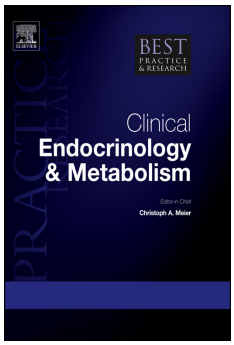

Accurate measurement of total and free testosterone levels for the diagnosis of androgen disorders

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Accurate measurement of total and free testosterone levels for the diagnosis of androgen disorders

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Key words: hypogonadism; testosterone deficiency; liquid chromatography tandem mass spectrometry; equilibrium dialysis; SHBG; bioavailable testosterone;

ABSTRACT

The circulating concentrations of total and free testosterone vary substantially in people over time due to biologic factors as well as due to measurement variation. Accurate measurement of total and free testosterone is essential for making the diagnosis of androgen disorders. Total testosterone should ideally be measured in a fasting state in the morning using a reliable assay, such as liquid chromatography tandem mass spectrometry, in a laboratory that is certified by an accuracy-based benchmark. Free testosterone levels should be measured in men in whom alterations in binding protein concentrations are suspected or in whom total testosterone levels are only slightly above or slightly below the lower limit of the normal male range for testosterone.

INTRODUCTION

The Endocrine Society's clinical practice guideline recommends making a diagnosis of hypogonadism only in men with signs and symptoms consistent with testosterone deficiency and unequivocally and consistently low serum concentrations of testosterone measured by a reliable assay.[1] Therefore, the evaluation of patients with suspected hypogonadism is predicated crucially upon careful ascertainment of the signs and symptoms, and accurate and precise measurement of circulating total and free testosterone. Although the diagnostic approach recommended by the Endocrine Society for the evaluation of men suspected of hypogonadism is conceptually uncomplicated, the non-specificity of symptoms, variations in the circulating testosterone levels over time due to biological factors, the inaccuracy and imprecision of some of the commonly used assays for the measurement of total and free testosterone levels, and the discordance of reference ranges among assays and laboratories can contribute to the diagnostic inaccuracy.[2]

This review offers steps that can be applied in clinical practice to improve the diagnostic evaluation of testosterone deficiency in men; these steps include: careful selection of patients in whom testosterone levels are measured; attention to the timing of blood sample collection; the use of accurate assays to measure total testosterone levels; measurement of free testosterone levels using an equilibrium dialysis method when binding protein alteration is suspected; application of rigorously-derived reference range to interpret total and free testosterone levels; and recognizing the influence of assay imprecision and inaccuracy in making treatment decisions especially when the testosterone levels are within two standard deviations of the diagnostic thresholds.

Current evidence does not support population level screening of men for testosterone deficiency. The symptoms of testosterone deficiency are often nonspecific and overlap with aging-related symptoms.

Sexual symptoms, such as low libido, loss of morning erections, and erectile dysfunction, are more robustly associated with low testosterone levels than physical and psychological symptoms .[3] The Endocrine Society's expert panel suggests evaluation for testosterone deficiency in men presenting with conditions that are associated with increased risk of testosterone deficiency, such as men presenting with low sexual desire, erectile dysfunction; infertility; gynecomastia; HIV-associated weight loss; osteoporosis or low trauma fracture; men using opioids, glucocorticoids, and androgenic-anabolic steroids; and men treated with cancer chemotherapeutic agents or pelvic radiation . [1]

THE TIMING AND FREQUENCY OF BLOOD SAMPLING FOR TESTOSTERONE LEVELS

Circulating testosterone levels vary over time due to its pulsatile secretion, and its circadian and circannual rhythms. Serum testosterone levels are higher in the morning than in the late afternoon. Testosterone levels also decline after a meal, especially after a glucose load. [4] Therefore, serum testosterone levels should be measured in the morning after an overnight fast on two or more separate days. Testosterone concentrations are suppressed during an acute illness and, therefore, diagnostic evaluation of testosterone deficiency should be avoided during an illness.

