During the last few years, there has been a dramatic surge in interest in testosterone testing of adults. Many clinicians are asking the laboratory to measure testosterone levels for the diagnosis of androgen excess states in women and in the identification and treatment of androgen deficiency in men. There are multiple methods available for assessing testosterone status beyond the simple measurement of total testosterone. Testosterone in the circulation is distributed in free, weakly-bound, and tightly-bound forms.

A number of analytical methods have been developed for measuring these pools of testosterone. Free testosterone can be determined by equilibrium dialysis, equilibrium ultrafiltration, and analogue immunoassay methods. The concentration of physiologically active testosterone can be also be estimated by calculation of free androgen index or by the measurement of bioavailable or salivary testosterone. With all of these choices, it is easy to understand why many clinicians and laboratorians are confused as to the most appropriate method for assessing testosterone status.

**Testosterone Physiology**

Testosterone is the principal androgen in men. The production of testosterone by the male testes is stimulated by luteinizing hormone (LH), which is produced by the pituitary. LH is secreted in a pulsatile manner in response to stimulation by gonadotropin hormone releasing hormone (GHRH), generated by the hypothalamus. LH secretion is, in turn, inhibited through a negative feedback loop by increased concentrations of testosterone and its metabolites.

Most of the testosterone in males is produced by the Leydig cells of the testes and is secreted into the seminiferous tubule, where it is complexed to a protein made by the Sertoli cells. This process results in the high local levels of testosterone that are required for normal sperm production. In tissues with high levels of the enzyme 5α-reductase, testosterone is converted to dihydrotestosterone (DHT), a significantly more potent androgen. The effects of androgen on prostate, scalp, and beard are primarily mediated by DHT. Together, testosterone and DHT play a key role in the development of the male genital tract and male secondary sex characteristics. Much smaller amounts of testosterone and dihydrotestosterone are produced in women than in men. Weaker adrenal androgens and ovarian precursor molecules including androstenedione, DHEA, and DHEA sulfate can have significant androgenic effects in women. The ovary and adrenal glands produce some testosterone, but the majority of the testosterone in women is derived from the peripheral conversion of other steroids.

In males, testosterone promotes normal growth and development of the sex glands and organs. Testosterone stimulates the muscle and laryngeal growth that are responsible for the physical maturation and change in voice that occurs as boys go through puberty. An increase in testosterone during puberty (and in some androgen excess states) may result in the development of acne. DHT produced in the hair follicle stimulates the growth and maintenance of axillary and pubic hair. Excessive DHT has been implicated in the loss of scalp hair that is referred to as male pattern balding or androgenic alopecia. Testosterone increases red cell production and has important behavioral effects, increasing libido, competitiveness, and aggression.

Testosterone levels change dramatically during the life cycle of males. At approximately 12 weeks after conception, testosterone concentrations rise in the male fetus due to stimulation of the developing testes by human chorionic gonadotropin (hCG). Testosterone levels then fall to low levels by the third trimester of pregnancy. Testosterone levels start increasing again in the male neonate after about three weeks of life, nearly reaching adult levels by the age of two months. Levels then gradually fall to less than 0.3 ng/ml by six months and remain at low levels until puberty. In females, testosterone levels remain low from conception until puberty.

Testosterone levels in females increase to adult levels during puberty, but never come close to the levels of adult males. Testosterone levels in males rise during puberty to the lifetime maximum levels achieved in young adulthood. As many men and women age, their testosterone levels gradually decrease to levels that are less than 50% of the maximal levels achieved during young adulthood.

