of hepatocellular carcinoma patients (30)</td>
<td>Reference intervals established by CALIPER using previous generation Abbott Diagnostics AFP assay (9)</td>
</tr>
<tr>
<td>Anti-Tg</td>
<td>Currently unknown</td>
<td>Monitoring of thyroid disease, including thyroid cancer (36)</td>
<td>95th percentile for individuals from birth to 20 years reported (24)</td>
</tr>
<tr>
<td>CA15-3</td>
<td>Currently unknown</td>
<td>Surveillance of breast cancer patients after diagnosis (30)</td>
<td>None reported</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Increased in some malignant germ cell tumors and immature teratomas (7)</td>
<td>Monitoring of pancreatic cancer (30); used, in combination with CEA, in diagnosis and follow-up of colorectal and gastrointestinal cancer patients (37)</td>
<td>Reference intervals available for children birth to 1.5 years (7)</td>
</tr>
<tr>
<td>CA125</td>
<td>Potential to aid in monitoring of patients with sacrococcygeal teratomas (3); increased concentrations observed in children with non-Hodgkins lymphoma (38)</td>
<td>Used, in combination with HE4, to aid in diagnosis and monitoring of ovarian cancer patients (4)</td>
<td>Reference intervals available for children birth to 1.5 years (7)</td>
</tr>
<tr>
<td>CEA</td>
<td>Currently unknown</td>
<td>Monitoring of colon cancer patients posttreatment (30)</td>
<td>None reported</td>
</tr>
<tr>
<td>HE4</td>
<td>Currently unknown</td>
<td>Used, in combination with CA125, to aid in diagnosis and monitoring of ovarian cancer patients (4); potential prognostic factor in lung cancer (39)</td>
<td>None reported</td>
</tr>
<tr>
<td>ProGRP</td>
<td>Currently unknown</td>
<td>Monitoring tool for lung cancer patients undergoing treatment and in remission (39)</td>
<td>None reported</td>
</tr>
<tr>
<td>SCC</td>
<td>Increased in various skin, esophageal, lung, head, and neck cancers (40)</td>
<td>Can help determine prognosis for cervical cancer patients (40)</td>
<td>None reported</td>
</tr>
<tr>
<td>Free and total PSA</td>
<td>Currently unknown</td>
<td>Used in prostate cancer screening efforts, prognosis, and monitoring (30)</td>
<td>95th percentile of total PSA for individuals aged birth to 18 years reported (22)</td>
</tr>
</tbody>
</table>

*Relates to cancers that typically occur in the adult population, although they may still occur in pediatric patient.

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4 Nonstandard abbreviations: SCC, squamous cell carcinoma antigen; CA, cancer antigen; CEA, carcinoembryonic antigen; AFP, α-fetoprotein; CALIPER, Canadian Laboratory Initiative in Pediatric Reference Intervals; HE4, human epididymis protein 4; proGRP, progastrin-releasing peptide; anti-Tg, antithyroglobulin; PSA, prostate specific antigen.
PARTICIPANT RECRUITMENT AND SAMPLE ACQUISITION

This study was approved by the Research Ethics Board at the Hospital for Sick Children, Toronto, Canada. Healthy children and adolescents from birth to 18 years of age were recruited from the greater Toronto and Hamilton areas from schools, community centers, and sports leagues. Participation involved completion of a short questionnaire, written informed consent, and donation of a blood sample. Samples were collected in serum separator tubes (Becton Dickinson) and centrifuged within 30 min to 4 h after collection. The separated serum was then aliquoted and stored at −80 °C until testing.