THE CONCEPT OF TOTAL, FREE, AND BIOAVAILABLE TESTOSTERONE CONCENTRATIONS

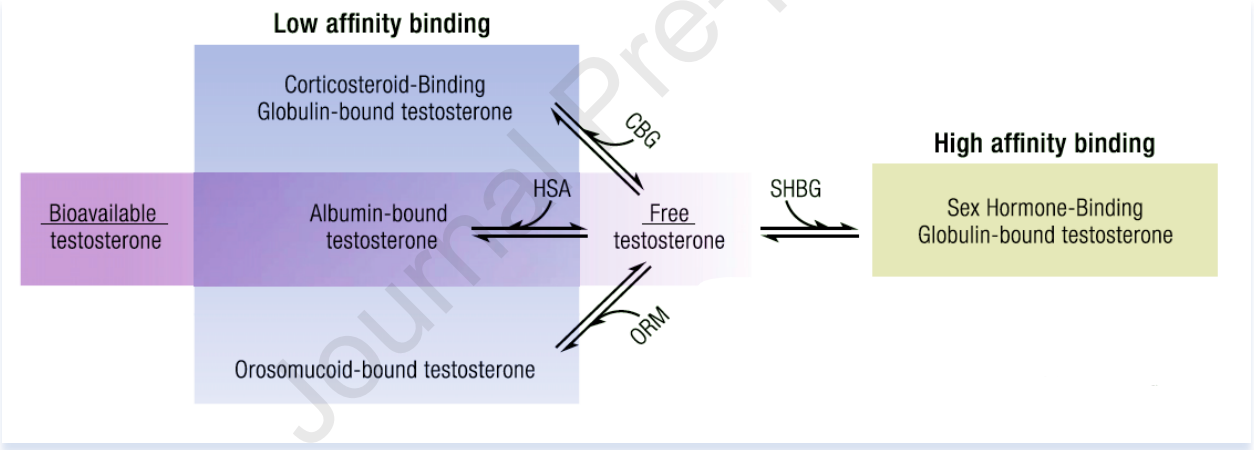
Testosterone and other steroid hormones are bound to plasma proteins that affect their circulating concentrations, distribution, metabolism, and tissue bioavailability (Figure 1). Circulating testosterone binds predominantly to sex hormone binding globulin (SHBG) and to human serum albumin (HSA) and, to a lesser extent, to corticosteroid-binding globulin and

orosomucoid; only 2% to 4% of circulating testosterone is unbound or free.[5,6] SHBG circulates as a homodimer with a single binding site on each of the two monomers. Recent studies using modern biophysical techniques and molecular modeling have shown that the binding of testosterone as well as estradiol to SHBG is a complex, multi-step, dynamic process that involves inter-monomeric allostery such that the binding affinities, conformations, and energy states of the two monomers are not identical in unbound or even fully bound states.[7,8] The fraction of circulating testosterone bound to SHBG varies in men and women, with approximately 44% of testosterone bound to SHBG in men and 66% in women.[8] The binding of testosterone to human serum albumin also is far more complex than had been recognized previously; our recent studies show that there are multiple, allosterically-coupled binding sites for testosterone on human serum albumin.[9] Testosterone shares these binding sites on human serum albumin with free fatty acids and many commonly used drugs such as ibuprofen and coumadin, which could displace testosterone from its binding sites under various physiological states or disease conditions, affecting its bioavailability .[9] It is generally believed that testosterone can rapidly dissociate from one or more binding sites on human serum albumin and become "bioavailable" in some target organs, especially in target organs with long transit times such as the liver and the brain; this premise has not been fully substantiated. The characteristics of testosterone binding to CBG and orosomucoid remain incompletely understood.

According to the free hormone hypothesis (FHH), the free testosterone is the biologically active fraction as it can diffuse into the cell and bind to the androgen receptor.[10] Since testosterone tightly bound to SHBG is not directly available for diffusion into the tissues, in situations with altered serum SHBG, free T (FT) levels would be expected to reflect tissue exposure more closely than total testosterone levels.[5,11-13,17] The pituitary gonadotropin,

luteinizing hormone (LH), responds to changes in FT concentrations; an increase in SHBG would lead to a decrease in free testosterone and a resultant rise in LH would restore FT levels back to normal. It has been postulated that the HSA-bound testosterone is so loosely bound that it can dissociate in the tissue capillaries and essentially become "free."[14,15] There is also a question of whether SHBG-bound testosterone can be internalized into the cell mediated by the membrane protein, megalin.[16] The tertiary complex is delivered to the lysosomal compartments where the testosterone dissociates from the binding proteins.

