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ORIGINAL ARTICLE

Importance of total and measured free testosterone in diagnosis of male hypogonadism: immunoassay versus mass spectrometry in a population of healthy young/middle-aged blood donors

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Abstract

Purpose To meet clinicians' request for adequate results and reliable reference ranges for testosterone, this study was planned with the aims (i) to verify the reliability of the reference interval for total testosterone (TT) declared by immunoassay manufacturer and adopted by laboratory, (ii) to compare results for serum TT obtained by immunoassay and LC–MS/MS and (iii) to verify if the cutoff values for low TT and measured free testosterone (FT), defined by Endocrine Society Guidelines for diagnosis of hypogonadism, are applicable to our study group.

Methods Sera from anonymous young/middle-aged male blood donors were selected for the study. TT was measured by immunoassay and LC–MS/MS. SHBG was measured by immunoassay and used with albumin concentration to calculate FT according to Vermeulen's formula.

Results The reference interval declared by the manufacturer and adopted by the lab was validated. The two methods for TT evaluation correlated very well. TT and FT lower limits at 5th and 2.5th percentile are below the cutoffs reported in the literature for the diagnosis of hypogonadism.

Conclusions The immunoassay currently used in our lab can be considered an adequate tool for TT, but it's essential that clinical data agree with the biochemical ones, particularly in the presence of TT values between the lower limit of reference range and the cutoff values recommended by scientific societies.

Keywords Testosterone · Hypogonadism · Immunoassay · Mass spectrometry · Men healthy donors

Introduction

Testosterone is the main male sex hormone that regulates fertility, muscle mass, fat distribution, and red blood cell production. It is primarily secreted by testicular Leyding cells and to a lesser extent by adrenal cortex, and its production is regulated by hypothalamus-pituitary-gonad axis negative feedback. Most circulating testosterone is tightly bound to sex-hormone-binding globulin (SHBG), a minor fraction is weakly bound to albumin and a small amount exists as free hormone. Albumin-bound testosterone dissociate freely in capillary blood becoming readily available for tissue uptake. For this reason, free testosterone and albumin-bound testosterone may be considered as bioavailable testosterone able to bind to the androgen receptor [1]. Total circulating testosterone (TT) levels are basilar to define the diagnosis of hypogonadism in men, a syndrome characterized by symptoms and signs of androgen deficiency that occurs in association with very low serum testosterone levels. Nevertheless, free or bioavailable testosterone (FT) concentration should be measured when serum TT levels are close to the lower limit of the normal range or when are suspected altered SHBG concentrations as in aging male [2–5]. Cutoff values of 3 ng/mL for TT and 50 pg/mL for calculated FT are recommended by Endocrine Society to

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define men hypogonadism [2–4]. In males, serum testosterone levels show a circadian variation with the highest level in the morning and the lowest level in late afternoon.

Total testosterone serum quantification is widely performed by rapid automated immunoassay instrument employing chemiluminescent detection [6], although it is known that these methods may be affected by interferences (in particular biotin interference is described in some immunoassay) and ranges and methods vary inter-laboratory. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) emerged as the method of choice for steroid hormones assessment. Advantages include its superior specificity compared to immunoassays, the possibility for multiplexing and low sample volume [7–9]. LC–MS/MS may also provide higher sensitivity (particularly at low concentrations of TT as in women, children and androgen-deficient men), specificity and accuracy than most immunoassays for the measurement of TT serum level. However, as showed by a large cohort study published in 2012, immunoassays may be considered sufficient for clinical application in eugonadal and hypogonadal men providing similar results to those obtained by mass spectrometry [10]. The reference method for determination of FT in serum is the equilibrium dialysis performed under standardized conditions. In most laboratories equilibrium dialysis is not available, so the estimation of FT concentration using TT, SHBG and albumin values is recommended, even because FT measurements by direct immunoassay are inaccurate and should not be used [2–4]. Moreover, since normal ranges vary significantly between laboratories depending on the method used and/or the assay kits employed, it is recommended to clinicians to measure testosterone in the same laboratory.

To respond to clinicians' request of adequate results and validated reference ranges for total testosterone, this study was planned with the aims (i) to verify the reliability of the reference interval for TT declared by immunoassay manufacturer and adopted by laboratory, (ii) to compare results for serum TT obtained by immunoassay and LC–MS/MS and (iii) to verify if the cutoff values for low TT and FT defined by Endocrine Society Guidelines for diagnosis of hypogonadism, are applicable to our study group.

