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# Establishment of reference intervals for thyroid hormones in premature infants beyond the first week of life using Beckman Coulter Unicel DxI 800

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ABSTRACT

*Background:* This 4-year retrospective cohort study aimed to establish reference intervals for free triiodothyronine (FT3), free thyroxine (FT4), and thyrotropin (TSH) in premature infants using the Beckman Coulter Unicel DxI 800 automated immunoassay system.

*Methods:* Study subjects included 605 preterm infants with a gestational age of 26–36 weeks (corrected: 29–38 weeks). Pearson correlation was used to evaluate the association between hormone levels and gestational and corrected gestational ages. A nonparametric method was used to establish reference intervals based on corrected gestational age.

*Results:* FT3 and FT4 levels were positively correlated with gestational and corrected gestational ages, respectively. TSH levels were slightly negatively correlated with gestational and corrected gestational ages. FT3 significantly differed according to corrected gestational age (29–33 weeks vs 34–38 weeks); however, the difference was smaller than the reference change value (RCV) for the FT3 test. Thus, we combined the FT3 reference intervals into a single reference interval: 2.65–4.93 pmol/L (29–38 weeks). The reference intervals of FT4 and TSH were 11.20–24.97 pmol/L (29–38 weeks) and 1.01–10.14 mIU/L (29–38 weeks), respectively.

*Conclusions:* Unlike those of full-term infants or adults, the reference intervals established in this study are applicable in premature infants. These results highlight the importance and complexity of establishing instrument-specific thyroid hormone reference intervals for preterm infants.

## 1. Introduction

Thyroid dysfunction is among the most common preventable causes of neonatal neurodevelopmental delay. Because of their immature hypothalamic-pituitary-thyroid axis, immature thyroid hormone synthesis and metabolism, systemic diseases, and physiological and non-physiological factors such as immature thyroid enrichment and iodine synthesis, premature infants likely have a wider thyroid hormone reference compared to full-term infants [1]. The use of established reference intervals for full-term infants or even those provided in the reagent instructions (typically established for adults) results in frequent and unnecessary retesting and intervention [2]. Mixing of reference intervals established by different detection systems is also well-known to be inappropriate. Therefore, it is essential to determine instrument-specific reference intervals for vulnerable populations such as premature infants. Chinese guidelines for the diagnosis and treatment of thyroid disease during pregnancy and postpartum [3] stipulate that early thyroid hormone screening should be carried out in newborns in China. Blood samples should be obtained from full-term neonates from 72 h up to 7 days post-birth, and the guidelines recommend delaying sampling until 7 days post-birth for premature neonates. However, few studies have examined the reference intervals of thyroid hormones in preterm infants after the first week of life. It is difficult to obtain a large number of blood samples from healthy preterm infants. Thus, in this study, we used the hospital database to obtain preterm infant inpatient medical records using appropriate screening methods and referred to the EP28-A3c file [4] revised by the Clinical and Laboratory Standards Institute (CLSI) of America and International Federation of Clinical

*Abbreviations:* Free triiodothyronine, FT3; Free thyroxine, FT4; Thyrotropin, TSH; Lower quartile, Q1; Upper quartile, Q3; Interquartile range, IQR

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Chemistry to establish thyroid hormone reference intervals in our region using the Beckman DxI 800 automated immunoassay system. All hospitalized premature infants were subjected to venous blood sampling for thyroid hormone testing 7 days after birth.

