Controversies in Testosterone Deficiency

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Chapter 1
What to Measure: Testosterone or Free Testosterone?

Christina Wang and Ronald Swerdloff

1.1 Why Serum Testosterone Is Necessary for the Diagnosis of Testosterone Deficiency?

Low serum testosterone (T) levels in adult men can lead to significant clinical symptoms and signs of testosterone deficiency (TD). These include sexual dysfunction, low energy and vitality, mood changes, sleep disturbances, loss of body hair, loss of muscle and bone mass, increased visceral fat, mild anemia, and, in more severe testosterone deficiency, small testes and infertility. In adolescent boys, TD presents as delayed sexual development, absence of axillary, pubic and facial hair, failure of the testes to increase in size, gynecomastia, and eunuchoidal proportions. The symptoms of testosterone deficiency are non-specific, and physical signs may not be clinically detectable in some older men with TD.

In addition, screening questionnaires to assess the symptoms of TD may be useful in large clinical studies; however, they have low specificity and are generally not useful in a clinic to diagnose an individual with TD [1–6]. Even with the use of screening questionnaire and symptoms of TD, the confirmation of TD must be based on the precise and accurate measurement of serum T concentrations [7, 8]. Because serum T can be transiently suppressed during acute illnesses, physical and mental stress, and intensive exercise, T should not be measured when these

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Table 1.1 Common conditions that may affect serum testosterone concentration

Acute	Chronic
Acute medical illness	Aging
Emotional stress	Obesity
Intense physical activity	Metabolic syndrome
Medications	Diabetes
Acute sleep disorders	Chronic medical illnesses
	Eating disorders
	Obstructive sleep apnea
	Medications (opiates and glucocorticoids)

conditions are present. More importantly, T concentrations may decrease with obesity, age, metabolic syndrome and diabetes, sleep disorders, eating disorders, chronic kidney and liver disease, and chronic use of opiates and glucocorticoids (Table 1.1) [9–14]. Thus, it is important to ascertain the general health of the patient as treatment of some of these conditions may restore serum T to the reference range.

1.2 What Methods Should Be Used for Serum Testosterone Measurements?

Immunoassays are used for measuring serum testosterone concentrations. Several publications in the early 2000s demonstrated that immunoassays used in many clinical laboratories with automated platforms depend on reagents and reference ranges provided by the manufacturers. Some of these automated platform assays provide results that are precise but not accurate with significant bias when compared to the "gold standard" of steroid assays – liquid chromatography-tandem mass spectrometry (LC-MS/MS) [15–17]. These tests had passing scores in proficiency testing when compared to the same method but differed significantly when compared against each other. The T measurements at low concentrations, e.g., in women and children, were most unreliable, which led the Endocrine Society to issue a position statement that emphasized the following : (1) mass spectrometry has better accuracy and specificity and may be the method of choice for measuring testosterone concentrations at low levels; (2) the clinician should know the method and the reference ranges of their laboratories [18]; and (3) standardization programs focusing on accuracy should be implemented and updated reference ranges be established for men, women, and children [18–20]. The Center for Disease Control and Prevention (CDC) Laboratory Science Division took up this challenge and established the Hormone Standardization Program with T measurement as the first hormone to have harmonized assays [21–23]. The goal of the program is not to define which method a laboratory should use but to create "testosterone measurements traceable to one accuracy basis allowing measurements to be comparable across methods, time and location" [22]. To date, not only reference and research laboratories participate in the program but also manufacturers of automated platforms and mass spectrometers. The CDC also assisted the College of American Pathology (CAP) to

Table 1.2 Accuracy-based testing of serum testosterone (ng/dL) from the College of American Pathologist 2020

