

Analytical and physiological factors affecting the interpretation of serum testosterone concentration in men

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Abstract

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Most hospital laboratories estimate the concentration of total circulating testosterone using a non-extraction method on an automated multi-channel immunoassay analyser supplied by a small number of multi-national diagnostic companies. Although these platforms offer advantages of quick turnaround times, small volume sampling and random access analysis, proficiency testing schemes suggest the quality of results produced remains similar to that of the early manual radioimmunoassay. An estimate of the bioavailable, non-sex hormone binding globulin (SHBG) bound fraction of circulating testosterone, be that the free or the free plus albumin-bound, may be a better index of gonadal status than total testosterone alone, especially when a borderline hypogonadal level of total testosterone is found, and may avoid misclassification of hypogonadal or eugonadal men. Free or bioavailable testosterone may be calculated or measured. The free androgen index may not give a true reflection of androgen status in men. In the interpretation of serum testosterone concentrations with results > 40 nmol/L, the possibility of exogenous administration or abuse needs to be considered. The marked diurnal rhythm in total testosterone should also be taken into account. There may be a diminution of testosterone secretion with advancing age, but the great majority of older men have a circulating total testosterone concentration well within the accepted reference intervals established for younger men. As testosterone concentration may fluctuate markedly both seasonally and from day to day, it may be judicious to measure levels on more than one occasion.

Provided that estimates of serum testosterone are unequivocally eugonadal (12.5–40 nmol/L) or hypogonadal (< 7.0 nmol/L), results produced by routine automated immunoassays will in all probability give a satisfactory assessment of androgen status in men.

Routine biochemical assessment of gonadal function in men should include measurement of early morning luteinizing hormone, follicle stimulating hormone, prolactin and SHBG together with total testosterone, and if necessary some estimate of bioactive testosterone.

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Introduction

Secreted by the testes, testosterone acts as an anabolic steroid promoting secondary sexual characteristics such as hair growth, muscle mass, penile enlargement and libido as well as sexual differentiation and spermatogenesis. Measurement of concentrations of serum testosterone in men is carried out mainly to assess gonadal status or to monitor replacement therapy.

Although it is generally recognized that erectile dysfunction (a common presenting feature) has, in the majority of cases, little to do with low circulating levels of testosterone, this needs to be excluded as a possible cause. Testosterone circulates in the blood stream in both protein-bound and non-protein-bound (free) moieties. In men, approximately 50% is loosely bound to albumin, 44% is avidly bound to sex hormone binding hormone (SHBG), 4% is bound to other proteins and

2% is free and non-protein bound. Control of testosterone secretion is via negative feedback to the hypothalamus where episodic secretion of gonadotrophin releasing hormone, in turn, promotes synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. LH is then responsible for stimulation of the testosterone-producing Leydig cells of the testes.

While for many years the non-protein bound fraction of a circulating steroid hormone has been considered to be the only biologically active form, recent work suggests that the albumin-bound fraction of testosterone may also be bioactive.

Problems associated with measuring serum concentrations of testosterone in specimens from adult females (where vulnerability to occasional method-related interferences is of particular concern) and prepubertal subjects (where analytical sensitivity at low concentrations is required) are beyond the scope of this review, which will reflect on the factors concerned with the estimation and the interpretation of testosterone levels in adult males. With the exception of the first few months of life, prepubertal children, be they male or female, have largely undetectable concentrations of serum testosterone.

The measured (estimated) concentration of testosterone circulating in human serum is dependent on numerous variables. The factors that play an important role are as follows:

- Method of measurement (including interferences) – analytical;
- Age – physiological;
- Time of day and season of year – physiological;
- Intra-individual variation – physiological;
- Nutritional status and weight – physiological;
- Influence of circulating LH concentrations – analytical/physiological;
- Presence and effect of relevant binding proteins – analytical/physiological.

Method of measurement

Unlike most other commonly measured hormones, the concentration of serum testosterone in normal adult males is at least four-fold higher than that in normal adult females. Furthermore, the generally accepted reference intervals for men of approximately 10–35 nmol/L^{1–4} span a wider concentration range. These intervals may vary a little from laboratory to laboratory but are unlikely to differ significantly, and in many cases reflect values supplied by manufacturers in reagent package leaflets. Expected values for many of these assays have been tabulated by Taieb *et al.*¹ In a

