

ORIGINAL ARTICLE

Comparison of Testosterone in Umbilical Cord Blood of Preterm Infants Measured by Immunoassay and Mass Spectrometry

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ABSTRACT

Background: Testosterone plays an important role in male sexual differentiation. In adult males, electrochemiluminescence immunoassays (ECLIA) are used globally to measure serum testosterone levels because of their efficiency and good correlation with liquid chromatography-tandem mass spectrometry (LC-MS/MS), despite their cross-reactivity potential. However, the accuracy and clinical significance of these methods in the early neonatal period remain unclear. This study compared umbilical cord testosterone levels measured using ECLIA and LC-MS/MS to discriminate sex differences between two methods in preterm infants with typical external genitalia.

Methods: Forty-eight preterm infants admitted to the neonatal intensive care unit of the Nihon University Itabashi Hospital between May 2020 and March 2024 were recruited. Umbilical cord serum testosterone levels were measured using ECLIA and LC-MS/MS. Sex differences in testosterone levels between males and females were compared using the Mann-Whitney U test for median values and interquartile ranges (IQRs) (p-value < 0.05). The Spearman's correlation analysis, a non-parametric method, was performed to evaluate the correlations between testosterone levels measured using ECLIA, and testosterone levels measured using LC-MS/MS. Method comparison between ECLIA and LC-MS/MS was performed using Passing-Bablok regression. Logistic regression and receiver operating characteristic (ROC) curve analyses were performed to assess the ability of testosterone levels measured using ECLIA and LC-MS/MS to predict sex of preterm infants with typical external genitalia, respectively.

Results: This study included 38 preterm infants with typical external genitalia (16 males and 22 females). Testosterone levels measured using ECLIA were significantly higher and exhibited wider distributions (males: 1.10 ng/mL, IQR 0.90 - 2.69; females: 0.95 ng/mL, IQR 0.71 - 1.18) than those measured using LC-MS/MS (males: 0.210 ng/mL, IQR 0.164 - 0.327; females: 0.026 ng/mL, IQR 0.0112 - 0.0526) (p-value < 0.05). The correlation between testosterone levels measured using ECLIA and those measured using LC-MS/MS was weak (Spearman's rank correlation coefficient $\rho(\text{rho}) < 0.5$). In all infants, the Passing-Bablok regression yielded a slope of 0.082 and an intercept of -0.021 (Figure 3A), indicating limited agreement. Among preterm infants with typical external genitalia, the ROC curve analyses showed that LC-MS/MS had superior diagnostic accuracy than ECLIA, clearly differentiating males from females (area under the curve = 0.96 vs. 0.715, respectively).

Conclusions: In preterm infants at birth, immunoassays (ECLIA) demonstrated limited accuracy in measuring serum testosterone levels. Therefore, we recommend using LC-MS/MS because of its superior precision, clearer sex differentiation, and greater clinical utility.

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INTRODUCTION

Testosterone, the major male hormone, plays an important role in the sexual differentiation and development of the brain, internal genitalia, and external genitalia during the fetal period [1], and influences behavior throughout later life [2]. In males, testosterone is primarily secreted by Leydig cells in the testes. In females, approximately 25% of testosterone is produced by the adrenal glands, 25% by the ovaries, and the remaining 50% by the peripheral conversion of precursor hormones [3]. Therefore, serum testosterone levels in females are significantly lower than those in males [3,4]. There are two main methods for measuring serum testosterone levels: immunoassay [5], which is commonly used and inexpensive, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6-8]. In the measurement of serum testosterone levels, immunoassays show cross-reactivity during antigen-antibody reactions. Women and children with significantly low testosterone are at risk of falsely elevated results [8,9], and the Endocrine Society has issued this caution [7]. Preterm infants secrete large amounts of fetal steroids derived from fetal adrenal glands [10-12]. Consequently, when measuring serum testosterone levels in neonates in the early stages of life, immunoassays can cause cross-reactivity, increasing the risk of falsely elevated testosterone levels, most likely due to cross-reactivity with adrenal metabolites [13,14]. In contrast, LC-MS/MS, which is becoming popular mainly in research facilities, can measure serum testosterone levels at low concentrations with high accuracy and low variability [7,15,16]. Furthermore, LC-MS/MS exhibits high sensitivity and specificity for measuring serum testosterone at low concentrations [17]. Previous reports on adults and children have shown a high degree of correlation between serum testosterone levels measured using electrochemiluminescence immunoassay (ECLIA) and LC-MS/MS [8]. However, according to Sigurdsson et al., a study measuring serum testosterone levels in 45 full-term infants on postnatal day 2 - 3 reported a correlation coefficient of 0.55 between ECLIA and LC-MS/MS methods, although the findings were presented as a conference report. This indicated only a moderate level of correlation [13]. Additionally, Hamer et al. reported that from birth to the first few days of life, immunoassays overestimate neonatal testosterone levels when compared with LC-MS/MS [14]. Serum testosterone levels in neonates, especially in those with ambiguous external genitalia, must be accurately measured because they are involved in sex determination. The United Kingdom Society for

Endocrinology guidelines recommend that serum steroids be measured using LC-MS/MS when considering disorders of sex development (DSD) [18].