Methods

Blood donor samples

Sera from anonymous blood donors (44 healthy males ranging from 29 to 58 years, median age 44 years, medium age 42 ± 8 years, weight 80.62 ± 13.11 kg, height 1.79 ± 0.09 m, BMI 25.04 ± 2.84 kg/m^2) were selected for the study (CEAVNO, protocol n. 16,726, 27/03/2018).

Immunometric analysis

Immunometric TT measurement was performed by Access Testosterone assay (Beckman Coulter Diagnostics, Brea, CA, USA) using Beckman Coulter UniCel DxI 600 highly automated platform; the same platform was also used to quantify serum concentration of SHBG by Access SHBG assay. For TT the declared reference interval was 1.75–7.81 ng/mL or 6.07–27.08 nmol/L for male ranging from 18 to 66 years, median age 41 years, precision expressed as CV% was $\leq 20\%$ at 0.5 ng/mL and $< 10\%$ from 2 to 10 ng/mL and LLoQ was 0.1 ng/mL.

For SHBG the declared reference interval was 13.3–89.5 nmol/L for male, precision was $< 7\%$ at concentrations > 2 nmol/L and analitycal sensitivity was 0.33 nmol/L.

FT was calculated by TT, SHBG and albumin values according to the formula described by Vermeulen et al. [11] and results were expressed in pg/mL.

LC–MS/MS analysis

LC–MS/MS testosterone measurement was performed using Steroid Hormones in Serum/Plasma by LC/MS kit from Eureka (Eureka Lab Division, Chiaravalle, Ancona, Italy), able to assay up to 19 steroids in human serum, by a AB-Sciex API 4000™ triple quadrupole mass spectrometer (Sciex, Concord, ON, Canada), equipped with an ESI source and coupled to an Agilent 1290 Infinity Series UHPLC system (Agilent, Santa Clara, CA, USA). Chromatographic separation was performed by an Agilent Zorbax RRHD Eclipse Plus C18 (50×2.1 mm, 1.8 µm) analytical column, kept at 60 °C. Samples, calibrators and controls preparation was performed as indicated by the manufacturer. The calibrator concentrations were 0.054, 0.116, 0.25, 0.77, 2.4, 10.7 ng/mL and control concentrations were 0.077, 4.6, 7.4 ng/mL.

Testosterone-d3 was used as an internal standard (IS) and the quantification transitions monitored were m/z 289.1 97.0 and 292.1 97.1, respectively for the analyte and the IS.

For testosterone the declared accuracy was $< 5\%$ and precision was $< 6\%$ at 0.1, 1.0 and 6.8 ng/mL, while the LLoQ was 0.006 ng/mL.

Data analysis and statistical methods

To validate the declared reference interval were followed the guidelines of document CLSI C28-A3, analyzing a subgroup of minimum 20 samples and evaluating the number of outliers [12].

To compare results obtained by immunoassay and LC–MS/MS, Passing-Bablok and Bland–Altman analysis were performed [13–15].

Results

The reference interval declared by manufacturer and adopted by the lab was validated using 44 samples from young/middle-aged healthy male subjects: only two outliers were found for each method, confirming the validity of the range also for LC–MS/MS (Fig. 1a, b) according to the guidelines of document CLSI C28-A3 [12].

The two methods for TT evaluation correlated well, as showed by non-parametric Passing-Bablok regression analysis ($y = -1.45 + 1.43x$). The value 0 included in 95% CI for intercept (systematic difference) and the value 1 included in 95% CI for slope (proportional difference), demonstrated the absence of proportional systematic error among two methods (Fig. 2a). Residual plots, representing the distribution of differences around fitted regression line, showed that residuals are randomly distributed above and below regression line, so differences among methods are not depending by concentration (data not shown). The Bland–Altman graphical analysis showed that average of