Participant demographic and health data collected by use of self-report questionnaires were entered into a Microsoft Access database. On the basis of the information provided in these questionnaires, participants were then selected for the study. First, participants were excluded on the basis of 3 criteria: history of chronic illness or metabolic disease, acute illness, or use of prescribed medication within the 2 weeks preceding donation. Next, the study population was selected randomly, ensuring a balanced age and sex distribution. Furthermore, efforts were made to ensure that the ethnic composition of the study participants matched that of the province of Ontario as reported by 2006 Canadian census data (11). To ensure a sufficiently large sample size for participants <5 years of age, in addition to CALIPER samples, samples from apparently healthy/metabolically stable children from the maternity wards of Women’s College Hospital and Mt. Sinai Hospital in Toronto and select outpatient clinics at the Hospital for Sick Children were used.

SAMPLE ANALYSIS

We analyzed serum samples for selected participants on the Abbott Architect ci4100 system for 11 key cancer biomarkers: AFP, CA15-3, CA125, CA19-9, CEA, human epidermidis protein 4 (HE4), progastrin-releasing peptide (proGRP), antithyroglobulin (anti-Tg), SCC, and total and free prostate specific antigen (PSA). Samples were analyzed in batches over a 10-month period. Analytical methods were controlled according to manufacturer’s instructions by preventive maintenance, function checks, calibration, and quality control. All samples tested underwent automated interference analysis for hemolysis, icterus, and turbidity (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue12).

STATISTICAL ANALYSIS AND DETERMINATION OF REFERENCE INTERVALS

We analyzed data in accordance with CLSI C28-A3 guidelines as outlined in Fig. 1. Statistical analysis was performed with Excel (Microsoft) and R software. Briefly, we used scatter and distribution plots to visually inspect the data, and extreme outliers were removed. Suspected partitions identified through visual inspection were tested by use of the Harris and Boyd method (12). Subsequently, the data in each partition were transformed by use of the Box–Cox transformation method. The normality of each of the partitions was then assessed by use of Q-Q plots (13). Outlier intervals is a particularly challenging task in the pediatric population. In addition to the challenges faced when establishing reference intervals in adult populations, the pediatric community presents a set of unique obstacles. Specifically, recruitment of large numbers of healthy children across the entire pediatric age range is difficult. Additionally, concentrations of many biomarkers fluctuate with the continuous physiological changes that occur throughout childhood. Development and growth profoundly influence reference intervals for many of the disease biomarkers measured in the laboratory and, as such, the need for age and sex partitioning becomes all the more crucial (8–10).

The Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) is a research initiative established by a Canadian team of investigators aimed at closing the gaps that currently exist in pediatric reference intervals by establishing a comprehensive database of reference values in Canadian children stratified by age, sex, and ethnicity. To date, the CALIPER project has published reference intervals for 40 common biochemical markers, protein biomarkers, lipids, and enzymes (8), as well as 23 endocrine and fertility hormones (9, 10). However, reference intervals for several pediatric disease biomarkers, including those for cancer, are still lacking.

The present study sought to close the gaps that currently exist in reference values for 11 key cancer biomarkers. It should be noted that these reference intervals were established on 1 platform only: the Abbott Architect ci4100. On the basis of observations from previous CALIPER studies, we hypothesize that circulating concentrations of tumor biomarkers are significantly influenced by child sex and growth (age); therefore, our major objectives were to assess population reference value distributions for key circulating tumor biomarkers, determine the effects of key covariates (age and sex), and develop a comprehensive database of covariate-stratified reference intervals for cancer biomarkers. These reference intervals will be useful not only in practice, by contributing to a more accurate assessment of tumor markers in cancer diagnosis and treatment, but also in research efforts aimed at determining the prognostic and/or diagnostic value of tumor markers in various types of cancers and populations.

Materials and Methods

PARTICIPANT RECRUITMENT AND SAMPLE ACQUISITION

This study was approved by the Research Ethics Board at the Hospital for Sick Children, Toronto, Canada. Healthy children and adolescents from birth to 18 years of age were recruited from the greater Toronto and Hamilton areas from schools, community centers, and sports leagues. Participation involved completion of a short
removal in normally distributed partitions was carried out by use of the Tukey test twice (14), and outlier removal in skewed partitions was carried out by use of the adjusted Tukey test twice (15). Finally, we calculated reference intervals for partitions with a sample size ≥120 by use of the nonparametric rank method and those for partitions with a sample size >40 and <120 by use of the robust method of Horn and Pesce (16). In addition, we calculated 90% CIs for the endpoints of each reference interval.