2003 survey on reference ranges, carried out by the United Kingdom National Quality Assurance Scheme for serum testosterone (UK NEQAS; J. Middle, personal communication), 27 out of 96 respondents quoted manufacturers data while 13 used 'in-house' derived data and 17 quoted 'historical' reference ranges. A recent report comparing results obtained from 10 immunoassays, including eight automated platforms, with an isotope dilution–gas chromatography–mass spectrometry (ID–GC–MS) method suggested that none of the immunoassays was sufficiently reliable to measure testosterone in serum at levels below 1.7 nmol/L.¹ An accompanying editorial⁵ concluded that current immunoassays for testosterone at such low concentrations produce results that are little better than generating random numbers. Although this is likely to be of more concern when assaying sera from women, and the study found that the quality of measurement of testosterone in male samples was better than that in women, three of the platforms examined produced results in the male range with mean differences of > 4.0 nmol/L from the ID–GC–MS method and the authors considered the results of two of the automated assays questionable at the lower limit of testosterone concentrations in men. The lack of agreement between results obtained on immunoassay platforms and those obtained by ID–GC–MS may be due to a matrix effect (seen more often in direct assays than in extraction assays), the effect of SHBG binding, methods used to displace protein-bound testosterone prior to immunoassay, or antibody recognition of steroids other than testosterone. Most clinical laboratories estimate the circulating concentration of total testosterone (i.e. the free, non-protein-bound fraction together with albumin-bound and SHBG-bound components). In order to quantify all three components together, a method for displacing testosterone from protein needs to be incorporated in the methodology. This may involve disruption of the protein, extraction of the steroid into an organic solvent or addition of a chemical agent that has a higher binding affinity than has testosterone for the binding protein. Prior to immunoassay, all the serum testosterone to be measured should be in the free, non-protein-bound state.

Currently, measurement of total testosterone in hospital laboratories is almost exclusively performed on fully automated immunoassay analysers, with fewer and fewer choices of assay available. Data produced by UK NEQAS (who, as well as supplying UK laboratories, distribute steroid samples to 111 laboratories overseas) indicate that five diagnostic companies with automated platforms provide more than 90% of testosterone assays. It is unlikely that a laboratory's choice of automated immunoassay platform is dictated by the ability to measure the small-number-requested analytes such as testosterone.

Compared with the original assays (mainly radioimmunoassay [RIA] with serum extraction) the current automated analysers provide a quick turn-around-time on small-volume samples, random access analysis and, if necessary, enable assay of many more samples as part of a profile, in batch mode. The quality of results produced by the automated analysers, however, appears little better than was reported by original workers in the field some 30 years ago^{6,7} using solvent extraction, manual pipetting, charcoal separation techniques and scintillation counting of radioactivity. Examination of inter-assay precision data provided by manufacturers¹ reveals coefficients of variation (CVs) ranging from 1.7% at 24.3 nmol/L on one platform to 7.8% at 34.3 nmol/L on another. Furthermore, in this study precision data for three out of eight automated platforms are little different from those quoted for two manual RIAs at male concentrations. Thus, in 1972, Ismail *et al.*⁷ described an assay with average within- and between-assay CVs of 7.8 and 8.2%, respectively, and a sensitivity of 0.6 nmol/L. Reviewing current published methods in 1970, Nugent and Mayes⁶ found assays capable of detecting 0.33 nmol/L and with CVs of 3.7%, 13% and 14% at concentrations of 1.6, 1.96 and 8.2 nmol/L, respectively.

Analytical interference in immunoassays may be characterized by a discrepant increase or decrease in the observed concentration of analyte.⁸ In testosterone assays, this is likely to be more obvious in female samples with unexpectedly high concentrations.^{9,10} It is more difficult to detect aberrant increases in total testosterone in men as most of the factitious increase could be masked by producing a testosterone level still within the broader reference range. The main cross-reactant in testosterone assays is likely to be 5 α -dihydrotestosterone (DHT), which will still be detected following solvent extraction. The normal circulating levels of DHT are, however, considerably less than those of testosterone, so unless abnormally raised, the interference would probably be minimal and (at normal male testosterone concentrations) virtually impossible to detect.

Personal experience would indicate that levels greater than 40 nmol/L in men who are not already known to be on testosterone replacement are likely to be of exogenous origin. In an unpublished series of 1987 unselected routine requests, levels greater than 40 nmol/L were found in only four samples. This corresponds with reference ranges from 96 laboratories subscribing to UK NEQAS where only two of the 96 laboratories quote a range up to 41 nmol/L. In cases where the total testosterone concentration exceeds 40 nmol/L it may be expected that gonadotrophins would be suppressed, and the misuse of drugs (testosterone) as a possible cause should be considered. Testosterone-secreting tumours of the testis are very rare, comprising around

1% of all testicular tumours, and serum testosterone concentrations tend to be normal.

Little is reported of spuriously low testosterone in men due to assay interference. In the past, some direct (non-extraction) assays have been affected by the level of SHBG present in the serum¹¹ or by autoantibodies that recognize the labelled antigen.^{9,12} Many of these interferences may well be overcome by incorporating a solvent extraction step prior to immunoassay. Simple solvent extraction can be carried out using diethyl ether at a volume 10 times that of the sample followed by snap freezing in dry ice. The ether layer may then be decanted and evaporated to dryness prior to reconstitution in steroid-free serum before immunoassay.