This study aimed to compare umbilical cord testosterone levels measured using immunoassay (ECLIA) [19, 20] and LC-MS/MS, analyze their correlations and differences, and evaluate the diagnostic performance and ability to discriminate sex differences between the two methods in preterm infants with typical external genitalia.

MATERIALS AND METHODS

Study design

This study was approved by the Ethics Committee of the Nihon University Itabashi Hospital (approval number: RK-230411-7, April 11, 2023). Study participants were premature infants born between 22 weeks 0 days and 36 weeks 6 days of gestation at the Nihon University School of Medicine affiliated to the Itabashi Hospital between May 2020 and March 2024. Infants with DSD were not eligible for enrollment. Preterm infants with typical male or female external genitalia were included. Typical external genitalia was defined as the absence of clinically apparent genital ambiguity on neonatal examination; sex was assigned at birth based on external genital findings. Karyotyping was not performed for sex determination in infants with typical external genitalia. Infants who were monochorionic twins or whose testosterone levels measured by LC-MS/MS were below the limit of detection (LOD) were excluded from the analysis. Monochorionic twins were excluded due to the potential impact of shared blood circulation on umbilical cord testosterone levels. Umbilical cord testosterone levels were measured using two analytical methods: immunoassay (ECLIA) and LC-MS/MS.

Blood sample collection and testosterone measurement

Immunoassay (ECLIA) for measuring umbilical cord testosterone levels

Umbilical cord testosterone levels were measured using ECLIA with the Elecsys® Testosterone II reagent (Roche Diagnostics, Basel, Switzerland). Analyses were performed by SRL, Inc. (Tokyo, Japan). The immunoassay used (Elecsys® Testosterone II, Roche Diagnostics) was also extensively evaluated in terms of analytical performance and cross-reactivity. Serum testosterone was measured using the Elecsys® Testosterone II assay (Roche) on a cobas e analyzer. This is a heterogeneous, competitive electrochemiluminescence immunoassay (ECLIA) employing a biotinylated anti-testosterone antibody, a ruthenium-labelled testosterone derivative (tracer), and streptavidin-coated microparticles with bound/free (B/F) separation. In this assay, endogenous testosterone in the sample competes with the ruthenium-labelled derivative for antibody binding sites, and the signal detected originates from the luminescence

emitted by the labelled derivative rather than the analyte itself. Signal is inversely proportional to analyte concentration [19,20].

Sample stability was validated for 5 days at room temperature (20 - 25°C), 14 days at refrigerated temperature (2 - 8°C), and 6 months at freezing temperature (-20°C ± 5°C). This is a two-step immunoassay that uses electrochemiluminescent detection. Initially, the samples were incubated with a biotinylated anti-testosterone sheep monoclonal antibody. Subsequently, ruthenium tris(2,2'-bipyridyl)-labeled (Ru[bpy]₃-labeled) testosterone and streptavidin-coated magnetic microparticles were added. The resulting immune complex binds to microparticles via biotin-streptavidin interactions.

After incubation, the mixture was transferred to a measuring cell, where the magnetic particles were immobilized on the electrode surface. After magnetic capture, unbound tracer and other reagents were removed by washing (bound/free separation), leaving only the particle-bound immune complexes for electrochemiluminescence detection. Oxidation at this electrode excites the Ru(bpy)₃ label, which subsequently emits luminescence in the presence of tripropylamine.

The emitted luminescence was quantified using a photomultiplier tube. Results were reported using an instrument-specific two-point calibration aligned to a predefined master calibration curve provided via the reagent barcode [19,20].

LC-MS/MS for measuring umbilical cord testosterone system

Measurement of umbilical cord testosterone levels using LC-MS/MS was conducted with instrumentation at the Chemical Analysis Laboratory, Research Center, Nihon University School of Medicine. The LC-MS/MS system consists of a LC assembly, ionization source, mass analyzer, and data acquisition system. An Acquity Ultra-Performance LC system coupled to a Xevo TQ-S micro-tandem quadrupole MS (Waters, Milford, MA, USA) was used. The reliability and validity of the LC-MS/MS system used in this study (Acquity UPLC and Xevo TQ-S; Waters) have been well established in previous endocrine analyses. LC conditions were as follows: column, Acquity HSH C18 (1.8 µm, 2.1 × 100 mm; Waters); mobile phase, (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile; column temperature, 40°C; flow rate, 0.3 mL/minute; gradient, 20% B (initial) → 80% B (6.0 minute) → 80% B (8.0 minute) → 20% B (8.1 minute). MS conditions included positive ion mode, capillary voltage 1.5 kV, source temperature 150°C, and desolvation temperature 500°C.

Chemicals and materials

Testosterone, testosterone-d4 (internal standard), pyridine-2-carboxylic acid (picolinic acid; PA), and 2-methyl-6-nitrobenzoic anhydride (MNBA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 4-Dimethylaminopyridine (DMAP), triethylamine (TEA), methyl tert-butyl ether (MTBE), and acetonitrile