**Results**

Between 400 and 700 pediatric samples from the CALIPER cohort, depending on the analyte in question, were tested for each of the 11 cancer biomarkers examined, and results were used to calculate age- and sex-specific reference intervals (Table 2). All analytes examined required some partitioning by age, sex, or both, with the exception of anti-Tg, which showed steady levels of expression across the entire pediatric age range.

Figs. 2–4 illustrate the fluctuations in test results throughout the entire pediatric age range and between boys and girls. It is important to note that Figs. 2–4 contain all data points (including outliers). Online Supplemental Figs. 1–3 indicate which data points were deemed outliers. Six of the 11 analytes examined (HE4, proGRP, SCC, CEA, CA19-9, and AFP) showed a similar, expected pattern for oncofetal antigens (Fig. 2). Specifically, high concentrations were observed at birth followed by a rapid decrease in concentration throughout the first 1 or 2 years of life. Two of these analytes also required some partitioning after the first year of life, namely, HE4 (2–<10 years and 10–<19 years) and proGRP (1–<12 years and 12–<19 years); it is important to note that fluctuations that occurred in the 1- to <19-year age range were not nearly as drastic as those observed during the first year of life.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Age</th>
<th>Lower limit</th>
<th>95th percentile</th>
<th>Upper limit</th>
<th>No. of samples</th>
<th>Lower limit</th>
<th>95th percentile</th>
<th>Upper limit</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP (ng/mL or μg/L)</td>
<td>0–&lt;1 month</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>113</td>
<td>0–&lt;1 month</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–&lt;6 months</td>
<td>9.8</td>
<td>1158</td>
<td>1359</td>
<td>4</td>
<td>9.8</td>
<td>1158</td>
<td>1359</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6 months–&lt;1 year</td>
<td>0.4</td>
<td>88.6</td>
<td>103.1</td>
<td>57</td>
<td>0.4</td>
<td>88.6</td>
<td>103.1</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>1–&lt;19 years</td>
<td>0.8</td>
<td>14.0</td>
<td>34.8</td>
<td>252</td>
<td>0.7</td>
<td>9.0</td>
<td>15.7–46.1</td>
<td>252</td>
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<tr>
<td>Anti-Tg (IU/mL, kIU/L)</td>
<td>0–&lt;19 years</td>
<td>0.4</td>
<td>12.6</td>
<td>17.7</td>
<td>387.0</td>
<td>0.3</td>
<td>12.7–32.4</td>
<td>0.3</td>
<td>12.7–32.4</td>
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<td>CA15-3 (IU/mL or kIU/L)</td>
<td>0–&lt;1 week</td>
<td>3.4</td>
<td>22</td>
<td>24</td>
<td>49</td>
<td>3.4</td>
<td>22</td>
<td>24</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>1 week–&lt;1 year</td>
<td>4.9</td>
<td>29</td>
<td>33</td>
<td>100</td>
<td>4.2</td>
<td>5.6</td>
<td>29–35</td>
<td>100</td>
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<td>1–&lt;19 years</td>
<td>3.9</td>
<td>18</td>
<td>21</td>
<td>252</td>
<td>3.6</td>
<td>4.6</td>
<td>18–23</td>
<td>252</td>
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<tr>
<td>CA19-9 (IU/mL or kIU/L)</td>
<td>0–&lt;1 year</td>
<td>&lt;2.0</td>
<td>59</td>
<td>64</td>
<td>122</td>
<td>53</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1–&lt;19 years</td>
<td>&lt;2.0</td>
<td>28</td>
<td>41</td>
<td>251</td>
<td>29</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA125 (IU/mL or kIU/L)</td>
<td>0–&lt;4 months</td>
<td>2.4</td>
<td>19</td>
<td>22</td>
<td>55</td>
<td>1.7</td>
<td>3.0</td>