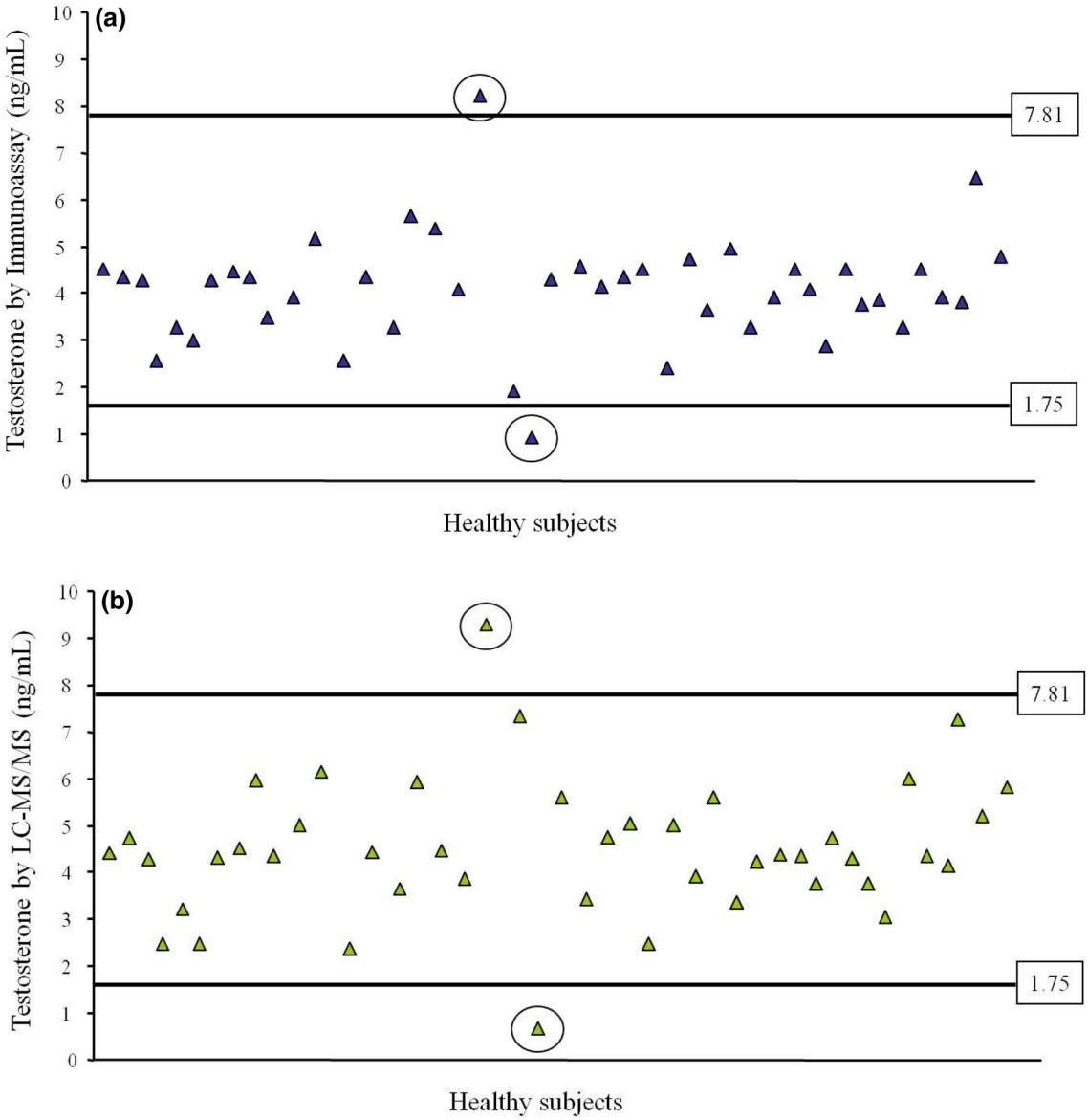

Fig. 1 Check of manufacturer declared reference range for TT measured by immunoassay (A) and LC–MS/MS (B). The range is acceptable with only two results slightly outside the limits for both methods

