Testosterone Assays

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KEYWORDS

• Testosterone assays • Hypogonadism • Total testosterone • Free testosterone

KEY POINTS

- Accurate and precise measurement of testosterone is necessary to diagnose and manage hypogonadism in men as well as other endocrine conditions.
- Measurement of free testosterone and the bioavailable fraction may be clinically useful in some patient populations and clinical scenarios, particularly in men with borderline low total testosterone.
- Mass spectrometry is the gold standard measurement modality for total testosterone but is not available in many standard clinical laboratories due to barriers related to cost and technical challenges.
- Testosterone measurements should be performed in the morning in a fasting state with any low serum testosterone measurements performed in duplicate.

The accurate detection and quantification of serum testosterone levels is necessary for the diagnosis of hypogonadism in men, evaluation of endocrine abnormalities, and monitoring and titration of testosterone therapy, among other clinical scenarios. Achieving accurate and precise measurements is accompanied by a host of challenges such as deficiencies in standardization across laboratories, variability in reference ranges, expenses related to gold standard equipment, and technical as well as logistical challenges in performing these assays. Total testosterone levels include testosterone that is specifically bound to sex hormone-binding globulin (SHBG), nonspecifically bound to albumin, corticosteroid-binding globulin, and orosomucoid, as well as a small percentage of unbound hormone within the serum. According to the free hormone hypothesis, the unbound fraction of testosterone represents the biologically active component of total testosterone, although this theory is debated. Other evidence suggests that the bioavailable fraction, which refers to free testosterone plus nonspecifically bound testosterone, is a better indicator of biological activity than the free testosterone. Measurement of total testosterone remains the most accessible for standard laboratories and can be accomplished by a variety of measures, which include immunoassays (IAs), which are relatively inexpensive, rapid, and technically facile; and mass spectrometry (MS), which is the gold standard but is associated with higher technological costs and is more technically challenging and time consuming. The measurement of free testosterone can be accomplished by equilibrium dialysis, IAs, and calculations that use algorithms to estimate the value based on measurements of total testosterone, SHBG, and albumin within the sample of interest. Equilibrium dialysis is the gold standard, which requires standardized conditions and is therefore more technically challenging and time consuming than the other methods. IAs are highly inaccurate and, although widely available and inexpensive, are not recommended. Bioavailable testosterone can be estimated via precipitation of SHBG-bound testosterone followed by assay of tracer-labeled testosterone and calculation. Reference ranges vary among society's guidelines, but the lower limit of normal testosterone in men is set at greater than 230 ng/dL by all major societies surveyed for this article. Efforts to adapt reference ranges to different populations, including age-adjusted populations, have augmented our understanding of normal testosterone levels in men. Further

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Box 1
Clinical Care Points

Testosterone levels in men should be assessed in the early morning in a fasting state.[23] Low testosterone values should be repeated.[45]

Mass spectrometry is the gold standard measurement assay for total testosterone, while certain immunoassays may be an inexpensive and widely available testosterone assay suitable for eugonadal men.[29]

Caution should be taken in the evaluation of low testosterone, including in women and children. Commercially available immunoassays may not be accurate at low levels of serum testosterone.[22]

Free testosterone measurement has demonstrated utility in men demonstrating borderline low levels of total testosterone.[22] Equilibrium dialysis followed by detection with mass spectrometry[23] or estimations via calculations should be utilized to measure free testosterone.[30] Immunoassays should not be used.[28,29]

The CDC Hormone Standardization Program can be utilized to assess the accreditation status of laboratories within the United States.[15]

For appropriate interpretation of assay results,[38] clinical urologists need to be up to date on published reference ranges, including age-related and sex-related standard hormone ranges.

collaboration aimed at establishing common reference ranges, as well as standardizing varied commercial products, is necessary to systematize the interpretation of testosterone assays, the diagnosis, and the monitoring of hypogonadism, as well as other endocrine conditions (**Box 1**).