<td>19–24</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>4 months–&lt;5 years</td>
<td>7.7</td>
<td>32</td>
<td>33</td>
<td>190</td>
<td>7.0</td>
<td>9.3</td>
<td>32–38</td>
<td>190</td>
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<tr>
<td></td>
<td>5 years–&lt;11 years</td>
<td>4.7</td>
<td>28</td>
<td>30</td>
<td>178</td>
<td>4.4</td>
<td>5.8</td>
<td>28–36</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>11–19 years</td>
<td>5.4</td>
<td>27</td>
<td>28</td>
<td>123</td>
<td>4.6</td>
<td>6.2</td>
<td>23–30</td>
<td>123</td>
</tr>
<tr>
<td>CEA (ng/mL or μg/L)</td>
<td>0–&lt;1 week</td>
<td>8.1</td>
<td>55</td>
<td>62</td>
<td>44</td>
<td>6.2</td>
<td>10.8</td>
<td>53.8–70.6</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>1 week–&lt;2 years</td>
<td>&lt;0.5</td>
<td>3.8</td>
<td>4.7</td>
<td>136</td>
<td>3.7</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2–&lt;19 years</td>
<td>&lt;0.5</td>
<td>2.3</td>
<td>2.6</td>
<td>516</td>
<td>2.5</td>
<td>2.8</td>
<td></td>
<td></td>
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<tr>
<td>Free PSA (ng/mL or μg/L)</td>
<td>0–&lt;12 yearsa</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
<td>69</td>
<td>0–&lt;19 years</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
<td>302</td>
<td>0.044–0.194</td>
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<tr>
<td></td>
<td>12–19 yearsa</td>
<td>&lt;0.008</td>
<td>0.239</td>
<td>0.279</td>
<td>122</td>
<td>0.223–0.552</td>
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<td></td>
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<tr>
<td>HE4 (pM/L)</td>
<td>0–&lt;1 week</td>
<td>159</td>
<td>567</td>
<td>618</td>
<td>44</td>
<td>137–182</td>
<td>549–689</td>
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<td></td>
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<td></td>
<td>1 week–&lt;6 months</td>
<td>55.7</td>
<td>165</td>
<td>178</td>
<td>68</td>
<td>51.1–61.1</td>
<td>163–193</td>
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<td></td>
<td>6 months–&lt;2 years</td>
<td>30.9</td>
<td>92.4</td>
<td>98.6</td>
<td>92</td>
<td>27.0–34.9</td>
<td>92.4–105</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2–&lt;10 years</td>
<td>27.3</td>
<td>63.4</td>
<td>69.7</td>
<td>181</td>
<td>25.7–28.7</td>
<td>63.4–81.7</td>
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</tr>
<tr>
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<td>10–19 years</td>
<td>22.5</td>
<td>52.7</td>
<td>61.8</td>
<td>233</td>
<td>21–24.4</td>
<td>53.0–75.4</td>
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<td></td>
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<tr>
<td>ProGRP (pg/mL or ng/L)</td>
<td>0–&lt;1 week</td>
<td>535</td>
<td>1749</td>
<td>1889</td>
<td>45</td>
<td>446–622</td>
<td>1710–2072</td>
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<td></td>
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<td>1 week–&lt;6 months</td>
<td>57</td>
<td>732</td>
<td>817</td>
<td>70</td>
<td>39–79</td>
<td>733–907</td>
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<tr>
<td></td>
<td>6 months–&lt;1 year</td>
<td>25</td>
<td>180</td>
<td>198</td>
<td>52</td>
<td>18–31</td>
<td>178–219</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1–&lt;12 years</td>
<td>22</td>
<td>108</td>
<td>129</td>
<td>257</td>
<td>21–24</td>
<td>106–134</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12–19 years</td>
<td>17</td>
<td>74</td>
<td>83</td>
<td>183</td>
<td>14–20</td>
<td>73–107</td>
<td></td>
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</tr>
</tbody>
</table>