Testosterone and DHT, like other steroid hormones, initiate their physiologic actions by forming complexes with specific cytoplasmic receptors within the cells of target tissues. The steroid-receptor complexes then enter the nucleus and cause changes in gene transcription and protein synthesis. Testosterone that is tightly bound to plasma proteins is not able to enter cells and produce androgenic effects. Only about 2% of the total testosterone in the plasma of men is free or nonprotein bound; about 1% in women. In most men and women, more than 50% of total circulating testosterone is bound to sex hormone-binding globulin (SHBG), and most of the rest is bound to albumin. SHBG-bound testosterone is not readily available for intracellular complex formation because of SHBG's high binding affinity for testosterone. Thus testosterone-bound SHBG is considered biologically inactive. Albumin has a much lower binding affinity for testosterone but binds a significant portion of the total testosterone because albumin is present at much higher plasma concentrations than SHBG. The rapid dissociation of weakly-bound testosterone from albumin, together with a relatively long transit time of albumin through target tissue capillary beds, result in the availability of essentially all albumin-bound testosterone for steroid-receptor interaction. The sum of the free and albumin-bound testosterone is often referred to as bioavailable testosterone.
The rate of testosterone production and the concentration of SHBG affect the concentration of bioavailable testosterone. SHBG levels are sensitive to changes in estrogen and testosterone. Decreased serum testosterone levels stimulate the production of SHBG by the liver. Increased estrogen levels that occur in pregnancy or with estrogen replacement therapy also increase SHBG production. SHBG levels can also be increased in patients with hyperthyroidism and liver disease. Increased SHBG-bound testosterone and cortisol can result in total testosterone levels in the normal range, despite a clinical deficiency of bioavailable testosterone. Conversely, SHBG levels tend to be low in androgen excess states, often resulting in total testosterone levels within normal limits and elevated bioavailable testosterone. Measurement of total testosterone alone can be misleading in many clinical situations.

**Testosterone Deficiency in Adults**

Diminished testosterone production is one of many potential causes of infertility in young adult men. Low testosterone concentrations in these men can be caused by testicular failure (primary hypogonadism) or inadequate stimulation by pituitary gonadotropins (secondary hypogonadism). Measurement of testosterone and gonadotropin levels can be useful in the differential diagnoses of these cases. Since men with hypogonadism often have high SHBG levels, the measurement of free or bioavailable testosterone has been advocated when total testosterone levels are normal in men with symptoms of androgen deficiency. The capacity of the testes to secrete testosterone can be assessed by injections of hCG or GHRH, two hormones that stimulate testosterone production in healthy males. In primary hypogonadism, injectable testosterone analogs can be used to maintain libido and male secondary sexual characteristics; however, diminished testosterone levels in the testes generally result in infertility. Administration of GHRH and/or hCG has been found effective in some patients with secondary hypogonadism.

Significant physiological changes occur in men as they age, in part due to a gradual decline in testosterone levels. It is generally accepted that the principal cause of this age-related decrease in testosterone production is testicular failure, although diminished gonadotropin production may play a role. By 75 years of age, the average male's testosterone level drops to 65% of that of the average level in young adults. Because serum levels of sex-hormone-binding globulin tend to increase with age, the reduction in bioavailable testosterone is even more dramatic. One in four men older than 75 years of age has a bioavailable testosterone level below the lower limit of normal in young adults. “Andropause” is a term that has been used to refer to the constellation of symptoms associated with the age-related decline in testosterone production in men. In contrast to the rapid decline in ovarian function in women at menopause, andropause consists of a more gradual decline of testicular function. While men can remain fertile until very old age, older men often develop symptoms that are similar to those observed in younger men with hypogonadism. These symptoms include loss of libido, erectile dysfunction, diminished muscular strength, fatigue, anemia, lethargy, and loss of motivation.

Although osteoporosis is less common in men than women, it is still a significant health problem in men as they age. The rate of osteoporotic hip fracture increases dramatically after the age of 60 years in men, and doubles with each decade thereafter. Testosterone deficiency associated with andropause is thought to be a significant cause of male osteoporosis.

Many clinicians feel that some men with marked symptoms of androgen deficiency may benefit from testosterone supplementation. A recent review in the American Journal of Medicine recommends that total testosterone measurement be used for initial screening of elderly men who present with signs and symptoms of hypogonadism. A level below 300 ng/dL was proposed as a cut-off for considering replacement therapy in symptomatic men. The authors go on to recommend that symptomatic men with values greater than 300 ng/dL be tested for free or bioavailable testosterone levels. Another recent article suggested that the lower limit of the reference range established for men between 20 and 40 years of age be used to diagnose testosterone deficiency in symptomatic men. Testosterone assessment is valuable in the diagnosis of hypogonadism in symptomatic men in large part because many of the symptoms can be caused by other organic causes that afflict men as they age. It is also important to note that while average testosterone levels decrease with age, there is a great deal of inter-individual variation. A quarter of men older than 75 years of age have total testosterone levels in the upper quartile of the reference interval for young men.