were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Anhydrous pyridine was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Umbilical cord blood samples were stored at -80°C and thawed at room temperature prior to analysis. Sample preparation followed the LC-MS/MS method described by Yamashita et al. [21] with the following modifications: increased amounts of derivatization reagents (DMAP, MNBA, and PA), substitution of pyridine for tetrahydrofuran as the reaction solvent, and an extended reaction time from 30 to 60 minutes at room temperature. Briefly, 100 µL of umbilical cord blood was mixed with internal standard (testosterone-d4) and MTBE as extraction solvent. After shaking, the aqueous phase was frozen, and the organic layer was carefully separated and dried under a gentle nitrogen stream at 55°C. The residue was dissolved in 100 µL of the derivatization reagent solution. This solution was prepared by dissolving DMAP (20 mg), MNBA (40 mg), and PA (50 mg) in 2 mL of pyridine. Subsequently, 20 µL of TEA was added, and the mixture reacted at room temperature for 60 minutes. Then, 300 µL of 2% HCl was added, followed by solid-phase extraction. The eluate was dried at 55°C under nitrogen and reconstituted in 100 µL of 40% acetonitrile. Finally, 20 µL of this solution was injected into the LC-MS/MS system. LC-MS/MS chromatograms and calibration curves were generated for the umbilical cord testosterone levels. Each sample was analyzed in duplicate, and the mean value was used for data analysis. The limit of detection (LOD) for testosterone measured using LC-MS/MS was 0.0036 ng/mL. Analytical precision of the LC-MS/MS assay was evaluated by measuring the intra-assay coefficient of variation (CV), expressed as a percentage (%). The CV was 12.9% at low testosterone levels (0.01 ng/mL) and 2.7% at high levels (2.5 ng/mL).

Disease definition

Maternal clinical factors included maternal age at delivery, pre-pregnancy body mass index, gestational weight gain, maternal height, and multiple gestations. Mono-chorionic twins were excluded due to potential cord blood interference resulting from shared placental blood flow. Clinical conditions, such as hypertensive disorders of pregnancy (HDP) [22], gestational diabetes mellitus (GDM) [23,24], and antenatal administration of betamethasone were recorded. According to our hospital's protocol, betamethasone (12 mg) was administered intramuscularly twice, 24 h apart, to pregnant women at high risk of preterm delivery before 33 weeks and 6 days of gestation. HDP was defined as hypertension occurring during pregnancy, with a blood pressure ≥ 140/90 mmHg.

GDM was defined as impaired glucose tolerance diagnosed or first recognized during pregnancy. An oral glucose tolerance test (OGTT) was performed, and GDM was diagnosed if ≥ 1 of the following criteria were met

Table 1.1. Maternal Clinical Characteristics (n = 38).

Clinical characteristics: mothers	n = 38
Age at delivery, years	34.5 (29.8 - 39.0)
Pre-pregnancy BMI, kg/m ²	21.9 (18.9 - 24.8)
Weight gain during pregnancy, kg	8.1 (4.6 - 11.0)
Height, cm	160 (155 - 161)
Di- and tri-chorionic births, n (%)	14.0 (36.8%)
Hypertensive disorders of pregnancy, n (%)	9 (23.7%)
Preterm use of betamethasone, n (%)	17 (44.7%)
Gestational diabetes mellitus, n (%)	3 (7.9%)

Data are presented as medians (interquartile range [IQR: Q1 - Q3]) or numbers (%). BMI: body mass index.

Table 1.2. Clinical Characteristics of Preterm Infants (n = 38).

Auxological characteristics	All infants (n = 38)	Males (n = 16)	Females (n = 22)
Gestational age at birth (weeks)	34.0 (31.8 - 35.0)	33.5 (32.0 - 35.0)	35.0 (31.0 - 36.0)
Birth weight (g)	1,874 (1,348 - 2,373)	1,931 (1,336 - 2,375)	1,850 (1,346 - 2,358)
Birth weight (SDS)	-0.47 (-1.54 - 0.382)	-0.35 (-1.98 - 0.653)	-0.57 (-1.34 - 0.208)
Birth length (cm)	43.3 (38 - 46.1)	43.0 (38.1 - 45.2)	43.8 (37.4 - 46.8)
Birth length (SDS)	-0.51 (-1.24 - 0.36)	-0.11 (-1.68 - 0.368)	-0.81 (-1.17 - 0.41)
Birth head circumference (cm)	31.1 (28.0 - 32.9)	31.1 (28.2 - 32.5)	31.1 (27.9 - 33.0)
SGA infants, n (%)	12 (31.6%)	6 (15.8%)	6 (15.8%)
Cesarean delivery, n (%)	38 (100%)	16 (100%)	22 (100%)

Data are presented as median (interquartile range [IQR: Q1 - Q3]) or numbers (%).
SDS: standard deviation score, SGA: small-for-gestational-age.

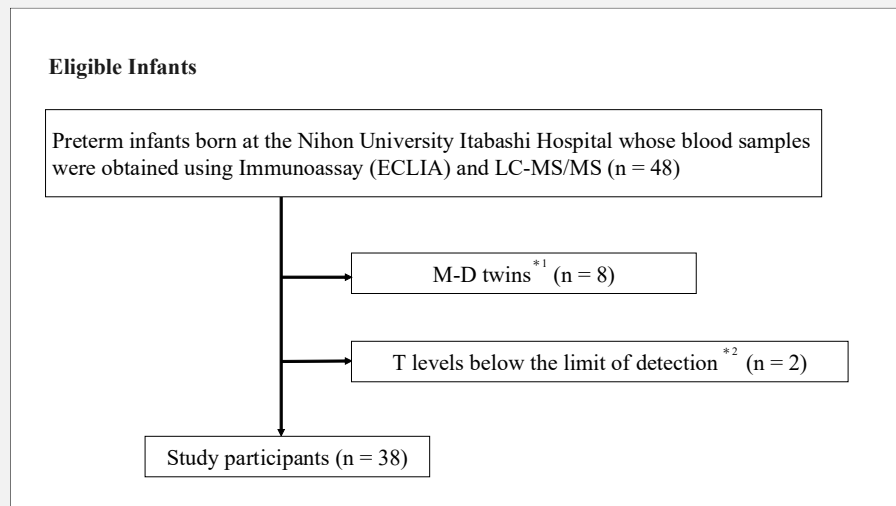


Figure 1. Eligibility and exclusion flowchart for study participants.