Age

It is generally assumed that, in men, advancing age is accompanied by a gradual diminution in total testosterone concentration.^{2,13–19} Nevertheless, many reports demonstrate concentrations of total serum testosterone in old men well into the reference range for young adults.^{20–27} A more recent report showed that as men aged, the lower limits of reference ranges decreased, but also that up to 50% of men aged 70–79 years had a total testosterone >15.1 nmol/L.²⁸

Time of the day and season of the year

Total serum testosterone displays a circadian rhythm (Figure 1), with the highest concentrations found in the morning and the lowest in the evening.^{29,30–36} This diurnal variation is reported^{14,37–39} to be blunted in elderly men, but a recent study⁴⁰ suggests that in fit healthy men this rhythm is maintained into the

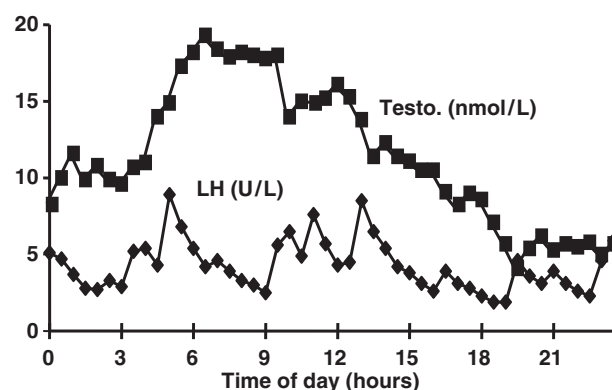


Figure 1 Example from one subject of the diurnal rhythm in total testosterone and corresponding LH in normal young men. Samples were taken every 30 min over a 24-h period. The figure illustrates the profound fall in testosterone concentration from morning to evening along with the pulsatile pattern of LH secretion and the lack of correlation between testosterone and LH

seventh decade. This report describes a decrease of at least 43% from peak to nadir in all the men studied. Similarly, both free and bioavailable testosterone concentrations display this marked variation between morning and evening. It would, therefore, not seem unreasonable when measuring testosterone in serum that strict attention is paid to the time of sampling.

Reports of seasonal variation in testosterone have shown considerable disagreement in both cross-sectional^{41–47} and longitudinal studies.^{48–55} More recently, in a cross-sectional study involving more than 1500 men, Svartberg *et al.*⁵⁶ found lowest total and free testosterone in months with the highest temperatures and longest hours of daylight. Samples analysed for this report were, however, collected at random times between 08:00 and 16:00 h. Little is reported on testosterone levels in shift workers. Peak and trough times of testosterone have been shown to be erratic with serum concentrations significantly decreased.⁵⁷ Other reports show no obvious modifications in testosterone concentration on exposure to bright light,⁵⁸ and the picture may be confounded by the influence of satisfaction or dissatisfaction with shift working since men dissatisfied with the pattern of work revealed lower testosterone concentrations.⁵⁹

Intra-individual variation

Disagreement also exists on the day-to-day constancy of testosterone concentration in normal men. Early work suggested marked day-to-day fluctuations in serum testosterone levels even though samples were collected at the same time each day in different studies.⁶⁰ One report⁶¹ of a cohort of 169 men aged 40–80 years, with serum testosterone measured eight times in 50 weeks, concluded that since the level in the initial sample correlated with the mean concentration, a single estimate was adequate. This reflected earlier work recommending measuring testosterone on three occasions throughout the day in order to establish an overall daily concentration.³¹ More recently, however, others have shown a marked week-to-week variability in both testosterone and bioavailable testosterone.⁶² These findings are in accordance with unpublished studies in the author's laboratory where testosterone in eight normal men aged 25–60 years showed marked intra-individual variability in total and bioavailable testosterone in both AM and PM samples (Figure 2). In some individuals, the values ranged from normal to hypogonadal. Furthermore, total testosterone measured 22 times in a single individual, collected under strict timing between 09:00 and 10:00 h over an eight-year period, revealed an intra-individual variation from 14.5 to 33 nmol/L with no chronological correlation. This evidence would suggest that un-

less the initial testosterone level is unequivocally normal (>12.5 nmol/L) or unequivocally abnormal (<7 nmol/L), more than one estimate of total testosterone should be obtained before classifying a result as indicating hypogonadism.

Nutritional status and weight

Concentrations of serum total testosterone are lower in obese men compared with those found in normal men of the same age.^{63–67} The major factor contributing to this decrease is reported to be the concomitant, positively associated decrease ($P < 0.001$) in SHBG concentrations.⁶⁶ We have found this inverse relationship between body mass index (BMI) and total testosterone in a group of men with a BMI between 17 and 29, the correlation being observed in both morning and evening samples.

Circulating levels of testosterone are lowered in critical illness such as burn injuries⁶⁸ or anorexia nervosa,⁶⁹ where nutrition may be compromised. This hypogonadism is probably secondary to hypothalamic/pituitary down-regulation in response to disease. More recent evidence showing a close temporal relationship between testosterone, LH and leptin suggests that low testosterone concentration seen in male anorectics may be related to low leptin levels. Changes of leptin levels over time were significantly correlated with those of gonadotrophins and testosterone.⁷⁰ The subject is comprehensively reviewed elsewhere.^{71,72} It is, however, unlikely that routine assessment of gonadal status would be paramount in such patients.

Influence of circulating LH concentrations

Testosterone, like all steroid hormones, is synthesized from cholesterol. The rate-limiting step in testosterone synthesis is the conversion of cholesterol to pregnenolone. LH regulates this reaction, thus controlling the overall rate of testosterone synthesis. As a general rule, a fall in circulating testosterone evokes a simultaneous rise in LH secretion and, in the clinical context, men with low circulating levels of testosterone tend to have elevated levels of LH unless the hypogonadism is secondary to a pituitary or hypothalamic cause. Attempts to correlate acute changes in testosterone and LH have, however, proved problematic. As an adjunct to a study of diurnal variation in testosterone⁴⁰ gonadotrophin levels were also measured every 30 min for 24 h in 10 young men aged 23–31 years. No concomitant cross-correlation was established between LH and total testosterone⁷³ (Figure 1). While total testosterone showed a significant circadian rhythm on Cosinor analysis, LH displayed a much less marked rhythm, with a typical episodic pattern of secretion.