INTRODUCTION

Testosterone is the primary androgenic hormone in humans.[1] The accurate detection and quantification of serum testosterone levels is an increasingly necessary component of standard clinical care for practicing urologists as well as for general practice medical providers.

Testosterone replacement therapy (TRT) as treatment of testosterone deficiency (TD), transgender health care, and endocrine abnormalities, among other clinical scenarios, requires precise and accurate detection of serum testosterone.

An accurate diagnosis of male hypogonadism depends on the reliable quantification of serum testosterone levels. In the case of TRT, clinical consensus guidelines consistently recommend that only men meeting criteria for TD should be treated.[2] Importantly, low testosterone alone does not define TD. Rather, a diagnosis of TD must include the presence of symptoms and/or signs associated with low testosterone in addition to documented low serum total testosterone levels.[3] To diagnose hypogonadism, the American Urological Association (AUA) requires symptoms and/or signs such as fatigue, cognitive dysfunction, loss of body hair, and depressed mood, as well as by total testosterone 300 ng/dL (10.4 nmol/L).[3] Furthermore, all patients receiving testosterone therapy require careful laboratory monitoring for safety and efficacy as well as to ensure that testosterone levels are titrated to target levels. A variety of laboratory assays and protocols exist to measure testosterone. Measurement by testosterone assays is a critical component of diagnosing hypogonadism in men, in addition to other endocrine conditions. Assay technologies, protocols, and target ranges can vary by the clinical or commercial laboratory running the samples. Recommended diagnostic and therapeutic ranges are also not standardized.

Challenges to obtaining accurate assays of serum testosterone include diurnal fluctuations in serum testosterone, a wide range of normal values of testosterone, and technical as well as logistical challenges in performing assays. In this article, we seek to elucidate the variety of testosterone assays available and the manner in which these assays are interpreted.

THE AVAILABILITY OF TESTOSTERONE IN THE SERUM AND THE FREE HORMONE HYPOTHESIS

Total testosterone refers to the sum of primary androgenic hormones circulating within the serum. Total testosterone levels include testosterone bound specifically to SHBG, testosterone bound nonspecifically to albumin, corticosteroid-binding globulin, and orosomucoid, and a small percentage of unbound hormone in the serum[4,5] (**Fig. 1**). The term *free testosterone* refers to this small unbound fraction. The term *bioavailable fraction* refers to the free testosterone as well as the nonspecifically bound testosterone. Measurement of the fraction of hormone that is available at the cellular level in target tissues, even in ideal laboratory settings, is currently limited to the estimation of the bioavailable fraction.

The *free hormone hypothesis* states that the unbound fraction of testosterone represents the biologically active component of total testosterone at target tissues.[6] The free hormone hypothesis

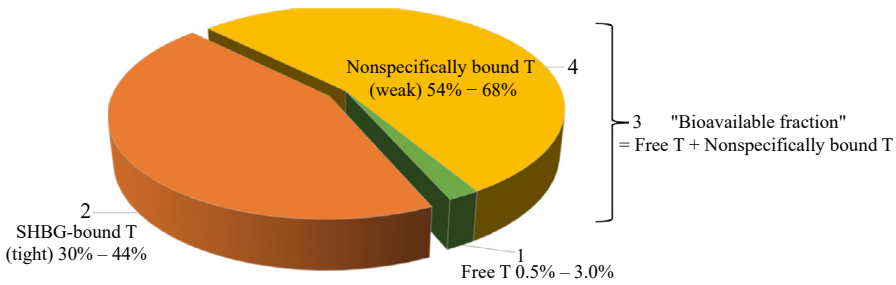

Fig. 1. The approximate fractions of testosterone circulating within the serum, including the unbound fraction (free testosterone), the nonspecifically bound fraction, and the tightly bound fraction.

continues to be highly debated.[7] Subsequent studies estimate that the bioavailable fraction more accurately represents the biologically active proportion of testosterone within the serum.[8,9] More recent evidence suggests that testosterone bound to SHBG may even exert a biological effect in certain tissues, such as in the prostate.[7,10]