	No. labs	Mean	SD	CV%	Median	Low	High
ABS-06 Method							
All	*68*	*494*	*58*	*11.8*	*499*	*356*	*628*
Abbott Architect	13	537	27	5.1	531	501	574
Beckman Unicel Dxl	7				426	382	446
Mass spectrometry	20	484	35	7.2	483	423	578
Roche Cobas e600	6				500	487	514
Roche Cobas e801	7				508	496	524
Siemens Advia Centaur XP/XPT	4				549	481	628
CDC reference method		460					
ABS-05 method							
All	*64*	*21.8*	*5.4*	*24.6*	*22*	*8*	*37*
Abbott Architect	13	25.3	2.5	10.0	25	20	29
Beckman Unicel Dxl	7				25	17	37
Mass spectrometry	20	21.9	2.8	12.7	22	12	26
Roche Cobas e600	6				16	13	17
Roche Cobas e801	7				15	12	17
Siemens Advia Centaur XP/XPT	4				25	24	32
CDC reference method		22.7					

introduce accuracy-based proficiency test for T assays with the participation of many clinical laboratories [24]. The results of the accuracy-based harmonization of T assays showed that with time (3.5 year) there was improvement in proficiency scores of both accuracy and precision in 65 participating clinical laboratories in State of New York [25]. The conclusion is that most clinical laboratories using automated platforms are aware that accuracy-based proficient testing should be implemented with improvement in the quality of the assay. The accuracy-based tests in recent CAP report showed very small difference within a method and between methods (Table 1.2). For the diagnosis and monitoring of T-replacement therapy in TD men, both immunoassay and LC-MS/MS methods are sufficient as the goal is to distinguish low concentrations from the population reference ranges of adult men. As shown in Table 1.2, with participation in accuracy-based external quality control, even low levels of testosterone showed good agreement within a method and between methods.

1.3 What Is the Reference Range for Serum Total Testosterone in Adult Men?

The generally accepted range of serum testosterone in adult men is 300–1000 ng/dL (10.4–34.7 nmol/L) based on immunoassays with a purification step before immunoassays [26]. There are many possible physiological explanations why men would

have such a large range of testosterone levels. Once a standardized method has been validated [22], reference ranges can be established based on cross calibrating assays to a reference method. Using data from four population-based studies in Europe and the United States (1656 younger community-dwelling men in the Framingham Heart Study; European Male Aging Study; Osteoporotic Fractures in Men Study; and Male Sibling Study of Osteoporosis), the harmonized serum testosterone reference range (2.5th to 97.5th percentile) for non-obese men between 19 and 39 years was established to be between 264 and 916 ng/dL (9.16 to 31.8 nmol/L) [27]. Most of these men identified themselves as white and it should be noted that geographical and ethnic/racial differences in sex hormone levels have been reported in some studies [28–30]. Population-based studies of harmonized T levels in other ethnic/racial group have not yet been reported.

1.4 What Are the Precautions to Reduce the Variability in Serum Testosterone Concentrations?

Serum T concentrations should be measured from a blood sample collected early in the morning between 7 am and 10 am. The diurnal variation of serum T results in T levels higher in the early morning than later in the day in both young and middle-aged men [31]. This diurnal variation is attenuated in older men [32]. Most laboratories establish reference ranges for healthy adult men based on morning samples. Thus, to compare a patient with possible TD, blood samples should be drawn in the morning between 7 am and 10 am. There are data showing that acute ingestion of glucose lowers serum total testosterone by 25% [33]. Thus, for diagnosis of TD, it is important to obtain a fasting morning blood sample. In general, serum samples should be used for assays. Because T concentrations are affected by acute illnesses, mental and physical stress, and medications, a repeat morning T concentration provides confirmatory evidence that the low T level is probably persistent. It should be noted that after oral testosterone undecanoate administration, the high levels of testosterone undecanoate in blood can be cleaved by non-specific esterases ex vivo, so a blood collection tube with inhibitors of non-specific esterases (plasma) [34] should be used or more conveniently a conversion factor (serum T = plasma T/1.2) be applied to calculate serum T concentrations [35].

Accurate and precise measurement of serum testosterone is adequate for the diagnosis and monitoring of testosterone replacement for most men with TD. Serum testosterone measurement should be obtained in the morning, preferably in fasting state, and a repeat sample for confirmation is advisable. The sample should be sent to a reliable laboratory that practices accuracy-based proficiency tests or external quality control programs and quotes a reference range of serum testosterone levels of adult men between 250 and 1000 ng/dL (8.7 to 34.7 nmol/L).