Figure 1. A model of testosterone's partitioning between various binding proteins (Reproduced with permission from Goldman et al, 2017)[17]



The Endocrine Society Clinical Practice Guideline recommends using total testosterone as well as free testosterone to diagnose hypogonadism in conditions in which alterations in sex hormone- binding globulin (SHBG) concentrations are suspected, or when total testosterone is borderline.[18] In men who have conditions that alter SHBG (Table 2) or whose initial total testosterone concentrations are at or near the lower limit of the normal range, clinicians should determine FT concentrations either directly from equilibrium dialysis assays or by calculations that use total testosterone, SHBG, and albumin concentrations. The conditions that are associated

with high or low SHBG levels are listed in Table 1 [19, 20, 21,22, 23, 24,25, 26,27, 28,29, 30,3132 33 34 35,36,37]. Obesity, diabetes mellitus, and metabolic syndrome are some of common conditions associated with low SHBG levels. Polymorphisms in the SHBG gene can cause elevated or decreased SHBG levels. Increased SHBG levels are associated with the variants rs6258 and rs12150660 and decreased SHBG levels are associated with the variants rs6257, rs6259, rs727428, rs1799941.[38-40] Some of the SHBG polymorphisms can also affect its binding to testosterone while others can affect its clearance and dimerization. SHBG levels increase with advancing age; therefore, the rate of age-related decline in FT levels us greater than that in total testosterone levels. Because SHBG levels are affected by numerous conditions and some of these conditions are highly prevalent in the general population, SHBG levels should be considered in interpreting total testosterone levels.

Table 1. Conditions associated with alterations in SHBG concentrations (Adapted with permission from Bhasin et al., 2017)[18]

Conditions associated with decreased SHBG concentrations	Conditions associated with increased SHBG concentrations
Moderate obesity	Weight loss
Nephrotic syndrome	Aging
Hypothyroidism	Hepatic cirrhosis and hepatitis
Use of glucocorticoids	Hyperthyroidism
Use of progestins	Use of anticonvulsants
Use of androgenic steroids	Use of estrogens
Acromegaly	HIV disease
Diabetes mellitus	Alcohol consumption
Polymorphisms in the SHBG gene: e.g., rs6257, rs6259, rs727428, rs1799941)	Polymorphisms in the SHBG gene (e.g., rs6258 and rs12150660)

Total testosterone is the sum of the concentrations of protein-bound and unbound or free testosterone in circulation. Only the unbound fraction of testosterone can enter the cell and exert its biological effects.[41,42] The term "bioavailable testosterone" refers to the sum of free testosterone plus albumin-bound testosterone and is based on the idea that testosterone bound to human serum albumin can dissociate rapidly in the tissue capillaries, especially in organs with long transit times, such as the liver and brain.

METHODS FOR THE MEASUREMENT OF TOTAL TESTOSTERONE IN HUMAN SERUM OR PLASMA

The methods of measuring serum total testosterone concentrations are antibody-based immunoassays, such as radioimmunoassays, enzyme-linked immunosorbent assays, immunofluorometric or immunochemiluminescent assays, aptamer-based assays, or mass spectrometry-based assays (**Table 2**). The immunoassays offer the benefits of wide availability, low cost, and rapid turnaround. However, comparison of serum total testosterone levels measured using commonly used automated and manual immunoassays with those obtained by liquid chromatography–tandem mass spectrometry (LC-MS/MS) show that immunoassays exhibit high imprecision and inaccuracy and significant bias relative to an LC-MS/MS method in the low range typical in hypogonadal men, women and children although their bias relative to LC-MS/MS is substantially lower in the normal male range.[43]

The LC-MS/MS assay starts with extraction of the serum or plasma using an organic solvent and followed by separation of compounds based on their polarity by high-pressure liquid chromatography.[44,45] The eluted compounds are transferred to the mass spectrometer, where they are separated based on their mass and charge. Gas chromatography–mass spectrometry provides even greater specificity than LC-MS/MS, but the latter offers higher throughput. The LC-MS/MS

assays have emerged as the method of choice with the highest specificity and sensitivity for the measurement of total testosterone[46-48] and have become widely available from many commercial laboratories.