## 2. Materials and methods

### 2.1. Study subjects

Preterm infants born between 2015 and 2018 in the neonatal department of Women and Children's Hospital, Xiamen city, Fujian Province of China, were selected. We carefully reviewed the infants' medical records, which included the basic condition and detailed disease information, mode of departure from the hospital (e.g., infant death), and the mother's health condition (e.g., diagnosed endocrine disease), with a special focus on the discharge summary. The infants had a gestational age of 26–36 weeks and corrected gestational age (i.e., gestational age plus age at the time of blood collection) of 29–38 weeks. The blood collection time was > 7 days after birth. In this study, we defined “normal” preterm birth as a common preterm birth complication [5]. For infants whose gestational age was > 33 weeks, the 5-min Apgar score should be ≥7; for gestational age ≤ 33 weeks, the 5-min Apgar score should be ≥6. The inclusion criteria for normal premature infants were a height, weight (between the 10th and 90th percentile of average birth weight), head circumference, and growth rate appropriate for gestational age. Exclusion criteria for infants were as follows: (1) thyroid abnormalities detected within 7 days of birth and treated with thyroxine, and clinically suspected of having thyroid diseases (feeding difficulties and long-term or recurrent jaundice); (2) major congenital abnormalities or significant clinical evidence of infection, such as necrotizing enterocolitis, respiratory distress syndrome, intraventricular hemorrhage, central nervous system diseases, and sepsis; (3) extrauterine growth retardation with growth values at or below the 10th percentile of intrauterine growth expectation based on estimated gestational age; (4) death within one month after birth; and (5) mother suffering from thyroid disease. These data were evaluated by neonatal physicians who did not participate in the study and were recorded in detail in the discharge summary. According to standard clinical protocols, nearly all mothers (approximately 90%) were administered prenatal glucocorticoid therapy to promote fetal lung maturation. This study was approved by the Ethics Committee of Xiamen Women and Children's Hospital, and all parents gave consent for their infants to participate in the study.

### 2.2. Instruments and reagents

A detection system, the UniCel DxI 800 chemiluminometer (Beckman Coulter, Brea, CA, USA), and its accompanying reagents were used. Free triiodothyronine (FT3) (kit code A13422), free thyroxine (FT4) (kit code 33880), and thyrotropin (TSH) (kit code 33820) levels were determined by conducting a competitive binding immunoassay, two-step competitive enzyme assay, and two-site enzyme immunoassay (sandwich method), respectively. We used two different levels of serum quality controls provided by Beckman. The intra-assay and inter-assay precision were < 6.25% and < 8.33%, respectively. According to the Westgard Sigma quality management model [6], when using the total allowable error in national standard, FT3, FT4 and TSH all levels of the sigma was > 4. We therefore used the $1_{3S}$ /$2_{2S}$ /$R_{4S}$ /$4_{1S}$ combination rules. The project passed the external quality assessment conducted by the Clinical Testing Center of the Ministry of Health of China and Clinical Testing Center of Fujian Province. The study was carried out in strict accordance with standard operating procedures. The accelerator vacuum tube composed of an inert colloidal material was used to collect venous blood in the morning. Serum separation was completed within 2 h, and the test was completed within 24 h.

### 2.3. Methods

#### 2.3.1. Normality analysis and transformation of data

Normality analysis of the data was performed using the skewness-kurtosis test. When the absolute value of the skewness and kurtosis was < 1.96-fold of the standard error of the sample, we considered the distribution as approximately normal. Non-normally distributed data were transformed by Box-Cox transformation, and normality analysis was repeated. The λ value in the transformation was obtained by maximum likelihood estimation.

#### 2.3.2. Elimination of outliers

Tukey's method was used to determine the lower quartile (Q1), upper quartile (Q3), and interquartile range (IQR) and to eliminate the data beyond (Q1–1.5 IQR) to (Q3 + 1.5 IQR) [7]. The cycle was continued until all outliers were removed.

#### 2.3.3. Grouping of reference intervals

*Z*-test [8] was used to calculate the Z-values (Formula 1) and Z* values (Formula 2) as follows:

$$Z = \frac{\bar{x}_1 - \bar{x}_2}{\left[\left(\frac{S_1^2}{n_1}\right) + \left(\frac{S_2^2}{n_2}\right)\right]^{1/2}} \quad (1)$$

$$Z^* = 3(n_{average}/120)^{1/2} = 3[(n_1 + n_2)/240]^{1/2} \quad (2)$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means of the two groups, $S_1$ and $S_2$ are the standard deviations of the two groups, and $n_1$ and $n_2$ are samples from each group. Grouping criteria were as follows: when $|Z| > Z^*$ or $S_2 > 1.5\,S_1$ or $|S_2/(S_2 - S_1)| < 3$. At least 120 cases were required per group, and those who could not be grouped were combined into one group. Because the days on which the blood was collected varied among newborns, and the corrected gestational age may affect the reference intervals of premature infants, the subjects were temporarily divided into three groups with corrected gestational ages: 29–33, 34–35, and 36–38 weeks.