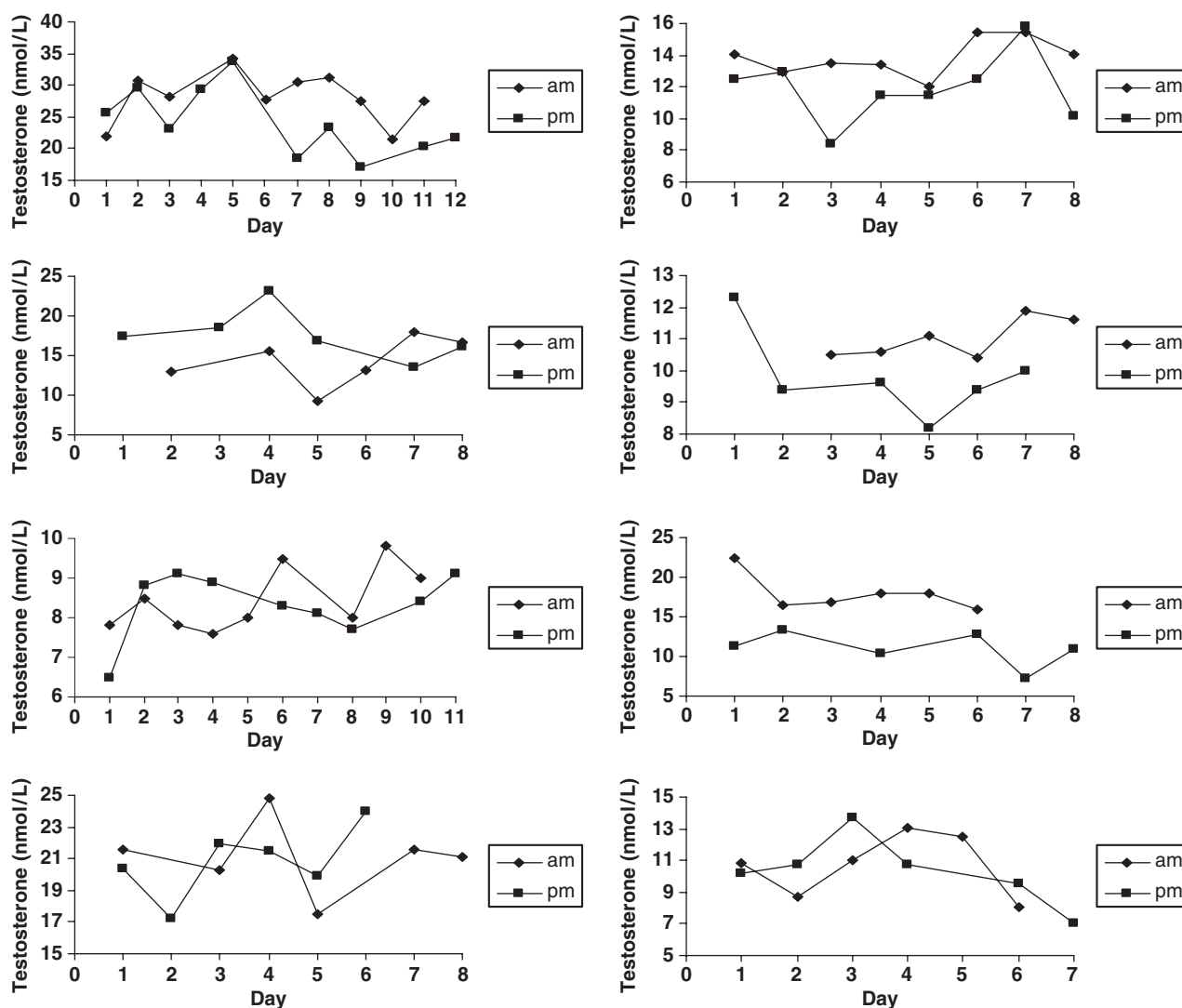


Figure 2 Intra-individual variability in serum total testosterone in eight normal men aged 25–60 years in samples taken between 09:00 and 10:00 h (—◆—) and between 16:00 and 17:00 h (—■—) on at least six separate days over a one-month period

Reports have shown rises in LH with age both without a decrease in testosterone²⁰ and in conjunction with decreased testosterone.^{19,74}

An increase in LH concentrations would be consistent with primary gonadal dysfunction, but although interstitial cell secretory reserve may be suboptimal, when elderly men were challenged with human chorionic gonadotrophin (HCG) they responded by normalizing testosterone levels.³

Presence and effect of relevant binding proteins

The precise function of specific binding proteins such as SHBG remains unknown (see Rosner W⁷⁵ for review), but some evidence suggests a role of active transport of steroids into certain target tissues such as prostate and

testis^{76,77} over and above the classical role of regulating the steady-state free hormone concentration in plasma.