Benchmarking of assays against national or international accuracy standards can improve the accuracy of the assays; many countries now have established such benchmarks. For instance, in the United States, the Centers for Disease Control and Prevention has established a Hormone Standardization Program for Testosterone (HoST) that performs a comparison of the methods and estimates the bias against a higher order benchmark. The HoST evaluation is based on assay accuracy and is available to laboratories around the world. The testosterone values reported by HoST-certified laboratories are very similar and the harmonized reference ranges for testosterone reported by Travison et al [49]can be used for results reported by any of the HoST-certified laboratories in any part of the world.

Table 2. Methods of measuring total and free testosterone

	Methods of measurement	Recommended method of choice	Harmonized Reference Ranges using method of choice[6,49]
Total testosterone	• Radioimmunoassays, enzyme-linked immunosorbent assay • Immunofluorometric or immunochemiluminescent assay • Aptamer-based assay • Mass spectrometry-based assays	Mass spectrometry-based assay	264-916 ng/dL (9.2 to 31.8 nmol/L)
Free testosterone	• Ultracentrifugation • Free androgen index • Analogue immunoassay • Salivary testosterone • Equilibrium dialysis	Measured using equilibrium dialysis or calculated using an algorithm that incorporates non-	Harmonized reference range is not available.

	• Calculated free testosterone	linear binding and allostery	

In summary, testosterone levels should be measured on two or more days in early morning hours in a fasting state, using a reliable assay, preferably a liquid chromatography tandem mass spectrometry-based (LC-MS/MS) assay in a laboratory that is certified by an accuracy-based benchmark, such as the CDC HoST Program.

REFERENCE RANGE FOR TOTAL TESTOSTERONE LEVELS

Reference range refers to the distribution of a hormone or analyte in the general population and rigorously derived reference ranges are essential for distinguishing normal from low and high levels of a hormone or analyte. To generate harmonized reference ranges for total testosterone in men that can be applied across laboratories, we measured serum total testosterone levels using LC-MS/MS assays in 9,054 community-dwelling men in four cohort studies in the United States and Europe: Framingham Heart Study; European Male Aging Study; Osteoporotic Fractures in Men Study; and Male Sibling Study of Osteoporosis .[49] Testosterone concentrations in 100 participants in each cohort were measured using a higher order reference method at the Centers for Disease Control and Prevention (CDC). Normalizing equations, generated using Passing-Bablok regression, were used to generate harmonized values. Harmonized normal range in a healthy nonobese population of European and American men, 19 to 39 years, is 264 to 916 ng/dL (9.2 to 31.8 nmol/L. This reference range can be applied to all assays certified by the HoST . [49]

The cut points defining the lower and upper limits of the normal range should not be viewed as absolute because of the imprecision of the assay. As an example, if the true value of circulating testosterone concentration were 260 ng/dL (9.0 nmol/L) using an assay that has an inter-assay coefficient of variation of 10%, the measured value has 95% probability of being

reported within 2 standard deviations of this true value, i.e. between 208 ng/dL (7.2 nmol/L) and 312 ng/dL (10.8 nmol/L). Thus, there is substantial risk of misclassification when the testosterone levels are slightly above or slightly below the cut point. Multiple low values reduce the likelihood of misdiagnosis but do not completely eliminate it. The consideration of additional clinical data, such as free testosterone levels, LH and FSH levels, testicular volume, and secondary sex characteristics can further aid in reducing the diagnostic error.

METHODS FOR THE MEASUREMENT OF FREE TESTOSTERONE IN HUMAN SERUM OR PLASMA

Free testosterone level should be measured when SHBG binding protein abnormality is suspected or when the total testosterone levels are at or near the lower limit of the normal range for men. [1] Free testosterone concentrations can be measured either directly using one of several available methods or estimated from total testosterone, SHBG, and albumin concentrations. The methods for the direct measurement of free testosterone include equilibrium dialysis,[50,51] centrifugal ultrafiltration,[52 53] steady-state gel filtration,[54] flow dialysis,[55] and direct tracer analog immunoassays.[56] Free testosterone concentration should be measured directly, preferably using an equilibrium dialysis assay in a reliable laboratory.[6] Lack of standardization of the equilibrium dialysis method has impeded efforts to generate harmonized reference ranges for free testosterone levels.[6]

If equilibrium dialysis assay is not available, free testosterone concentration should be estimated using an equation that provides a close approximation of values derived using the equilibrium dialysis method. [57] Free androgen index does not provide an accurate or a rational

estimate of free testosterone concentration and its use is not recommended. Tracer analog methods have been shown to be inaccurate and should be avoided.