Evidence from a Large Epidemiological Study

The European Male Aging Study is a multicenter, prospective cohort study examining aging-related symptoms in men, specifically focusing on the effects of age-related changes in hormone levels and their related symptoms.[11] A significant association was found between decreased libido, morning erections, and erectile dysfunction and testosterone levels of < 11 nmol/L (317 ng/dL) and free testosterone levels of < 280 pmol/L in this large-scale, population-based study.[11,12]

These threshold values indicate the likelihood of sexual symptoms occurring more significantly in men with these levels compared to men with normal values of testosterone. However, the probability of these sexual symptoms increased with decreasing levels of total testosterone and free testosterone, therefore revealing an inverse relationship. The results of this study suggest that there may be utility in measuring free testosterone for diagnostic purposes in symptomatic men with normal or borderline low total testosterone levels.

TESTOSTERONE MEASUREMENTS

Measurement of total testosterone is typically preferred in standard clinical settings due to the challenging nature of measuring free testosterone. Equilibrium dialysis methods of free testosterone quantification remain the current gold standard, but these assays are generally too complex for standard laboratories, as their performance can be affected by assay conditions that result in high assay variability.[6,7,13] In a 2017 study by Cao and colleagues,[14] in which four samples were distributed to 142 accredited laboratories to study assay performance compared to target values determined by using the reference measurement procedures operated by the Centers for Disease Control and Prevention Clinical Reference Laboratory, significant variability in accuracy and precision between the types of assays performed and the laboratories performing the assays was reported. Cao and colleagues[14] ultimately concluded that incorrect assay calibration and insufficient analytical specificity were most likely responsible for the high variability.

Furthermore, the lack of common reference intervals, which rely on faulty models of testosterone to SHBG ratios, fosters potential misinterpretation of estimates of free testosterone.[7] Similar challenges impede the measurement of bioavailable testosterone. Available assays are technically difficult and not commonly performed in typical clinical laboratories.[7] Because of the significant interassay variability, programs such as the CDC Hormone Standardization Program (CDC HoSt) were established to accredit the performance of specific laboratories and assays.

Regarding testosterone, CDC HoSt certifies the performance of assays at distinct laboratories within the concentration range of 2.50 to 1000 ng/dL for total testosterone and designates those laboratories that meet the performance criterion of ±6.4% mean bias when compared to the CDC reference measurement procedure for total testosterone.[15] Moreover, the AUA guidelines recommend measuring total testosterone levels at the same laboratory and using the same assay on two separate occasions in an early morning fashion to ensure comparable measurements.[3]

TOTAL TESTOSTERONE

Immunoassays

IAs have been widely utilized in clinical practice, given their relative ease of use, simplicity, cost, acceptable performance at normal testosterone levels, and scalability (**Table 1**).

Table 1
A select summary of assays available to measure total testosterone

Assays for Total Testosterone					
Assays	**Mechanisms**	**Units**	**Coefficients of Variation**	**Advantages**	**Disadvantages**
Immunoassay (including radioimmunoassay and enzyme immunoassay)	• Serum mixed with T antibodies and tracer • Tracer can be radioisotope (RIA), enzyme (EIA), fluorescent, or chemiluminescent compound	ng/dL	• Intraassay: −14% to +19% • CV most pronounced at low T values (40% in samples with TT < 100 ng/dL)	• Rapid and simple • Inexpensive • Commonly utilized • High-throughput • Reference range data available	• Significant interassay variability • Reduced accuracy at low/high T levels • Interfering factors (heterophile antibodies in serum)
Liquid chromatography-mass spectrometry	• Ionizes molecules and measures mass-to-charge ratios	ng/dL	• ±6.4% (to maintain CDC approval status)	• Gold standard • Excellent sensitivity and specificity, even at low T concentrations (<40 ng/dL) • Simultaneous measurement of multiple steroids	• Not FDA approved • Low-throughput • Labor intensive • Expensive