Continued on page XX
Two of the analytes examined, CA15-3 and CA125, showed atypical patterns of expression during the first year of life given their suspected status as oncofetal antigens (Fig. 3). CA15-3 concentrations were lower during the first week of life, with the upper limit of the reference interval falling at 24 U/L followed by an increase in concentration with the upper limit of the 1-week to <1-year reference interval falling at 33 U/L (Table 2). Similarly, the upper limit of the 0- to <4-month reference interval for CA125 fell at 22 U/L, whereas the upper limit of the 4-month to <5-year reference interval was at 33 U/L (Table 2).

Three of the markers examined—CA125, total PSA, and free PSA—required sex-specific partitions (Fig. 4). CA125 required sex-specific partitions in the teenage group. Specifically, the upper limit of the reference interval for girls aged 11–19 years was significantly higher than that of boys (39 vs 28 U/L).

In contrast to CA125, for which only 1 age partition demonstrated sex differences, in the case of total and free PSA, given the vastly different patterns of expression observed in boys and girls, age partitioning for the 2 sexes was carried out separately. Several notable trends were observed with respect to these 2 markers. First, for total PSA, higher upper limits were observed in the 0- to <1-week reference interval compared to the 1-week to <6-month reference interval in boys (0.047 vs 0.038 ng/mL). Similarly, higher upper limits were observed in the 0- to <1-week reference interval compared to the 1-week to <1-year age partition in girls (0.039 vs 0.010 ng/mL). In addition, for both free and total PSA, increases in concentration were observed in boys 12 years old. Both total and free PSA required a unique partition for males age 12–19 years. In contrast to the dynamic changes observed in total PSA concentrations in boys and girls and in free PSA concentrations in boys, only 1 free PSA reference interval spanning the entire pediatric age range was required for girls.

**Discussion**

Cancer biomarkers have become an important tool in the treatment of cancer patients, particularly in the monitoring of patients during and after treatment (Table 1). However, there remains a paucity of information regarding the application of these markers to pediatric cancers. Specifically, little is known about how concentrations of these markers fluctuate in a healthy reference population and, as previous studies have demonstrated (2, 3, 17), the potential of these markers remains limited without rigorously established reference intervals that have been properly stratified by key covariates like age and sex. By establishing age- and sex-specific pediatric reference intervals for 11 key bio-

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**Table 2. Age- and sex-stratified reference intervals for 11 serum immunoassay cancer biomarker analytes analyzed on Abbott Architect ci4100.**

(Continued from page XX)
markers, the present study has taken an important first step toward harnessing the full potential of these markers in the pediatric population. As expected, significant fluctuations in biomarker concentrations by age and sex were observed in 10 of the 11 biomarkers investigated. Age partitioning was required for CA15-3, CA125, CA19-9, CEA, SCC, proGRP, total and free PSA, HE4, and AFP, whereas sex partitioning was also required for CA125 and total and free PSA (Table 2).

Several common expression patterns were observed between the various analytes examined. Six of the oncofetal antigens (AFP, HE4, proGRP, CEA, CA19-9, and SCC) exhibited the expected pattern of expression with high concentrations at birth followed by rapid decreases to significantly lower concentrations (Fig. 2). This pattern is likely explained by the fact that many of these analytes are expressed during fetal development and/or play an important role in early postnatal development (17–19). It is also possible that these analytes are found in higher concentrations in the serum of neonates due to immature hepatic function (7). That is, if the liver properly processes these proteins, the high serum concentrations observed here may be the result of inefficiency in immature neonatal livers.