As a general concept, as with other specific binding proteins, when the circulating concentrations of these proteins rise, a concomitant rise is also observed in the total concentration of hormone. For example, when the oestrogen-induced rise in thyroxine-binding globulin (TBG) and cortisol-binding globulin (CBG) occurs in pregnancy, a rise in total thyroxine and in total cortisol is seen. This is as a result of the negative feedback mechanism maintaining the constant level of free hormone. Similarly, it is widely accepted that a rise or fall in SHBG results in an increase or decrease in total testosterone. These changes do not, however, occur acutely, as evidenced by the profound decrease in testosterone concentration throughout the day while levels of SHBG remain almost constant. In one study⁴⁰ although cosinor analysis revealed a significant

diurnal rhythm in SHBG, with an acrophase (period of highest levels) in mid-afternoon, the changes were relatively small (~10%) and probably reflected the shift in body fluid between compartments as subjects lay down at night and rose in the morning. It seems clear that SHBG levels change very little throughout the day, altering only with posture as with other plasma proteins like albumin.^{29,78} This lack of a significant relationship between SHBG and testosterone is also illustrated in a study in the author's laboratory where no correlation was found between SHBG and testosterone concentrations measured in a single individual on 22 occasions. The marked diurnal rhythm seen in testosterone is, therefore, not attributable to a change in binding proteins.

A further paradox exists between SHBG and testosterone. As mentioned above, many reports indicate a decrease in testosterone with advancing age. At the same time it is universally acknowledged that SHBG increases as men grow older. This results in a marked diminution of both free and bioavailable testosterone as men age. With the well-recognized negative feedback in the hypothalamic–pituitary–testicular axis, a rise in LH secretion might be expected in order to reset the free testosterone level and, as SHBG increases with advancing age, the total testosterone level might increase simultaneously and to the same degree. This anomaly may well be explained by a decreased testosterone production rate in older men.⁷⁹

The main reason for estimation of serum testosterone in men is detection of hypogonadism. In cases of proven hypogonadism satisfactory replacement of deficient testosterone is relatively easily achieved, the main routes of administration being by bolus intra-muscular injection of mixtures of testosterone esters or by transdermal patches of testosterone. Shortly after injection, supra-physiological circulating levels (>40 nmol/L) are achieved, the concentration gradually diminishing to hypogonadal levels within 2–3 weeks and prior to the next dose. Replacement of testosterone may not be without risks⁸⁰ and, therefore, treatment should be confined to those men with proven frank hypogonadism. This group would encompass those men with clinical signs whose total, free and/or bioavailable testosterone are unequivocally low be that as a result of primary gonadal failure or secondary to hypothalamic/pituitary dysfunction. In some cases, men may show an equivocally low total testosterone, but when free or bioavailable testosterone is estimated these may be normal. Replacement in these men may, therefore, not be warranted.

Total, bioavailable or free testosterone?

In some cases, the concentration of total serum testosterone may not always represent a true reflection of the

androgen status. If, for example, two men have exactly the same total testosterone concentration and one has SHBG 10 nmol/L while the other has SHBG 80 nmol/L, then they will have entirely different levels of bioactive testosterone. In all probability testosterone bound to SHBG is biologically inactive,⁸¹ and therefore some estimate of the non-SHBG-bound fraction may be a more appropriate measure. Methods of assessment of the non-SHBG-bound fraction of testosterone include estimation of the free (non-protein bound) concentration by various methods including calculation of the free androgen index (FAI), equilibrium dialysis, centrifugal ultrafiltration, direct analog RIA, assay of bioavailable (free plus albumin bound) testosterone or calculation of the free and/or bioavailable fraction.

Many laboratories measure SHBG in conjunction with total testosterone and produce an FAI using a simple arithmetical formula:

$$\text{FAI} = [\text{testosterone}] \times 100 / [\text{SHBG}].$$

Although this index has found favour in discriminating hyperandrogenic females with normal total testosterone concentrations,^{82–84} its use as a reliable index of bioavailable testosterone in men has recently been questioned.^{85,86} The 'reference method' for estimating free testosterone is generally accepted as equilibrium dialysis^{85,87–90} or centrifugal ultrafiltration,⁹¹ but these methods are time-consuming and labour-intensive and are not suitable for a routine clinical service.

Reports have suggested that use of analog RIA direct methods for measurement of free testosterone produces results at variance with those generally accepted as reflecting the free hormone concentrations.^{85,92–95} An estimate of free testosterone concentration may be achieved by use of a simple equation⁸² if the total testosterone and SHBG are measured:

$$\begin{aligned} \text{Free testosterone (\% of the measured total)} \\ = 6.11 - 2.38 \log_{10}[\text{SHBG}] \end{aligned}$$

Otherwise more complicated formulae requiring computer-assisted calculation may be used.^{85,96} The results produced by any of the formulae show similar concentrations of free testosterone.⁹⁷ The website of the International Society for the Study of the Ageing Male (www.ISSAM.ch) reveals a page requesting entry of total testosterone, SHBG and albumin concentrations and selection of the appropriate units of measurement will automatically calculate the free testosterone concentration by simply clicking the 'return' key. This method of calculating the free testosterone concentration has recently been validated by Vermeulen *et al.*⁸⁵