1.5 What Is Free Testosterone?

In men, T and estradiol circulate bound tightly to the sex hormone binding globulin (SHBG) and loosely to albumin, and they also circulate in free or unbound form. This led to the free hormone hypothesis postulating that free and the loosely bound testosterone or estradiol can freely diffuse out to the capillaries into the cells of the target tissues to initiate appropriate responses to these sex hormones [36]. SHBG is a glycoprotein synthesized in the liver with high-affinity binding sites for T and estradiol [37, 38]. In men, about 50–55% of testosterone is bound to SHBG and the rest to albumin (45–48%), and the fraction of free testosterone is <2%. SHBG concentrations are affected by a number of factors (Table 1.3) [39]. Androgens (testosterone and its esters) and androgenic progestins (levonorgestrel, desogestrel, and progestins commonly used in female contraceptive pills) decrease SHBG levels. SHBG concentration is a good biomarker for androgen activity in the body. TD men have higher SHBG concentrations which decrease with androgen treatment. Since total T concentration is a measure of both bound and free testosterone, conditions where SHBG may be increased or decreased will affect the total T concentration. It is under these conditions that measurement of free T concentrations may be warranted (Table 1.3) [39]. For example, in an older overweight man with some symptoms of TD, aging increases and obesity decreases SHBG; in such circumstances, thus, to assess whether the older man has TD, measurement of serum free T may be indicated. Free T measurements may also be helpful in symptomatic men with borderline low T concentrations, e.g., between 250 and 350 ng/dL (8.7–12.2 nmol/L) [7]. The binding of T to SHBG is complex, which results in many different methods that directly measure or calculate free T. Some of these methods do not measure free fraction of T and some formulae may provide less accurate results [40].

Table 1.3 Factors that affect SHBG concentrations

Increase SHBG	Decrease SHBG
Estrogens	Androgens
Male hypogonadism	Androgenic progestins
Thyroid hormone, hyperthyroidism	Hypothyroidism
Pregnancy	Obesity, metabolic syndrome, type 2 diabetes
Aging	Hyperprolactinemia
Anticonvulsants	Acromegaly
Rifampin	Nephrotic syndrome
Alcoholic liver disease	End-stage liver disease

1.6 Are There Reliable Methods to Estimate Free Testosterone Concentrations?

The different methods of estimating free testosterone are shown in Table 1.4.

1.6.1 Free Testosterone by Equilibrium Dialysis

Free T can be measured by equilibrium dialysis where test serum or plasma is mixed with isotope-labeled T and dialyzed overnight into buffer solution. The free non-protein-bound fraction of the labeled T outside of the dialysis cell is the active "free fraction" of T. The free fraction (percent free T, usually between 1 to 2%) is multiplied by the total T measured by a reliable method to provide the free T concentration [41, 42]. This gold standard method of measuring free T by equilibrium dialysis is not automated, is cumbersome, and requires technical skill. Equilibrium dialysis methods are too complex for use in routine clinical chemistry laboratories. The methods are not harmonized and thus there are no common reference intervals to help clinicians to interpret free T concentrations [44]. Using this method, it has been found that concentrations of free T are normal even when the total testosterone is low in clinical conditions such as obesity [43] and other circumstances where SHBG is abnormal. Ultrafiltration methods are simpler but are sensitive to temperature changes, and difficult to standardize with reproducible data, and, therefore, are not commonly utilized today [45]. Recent studies

Table 1.4 Methods to measure serum free testosterone

Method	Advantages	Potential problems
Equilibrium dialysis	Gold standard to measure free testosterone	Not automated, required technical skills Require accurate testosterone assay Higher variability
Bioavailable testosterone	Technically easier	Not automated Require accurate testosterone assay
Salivary testosterone	Technically easy Does not require a blood draw	Collection issues may influence results
Calculated free testosterone	Can be automated Technically easy	Require accurate testosterone and SHBG assays Different formulae. Different algorithms may be better for different clinical questions
Analog free testosterone	Can be automated Technically easy	Does not measure free T Values are 1/5 of that from equilibrium dialysis Provides no additional information than total testosterone Recommend not to be used

indicate that the method has better consistency in some laboratories, and their usefulness as a modified methodology will need further validation across laboratories [46, 47].