In agreement with our data, similar trends have been previously demonstrated for AFP, proGRP, and CA19-9 (7, 9, 18). Reference intervals were also previously established for both AFP and CA19-9. AFP reference intervals were established on earlier-generation AFP Abbott Architect assay by CALIPER in 2013. The reference intervals established in the current study had, overall, higher upper limits than those established in the previous CALIPER study. It should be noted that this may be due to differences in the 2 assays, specifically related to a higher limit of detection in the AFP assay used in the present study. Regarding CA19-9, the upper limit of the 0- to <1-year reference interval established in the current study (64 U/mL) was vastly different from the upper limit of the 95th percentile CI (22 U/L) established by Lahdenne et al. for the 0.1- to 0.5-year age range (7). This may be due, in part, to the fact that Lahdenne et al. used an RIA method and reagents (including antibodies) distinct from the ones used in the present study (7).

Surprisingly, CA125 and CA15-3 showed a distinct pattern of expression compared to the first group of analytes (Fig. 3). Both CA15-3 and CA125 showed slightly lower concentrations in the early days and

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**Fig. 2.** Age-dependent scatter plots, by sex, of analytes with high concentrations at birth. (A), AFP; (B), CA19-9; (C), CEA; (D), HE4; (E), proGRP; (F), SCC.
months of life, respectively. Although the status of both markers as oncofetal antigens remains controversial, given that both markers are known to be expressed in fetal and neonatal tissue, this pattern of expression is unexpected; however, in the case of CA125, it has been shown that concentrations in both maternal serum and amniotic fluid are higher in the early stages of pregnancy and tend to attenuate by birth (20). It is possible, therefore, that CA15-3 and CA125 may play a role in fetal development at the early stages of pregnancy and may again become important to neonatal development after the first week and months of life, respectively. Patterns similar to the ones observed here have been previously demonstrated for CA125. In addition, reference intervals were established for birth to 1.5 years (7). Again, although the expression patterns were similar, the established values differed greatly from those of Lahdenne et al. (7), who used an RIA method and reagents/antibodies distinct from those used in the present study. Whereas the upper limit of the 0- to <4-month reference interval established in the current study was 22 U/mL, the upper limit of the 95th percentile CI established by Lahdenne et al. for the 0.1- to 0.5-year age range was 45 U/L (7).

Three analytes demonstrated sex differences according to our analysis: free PSA, total PSA, and CA125 (Fig. 4). Specifically, free and total PSA concentrations were higher in males than in females after puberty. This is expected, as PSA concentrations are known to be controlled by insulin-like growth factor 1 and testosterone, both of which increase during puberty. Additionally, previous studies have demonstrated that PSA concentrations increase with Tanner stage (21), lending further support to the hypothesis that changes linked to puberty cause an increase in PSA concentrations in young males. Randell et al. (22) had previously established reference intervals for total PSA in males and females from birth to 18 years. Because this study created 7 different age partitions for total PSA in males and females from birth to 18 years. Because this study created 7 different age partitions for total PSA in males and females from birth to 18 years.
age 11 to <19 years compared with males. There are a variety of explanations for this observed elevation in the female population, including development of the female reproductive system at puberty, phase of menstrual cycle, and pregnancy (23). However, to our knowledge, this is the first study to uncover this sex difference in CA125 concentrations; thus, further work examining the relationship between phase of the menstrual cycle and CA125 concentrations, as well as Tanner stage and CA125 concentrations, should be undertaken.

Anti-Tg was the only marker that did not require any age or sex partitioning. This contradicts a recent report by Taubner et al., who observed sex differences, with higher concentrations of anti-Tg in girls aged 6–20 years (24). This may be because that study was carried out by use of a different immunoassay on the Roche Modular System. The poor concordance between anti-Tg assays has been previously noted (25) and highlights the importance of establishing reference intervals specific to each platform. This is crucial not only for anti-Tg but for all tumor marker assays. The U.K. National External Quality Assessment Service has reported between-method CVs in excess of 20% for some tumor markers (26). Therefore, it is important to consider that these reference intervals are specific to the Abbott Architect ci4100 platform, and future studies will be required to translate these reference intervals to additional platforms.