Reports from Pardridge's group^{98–100} and others¹⁰¹ have led to the belief that bioavailable testosterone, more than total testosterone, best reflects androgen status and this is now widely advocated.^{78,102–113}

The first Pardridge hypothesis suggests that sex steroids are available to nearly all tissues from the pool of albumin-bound steroid. During capillary transit, provided the $t_{1/2}$ of the hormone-albumin dissociation is less than the transit time, the hormone undergoes dissociation and subsequent transport across the cell membrane via a 'free intermediate mechanism'. Pardridge's second hypothesis involves transport from the plasma protein-bound fraction via a specific tissue-mediated enhanced dissociation of the hormone from the protein by either a non-competitive inhibition of hormone binding to the plasma protein or by a putative cell surface receptor, followed by rapid dissociation of the protein-hormone complex.

These hypotheses advanced by Pardridge have been strongly criticized and the conclusions questioned.^{114,115} Nevertheless, an ever-increasing volume of literature presenting data on bioavailable testosterone has appeared with a 10-fold rise over the past decade. A strong correlation between bioavailable testosterone and calculated free testosterone has been demonstrated⁸⁵ and between bioavailable testosterone and such clinical indices as muscle strength and bone density,²⁷ suggesting clinical usefulness of the measurement.

Although more technically demanding than measurement of total testosterone (especially if total testosterone is measured on an automated platform), bioavailable testosterone data based on the method of Tremblay and Dube¹⁰⁷ have been reported by several groups.^{29,98,103,112} This technique involves precipitation of SHBG by saturated ammonium sulphate, leaving the free plus albumin-bound fractions in the supernatant. Using a radioactive testosterone tracer as a measurable endpoint, the percentage of the total testosterone found in the supernatant may be determined, thereby representing the 'bioavailable', or non-SHBG-bound fraction. The concentration of bioavailable testosterone can then be calculated from the concentration of total testosterone. The measurement has recently⁸⁵ been advocated as a valid marker of bioactive androgen, correlating well with the 'reference' equilibrium dialysis free testosterone assay.

In light of the ever-growing body of evidence of a better correlation of bioavailable or free rather than total testosterone with such clinical findings as hypogonadism, bone mineral density and cognitive function in the elderly, it would seem not unreasonable for laboratories to adopt some measure of non-SHBG-bound testosterone as a marker of androgen status. As measurement of bioavailable or free testosterone will be beyond the scope of routine clinical laboratories, the simple derivation of both free and bioavailable testosterone on the ISSAM Website might well be advocated. As an alternative, laboratories using an automated platform capable of measuring both total testosterone and SHBG might

choose to incorporate an automatic calculation of free testosterone into their laboratory computer software.

Conclusion

Laboratories should be aware of the limitations of an automated direct estimate of serum testosterone. Provided that the level is unequivocally in the reference range (>12.5 nmol/L) or unequivocally low (<7.0 nmol/L), this will probably give an acceptable reflection of gonadal status.

When a borderline result is obtained, (between 7 and 12.5 nmol/L) the factors mentioned above which influence serum testosterone concentration should be borne in mind and in these cases a further, early-morning sample should be analysed for total testosterone, SHBG, LH, FSH and prolactin, along with an estimate of free or bioavailable testosterone.

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References

- 1 Taieb J, Mathian B, Millot F, *et al.* Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 1116 men, women and children. *Clin Chem* 2003; **49**: 1381-95
- 2 Harman SM, Metter JE, Tobin JD, Pearson J, Blackman MR. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. *J Clin Endocrinol Metab* 2001; **86**: 724-31
- 3 Kaufman JM, Vermeulen A. Declining gonadal function in elderly men. *Clin Endocrinol Metab* 1997; **11**: 289-309
- 4 Ismail AAA, Astley P, Burr WA, *et al.* The role of testosterone measurement in the investigation of androgen disorders. *Ann Clin Biochem* 1986; **23**: 113-34
- 5 Herold DA, Fitzgerald RL. Immunoassays for testosterone in women: better than a guess? Editorial. *Clin Chem* 2003; **49**: 1250-1
- 6 Nugent CA, Mayes D. Reliability of plasma testosterone assays by competitive protein binding methods. *Acta Endocrinol* 1970; **147**(Suppl.): 257-71
- 7 Ismail AAA, Niswender GD, Midgley AR. Radioimmunoassay of testosterone without chromatography. *J Clin Endocrinol Metab* 1972; **34**: 177-84
- 8 Ismail AAA, Walker PL, Cawood ML, Barth JH. Interference in immunoassay is an underestimated problem. *Ann Clin Biochem* 2002; **39**: 366-73
- 9 Torjesen PA, Bjoro T. Antibodies against [125 I] testosterone in patient's serum: a problem for the laboratory and the patient. *Clin Chem* 1996; **42**: 2047-8
- 10 Hill RP, Henson DB, Selby C, Saihan E, Thomson GA. Grossly elevated testosterone in three female patients: an assay artefact on the Bayer Immuno-1 Proceedings of the ACB National Meeting, 1998:38 (Poster 45)