* ¹ Monozygotic diamniotic twins.

* ² Limit of detection (LOD) for the umbilical cord testosterone measured using LC-MS/MS was 0.0036 ng/mL.

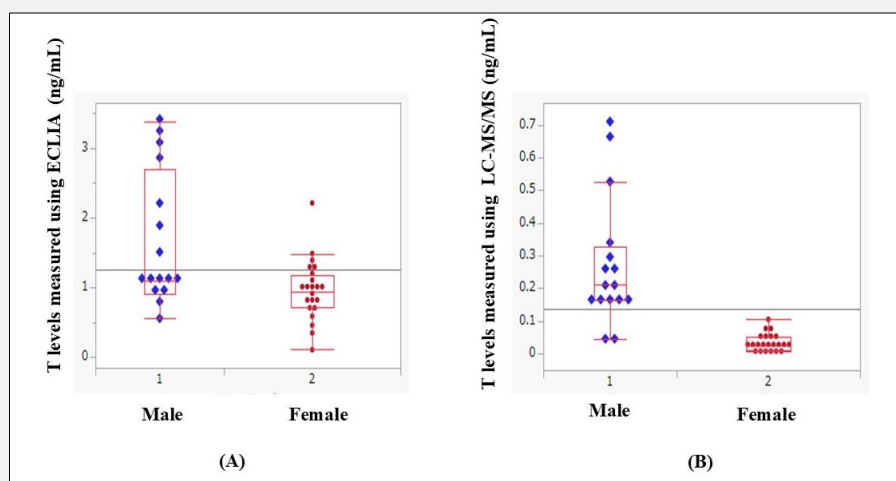


Figure 2. Comparison of umbilical cord testosterone T levels between male and female preterm infants measured using (A) ECLIA and (B) LC-MS/MS methods.

Boxes represent the interquartile range (IQR, Q1 - Q3), with median values indicated by the line within each box. Individual data points have been presented. The LC-MS/MS method demonstrates clearer differentiation of testosterone levels between the sexes than the ECLIA method. Male infants are indicated by blue diamonds, and female infants are indicated by red circles. T: Umbilical cord testosterone, ECLIA: electrochemiluminescence immunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry.

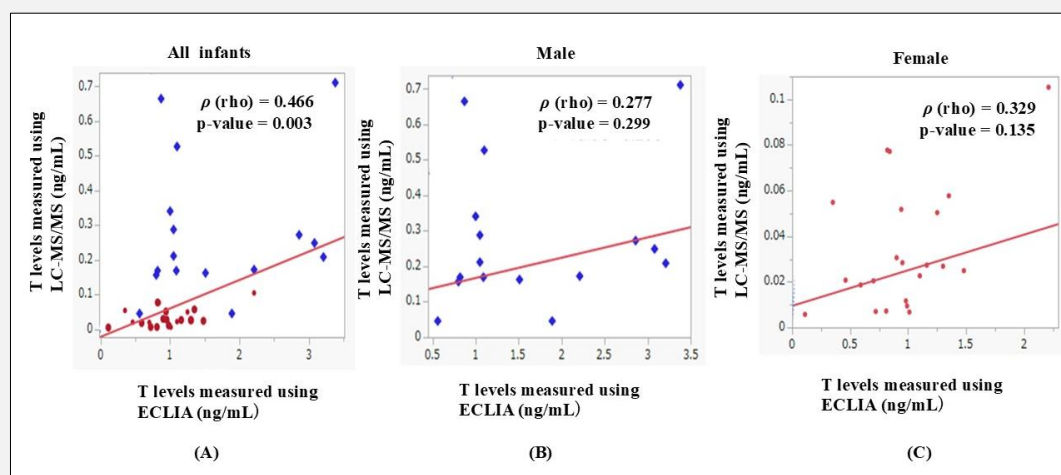


Figure 3. Correlation between testosterone T levels measured using ECLIA and LC-MS/MS.

Scatter plots illustrating correlations between umbilical cord testosterone (T) levels measured using ECLIA and LC-MS/MS in (A) all preterm infants (n = 38), (B) male infants (n = 16), and (C) female infants (n = 22).

The red line shows the Passing-Bablok regression estimating LC-MS/MS (y) from ECLIA (x), while the scatter points show measured values.

A: Estimated LC-MS/MS T levels (ng/mL) = $-0.021 + 0.082 \times \text{T levels measured using ECLIA (ng/mL)}$.

B: Estimated LC-MS/MS T levels (ng/mL) = $0.110 + 0.057 \times \text{T levels measured using ECLIA (ng/mL)}$.

C: Estimated LC-MS/MS T levels (ng/mL) = $0.010 + 0.016 \times \text{T levels measured using ECLIA (ng/mL)}$.