For these reasons, most of the total testosterone reference ranges were established by using these methods. In short, IAs rely on tracer-linked testosterone molecules that compete with native testosterone in samples for the binding of testosterone antibodies. The tracer molecule may be an enzyme (*enzyme immunoassay*, *EIA*), a radioisotope (*radioimmunoassay*, *RIA*), chemiluminescent (chemiluminescent immunoassay), or fluorescent (*fluoroimmunoassay*, *FIA*) compound. The disadvantages of performing IA include the technical expertise and additional time needed for testosterone extraction/chromatography as well as reduced accuracy at low or high testosterone levels. RIA are accompanied by the additional disadvantage of generating radioactive waste.[16]

Wang and colleagues evaluated the results of four common automated IA and two manual IA related to 62 eugonadal and 60 hypogonadal men. As demonstrated by Wang and colleagues,[17] who used liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the gold standard, none of the six different IAs tested were of sufficient accuracy at low serum testosterone levels. Despite these findings, Wang and colleagues[17] stated that, from a clinical standpoint, several of the IAs in the study would be appropriate for use in adult males with low testosterone (100 ng/dL) levels, as these men would have been diagnosed with hypogonadism and treated accordingly. In a similar study, Taieb and colleagues[18] investigated commercially available testosterone IAs for accuracy in the measurement of serum testosterone in 50 men, 55 women, and 11 children. Compared with the gas chromatography-mass spectrometry analysis, which is considered the gold standard in this study, none of the 10 IAs tested were sufficiently reliable in women and children whose testosterone levels were low (<1.7 nmol/L, <49.05 ng/dL) or very low (0.17 nmol/L, 4.9 ng/dL).[18]

Mass Spectrometry

MS-based assays remain the gold standard for quantification of total testosterone levels. Despite higher costs, MS has become increasingly more utilized in clinical practice in part due to its higher sensitivity and specificity at both low and high testosterone levels compared to IAs, which have been shown to vary significantly, particularly at low testosterone levels.[19,20] From 2012 to 2015, nearly a fivefold increase in the use of MS-based assays was reported by the College of American Pathologists.[14]

Additional advantages of MS include the ability to measure multiple steroid levels simultaneously, simple sample preparation wherein nonderivatized steroids can be analyzed directly; high recovery with improved signal-to-noise ratio; and lower interference.[17]

In contrast to IA, MS involves the ionization of serum compounds and subsequent measurement of their mass-to-charge ratios or molecular weight. Preanalysis extraction or chromatography (gas or liquid) prior to MS can be performed to separate hormones and proteins that could otherwise affect the accurate measurement of testosterone. The MS subtype, LC-MS/MS, which couples the liquid chromatography technique of chemical separation to the MS technique, has been an increasingly adopted high-throughput and accurate testosterone assay in clinical practice and research.[19] Although considerable interlaboratory variability exists with MS, this remains a significant improvement from the variability inherent to commercially available IAs.[21] The high complexity of laboratory equipment required and the relatively high expense of running MS remain the predominant obstacles to its widespread adoption.[22]

FREE TESTOSTERONE

Equilibrium Dialysis

Equilibrium dialysis, if performed under standardized conditions, represents the gold standard for detection of free testosterone[23] (**Table 2**). This method utilizes a semipermeable membrane to isolate testosterone bound to protein by molecular weight.[24] Free testosterone, which is not bound to protein, is able to equilibrate across the semipermeable membrane due to its lower molecular weight. Testosterone present within the dialysate can then be measured via direct or indirect methods.[7] Direct methods include LC-MS/MS to measure the amount of free testosterone within the dialysate. Alternatively, free testosterone can be measured directly via an IA or indirectly via assessment with the addition of a small quantity of testosterone radiotracer.[7] Analytical performance during the measurement step highly impacts the accuracy of this measurement.