Side effects of testosterone supplementation have been shown to be minimal when plasma testosterone levels are kept within the physiological range. Clinical studies indicate that giving testosterone to a normal man with no prior evidence of prostate cancer will not increase his chances of developing prostate cancer in the future. This conclusion is supported by the fact that there is no correlation between plasma testosterone levels and the risk of developing prostatic cancer or benign prostatic hypertrophy in untreated men. Testosterone, however, should not be given to men who have been diagnosed with prostate cancer. Testosterone can markedly speed up the rate of cancer progression in men diagnosed with prostatic carcinoma. In fact, treatment protocols for prostate cancer often involve surgery or drugs meant to lower testosterone levels.

Testosterone supplementation has been considered for women with symptoms of androgen deficiency, especially during menopause. Women with low bioavailable testosterone relative to reference intervals established in women in their 20s and 30s have been found to suffer from symptoms similar to those observed in hypogonadal men. These symptoms include diminished libido and persistent fatigue. Testosterone supplementation has been shown to improve sexual function and psychological well being in women with decreased testosterone caused by surgical removal of their ovaries. Some clinicians feel that positive benefit may also be gained by increasing testosterone levels in women with other causes of low testosterone. Although
positive results have been reported when testosterone levels are increased from subnormal to normal levels, side effects such as increased facial oiliness, acne, hirsutism, and alopecia can occur when testosterone is increased to greater than normal physiologic levels.\textsuperscript{9,16}

**Testosterone Excess in Women**

Often, the first sign of androgen excess in women is the development of male pattern hair growth, which is referred to as hirsutism.\textsuperscript{1,6,18} It should be noted that some women experience hair growth similar to that caused by increased testosterone due to racial or genetic causes and not due to excessive androgens.\textsuperscript{6,7} Measurement of the testosterone may help to distinguish racial or genetic causes of hirsutism from the abnormal pathlogy, particularly in women with mixed ethnic backgrounds.\textsuperscript{1,6,7} Women with more excessive androgen levels may also experience virilization, with symptoms including increased muscle mass, redistribution of body fat, enlargement of the clitoris, deepening of the voice, acne, and increased perspiration.\textsuperscript{4} These women can also suffer from androgenic alopecia, the female equivalent of male pattern baldness.\textsuperscript{2}

Many women with slowly progressive androgenic symptoms are diagnosed as having polycystic ovary syndrome (PCOS).\textsuperscript{2,6,20} PCOS is relatively common, affecting approximately 6% of women of reproductive age.\textsuperscript{1} Women with this complex syndrome experience symptoms of androgen excess associated with menstrual abnormalities and infertility. Most women with the syndrome have polycystic ovaries that can be detected by ultrasonography, although this finding is not essential for diagnosis.\textsuperscript{2,6,18} Chronic anovulation experienced by patients with PCOS increases their risk of developing endometrial cancer. Women with PCOS are often overweight and are likely to suffer from insulin resistance, putting them at increased risk for developing type 2 diabetes mellitus.\textsuperscript{2,20} Obesity and insulin resistance can result in acanthosis nigricans, a skin condition that is characterized by hyperpigmented, velvety plaques of body folds.\textsuperscript{5} Lipid abnormalities, including decreased high-density lipoprotein cholesterol levels and elevated triglyceride levels as well as impaired fibrinolysis are seen in women with PCOS.\textsuperscript{20} Cardiovascular disease is more prevalent, and women with PCOS have a significantly increased risk for myocardial infarction.\textsuperscript{10}

The measurement of testosterone levels can be useful for the investigation of hirsutism and virilization in women.\textsuperscript{1,6,18} Many women with these conditions, however, have normal serum androgen levels, suggesting increased end organ sensitivity to androgens. Increased LH levels or an increase in the LH/FSH ratio have also been associated with PCOS.\textsuperscript{5} Measurement of other androgens is recommended to rule out adrenal hyperplasia or tumor as the cause of androgen excess.\textsuperscript{2} The early diagnosis of PCOS is very important because it allows the clinician to prescribe treatment to prevent many of the complications associated with the syndrome.\textsuperscript{2}