#### 2.3.4. Establishment of biological reference intervals

The nonparametric method was used to establish the reference intervals. In the interval in which 2.5–97.5% of the values were located, the size of the 95% distribution range was selected to represent the reference intervals.

### 2.4. Other statistical analyses

Statistical analyses of the data were performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA) with the skewness-kurtosis normality test, Box-Cox transformation, outlier elimination, and Pearson correlation analysis. The mean ± standard deviation ($\bar{x} \pm S$) was used to analyze data conforming to the normal distribution, while the median (IQR) was used to analyze data that did not show a normal distribution. $P < .05$ was considered to indicate a statistically significant difference.

## 3. Results

### 3.1. Demographic results of the study cohort

We extracted 2235 neonatal thyroid data from 2015 to 2018. After carefully reviewing the medical records according to the inclusion and exclusion criteria, 605 “normal” preterm infants (361 males and 244 females) were included in the study. The mean (SD) gestational age of this cohort was 32.7 weeks (2.1). The mean (SD) corrected gestational age was 34.6 weeks (2.0) at the time of testing. The median age at the time of testing was 11 days (IQR = 9–15). Other demographic characteristics are shown in Table 1.

**Table 1**
Demographic characteristics of the study cohort.

| Total number of infants (n) | 605 |
|---|---|
| Male/female (n) | 361/244 |
| Birth weight, g (mean ± SD) | 1923.3 ± 405.5 |
| Length, cm (mean ± SD) | 43.5 ± 4.2 |
| Head circumference, cm (mean ± SD) | 32.9 ± 2.1 |
| Birth Apgar score at 1 min (mean ± SD) | 8.7 ± 1.4 |
| Birth Apgar score at 5 min (mean ± SD) | 9.2 ± 0.9 |
| Birth Apgar score at 10 min (mean ± SD) | 9.3 ± 0.7 |
| Gestational age, weeks (mean ± SD) | 32.7 ± 2.1 |
| Corrected gestational age at the time of testing, weeks (mean ± SD) | 34.6 ± 2.0 |
| Age at testing in days, median (IQR) | 11 (9–15) |
| Hospital stay in days, median (IQR) | 22 (14–32) |

### 3.2. Inclusion data normality analysis and transformation

The skewness-kurtosis test showed that FT3, FT4, and TSH were not normally distributed. Thus, the variables were converted to a normal distribution by Box-Cox test. The results are shown in Table 2.

### 3.3. Correlation analysis results

Pearson correlation analysis showed that FT3 and FT4 were positively correlated with gestational age and corrected gestational age, respectively. TSH was negatively correlated with gestational age and corrected gestational age, and the linear regression coefficient β was determined, as shown in Table 3.

### 3.4. Outliers and grouping results of reference intervals

The outliers after normal transformation included five cases in FT3, two in FT4, and eight in TSH. The *Z*-test results revealed no significant difference between the FT3 levels in the group with a corrected gestational age of 34–35 weeks and the group with a corrected gestational age of 36–38 weeks, with all levels showing significant differences from those in the group with a corrected gestational age of 29–33 weeks. However, we found that the difference in the lower limit of normal and upper limit of normal of the FT3 hormone between 29 and 33 weeks and 34–38 weeks of corrected gestational age (9.5–10.2%) was smaller than the RCV for the FT3 test (29.4%). Thus, we combined the FT3 reference intervals into a single reference interval (29–38 weeks). For the corrected gestational age, combining the 34–35 week group and 36–38 week group did not significantly change the reference intervals for FT4 and TSH; thus, these groups can use a single reference interval. No group met the criterion of $S_2/S_1 > 1.5$ or $|S_2/(S_2 - S_1)| < 3$, as shown in Table 4.

