Letter to the Editor

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Falsely elevated plasma testosterone concentrations in neonates: importance of LC-MS/MS measurements

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To the Editor,

In newborns with atypical genitalia, suspicious for a disorder of sex development (DSD), measurement of testosterone is an essential part in the diagnostic workup [1].

Previously, direct testosterone immunoassays have proven to be inaccurate because they tend to overestimate testosterone concentrations in the lower ranges, such as those in females and infants [2], but specifically also in neonates [3, 4]. Based on the concern for cross-reactivity in neonatal samples, the recently revised UK guideline on the initial evaluation of DSD from the UK Society for Endocrinology recommends that steroids in plasma or serum are measured by either LC-MS/MS or immunoassays after organic solvent extraction [1]. The use of LC-MS/MS was considered superior by a recent consensus meeting of DSD experts across Europe, although validation and quality control remain challenging [5].

The accuracy of testosterone immunoassays has improved significantly with the introduction of second-generation testosterone assays [2]. These second-generation assays generally show high correlation coefficients with LC–MS/MS data, at both low and high concentrations [2, 6].

The aim of the present study was to assess whether second-generation immunoassays are able to determine testosterone concentrations in neonates accurately. We compared plasma testosterone concentrations measured with two widely used second-generation immunoassays to those measured with LC-MS/MS in infants directly after birth up until 6 months of age.

For measurements of plasma testosterone, leftover heparin plasma samples were anonymously selected from infants born at term (≥37 weeks) with normal external genitalia. Ages varied between the day of birth and 6 months. For comparison of the Architect® second-generation testosterone assay with LC-MS/MS, samples from 33 male and 45 female neonates were collected at the VU University medical center. For comparison of the Elecsys® second-generation testosterone assay with LC-MS/MS, samples from 16 male and 4 female neonates were collected at the Radboud University medical center. For additional analysis of 11β-hydroxytestosterone, a metabolite with known high cross-reactivity in both testosterone immunoassays, leftover samples were used from male (n = 27) and female (n = 16) infants born at term aged 0–2 days or >6 months. Use of anonymized leftover samples is approved by the Medical Ethics Committees of the respective University Medical Centers.

The total plasma testosterone concentration was measured with an automated chemiluminescent microparticle immunoassay, the Architect® second-generation testosterone assay (Abbott Diagnostics, Abbott Park, IL, USA) [2, 6], or with an automated chemiluminescent microparticle immunoassay, the Elecsys® second-generation testosterone assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland [7]. In all samples, total testosterone was also measured with isotope-dilution LC–MS/MS as described previously by our research group [6].

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Table 1: Median and absolute range of plasma testosterone concentrations (nmol/L) measured with a second-generation immunoassay (Architect®) and LC-MS/MS in male and female neonates between 0 days and 6 months of age.

Age, days	Boys			Girls		
	n	Second-generation immunoassay	LC-MS/MS	n	Second-generation immunoassay	LC-MS/MS
0	3	5.0 (4.0–13.5)	4.8 (1.3–8.7)	2	3.8 (2.2–5.4)	0.2 (0.1–0.3)
1	4	7.0 (2.1–9.0)	3.7 (1.1–7.3)	3	3.1 (1.5–5.8)	0.3 (0.1–0.3)
2	3	4.1 (2.4–4.5)	1.9 (1.7–2.1)	3	1.6 (0.7–2.3)	0.1 (0.1–0.2)
3–4	3	1.5 (1.4–1.5)	0.7 (0.4–0.9)	9	1.1 (0.3–2.7)	0.1 (0.1–0.5)
5–7	6	1.4 (0.8–2.1)	0.4 (0.2–1.2)	8	0.8 (0.2–1.2)	0.2 (0.1–0.3)
8–30	4	5.9 (4.9–7.6)	6.4 (4.7–7.5)	14	0.8 (0.3–2.2)	0.2 (0.1–0.4)
31–120	6	4.3 (2.7–9.4)	5.0 (1.7–7.8)	3	0.4 (0.1–0.6)	0.1 (0.1–0.2)
121–180	4	0.4 (0.2–1.4)	0.5 (0.2–1.6)	3	0.2 (0.1–0.2)	0.1 (0.05–0.1)

Table 1 shows the median concentrations and ranges of testosterone in plasma in boys and girls from all age categories up until 6 months of age measured with the Architect® second-generation immunoassay and LC-MS/MS. In boys (n = 10), the median (range) plasma testosterone concentration during the first 3 days of life was 4.7 nmol/L (2.1–13.5 nmol/L) and 2.0 nmol/L (1.1–8.7 nmol/L) when measured with the Architect® second-generation immunoassay and LC-MS/MS, respectively. In girls of the same age (n = 8), the median (range) plasma testosterone concentration was 2.3 nmol/L (0.7–5.8 nmol/L) and 0.1 nmol/L (0.1–0.3 nmol/L) when measured with the Architect® second-generation immunoassay and LC-MS/MS, respectively.