Advances in transvaginal ultrasonography and infertility treatments, including newer medications, have facilitated assisted reproduction in patients with PCOS.\textsuperscript{20} Ongoing pharmacological research focusing on the treatment of insulin resistance appears promising in reversing the long-term complications of the syndrome.\textsuperscript{20}

**Methods for Measuring Testosterone**

Many clinicians and laboratorians are confused about the most appropriate method for measuring testosterone status.\textsuperscript{4,5,22} There are numerous schools of thought as to which form of the hormone should be measured and which analytical method provides the most accurate assessment of biological activity. Since there is not a clear consensus on this issue, it is important, at least, to understand the analytical basis for the various methods available. Some of the different approaches currently used for measuring testosterone status include (1) total testosterone, (2) androgen index calculation, (3) Free testosterone by equilibrium dialysis or equilibrium ultrafiltration, (4) free testosterone by analog tracer immunoassay, (5) bioavailable testosterone, and (6) salivary testosterone. Each is described below.

- **Total Testosterone.** Most clinical laboratories performing total testosterone testing use automated methods based on immunoassay.\textsuperscript{4} In order to measure total testosterone these instruments must first displace bound testosterone from SHBG and albumin. This is achieved by a number of mechanisms, including the addition of low-pH buffers, surfactants, salicylates, or a competing steroid that does not bind to the anti-testosterone antibody used in the immunoassay. The testosterone antiserum used in commercial methods typically cross-react to some extent with other steroids, particularly DHT.

  Historically, solvent extraction and/or chromatography have been used to remove these interfering compounds prior to testosterone measurement. These purification techniques, however, cannot be readily incorporated into the methods on automated analyzers. Fortunately, plasma levels of DHT are only about one tenth of testosterone levels, and the cross-reactivity is typically less than 5%.\textsuperscript{4} In the great majority of cases, the interferences observed in commercial assays do not detract from the clinical utility of the results generated.\textsuperscript{4}

- **Androgen Index Calculation.** The concentration of testosterone in the various free and bound forms is essentially a function of total testosterone concentration and the relative concentrations of SHBG and albumin. It can be predicted that increased SHBG will decrease the concentration of both free and bioavailable testosterone for a given total testosterone concentration. Many clinicians use a calculated free androgen index to estimate physiologically active testosterone.\textsuperscript{5,6} This index is typically calculated as the ratio of total testosterone divided by SHBG and multiplied by 100 to yield numerical results comparable in free testosterone
concentration. Alternatively, more complicated mathematical algorithms can be used to estimate the percentage of free testosterone from the SHBG concentration alone or in combination with albumin concentration. The precision of these algorithms is subject to the combined errors of the individual tests performed but a number of authors have shown them to be useful in the assessment of testosterone status.

- **Free Testosterone by Equilibrium Dialysis or Equilibrium Ultrafiltration.** The measurement of free testosterone in serum is technically demanding. The concentration of free testosterone is very low, typically less than 2% of the total testosterone concentration. Routinely available assay methods are not sensitive enough to quantitate free testosterone directly. Instead, free testosterone is often estimated by indirect methods. In these methods, tritiated testosterone is added to the sample and allowed to come to equilibrium with testosterone in the serum at physiological temperature. The amount of the added radiolabeled testosterone must be low enough to ensure that the addition will not significantly increase the total testosterone concentration. Once equilibrium is achieved the free testosterone is separated from the bound by filtration through a membrane. This filtration can be accomplished by equilibrium dialysis or by centrifugal ultrafiltration. The radioactivity of the protein-free ultrafiltrate is measured and used to calculate the percentage of free testosterone. The concentration of free testosterone can then be calculated by multiplying the percentage of free testosterone by the total testosterone concentration. Measurement of free testosterone by these methods is not available in most clinical laboratories due to the complicated nature of the testing and the requirement of a scintillation counter to measure the tritiated testosterone concentration.

The results of equilibrium dialysis and centrifugal ultrafiltration methods have been shown to be quite comparable. While equilibrium dialysis is often considered to be the "gold standard," centrifugal ultrafiltration is somewhat simpler to perform and may theoretically be more accurate due to the fact that the equilibrated sample is not diluted with dialysis buffer.