Challenges related to equilibrium dialysis include the impact of dilution of samples as well as the susceptibility of dialysis based on temperature and pH.[25] These challenges highlight the crucial maintenance of a standardized environment. The utility of equilibrium dialysis is also impacted by the time-consuming movement of dialysate across the membrane, given separation can take up to 16 hours to complete. A modified method of equilibrium dialysis, known as *ultrafiltration,* utilizes centrifugation to force the sample through the semipermeable membrane. This method reduces the time necessary for the sample

Table 2
A select summary of assays available to measure free testosterone

Assays for Free Testosterone					
Assays	**Mechanisms**	**Units**	**Coefficients of Variation**	**Advantages**	**Disadvantages**
Equilibrium dialysis	• Serum placed in dialysis chamber • Tracer-labeled T added to serum • Equilibrium achieved • Low molecular weight permeable membrane restricts passage of small molecules • Proportion of bound & free-labeled T assessed	pg/dL	• Interassay: 6.8% • Intraassay: 10.0%	• Gold standard • Excellent sensitivity and specificity • Reproducible	• Time/labor-intensive • Low-throughput • Technically challenging • Expensive • Relies on accuracy of TT assay • Tracer impurities may compromise results
Calculated FT	• Law of mass action (Nanjee & Wheeler, Sodergard, Vermeulen)	pg/mL	• Interassay: 18–30%	• Simple • Rapid • Has correlated well in some series with equilibrium dialysis	• Relies on TT and SHBG assay accuracy • Accuracy relies on equilibrium dissociation constants for binding of SHBG and albumin to testosterone • High interassay variability • Tends to overestimate true value
Direct (Ultracentrifugation, Analog)	• Radiolabeled T is added to sample and allowed to reach equilibrium with T in serum • Free T is separated by centrifugal ultrafiltration • Radioactivity of protein-free ultrafiltrate is measured and used to calculate % free T	pg/mL	• Interassay: 8.9% • Intraassay: 10.3%	• Method shows promise but additional studies required to measure assay performance across the range of free T values	• Low-throughput • Technically challenging • Relies on accuracy of TT measurement

to reach equilibrium. Analytical performance has been noted to be generally comparable to standard equilibrium dialysis while requiring significantly shorter operating times.[26,27] An additional challenge with ultracentrifugation is the adsorption of samples to the ultrafiltration filter.[25] The percentage of samples lost in this manner likely varies according to the commercial filter being used.

Analogue Immunoassays

Use of commercially available IA kits for the measurement of free testosterone confers many of the same benefits as described earlier. IAs, which are widely available at large and small laboratories, are relatively inexpensive and easily performed over short operating times. Measurements of free testosterone, which is present at relatively low concentrations, are often inaccurate due to alterations in the levels of SHBG.[28] For these reasons, the use of IAs is not recommended for the measurement of free testosterone.[29]

Calculated Free Testosterone

Free testosterone can be measured indirectly via several established calculations using algorithms to estimate bioavailable testosterone, free androgen index (FAI = TT/SHBG × 100), and free testosterone index, for example. Additional examples include the Sodergard, Nanjee–Wheeler, Vermeulen, and Ly–Handelsman methods that estimate free testosterone by using measured testosterone, albumin, and SHBG concentrations.[30] Several studies have investigated the predictive accuracy of calculated free testosterone, including one by Morris and colleagues,[31] which demonstrated high predictability by using and comparing total testosterone to other tested modalities in assessing for biochemical hypogonadism. Using multiple linear regression analysis on their training cohort, Morris and colleagues derived an equation, lnBioT = −0.266 + (0.955 × lnTT)–(0.228 × lnSHBG), where ln = natural log, BioT = bioavailable testosterone, TT = total testosterone, and SHBG = sex hormone-binding globulin, and demonstrated a high correlation between derived values and true values for bioavailable testosterone.[31] Calculation of free testosterone relies on the measurement of total testosterone, SHBG, and albumin within the sample of interest. Calculation of free testosterone is performed according to the law of mass action while utilizing the specific dissociation constants of the other analytes measured within the sample. In this method, the measurement accuracy of the concentrations of analytes determines the accuracy of the calculated free testosterone value. Multiple algorithms exist within the literature, including examples derived from equilibrium binding[4] and empirically derived examples devised from the results of computer modeling based on known concentrations of the analytes of interest.[32,33]