- 11 Micallef JV, Hayes MM, Latif A, Ahsan R, Sufi SB. Serum binding of steroid tracers and its possible effects on direct steroid immunoassay. *Ann Clin Biochem* 1995; **32**: 566-74
- 12 Slaats EH, Kennedy JC, Kruijswijk H. Interference of sex-hormone binding globulin in the 'coat-a count' testosterone no-extraction radioimmunoassay. *Clin Chem* 1987; **33**: 300-2
- 13 Vermeulen A, Rubens R, Verdonck L. Testosterone secretion and metabolism in male. *J Clin Endocrinol Metab* 1972; **34**: 730-5
- 14 Bremner WJ, Vitiello MV, Prinz PN. Loss of circadian rhythmicity in blood testosterone levels in normal men. *J Clin Endocrinol Metab* 1983; **56**: 1278-81
- 15 Korenman SG, Morley JE, Mooradian AD, *et al.* Secondary hypogonadism in older men: its relation to impotence. *J Clin Endocrinol Metab* 1990; **71**: 963-9
- 16 Gray A, Berlin JA, McKinlay JB, Longcope C. An examination of research design effects on the association of testosterone and male aging: results of a meta-analysis. *J Clin Epidemiol* 1991; **44**: 671-84
- 17 Simon D, Preziosi P, Barrett-Connor E, *et al.* The influence of aging on plasma sex hormones in men: the telecom study. *Am J Epidemiol* 1992; **135**: 783-91
- 18 Tennekoon KH, Karunanayake EH. Serum FSH, LH and testosterone concentrations in presumably fertile men; effect of age. *Int J Fertil* 1993; **38**: 108-12
- 19 Morley JE, Kaiser FE, Perry HM, *et al.* Longitudinal changes in testosterone, luteinising hormone and follicle stimulating hormone in healthy older men. *Metabolism* 1997; **46**: 410-3
- 20 Harman SM, Tsitouras PD. Reproductive hormones in aging men. Part 1: measurement of sex steroids, basal luteinising hormone, and Leydig cell response to human chorionic gonadotrophin. *J Clin Endocrinol Metab* 1980; **35**: 35-40
- 21 Sparrow D, Bosoe R, Rowe JW. The influence of age, alcohol consumption and body build on gonadal function in men. *J Clin Endocrinol Metab* 1980; **51**: 508-12
- 22 Nankin HR, Calkins JH. Decreased bioavailable testosterone in aging normal and impotent men. *J Clin Endocrinol Metab* 1986; **63**: 1418-20
- 23 Schiavi RC, White D, Mandeli J. Pituitary-gonadal function during sleep in healthy aging men. *Psychoneuroendocrinol* 1992; **17**: 599-609
- 24 Mulligan T, Iranmahesh A, Gheorghius S, Godschalk T, Veldhuis JD. Amplified nocturnal luteinising hormone (LH) secretory burst frequency with selective attenuation of pulsatile (but not basal) testosterone secretion in healthy aged men: possible Leydig cell desensitisation to endogenous signalling-a clinical research center study. *J Clin Endocrinol Metab* 1995; **80**: 3025-31
- 25 Ongphiphadhanakul B, Rajatavin R, Chailurkit L, *et al.* Serum testosterone and its relation to bone mineral density and body composition in normal males. *Clin Endocrinol* 1995; **43**: 727-33
- 26 Janssen JAMJL, Stolk RP, Pols HAP, Grobbee DE, de Jong FH, Lamberts SWJ. Serum free IGF-1, total IGF-1, IGFBP-1 and IGFBP-3 levels in an elderly population: relation to age and sex steroid levels. *Clin Endocrinol* 1998; **48**: 471-8
- 27 van den Beld AW, de Jong FH, Grobbee DE, Pols HAP, Lamberts SWJ. Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *J Clin Endocrinol Metab* 2000; **85**: 3276-82
- 28 Mohr BA, Guay AT, O'Donnell AB, McKinlay JB. Normal, bound and unbound testosterone levels in normally ageing men: results from the Massachusetts Male Ageing Study. *Clin Endocrinol* 2005; **62**: 64-73
- 29 Cooke RR, McIntosh JEA, McIntosh RP. Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action mode. *Clin Endocrinol* 1993; **39**: 163-71
- 30 Resko JA, Eik-Ness KB. Diurnal testosterone levels in peripheral plasma of human male subjects. *J Clin Endocrinol Metab* 1966; **26**: 573-5
- 31 Neischlag E, Ismail AAA. Diurnal variations of plasma testosterone in normal and pathological conditions as measured by the technique of competitive protein binding. *J Endocrinol* 1970; **46**, ii-iv
- 32 Faiman C, Winter JSD. Diurnal cycles in plasma FSH, testosterone and cortisol in men. *J Clin Endocrinol Metab* 1971; **33**: 186-92
- 33 Rose RM, Kreuz LE, Holaday JW, Sulak KJ, Johnson CE. Diurnal variation of plasma testosterone and cortisol. *J Endocrinol* 1972; **54**: 177-8
- 34 Rowe PH, Lincoln GA, Racey PA, *et al.* Temporal variations of testosterone levels in the peripheral blood plasma of men. *J Endocrinol* 1974; **61**: 63-73
- 35 Campbell IT, Walker RF, Riad-Fahmy D, Wilson DW, Griffiths K. Circadian rhythms of testosterone and cortisol in saliva: effects of activity-phase shifts and continuous daylight. *Chronobiologia* 1982; **9**: 389-96
- 36 Montanini V, Simoni M, Chiossi G, *et al.* Age-related changes in plasma dehydroepiandrosterone sulphate, cortisol, testosterone and free testosterone circadian rhythms in adult men. *Horm Res* 1988; **29**: 1-6
- 37 Marrama P, Carani C, Baraghini GF, *et al.* Circadian rhythm of testosterone and prolactin in the ageing. *Maturitas* 1982; **4**: 131-8
- 38 Deslypere JP, Vermeulen A. Leydig cell function in normal men: effect of age, lifestyle, residence, diet and activity. *J Clin Endocrinol Metab* 1984; **59**: 955-62
- 39 Plymate SR, Tenover JS, Bremner WJ. Circadian variation in testosterone, sex hormone-binding globulin and calculated non sex hormone-binding globulin bound testosterone in healthy young and elderly men. *J Androl* 1989; **10**: 366-71
- 40 Diver MJ, Imtiaz KE, Ahmad AM, Vora JP, Fraser WD. Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clin Endocrinol* 2003; **58**: 710-7
- 41 Bellastella A, Esposito V, Mango A, D'Allessandro B. Temporal relationship between circannual levels of luteinising hormone and testosterone in prepubertal boys with constitutional short stature. *Chronobiologia* 1982; **9**: 123-5
- 42 Nicolau GY, Haus E, Lakatua DJ, *et al.* Circadian and circannual variations of FSH, LH, testosterone, dehydroepiandrosterone-sulfate (DHEA-S) and 17-hydroxyprogesterone (17 OH-Prog) in elderly men and women. *Endocrinologie* 1985; **23**: 223-46
- 43 Reinberg A, Smolensky MH, Hallek M, Smith KD, Steinberger E. Annual variation in semen characteristics and plasma hormone levels in men undergoing vasectomy. *Fertil Steril* 1988; **49**: 309-15
- 44 Dabbs Jr JM. Age and seasonal variation in serum testosterone concentrations among men. *Chronobiol Int* 1990; **7**: 245-9
- 45 Perry III HM, Millar DK, Patrick P, Morley JE. Testosterone and leptin in older African-American men: relationship to age, strength, function and season. *Metabolism* 2000; **49**: 1085-91