### 3.5. Comparison of reference intervals established in this study with the reference intervals from Beckman and Roche

The nonparametric ranking method used values of 2.5% and 97.5% as the lower and upper limits of the reference intervals, respectively. The reference intervals established in this study showed large differences from those provided by Beckman (UniCel DxI 800) and Roche (Cobas e 411) [9], as shown in Table 5.

Owing to the lack of reference intervals for thyroid hormone levels of premature infants, if a clinician uses the adult reference intervals provided by Beckman, 581 of 605 infants in this cohort would be considered to have abnormal levels, which is unreasonable. If the reference intervals established in this study are used, the number of infants who require repeat testing or intervention is reduced to 85.

## 4. Discussion

According to the recommendations of CLSI, healthy subjects should be selected and a direct reference method should be used to establish a reference interval; however, this would be costly. Moreover, samples must be collected from a vulnerable group of premature infants, which is difficult to achieve. Because of ethical and practical considerations, we retrospectively reviewed medical records provided by the hospital database to identify newborns who were “healthy except for prematurity.” We therefore excluded premature infants who were considered “relatively unhealthy”, such as those larger or smaller than gestational age standards and those with extrauterine growth retardation. The lack of consistency in the definition of “normal” preterm infants made this process difficult. Premature infants are at a higher risk for certain diseases, and maturity changes in FT4 levels often occur during the last trimester of pregnancy. Non-thyroid diseases such as respiratory distress syndrome may affect thyroid hormone levels [10]. Williams et al. [11] reported that delayed sepsis significantly reduced thyroxine, total triiodothyronine, and thyroxine binding globulin levels. Furthermore, they found that intraventricular hemorrhage, necrotizing enterocolitis, and the onset of acute inflammatory cytokine response affected the thyroid hormone levels and caused thyroid dysfunction. In our previous study, we also found that maternal thyroid disease was a risk factor affecting the results of neonatal TSH screening [12]. Some studies evaluated a cohort comprising both diseased preterm infants and “normal” preterm infants, which may have increased the reference interval range [13].

Because of immature thyroid axis development in premature infants, the production and secretion of thyrotropin-releasing hormone in the hypothalamus are reduced, eventually leading to decreased levels of total triiodothyronine, thyroxine, and FT4 in preterm infants. However, within 6–8 weeks after delivery, these levels increase to levels comparable to those in full-term infants [14]. We found that FT3 and FT4 levels were positively correlated with gestational and corrected gestational ages, respectively, and TSH showed a weak negative correlation with gestational and corrected gestational ages, which is consistent with the results of some previous studies [15,16]. Because the blood collection days varied among newborns, some studies [9,17] directly grouped the gestational age or days at the time of testing or did not distinguish full-term from preterm infants, which may have affected the final result. This issue was taken into consideration in our study. We grouped variables according to the corrected gestational age at the time of testing and found that FT3 should be grouped by 29–33 and 34–38 weeks (the difference was smaller than the RCV), while FT4 and TSH did not require grouping. We also performed statistical analysis by gender, consistent with previous literature [16], but there were no significant differences in hormone levels by gender.

Some studies recommend 11.58–29.60 pmol/L FT4 as a reference

**Table 2**
Normality analysis of each hormone and Box-Cox transformation results.

| Hormone | Number of cases (n) | Parameter λ | Before transformation | | | | After transformation | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Standard deviation | Kurtosis | Skewness | Mean | Standard deviation | Kurtosis | Skewness |
| FT3 | 605 | 0.53 | 3.76 | 0.63 | 0.482 | 0.292 | 1.90 | 0.34 | 0.304 | 0.018 |
| FT4 | 605 | 0.15 | 17.45 | 3.85 | 0.303 | 0.513 | 3.54 | 0.34 | −0.190 | −0.001 |
| TSH | 605 | 0.07 | 4.03 | 2.85 | 12.955 | 2.909 | 1.27 | 0.67 | 1.017 | 0.014 |