BIOAVAILABLE TESTOSTERONE

Ammonium Sulfate Precipitation of Sex Hormone-Binding Globulin-Bound Testosterone

As a technique used to measure bioavailable testosterone, ammonium sulfate precipitation involves the mixing of tracer-labeled testosterone with serum followed by the precipitation of SHBG via the addition of ammonium sulfate (**Table 3**). Protocols often utilize saturated ammonium sulfate solution in a 1:1 ratio with the sample specimen. The remaining tracer-labeled testosterone is then multiplied by total testosterone to yield an estimation of bioavailable testosterone in serum. Although this technique correlates well with equilibrium dialysis, it has several disadvantages such as its reliability on the accuracy of the total testosterone assay and the potential impact of tracer impurities on results.[19]

An alternative to precipitation of SHBG with ammonium sulfate has been proposed with the use of concanavalin A separation.[34] Early evidence suggests that this method may have increased specificity over ammonium sulfate.[35] Giton and colleagues compared the results of 131 samples assessed for bioavailable testosterone using both ammonium sulfate precipitation and concanavalin A separation methods. They found similar results from both methods.

Concanavalin A has the benefit of eliminating errors associated with nonspecific albumin precipitation that may occur in poorly controlled assay conditions.[35] Further evaluation of this relatively novel method should precede its adoption into clinical practice.

REFERENCE RANGES

Since a standard reference range for the distribution of circulating concentrations of testosterone in healthy men remains variable and therefore undefined, there is no consensus on the accepted lower testosterone limits. Rigorously derived reference ranges serve as the mainstay of the contemporary approach to making medical diagnoses, including hypogonadism. According to a guideline supported by the European Association of Urology, International Society of Andrology, International Society for the Study of Aging Male,

Table 3
A select summary of assays available to measure bioavailable testosterone

Assays for Bioavailable Testosterone					
Assays	**Mechanisms**	**Units**	**Coefficients of Variation**	**Advantages**	**Disadvantages**
Ammonium sulfate precipitation	• Tracer-labeled T added to serum • SHBG precipitated via addition of ammonium sulfate • Remaining tracer multiplied by TT	ng/dL	• Interassay: 7.9% • Intraassay: 7.2%	• Excellent sensitivity and specificity • Correlates well with equilibrium dialysis	• Time/labor intensive • Technically challenging • Low throughput • Relies on accuracy of TT assay • Tracer impurities may compromise results

European Academy of Andrology, and the American Society of Andrology, TT levels of < 230 ng/dL (8 nmol/L) in young men would benefit from TRT, while TT > 350 ng/dL (12.1 nmol/L) does not require treatment.[36] In contrast, Endocrine Society guidelines endorse a lower TT threshold for consideration of TRT at 280 to 300 ng/dL (9.7 to 10.4 nmol/L), whereas the AUA guidelines support < 300 ng/dL (10.4 nmol/L) as a reasonable cut-off level [3291].

Given the widespread adoption of MS in clinical practice, reference ranges for testosterone concentrations in healthy men have been established to clearly define the diagnosis of androgen deficiency. For example, Bhasin and colleagues[37] described reference ranges in a cohort of 456 men (aged 19–40 years) from the Framingham Heart Study Generation 3. In this community-based sample of nonobese, healthy men without significant risk factors or comorbidities, such as diabetes mellitus, hypertension, cardiovascular disease, tobacco use, or dyslipidemia, the mean TT was 724 ng/dL (25.1 nmol/L). The upper (97.5%) and lower (2.5%) intervals were 1197 ng/dL (41.5 nmol/L) and 348 ng/dL (12.1 nmol/L), respectively.[37]

Similar data have been published in other populations.