In each panel of Figure 3, Spearman's rank correlation coefficient ρ (rho) and the corresponding p-value are shown to indicate the strength of the association between testosterone levels measured using ECLIA and those measured using LC-MS/MS, as well as its statistical significance. X-axis: T levels measured using ECLIA (ng/mL), Y-axis: T levels measured using LC-MS/MS (ng/mL). Male infants are indicated by blue diamonds, and female infants are indicated by red circles. ECLIA, electrochemiluminescence immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

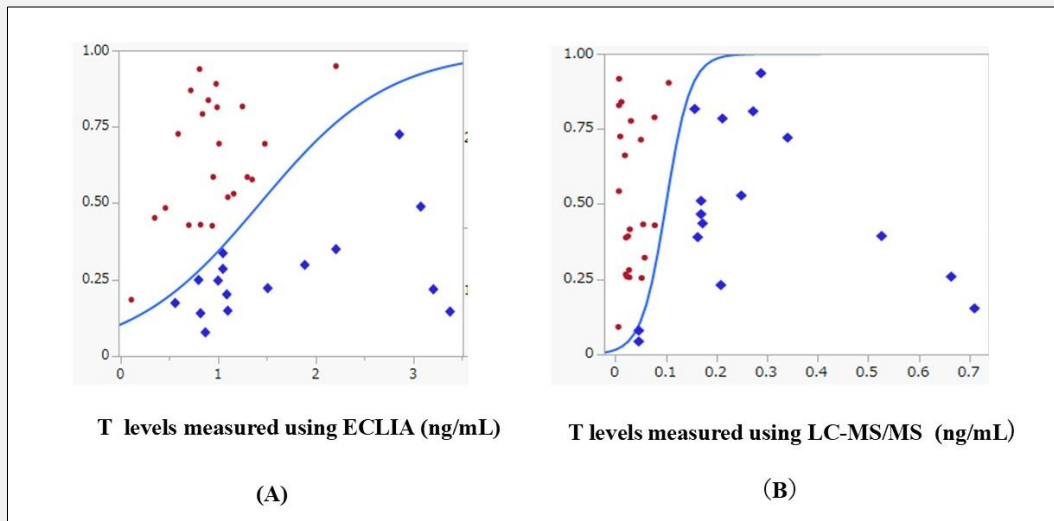


Figure 4. Logistic regression predicting male sex using umbilical cord testosterone levels.

Logistic regression analysis demonstrated clearer discrimination between male and female neonates when T levels were measured using LC-MS/MS than when using ECLIA. Preterm infants ($n = 38$). (A) ECLIA; X-axis: T levels measured using ECLIA (ng/mL), Y-axis: Predicted probability of being male. (B) LC-MS/MS, X-axis: T levels measured using LC-MS/MS (ng/mL), Y-axis: Predicted probability of being male. Male infants are indicated by blue diamonds, and female infants are indicated by red circles. T, umbilical cord testosterone; ECLIA, electrochemiluminescence immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

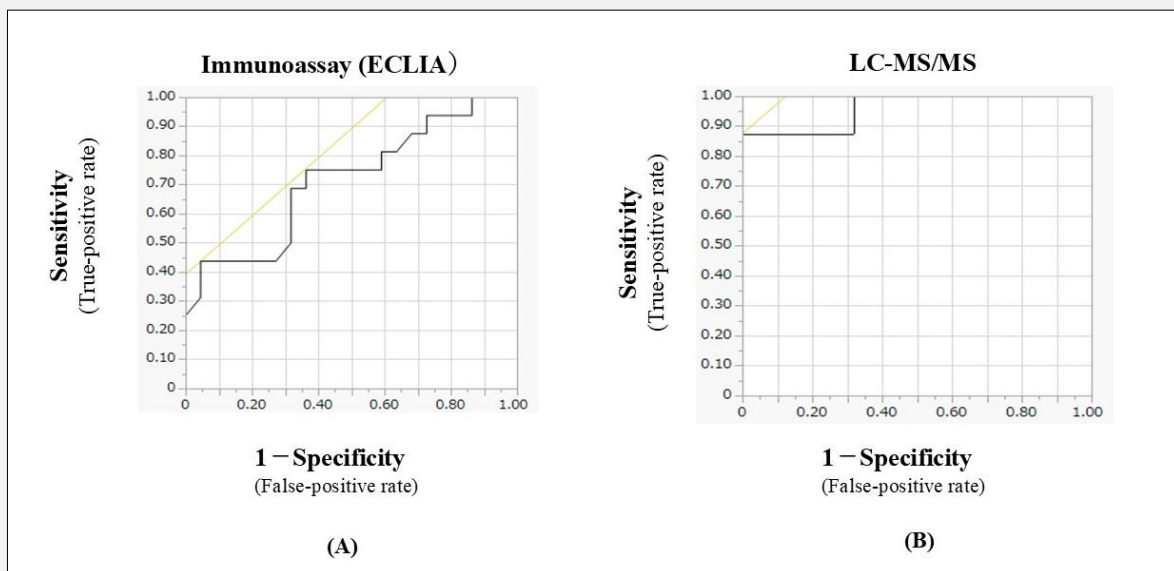


Figure 5. ROC curves for predicting male sex using umbilical cord testosterone levels.