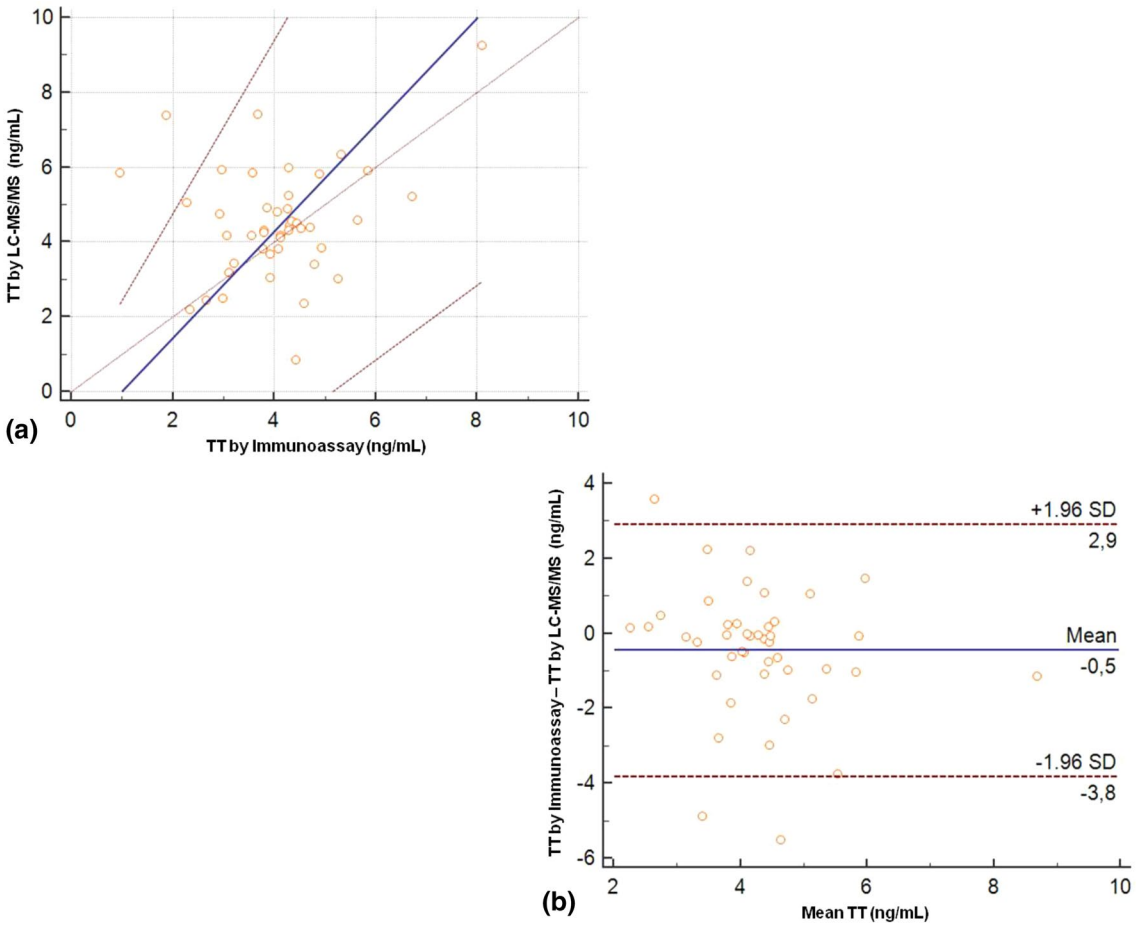

Fig. 2 Passing-Bablok regression curve (A). The CI 95% for intercept is − 5.17–0.12 and the CI 95% for slope is 0.99–2.32. The solid line represents the regression line, the dashed lines represent the confidence interval and the dotted line is the identity line. Bland–Altman plot (B). The differences between observation pairs are plotted against their mean and the average of the differences (bias) and its 95% CI lines (agreement range) are drawn on the same plot. The solid line represents the mean of the differences and the dashed lines represent the SD of that mean

differences is near to zero and the 0 value is included in CI 95%, excluding a systematic significant error (Fig. 2b).

As mentioned in the Introduction, cutoff values of 3 ng/mL for TT and 50 pg/mL for calculated FT are recommended by Endocrine Society to define men hypogonadism [2–4]. The distribution of TT and FT in our study group of healthy men is described in Table 1. TT and calculated FT levels at 5th and 2.5th percentile are

Table 1 Distribution of TT and calculated FT values in our study group of young/middle-aged men donors

	TT (ng/mL)		Calculated FT (pg/mL)	
	Immunoassay	LC–MS/MS	Immunoassay	LC–MS/MS
Mean	4.05	4.51	76.93	82.18
SD	1.23	1.52	28.30	21.90
CI 95%	3.68–4.42	4.05–4.97	68.33–85.53	75.52–88.84
Median	4.10	4.37	76.10	81.55
97.5th percentile	6.65	7.41	134.48	114.93
95th percentile	5.82	7.23	125.45	113.4
5th percentile	2.28	2.37	35.63	53.60
2.5th percentile	1.90	2.20	18.35	47.37

Mean, SD, CI 95% of the mean, median and 2.5th to 97.5th percentile values are reported

below the literature cutoffs, with only a value of FT at 5th percentile that is slightly higher.