**Table 3**
Correlation of test results of each hormone with gestational age and corrected gestational age.

| Hormone[a] | Gestational age | | | | Corrected gestational age | | | |
|---|---|---|---|---|---|---|---|---|
| | *r* | *P*[b] | *β* | *P*[c] | *r* | *P*[b] | *β* | *P*[c] |
| FT3 | 0.404 | 0.000 | 0.066 | 0.000 | 0.410 | 0.000 | 0.071 | 0.000 |
| FT4 | 0.242 | 0.000 | 0.039 | 0.000 | 0.148 | 0.000 | 0.026 | 0.000 |
| TSH | −0.103 | 0.011 | −0.033 | 0.011 | −0.177 | 0.000 | −0.060 | 0.000 |

[a] Box-Cox transformed result.
[b] *P* value of correlation coefficient.
[c] *P* value of regression coefficient.

interval for full-term infants [16]; the upper limit of this range is higher than that found in our research. Some authors [18] reported the normal TSH concentration at 2–6 weeks of age as 1.7–9.1 mIU/L; however, our TSH range was wider than these values. Zhu et al. [9] provided reference intervals based on the corrected gestational age for the second week of preterm birth; the ranges for FT3 and FT4 were similar to those found in our study, but the TSH range was wider (0.68–12.53 mIU/L). We found no records of outlier removal in their reports, which may explain the wider reference interval. Our upper and lower limits of FT3 were lower, upper and lower limits of TSH were higher, and range of FT4 was significantly wider than the reference intervals provided by the kit.

The elimination of outliers is an indispensable step in establishing reference intervals. Although the Dixon test is recommended by CLSI as a method of culling outliers, the EP28-A3c guidelines also point out that if there are several similar outliers on the same side of the data, they will mask each other and render the Dixon test less effective. In this case, the data analyst must calculate the outlier with the smallest dispersion to ensure that all outliers can be eliminated. However, the method for determining the outlier with the smallest dispersion when using the Dixon test is not described in the EP28-A3c guidelines. Therefore, we chose the Tukey method, which uses only 50% of the sample data center to reduce or limit the masking effect of multiple simultaneous outliers on one side. For this test, we determined the lower quartile (Q1), upper quartile (Q3), and interquartile range (IQR) and eliminated the data beyond (Q1–1.5 IQR) to (Q3 + 1.5 IQR). The cycle was continued until all outliers were removed.

The comparison between the Beckman and Roche platforms showed that there are still large differences in neonatal reference intervals established for different detection platforms (e.g., TSH: (1.01–10.14) vs (4.55–20.00)). These differences may be explained as follows. First, Roche did not distinguish premature and term infants, whose thyroid hormone levels are different. Second, they did not distinguish corrected gestational ages. It is well known that thyroid hormone levels are different between the first week of life and at 2–4 weeks of life [19]. Thus, the reference intervals established by Roche may be wide, which prompted us to establish laboratory-specific reference intervals for specific neonatal groups.

There were several limitations to our study. Although we adopted the most stringent inclusion and exclusion criteria, drugs such as dopamine, caffeine, and steroids used to treat premature infants may have caused transient thyroid dysfunction and affected the test results. Given that there is currently no consistent interaction report [20–22] and these drugs are routinely used in common complications of preterm infants, these factors likely did not affect the reference intervals established for this population. We retested some outliers of clinical concern (i.e., TSH > 20 mIU/L). However, because this was a retrospective analysis, we did not retest all outliers. In addition, because of the large number of subgroups of the inclusion and exclusion criteria, some premature neonates belonged to multiple subgroups. Although we have thyroid data for these premature neonates, there are inherent difficulties in data analysis.

Premature infants have a certain level of hyperbilirubinemia. The "INTERFERENCES" section of the Beckman instructions states the following: Samples containing up to 20 mg/dL (342 μmol/L) bilirubin and 10 mg/dL (119 μmol/L) conjugated bilirubin do not affect the concentration of free T3 assayed. Samples containing up to 10 mg/dL (171 μmol/L) bilirubin do not affect the concentration of free T4 or TSH assayed. In our cohort, 67 neonates (11.1%) had higher bilirubin concentrations than the upper limit of the interference test, with a median of 213.7 μmol/L (IQR = 192.1–278.7). However, the manufacturer's instructions did not further elaborate on how hyperbilirubin interferes with the test. In addition, the purpose of this study was to provide reference intervals for thyroid function assessment in preterm neonates that can be extended to the entire preterm neonatal population, in which the proportion of hyperbilirubin should be no different from that of the study population. Thus, the current clinically available methods for assessing neonatal thyroid function are inevitably affected by uncertain effects of hyperbilirubin.