Bioavailable testosterone can be measured directly using the ammonium sulfate precipitation method that precipitates SHBG-bound testosterone or can be calculated from total testosterone, SHBG, and albumin. The high level of imprecision of ammonium sulfate precipitation method and the lack of a rigorously derived reference range, limits its utility in clinical practice.

COMPLEXITIES OF CALCULATING VERSUS MEASURING FREE TESTOSTERONE (FT)

Due to the methodological complexity of FT measurements by the equilibrium dialysis method, the Endocrine Society has suggested the use of calculating FT (cFT) from the TT, SHBG, and albumin levels as an acceptable approach.[18] Two categories of algorithms to calculate FT from the TT, SHBG, and albumin levels have been published; the linear equations that are based on assumption of a simple linear model of testosterone's binding to SHBG and human serum albumin with a single fixed Kd, and those that are empirically derived from regression analyses of the relation between free testosterone and total testosterone and SHBG levles. The linear equations for FT estimation are based on the assumption that each SHBG dimer binds two testosterone molecules and that the two binding sites on SHBG have similar binding affinity; these assumptions are not supported by our current understanding of the dynamics of testosterone's binding to SHBG. cFT values obtained using a multi-step, allosteric ensemble model is much closer to those obtained using equilibrium dialysis.[7,49]

The lack of standardization of the equilibrium dialysis method among laboratories has been a barrier to the generation of a harmonized reference range for free testosterone levels; until

such rigorously-derived harmonized references ranges become available, the clinicians currently must rely on reference ranges provided by a laboratory[6] or those published from the analyses of large epidemiologic studies.[7]

IMPORTANT POINTS FOR CLINICAL CARE

- In men with signs and symptoms of testosterone deficiency, measure total testosterone levels on two or more occasions in the early morning and in a fasting state using an LC-MS/MS assay, if available, in a laboratory that is certified by an accuracy-based benchmark, such as the CDC HoST Program.
- Avoid measuring testosterone levels during an acute illness.
- Measure free testosterone concentration when a binding protein abnormality is suspected or when the total testosterone levels are at or near the lower limit of the normal range for men. Use an equilibrium dialysis method for the direct measurement of free testosterone level in a reliable laboratory.

RESEARCH AGENDA

- The availability of high-quality testosterone assays, especially LC-MS/MA assays, has remained constrained in most countries outside the US. Increasing access to high-quality, affordable assays; harmonization of assays across laboratories; and generation of reference ranges that can be applied to people of various racial and ethnic groups in different regions of the world is necessary to improve quality of care and health outcomes for men across the globe.

- The dynamics of the binding of testosterone to SHBG, human serum albumin, orosomucoid, and corticosteroid-binding globulin and the roles of these binding proteins in regulating testosterone's bioavailability to the tissues remain incompletely understood and need further investigation.
- The lack of standardization of the equilibrium dialysis method for the measurement of free testosterone has been a barrier to the generation of rigorously derived harmonized reference ranges for free testosterone levels in men. The reference ranges for free testosterone levels measured using a standardized equilibrium dialysis method that can be applied across laboratories is an unmet need.
- The risk of misdiagnosis of testosterone deficiency is high when testosterone levels are close to the lower limit of the normal range. Validation of additional biomarkers of testosterone deficiency is needed to improve the diagnostic accuracy especially in men with testosterone levels that are only slightly below or slightly above the lower limit of the normal range

CONCLUSION

Accurate measurement of total and free testosterone concentrations is central to the accurate diagnosis of hypogonadism and other androgen disorders. Only a small proportion of men receiving testosterone therapy undergo appropriate evaluation and monitoring, which may lead to suboptimal outcomes.[58] Testosterone levels should be measured preferably in a CDC-certified laboratory using validated assays; in cases of equivocal TT concentration and/or abnormal SHBG levels, free testosterone levels should be measured using equilibrium dialysis or

calculated using an equation based on our current understanding of the dynamics of testosterone binding, such as the ensemble allostery model.

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