1.6.2 Bioavailable Testosterone

The concept of bioavailable T is based on the theory that the free fraction and the loosely albumin-bound fraction of circulating T are available to the target organs and tissues [48]. Thus, not only the free but also the albumin-bound T is biologically active in tissues. Bioavailable T – the sum of albumin-bound and free testosterone – is generally measured in the serum after adding saturated ammonium sulfate solution to precipitate the SHBG-bound fraction. The separation of SHBG-bound testosterone can also be done by adding concanavalin A [49]. These methods are not automated and standardized and thus not generally available in routine laboratories but are available in reference laboratories [44]. These methods require accurate and precise measurment of T in the non-SHBG bound fraction after ammonium sulfate precipitation or ultracentrigfugation. Measurement of bioavailable T may be useful in men whose SHBG binding or concentration may be abnormal such as in older [50] and obese men [50].In most men with TD, measurement of bioavailable T is not usually required for diagnosis [51].

1.6.3 Salivary Testosterone

Because saliva does not contain SHBG, salivary T has been used as a surrogate for serum-free T [52, 53]. Salivary T can be measured by immunoassays or LC-MS/MS [54]. The concentration of T in the saliva may be influenced by the flow and also presence of blood in the sample. For these reasons and non-familiarity of use of saliva as a matrix, this simple method is not commonly used for free T determinations [55]; exceptions include the use of salivary T in studies on athletes and prepubertal children and infants where a blood draw can be avoided [56–58].

1.6.4 Calculated Free and Bioavailable Testosterone

Because of the technical difficulty, lack of automation, and absence of reference intervals for free T measured by equilibrium dialysis and bioavailable testosterone measured by ammonium sulfate precipitation methodologies (described above), these methods may not be suitable nor available in most routine clinical chemistry laboratories. This creates a widely utilized niche for calculated free T determinations. Calculated free and bioavailable T can be determined by using accurate and

precise assays of T and SHBG [50, 59, 60]. Using different equations/algorithms, both free and bioavailable T may be calculated [59, 60], which have been recommended as suitable for routine clinical use [18, 44, 59]. The most commonly used equations based on the law of mass action [41, 60–62] are used to calculate both the free and bioavailable T. Other investigators have recommended the use of empirically derived equations that fitted the clinical populations studied [63, 64]; but these empirical equations are not widely used. The calculated free T using law of mass action formula overestimates free T measured by equilibrium dialysis. Empirical formulae are free from assumptions and may be more concordant with the measured free T [63]. Recent evidence suggests that the law of mass action formula which is based on the assumption that two T molecules bind to two binding sites on the SHBG with similar binding affinity may be incorrect. And further argues that the binding of T to SHBG may be a multistep, dynamic process with complex allosteric characteristics [65]. Based on this new model, investigators used a new formula to calculate free T in younger men in the Framingham Heart study and showed that the newly calculated values were similar to those measured by equilibrium dialysis. They further verified that the calculated free T values had clinical diagnostic validity using data from the European Male Aging Study. They demonstrated that men with calculated free T below the reference range had higher risk of sexual symptoms and elevated LH suggestive of primary TD. While enticing, the use of this new formula must be further validated by other laboratories. The Endocrine Society suggests that calculated free T may be the most practical method to measure free T using accuracy and precise total T and SHBG measurements [7, 18]. Currently the CDC is developing a harmonized method for free T based on calculated free T using revised formulae. This may bring the measurement of free T to a referable standard in clinical laboratories and common reference intervals that all clinicians can use.

1.6.5 Analog-Based Immunoassays and Why They Are Not Recommended by Most Experts in the Field

Analog-based free T assaysare offered by most clinical laboratories, are measured on automated platforms, and are widely used by clinicians. However, many studies have shown that free T concentrations measured by analog-based immunoassay are about one-fifth the concentrations measured by equilibrium dialysis and are related to SHBG [59, 66] or total T concentration [67] and do not reflect free T concentrations in clinical conditions such as hirsutism and hyperthyroidism [18, 68]. Experiments using varying concentrations of SHBG and T showed that analog-based free T immunoassay reported free T results that were related primarily to total T and concluded that free T analog assays do not detect serum free T [67]. Some argue that free T concentrations measured by immunoassays are as good as calculated free T but failed to note that the free T values obtained by immunoassays are