Because the measurements of many laboratories are not yet comparable, reference intervals are usually established for each assay and are considered assay-specific. However, healthcare providers who

**Table 4**
Relationship between test results of each hormone and corrected gestational age.

| Hormone[a] | Corrected gestational age (weeks) | Number of cases (n) | Mean | Standard deviation | Judging criteria | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $\lvert Z\rvert$ | $Z^*$ | $S_2/S_1$ | $\lvert S_2 / (S_2–S_1)\rvert$ | Remarks |
| FT3 | ① 29–33 | 200 | 1.75 | 0.30 | 6.94 | 4.16 | 0.98 | 44.98 | ① and ② comparison[b] |
| | ② 34–35 | 261 | 1.95 | 0.30 | 2.87 | 3.87 | 1.12 | 9.68 | ② and ③ comparison |
| | ③ 36–38 | 139 | 2.04 | 0.33 | 8.27 | 3.57 | 0.92 | 11.00 | ③ and ① comparison[b] |
| FT4 | ① 29–33 | 200 | 3.47 | 0.33 | 4.07 | 4.16 | 1.03 | 33.84 | ① and ② comparison |
| | ② 34–35 | 262 | 3.60 | 0.34 | 1.87 | 3.89 | 0.95 | 19.18 | ② and③ comparison |
| | ③ 36–38 | 141 | 3.53 | 0.32 | 1.77 | 3.58 | 1.02 | 48.52 | ③ and ① comparison |
| TSH | ① 29–33 | 198 | 1.39 | 0.66 | 2.40 | 4.15 | 0.92 | 11.45 | ① and ② comparison |
| | ② 34–35 | 262 | 1.25 | 0.60 | 1.16 | 3.87 | 1.01 | 90.93 | ② and ③ comparison |
| | ③ 36–38 | 137 | 1.18 | 0.61 | 3.11 | 3.54 | 1.08 | 14.27 | ③ and ① comparison |

[a] Box-Cox transformed result.
[b] $\lvert Z\rvert > Z^*$.

**Table 5**
Comparison of reference intervals for FT3, FT4, and TSH.

| Hormone | UniCel DxI 800 (Beckman Coulter, Brea, CA, USA) | | Cobas e 411 (Roche Diagnostics GmbH, Mannheim, Germany) |
|---|---|---|---|
| | Reference intervals established in this study for 29–38 weeks (corrected gestational age) | Manufacturer reference intervals | 0–30 days |
| FT3 (pmol/L) | 2.65–4.93 | 3.85–6.01 | 2.60–8.10 |
| FT4 (pmol/L) | 11.20–24.97 | 7.85–14.40 | 8.50–39.80 |
| TSH (mIU/L) | 1.01–10.14 | 0.34–5.60 | 4.55–20.00 |

require test results from different laboratories often face challenges owing to different reference intervals. Thienpont et al. [23] reported a multiassay method comparison study with clinical serum samples and target setting with a robust factor analysis method for TSH, which provided evidence that harmonization may enable manufacturers to achieve more uniform reference intervals in the near future. The researchers pointed out that a uniform reference interval does not mean "one size fits all" and may be affected by factors such as age, ethnicity, and iodine intake. We anticipate that our study of premature infants will contribute to the uniformity of thyroid hormone assays and better consistency of future results.

In conclusion, we used the Beckman UniCel DxI 800 immunoassay system to establish reference intervals for FT3, FT4, and TSH after one week of birth in preterm infants in our region. These reference intervals were significantly different from those of term infants and those provided with the kit. Furthermore, these results can be used to avoid unnecessary retesting and intervention of preterm infants and will be valuable for clinical evaluation of thyroid function in preterm infants and differential diagnosis of thyroid diseases.

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## Declarations of Competing Interest

None.

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