- **Free Testosterone by Analog Tracer Immunoassay.** There are a number of commercial kits available for the direct estimation of free testosterone in serum. These kits use a labeled testosterone analogue that has a low binding affinity for both SHBG and albumin but is bound by antitestosterone antibody used in the assay. Since the analogue is unbound in the plasma, it competes with free testosterone for binding sites on an antitestosterone antibody that is immobilized on the surface of the well or assay tube. The first kits developed used a radiolabeled testosterone analogue to compete with free testosterone for binding sites on an antibody-coated polypropylene tube. More recently developed kits employ an enzyme-labeled analogue that can be measured after competitive binding to antitestosterone antibodies coated to microtiter wells. These analogue methods are technically less demanding than equilibrium dialysis or centrifugal ultrafiltration and require substantially less sample. The analogue methods also offer the advantage of direct estimation of free testosterone concentration without the need to measure total testosterone. The enzymatic methods can be readily performed by many laboratories because they are nonisotopic.

Most published studies comparing analogue free testosterone measurement to other methods of assessing free testosterone status have tested the radiolabeled analogue method simply because the enzymatic assays have only recently become available. These studies have produced inconsistent and sometimes contradictory results. Several authors indicated that the analogue method had good correlation with equilibrium dialysis but found that the analogue results were only about one-fourth as high. Another group found that the analogue method produced results directly comparable to equilibrium dialysis without multiplication by a factor. More recently, Winters and colleagues have found the analogue method to correlate better with total testosterone levels than with bioavailable testosterone determined by the ammonium sulfate precipitation method. They suggested that the analogue free testosterone results might be misleading in men with low SHBG concentration. Ooi suggested that the problems observed by Winters might, in large part, be resolved simply by using a more appropriate population-based reference interval. Vermeulin and colleagues found that the analogue free testosterone method correlated well with free testosterone by equilibrium dialysis but did not correspond with a free testosterone calculated from total testosterone and SHBG.

- **Bioavailable Testosterone.** Bioavailable testosterone is a term applied to the sum of circulating free testosterone and albumin-bound (weakly bound) testosterone. A commonly used method for determining bioavailable testosterone involves the selective precipitation of SHBG with ammonium sulfate. As in the free testosterone methods described above, tritiated testosterone is added to serum that is then allowed to come to equilibrium at physiologic temperature. Testosterone bound to SHBG is then selectively precipitated with 50% ammonium sulfate, leaving free and albumin-bound testosterone in solution. The percentage of tritiated label not bound to SHBG is multiplied by the total testosterone to produce the bioavailable testosterone. Alternatively,
the concentration of bioavailable testosterone can be measured directly by radioimmunoassay in the supernatant after solvent extraction.\textsuperscript{26}

Another approach to measuring bioavailable testosterone has been described.\textsuperscript{25,26} This technique involves saturating SHBG binding sites with DHT. SHBG has a significantly stronger affinity for DHT than for testosterone. Addition of excess DHT to the sample effectively forces all the SHBG-bound testosterone into solution. The non-protein-bound fraction is then measured after equilibrium dialysis\textsuperscript{25} or centrifugal ultrafiltration.\textsuperscript{26}

- **Salivary Testosterone.** Using salivary samples for the estimation of plasma-free testosterone levels is an attractive concept because of the ease of sample collection. In general, steroid levels in saliva are thought to reflect the free levels in the blood.\textsuperscript{26,33} Despite the fact that a number of laboratories offer salivary testosterone testing,\textsuperscript{33} this methodology has not gained widespread acceptance for routine clinical applications.\textsuperscript{33} Salivary testosterone levels are very low, especially in women. Currently available salivary testosterone methods have been effectively used in studies where ease of sample collection is a priority and the mean testosterone levels of large populations are compared; however, salivary testosterone methods have not been shown to be sensitive enough to produce diagnostically accurate results for the clinical assessment of individual patients, especially women.\textsuperscript{33} Salivary testosterone measurement may play a more significant role in the future as more sensitive techniques are developed and appropriately validated.\textsuperscript{34} Ultimately, the clinical utility of salivary testosterone measurement will depend on its analytical correlation with other, more established assays of testosterone status.

**Conclusion**

It is clear that effective methods for assessing testosterone status will continue to be required as clinicians expand their understanding of the role of androgens in the pathophysiology experienced by many adults. With the many options available, it is critical that both clinicians and laboratorians have a thorough understanding of the analytical basis for each of the methods used to assess testosterone status.

**References**


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