Age-Specific Reference Ranges

Male testosterone levels have been demonstrated to decrease with age. Originally a controversial conclusion, the decrease in bioavailable testosterone in aging men compared to their 40-year-old counterparts has been shown in a large series of cross-sectional and longitudinal studies.[38] The natural decline in TT levels with age and the lack of defined, age-specific thresholds for distinct symptom complexes add to the challenge of establishing reference ranges.[39]

For example, in a 2012 study that included 3690 elderly, community-dwelling men (>70 years, mean age 77 years), Yeap and colleagues[39] reported a mean TT of 378 ng/dL (13.1 nmol/L) with upper (97.5%) and lower (2.5%) reference ranges of 693 ng/dL (24 nmol/L) and 145 ng/dL (5 nmol/L), respectively. More specifically, in a subset of the study population that included 394 men (aged 76.1 years $\pm$ 3.2 years) who described themselves as in excellent or very good health, without cancer, cardiovascular disease, depression, dementia, or diabetes, and with no history of smoking, the mean as well as the upper and lower reference ranges were similar to the entire cohort.[39] In this reference group, the mean, 97.5%, and 2.5% TT levels were 406 ng/dL (14.1 nmol/L), 739 ng/dL (25.6 nmol/L), and 184 ng/dL (6.4 nmol/L), respectively.[39] Using these reported cutoffs for the entire cohort of 3690 men, those with hypogonadism, defined as below the 2.5% reference range, had increased odds of diabetes, frailty, and cardiovascular disease.[39] The men categorized as hypogonadal in this sample population also had a higher odds ratio for these outcomes.[39]

As evidenced by Yeap and colleagues and other researchers, application of one reference range to all age groups may overestimate the prevalence of low testosterone in elderly populations compared with a standard group within the corresponding age bracket. Furthermore, laboratory reference ranges are not standardized to consider age-related testosterone standards.[40] While some laboratories may apply age-related standards, others may not. Age-related discrepancies are further complicated by the fact that while serum testosterone levels decline with age, there is an age-associated increase in SHBG of about 1.3–1.6% per year.[41] This can further exacerbate the decrease in bioavailable testosterone in aging men.[38] In the population-based Massachusetts Male Aging Study, bioavailable testosterone decreased by 2–3% per year.

Standardization of Reference Ranges

The distribution of testosterone levels has been shown to vary across populations of men from different geographic regions. In addition to biological or environmental factors, interassay and interlaboratory differences also contribute to these reported variations in reference ranges.[42] It is unclear whether reported reference ranges from one study population can be applied more broadly to other populations of men from different parts of the world. Additionally, certain disease processes and therapeutics can alter testosterone levels and further complicate the applicability of reference ranges. Diabetes mellitus, thyroid disease, human immunodeficiency virus, pituitary disorders, long-term narcotic use, and obesity can all have an effect on testosterone levels, and including men with such comorbidities in studies can distort reported reference ranges.[3,42] Ultimately, whether reported reference ranges that have been established in healthy men are appropriate for men in various diseased states remains an area of active investigation.

Specifically, with regard to reference range variations related to differences in assay technologies, researchers have investigated ways to address these systemic differences and minimize their influence on reference range calculations. For example, normalizing equations derived through the harmonization of all measurements to a higher-order standard before the calculation

of reference ranges have reduced intercohort variation of testosterone measurements, suggesting assay differences as major factors in observed geographical variations.[42] By cross-calibrating assays to a reference method and standard, these harmonized reference ranges can be applied across laboratories to reduce intercohort variations of testosterone measurements.[42]