It is important to note that key differences were observed between the pediatric reference intervals established here and recommended cutoffs in the adult population. It is also important to note that reference intervals and cutoff values that we have used for comparison are 2 distinct types of measurements; although, depending on the method used for the establishment of cutoff values, a reference interval upper limit and cutoff value may have similar or identical definitions (27). Regardless of the method used for determination of cutoff values, both reference intervals and cutoff values can help provide an indication of test results that are likely to be indicative of a normal, healthy individual in comparison to a diseased patient and are, therefore, useful for general comparisons. Specifically, both anti-Tg and total PSA remained well below recommended adult cutoffs of 22 IU/mL and 2–4 ng/mL throughout the entire pediatric age range (24, 28). Not surprisingly, 4 of the oncofetal antigens examined (SCC, HE4, CEA, and AFP) fell above the recommended adult cutoffs during the neonatal period and subsequently dropped to below or approximately at the adult cutoff (23, 29–31). On the other hand, 2 of the oncofetal antigens examined (proGRP and CA19-9) were persistently higher than the recommended adult cutoffs (23, 32). Finally, at birth, the upper limit of the reference intervals for both CA15-3 and CA125 (24 and 22 U/mL) fell below the recommended adult cutoffs of 25 and 35 U/mL, respectively. However, although the CA15-3 reference interval falls below the adult cutoff at around 1 year of age, concentrations of CA125 in teenage females are higher than the adult cutoff of 35 U/mL, with an upper limit of 39 U/mL (Table 2). These differ-

![Fig. 4. Age-dependent scatter plots by sex of analytes with sex-specific reference intervals. (A), Total PSA; (B), free PSA. CA125 (Fig. 3A) is an additional analyte with sex-specific reference intervals.](https://example.com/fig4.png)
ferences highlight the importance of establishing reference intervals specific to the pediatric population.

There are certain limitations to the present study that are important to recognize. First, many of the samples used in this analysis have been stored at −80 °C for up to 3 years. The CALIPER project has previously examined the stability of 57 biochemical markers also stored at −80 °C over a 10- to 13-month period and discovered that all analytes with the exception of parathyroid hormone were very stable (33). However, with the exception of AFP, none of the cancer markers examined in the present study were included in that stability study. Second, the data for several of the analytes examined in the present study were highly skewed. Although transformations were carried out to mitigate any potential issues in the statistical analysis, in many cases, the skewness of the data resulted in wide confidence intervals, specifically around the upper limit of the reference interval (Table 2). Finally, owing to the difficulty in collecting a large sample volume of blood from the neonatal population, we were limited in our ability to partition free PSA data in the first year of life even though the scatterplot suggests the need for a 0- to <1-week age partition.

In addition to the establishment of reference intervals for other cancer biomarkers, it will be important to carry out a transference study to establish reference intervals for the 11 markers examined on additional analytical platforms (34). If transference is not possible, a full reference interval study will need to be carried out on additional platforms. Finally, if these markers are to be used for monitoring cancer patients, it will be important to establish key biological variation parameters such as reference change values to make informed choices about what constitutes a significant change between serial measurements (35).

The establishment of pediatric reference intervals for tumor biomarkers will aid in harnessing the true predictive, diagnostic, and monitotrial potential of tumor markers in a pediatric population. These reference intervals will be useful not only in practice, by contributing to a more accurate assessment of tumor markers in cancer diagnosis and treatment, but also in research aimed at determining the prognostic and/or diagnostic value of tumor markers as well as the establishment of cutoff values in pediatric cancers.

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