ROC curves comparing the clinical utility of ECLIA and LC-MS/MS in predicting male sex based on umbilical cord testosterone levels in pre-term infants ($n = 38$). Sensitivity, specificity, and area under the curve (AUC) were calculated. Male infants are indicated by blue diamonds, and female infants are indicated by red circles. A: ECLIA, X-axis: 1 - Specificity (false-positive rate), Y-axis: Sensitivity (true-positive rate). B: LC-MS/MS, X-axis: 1 - Specificity (false-positive rate), Y-axis: Sensitivity (true-positive rate). ECLIA: electrochemiluminescence immunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, ROC: receiver operating characteristic.

in a 75-g OGTT: fasting glucose ≥ 92 mg/dL, 1-hour glucose ≥ 180 mg/dL, or 2-hour glucose ≥ 153 mg/dL. Small-for-gestational-age (SGA) infants were defined as those with a birth weight and birth length below -2.0 standard deviations [25]. Neonatal clinical factors included gestational age (weeks), birth weight (g), birth weight standard deviation score (SDS), birth length (cm), birth length SDS, birth head circumference (cm), proportion of SGA infants (%), and proportion of cesarean section deliveries (%).

Statistical analysis

Study 1: Comparison of umbilical cord testosterone levels by measuring ECLIA and LC-MS/MS

The Mann-Whitney U test was used to compare the median testosterone levels between males and females for both ECLIA and LC-MS/MS, and p-values < 0.05 were considered statistically significant. Interquartile ranges (IQRs) were analyzed to assess data dispersion. IQR is presented as Q1 - Q3, corresponding to the 25th to 75th percentiles.

Study 2: Relationships between testosterone levels by ECLIA and testosterone levels using LC-MS/MS

Spearman's rank correlation coefficient ρ (rho) was calculated between the testosterone levels measured using ECLIA and measured using LC-MS/MS. As the measured values were not normally distributed, Spearman's rank correlation was used as a non-parametric method. Method comparison between ECLIA and LC-MS/MS was performed using Passing-Bablok regression [26, 27] to estimate constant (intercept) and proportional (slope) bias between the two methods, a standard non-parametric approach used in method-comparison studies. No regression-corrected ("calculated") values were used in the statistical analyses.

Study 3: To predict the probability of being male based on testosterone levels measured using ECLIA and LC-MS/MS

Logistic regression analysis was conducted using sex (male = 1, female = 0) as the dependent variable and testosterone levels measured using ECLIA as the independent variables. Based on these data, ROC curves were created for testosterone levels measured using ECLIA and the probability of being a boy or a girl (male = 1 and female = 0). Similarly, these analyses were performed for testosterone levels measured using LC-MS/MS. The Youden's index is equal to the value at which sensitivity + specificity - 1 is maximized.

All statistical analyses were performed using JMP ver18.0pro (JMP Statistical Discovery LLC, Tokyo, Japan), and p-values < 0.05 were considered statistically significant.

RESULTS

Among the 48 initially recruited preterm infants, ten were excluded from the analysis because their mono-chorionic twins (n = 8) and testosterone levels measured

using LC-MS/MS were below the LOD (LOD: 0.0036 ng/mL; n = 2). The final analysis included 38 preterm infants (16 males and 22 females), as shown in Figure 1. Characteristics of the study participants are summarized in Tables 1.1 and 1.2.

In Study 1, Figure 2 shows comparisons of umbilical cord testosterone levels in male and female preterm infants measured using the two methods. It shows median (IQR: Q1 - Q3) testosterone levels measured using ECLIA were higher in males (1.10 ng/mL [0.90 - 2.69]) than those in females (0.95 ng/mL [0.71 - 1.18]) (p-value < 0.05). However, the distribution of testosterone levels measured using ECLIA exhibited considerable overlap between the sexes, indicating a limited differentiation ability. In contrast, the testosterone levels measured using LC-MS/MS were significantly higher in males (0.210 ng/mL [0.164 - 0.327]) than those in females (0.026 ng/mL [0.0112 - 0.0526]) (p-value < 0.05), with the median testosterone levels in males being approximately 10-fold higher than those in females. Based on these results, sex differences were more evident when measured using LC-MS/MS than when using ECLIA alone.

In Study 2, Figure 3 shows the correlations between testosterone levels measured using ECLIA and LC-MS/MS. The Spearman's rank correlation coefficient ρ (rho) revealed weak correlations for all preterm infants (ρ (rho) = 0.466, p-value = 0.003), males (ρ (rho) = 0.277, p-value = 0.299), and females (ρ (rho) = 0.329, p-value = 0.135). Considering the low correlation coefficients, method comparison was performed using Passing-Bablok regression. In the overall sample, the regression yielded a slope of 0.082 and an intercept of -0.021 (Figure 3). Spearman's rank correlation coefficient ρ (rho) was small, indicating limited agreement. For visualization only, the Passing-Bablok regression line estimating LC-MS/MS (y) from ECLIA (x) is shown in Figure 3; data points represent measured values. No regression-corrected values were used in the statistical analyses.