Discussion

Physicians are very sensitive to problems related to laboratory dosage of testosterone for diagnosis of hypogonadism in men, and this study was planned specifically to meet their request for more accurate results and properly validated reference ranges. The study included the aims to verify the reliability of the reference interval for TT declared by immunoassay manufacturer and adopted by laboratory, to compare results for serum TT obtained by immunoassay and LC–MS/MS and to verify if the cutoff values for low TT and FT, defined by Endocrine Society Guidelines for diagnosis of hypogonadism, are applicable to our study group.

The reference interval adopted by the lab was validated and the obtained results permitted to declare that in use immunoassay provided a reliable measurement of serum TT levels sufficient for clinical application, especially because of the good correlation with the results obtained by LC–MS/MS, the technique recognized by the scientific community as the gold standard method for steroids measuring. Our data were also supported by those obtained by the European Male Aging Study (EMAS) research consortium that measured testosterone and estradiol concentrations in samples from a large cohort of middle-aged/elderly men by both immunoassay and LC–MS/MS. This research indicated that clinically relevant results on serum testosterone for diagnosis of hypogonadism were obtained both with well-validated immunoassay and LC–MS/MS and did not support a mandatory move towards this high-tech approach [10].

The last aim of our study concerns an interesting and debated argument in scientific and medical community that is oriented to find a shared and harmonized reference range for TT. With the goal to harmonize reference ranges from different methods by cross-calibrating them using a higher order standard and a higher order assay, in more than 400 samples from four big American and European cohort studies serum total testosterone levels were measured at the CDC Clinical Reference Laboratory using a reference LC–MS/MS method [16]. There was a good concordance in age-adjusted harmonized TT levels among men in four geographically distinct cohorts, suggesting that intercohort variation may be influenced by inter assay variation. The suggested range for healthy non obese men 19–39 years was 2.64–9.16 ng/mL.

Biochemical parameters used to identify hypogonadism in men include TT, calculated FT, FT, bioavailable testosterone and free androgen index [17, 18]. Bioavailable testosterone is the most accurate parameter, but usually morning TT is the accepted substitute in the presence of normal values of SHBG. The measurement of SHBG is recommended when TT value is low or borderline, particularly in obese or older men [19], to calculate FT values and to avoid inaccuracies in diagnosis [11, 20]. Since normal ranges vary significantly between laboratories depending on employed methods and assays, it's better to measure testosterone always in the same laboratory. Besides the variation due to methods and instrumentations, cutoff values for low TT and FT are different between studies and scientific societies [18]. For example the Endocrine Society recommends using a TT level below 3 ng/mL and a calculated FT value below 50 pg/mL in symptomatic patients to make hypogonadism diagnosis and evaluate a replacement therapy prescription [2–4].

As reported in results, TT and FT lower limits at 5th and 2.5th percentile are below the cutoffs reported in the literature for the diagnosis of hypogonadism [2–4] and therefore subjects with normal values of TT and FT may need replacement therapy in the presence of clinical signs. Hence the importance of always associating consistent clinical signs and symptoms with laboratory hormonal data as recommended by the main international guidelines [2–4]. In a very recent review the evolution of clinical guidelines with respect to testosterone replacement therapy was described [18] to stress the importance for clinicians to follow a well-defined protocol to avoid the misuse of testosterone.

In conclusions, the immunoassay currently used in our lab can be considered as an adequate tool for TT evaluation, also for the good agreement with the high specificity, sensitivity and accuracy method LC–MS/MS. The reference range for TT declared by manufacturer is reliable for our laboratory. Finally, obtained results confirm the need of concordance between clinical and biochemical data to diagnose hypogonadism in men, particularly in the presence of TT values placed in a grey zone between the lower limit of reference range and the cutoff value.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Statement of human rights The study have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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