Travison and colleagues[42] demonstrated the feasibility of harmonization procedures in a study that compared testosterone concentrations in 100 men from four cohorts in Europe and the United States: the Framingham Heart Study, the European Male Aging Study, the Osteoporotic Fractures in Men Study, and the Male Sibling Study of Osteoporosis. Travison and colleagues[42] were able to construct normalizing equations that generated harmonized values used to derive standardized, age-specific reference ranges by measuring testosterone concentrations calibrated to a higher-order benchmark, such as that provided by the CDC Reference Laboratory. A remarkable concordance in age-adjusted, harmonized testosterone levels among men in the four geographically distinct cohorts was demonstrated.[42] The harmonized normal reference range in a healthy, nonobese population of European and American men (aged 19–39 years) was reported to be 264 ng/dL–916 ng/dL (9.15–31.75 nmol/L).[42] Specifically, the harmonized 2.5th, 5th, 50th, 95th, and 97.5th percentile values were 264, 303, 531, 852, and 916 ng/dL, respectively.[42] This data from Travison and colleagues[42] demonstrate the feasibility and promise of calculating reference ranges using harmonized values that can be applied to laboratories that use calibrators such as those available from the National Institute of Standards and Technologies.

The application of reference ranges across laboratories and geographic regions has been and remains to be a formidable challenge. It not only requires mechanisms for the implementation of standardizing assays but also requires a fundamental understanding of the biological as well as social differences in analyte distribution. Furthermore, validation of the harmonized reference ranges using outcome-related data from randomized trials and longitudinal studies remains a complex yet crucial step in the clinical application of standardizing reference ranges.

OTHER FACTORS AFFECTING ACCURACY OF TOTAL TESTOSTERONE MEASUREMENT

Timing of Laboratory Testing

Circadian variation in testosterone levels is a well-documented phenomenon, with the highest levels of testosterone released in the morning and relatively lower levels of testosterone released in the afternoon and evening,[43] while trough levels of testosterone are observed approximately 12 hours after peak.[44] This diurnal variation in testosterone levels has been reported to be blunted in older populations of men. A 2009 study reported a 20–25% difference in testosterone levels in younger men (aged 30–40 years) between the hours of 08:00 and 16:00 compared to a 10% difference in testosterone levels between the same hours for older men (aged 70 years).[44]

Notably, this study of 66 men included many subjects with normal morning (before 12:00) testosterone levels ($\geq$300 ng/dL) and low testosterone levels (<300 ng/dL) in the afternoon. For these reasons, testosterone measurements should ideally be performed as close to waking as possible. In clinical practice, this may translate to sample procurement between 07:00 and 09:00.

Repeat Laboratory Testing

Testosterone laboratory values should be confirmed with at least one duplicate measurement for the diagnosis of low testosterone. In one community-based study, 30% of men with initial testosterone values considered to be in the hypogonadal range were found to have normal testosterone values on repeat measurements.[45] There has been evidence reported that week-to-week variability can account for some of this variation.[46] Another study, which employed frequent testing of testosterone levels at 20-minute intervals, found that 3 of 10 healthy subjects intermittently registered testosterone concentrations below the normal range.[47]

Repeat measurements have been noted to vary widely (65–153%) depending on the assay utilized. Conducting two or more repeat measurements can mitigate some of this variability.[45]

SUMMARY

Because the accurate diagnosis of male hypogonadism is dependent on the reliable assessment of testosterone levels, accurate detection and quantification of serum testosterone levels remains a necessary component of standard clinical care for the practicing urologist. Consensus guidelines routinely recommend that only men meeting criteria for TD should be offered TRT. The accurate quantification of testosterone levels remains a challenge, in part due to the diurnal fluctuations in serum testosterone, the wide range of normal testosterone values, and the technical as well as

logistical challenges of performing assays. Although IAs and MS-based assays are commonly utilized in clinical practice, both these assay systems exhibit unique flaws that erroneously hinder the establishment of reference ranges. Moreover, reported reference ranges from distinct populations and cohorts cannot be applied more broadly to other populations around the world, but feasible measures, such as harmonization procedures, offer promise in the standardization of reference ranges.

CLINICS CARE POINT

- A diagnosis of hypogonadism should only be made in men with symptoms and signs consistent with testosterone deficiency and unequivocally low serum T concentrations on at least two early morning tests.
- The use of accurate assays for the measurement of testosterone and rigorously derived reference ranges for the interpretation of testosterone levels is necessary for the diagnosis of hypogonadism.

DISCLOSURE

The authors have nothing to disclose.

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