In Study 3, Figure 4 shows logistic regression curves predicting male sex based on cord testosterone levels measured using ECLIA and LC-MS/MS. The ECLIA method (Figure 4A) showed considerable overlap between the sexes, reflecting its limited predictive ability. In contrast, the LC-MS/MS method (Figure 4B) exhibited a clear, steep curve, indicating high predictive accuracy with a 92.2% probability of male sex when testosterone levels were ≥ 0.157 ng/mL. Figure 5 shows the ROC curves for predicting male sex based on cord testosterone levels. Cutoff values were determined using the Youden's index. ECLIA yielded a cutoff value of 1.51 ng/mL (sensitivity 0.438, specificity 0.545, AUC = 0.715). In contrast, LC-MS/MS provided superior diagnostic accuracy with a cutoff value of 0.157 ng/mL (sensitivity 0.875, specificity 1.0, AUC = 0.96). Based on these results, and according to the evaluation using the aforementioned curve for predicting male infants with typical external genitalia, the LC-MS/MS method

demonstrated significantly greater reliability and accuracy than the ECLIA method in predicting male sex using umbilical cord testosterone levels in preterm infants.

DISCUSSION

This is the first report comparing umbilical cord testosterone levels measured using immunoassay (ECLIA) and LC-MS/MS methods in preterm infants.

In neonates with ambiguous genitalia, serum testosterone levels, together with other steroid hormones, anti-Müllerian hormone and imaging findings such as ultrasonography and magnetic resonance imaging, are used to assess the presence and function of testicular tissue, thereby directly influencing clinical decision-making in the diagnosis and management of DSD [18]. Therefore, achieving a high accuracy in measuring serum testosterone levels is critical.

Characteristics of immunoassay and LC-MS/MS for measuring umbilical cord testosterone levels

A previous report in adults and children indicated that testosterone levels measured using ECLIA were significantly higher than those measured using LC-MS/MS [8]. In study 1 (Figure 2), the results of the IQR analysis indicated that in preterm infants, the distribution of testosterone levels measured using ECLIA was more dispersed than that measured using LC-MS/MS in both male and female infants. Although the timing of measurement in our study (at birth) differed from that in previous reports, our findings are consistent with those of previous studies. This may be related to the fact that immunological assays show falsely elevated testosterone levels due to cross-reactivity with chemically similar substances, whereas LC-MS/MS specifically measures only the target substance and does not show such cross-reactivity [17]. Furthermore, the distribution of testosterone levels in males showed greater variability than that in females in both methods, and this difference was considered to reflect differences in the amount of testosterone secretion between males and females. A meta-analysis examining sex differences in umbilical cord testosterone levels consistently reported higher testosterone levels in males than that in females [28].

Limitations of immunoassay

Umbilical cord serum testosterone levels in preterm infants are much lower than those observed in adult men. In the very low concentration range, immunoassay-based testosterone measurements tend to show a positive bias relative to LC-MS/MS [5,7-9,14,20,29]. The Elecsys Testosterone II is a competitive, heterogeneous ECLIA in which the emitted signal is inversely proportional to analyte concentration; documented cross-reactivity and the bound/free (B/F) separation inherent to this format can contribute to upward bias at the low end of the measuring range [19,20]. A fundamental limita-

tion of immunoassays such as ECLIA is that the detected signal does not originate from the analyte itself but from a labelled derivative (tracer). Consequently, the measurement reflects the competitive binding between the analyte and the tracer, which may be affected by cross-reactivity or variations in antibody affinity. The platform's instrument-specific two-point calibration aligned to a predefined master curve harmonizes instrument response but does not itself address antibody specificity [19]. By contrast, LC-MS/MS chromatographically separates interfering substances and quantifies the native analyte against an isotope-labelled internal standard, supporting accurate measurement in the low range [6,15-17,21]. Such overestimation of these values in the very low concentration range may lead to a misinterpretation of endogenous testosterone secretion. This could potentially mislead clinicians to assume that higher concentrations have been secreted than are actually present. Therefore, values near decision thresholds should be interpreted with caution, and LC-MS/MS is preferred in equivocal cases.

Correlations of umbilical cord testosterone levels: Immunoassay vs. LC-MS/MS

A previous report on adults and children showed a strong Pearson's correlation coefficient ($r = 0.819$) between serum testosterone levels measured using immunoassay (ECLIA) and LC-MS/MS [8]. According to Sigurdsson et al., although the findings were presented only in a conference report, a study measuring serum testosterone levels in 45 full-term infants on postnatal day 2 - 3 reported a correlation coefficient of 0.55 between the ECLIA and LC-MS/MS methods, which reflects only a moderate level of correlation [13]. Additionally, findings from a small number of full-term infants reported by Hamer et al. clearly indicate that immunological assays overestimate testosterone levels in neonates from birth to the first few days of life compared with LC-MS/MS [14].

According to findings from study 2 (Figure 3), umbilical cord testosterone levels in preterm infants showed only a weak correlation (Spearman's rank correlation coefficient ρ (ρ) < 0.5) between measurements obtained using ECLIA and LC-MS/MS. The agreement between ECLIA and LC-MS/MS was poor. As the primary method-comparison analysis, Passing-Bablok regression demonstrated a slope far below unity and a small Spearman's rank correlation coefficient ρ (ρ), indicating limited agreement; therefore, Bland-Altman analysis was not performed.