In a second cohort (16 male and four female samples), testosterone concentrations were measured with the Elecsys® second-generation testosterone immunoassay and compared to LC-MS/MS. In boys (n = 8), median (range) plasma testosterone concentrations during the first 3 days of life were 12 nmol/L (9.3–22 nmol/L) and 5.2 nmol/L (1.7–18 nmol/L) when measured with the Elecsys® second-generation immunoassay and LC-MS/MS, respectively. In girls of the same age (n = 2), median (range) plasma testosterone concentrations were 10 nmol/L (7.6–13 nmol/L) and 0.7 nmol/L (0.6–0.7 nmol/L) when measured with the Elecsys® second-generation immunoassay and LC-MS/MS, respectively.

Absolute differences in testosterone concentrations were highest during the first days after birth (Figure 1). Differences of up to 5.4 and 5.7 nmol/L in boys and girls, respectively, were found when the Architect® second-generation immunoassay was compared to LC-MS/MS. Differences of up to 12.8 and 12.2 nmol/L were found in boys and girls, respectively, when the Elecsys® second-generation immunoassay was compared to LC-MS/MS.

Pooled plasma samples from male (day 0, day 1, day 2 and >6 months) and female (day 0–1, day 2 and >6 months) infants were analyzed using the ACQUITY UPC²-MS/MS (Waters Corporation Milford, USA) for measurement of 11β-hydroxytestosterone as described previously [8]. In boys, 11β-hydroxytestosterone concentration at birth (day 0) was 5.4 nmol/L, declining to levels below the LOQ (4.8 nmol/L) from day 1 on. In girls, these concentrations were negligible in all samples.

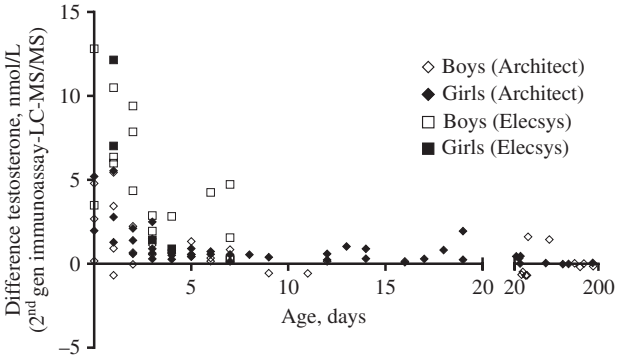

Figure 1: Absolute differences in testosterone concentrations (nmol/L) between LC-MS/MS and immunoassays in boys (open icons) and girls (closed icons).
Testosterone was measured with two second-generation immunoassays (Architect [Abbott Diagnostics] [◊] and Elecsys [Roche diagnostics] [□]) and LC-MS/MS in male and female neonates between 0 days and 6 months of age.

The presented data clearly show that second-generation immunoassays overestimate testosterone concentrations in newborns, particularly in the first days after birth, when compared to LC-MS/MS.

Although the present data should be interpreted with caution due to the low sample size, the course of testosterone concentrations measured with LC-MS/MS in boys is consistent with previous publications [4, 9, 10]. In boys, testosterone concentrations were high at birth, rapidly decreased to <1 nmol/L within the first few days,

followed by a rise starting from the second week with a peak around age 1–3 months, with a subsequent fall to prepubertal levels. In girls, testosterone concentrations are consistently low when measured with LC-MS/MS. By contrast, measurements with second-generation immunoassays show relatively high testosterone concentrations in the first days after birth. These high postnatal testosterone concentrations in girls have also been published previously when measured with direct immunoassays [4, 9, 10].

In both boys and girls, higher testosterone concentrations were found when measured with both widely used second-generation immunoassays compared to LC-MS/MS, mainly in the first days after birth.

Positive interference due to cross-reactivity with other steroids is a known problem for immunoassays [2], especially in neonatal samples [3, 4]. These publications report that purification and extraction steps should be performed before measurement with traditional radioimmunoassays. However, despite these purification steps, the published postnatal testosterone concentrations in girls are still higher compared to our present findings as determined with LC-MS/MS [4, 9, 10].

The precise nature of the interfering compounds in neonates has yet to be elucidated. We additionally analyzed 11β-hydroxytestosterone levels because this metabolite has been shown to have a cross-reactivity of 30.6% and 18.0% in the Architect® and Elecsys® second-generation immunoassays, respectively, as reported by the manufacturer. In boys, the cross-reactivity may partly be explained by the 11β-hydroxytestosterone levels measured at day 0. However, in girls, levels of this steroid were negligible. It can therefore be concluded that other interfering metabolites in girls, and also in boys, remain unknown.

In cases of DSD, it is of utmost importance that gender assignment is based on accurate measurements of testosterone, which can be performed within the first few days after birth. Within this period, a clear discrimination between boys and girls can only be made when using LC-MS/MS.

In conclusion, even when using the second-generation immunoassays, falsely high testosterone concentrations are measured in neonates during the first weeks after birth, which could lead to diagnostic confusion. An LC-MS/MS method should therefore be used to accurately determine testosterone concentrations in neonates in the first month of life.

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Article note: Previous presentations: Dutch Endocrine Meeting 2017.