One possible explanation for this may be cross-reactivity in early neonates due to steroids derived from the fetal adrenal glands, as discussed by Sigurdsson et al. and Hamer et al. Furthermore, umbilical cord blood is likely to contain substantial amounts of maternal estrogen. These factors, along with differences in measurement accuracy between the two assay methods, mainly arising from the methodological limitations of ECLIA compared with the higher accuracy of LC-MS/MS, are con-

sidered to have contributed. Consequently, they likely explain the weak correlation observed between the two methods.

Sex differences in umbilical cord testosterone: Immunoassay vs. LC-MS/MS

Turning to sex differences in testosterone levels, umbilical cord testosterone levels in the present study were higher in males than those in females when measured using both the ECLIA and LC-MS/MS methods, which is consistent with previous reports (Figure 2) [28]. A previous study by Chafkin JE et al., which analyzed salivary testosterone concentrations in 207 adolescents aged 13 - 16 years, reported that chemiluminescent immunoassay tended to overestimate hormone levels, particularly testosterone levels in females, compared with LC-MS/MS, thereby obscuring sex differences [29]. According to the findings shown in Figures 2 and 4 of this present study, sex differences in umbilical cord testosterone levels were more clearly observed when measured using LC-MS/MS than those using immunoassay (ECLIA), and these results are consistent with previous reports. Figures 4 and 5 suggest that LC-MS/MS not only measures testosterone with greater biochemical accuracy but also provides better clinical discrimination between male and female neonates. The LC-MS/MS method (Figure 4B) exhibited a clear, steep curve, indicating high predictive accuracy with a 92.2% probability of male sex when testosterone levels were ≥ 0.157 ng/mL. Overestimation and broader variability in testosterone values observed with ECLIA may obscure subtle but clinically significant sex differences. The UK Society for Endocrinology recommends measuring serum steroids using LC-MS/MS in patients with suspected DSD [18]. In conclusion, our findings of this study underscore the importance of adopting LC-MS/MS for testosterone measurement in preterm neonates, whose hormone levels are inherently low and susceptible to assay interference and cross-reactivity. Moreover, our findings show that the choice of assay has direct clinical implications. When serum testosterone levels in umbilical cord blood are measured using ECLIA, overestimation of these values can lead to misinterpretation of endogenous testosterone secretion and may cause clinicians to misperceive endogenous testosterone levels as higher than they actually are. This could affect clinical judgments related to the evaluation of neonatal endocrine function, and conditions that are generally associated with impaired androgen secretion. By contrast, LC-MS/MS demonstrates higher specificity and provides a more reliable basis for accurate general clinical decision-making. Taken together, our results are consistent with previous studies in other populations and extend this evidence by directly demonstrating these methodological differences, thereby providing novel evidence in preterm infants.

Limitations

This study used a small number of cord blood samples. However, there was no bias in the number of weeks of gestational age or birth weight of the preterm infants who were included in the study, and the proportion of males and females was equal in terms of SGA infants with respect to body size. In this study, luteinizing hormone, follicle-stimulating hormone, and sex hormone-binding globulin were not measured simultaneously when measuring serum testosterone levels in cord blood. Importantly, this study included male infants with typical external genitalia as the study population. The absence of cytogenetic confirmation in infants with typical external genitalia, and thus, the rare discordance between phenotypic sex at birth and chromosomal sex cannot be excluded. However, our outcome of interest was sex assigned at birth, which is clinically relevant for the intended use of umbilical cord serum testosterone, and this limitation does not alter the method-comparison conclusions between ECLIA and LC-MS/MS. Moreover, in clinical practice, when evaluating newborns with ambiguous genitalia suspected of DSD, sex assignment is not determined by testosterone cutoff values or ROC curve analysis. Instead, it is made comprehensively by incorporating information from anti-Müllerian hormone levels, SRY gene testing, imaging studies, and steroid profiling. The timing of measurement in this study was limited to umbilical cord blood collected immediately after birth; thus, caution is required when generalizing these results to the entire neonatal period. These limitations should be considered when interpreting our findings.

CONCLUSION

In preterm infants at birth, we recommend using LC-MS/MS rather than ECLIA for the measurement of serum testosterone levels because of its better accuracy and clearer distinction between sexes.

Further directions

Further studies are required to clarify the relationship between umbilical cord testosterone levels and maternal clinical factors, as well as neonatal clinical factors in both preterm and term infants. Further investigations are also necessary to obtain comprehensive steroid profile data, including sex steroids beyond testosterone, and comparisons of umbilical cord testosterone levels between healthy neonates and those with DSD or infants born with fetal growth restriction. Additionally, more detailed studies are needed to determine the postnatal day at which the correlation between serum testosterone levels measured using ECLIA and LC-MS/MS becomes statistically significant. These efforts will contribute significantly to advancing our understanding of the suspected abnormalities in testosterone secretion dynamics during the early neonatal period.

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Institutional Review Board Statement:

This study was approved by the Ethics Committee of Nihon University School of Medicine (Approval No. RK-230411-7, Date: April 11, 2023). This study was conducted in accordance with the relevant guidelines and regulations.

Declaration of Generative AI and AI-assisted technologies in the writing process:

During the preparation of this manuscript, the authors used ChatGPT (GPT-4o and GPT-4.5) to assist with proofreading and enhancing the clarity of the English text. After using these tools for language refinement, we thoroughly reviewed and revised the content as necessary, and we take full responsibility for the final version of the manuscript.

Declaration of Interest:

The authors declare no competing interests.

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