- 46 Dai WS, Kuller LH, LaPorte RE, Gutai JP, Falvo-Gerard L, Caggiola A. The epidemiology of plasma testosterone levels in middle-aged men. *Am J Epidemiol* 1981; **114**: 804-16
- 47 Abbatachio G, de Fini M, Giagulli VA, Santoro G, Vendola G, Giorgino R. Circannual rhythms in reproductive functions of human males, correlations among hormones and hormone dependent parameters. *Andrologia* 1987; **19**: 353-61
- 48 Reinberg A, Lagoguey M, Chauffournier JM, Cesselin F. Circannual and circadian rhythms in plasma testosterone in five healthy young Parisian males. *Acta Endocrinol* 1975; **80**: 732-4
- 49 Smals AG, Kloppenberg PW, Benraad TJ. Circannual cycle in plasma testosterone levels in man. *J Clin Endocrinol Metab* 1976; **42**: 979-82
- 50 Nicolau GY, Lakatua D, Sackett-Lundeen L, Haus E. Circadian and circannual rhythms of hormonal variables in elderly men and women. *Chronobiol Int* 1989; **1**: 301-9
- 51 Meriggiola MC, Noonan EA, Paulsen CA, Bremner WJ. Annual patterns of luteinising hormone, follicle stimulating hormone, testosterone and inhibin in normal men. *Hum Reprod* 1996; **11**: 248-52
- 52 Valero-Polti J, Fuentes-Arderiu X. Annual rhythmic variations of follitropin, lutropin, testosterone and sex hormone binding globulin in men. *Clin Chim Acta* 1998; **271**: 57-71
- 53 Baker HW, Burger HG, de Kretser DM, *et al.* Changes in pituitary-testicular system with age. *Clin Endocrinol* 1976; **5**: 349-72
- 54 Martikainen H, Tapanainen J, Vakkuri O, Leppaluoto J, Huhtaniemi I. Circannual concentrations of melatonin, gonadotrophins, prolactin and gonadal steroids in males in a geographic area with a large variation in daylight. *Acta Endocrinol* 1985; **109**: 446-50
- 55 Maes M, Mommen K, Hendrickx D, *et al.* Components of biological variation, including seasonality, in blood concentrations of TSH, TT3, FT4, PRL, cortisol and testosterone in healthy volunteers. *Clin Endocrinol* 1997; **46**: 587-9
- 56 Svartberg J, Jorde R, Sundsfjord J, Bonaa KH, Barrett-Connor E. Seasonal variation of testosterone and waist to hip ratio in men: the Tromso study. *J Clin Endocrinol Metab* 2003; **88**: 3099-104
- 57 Touitou Y, Motohashi Y, Reinberg A, Touitou C, *et al.* Effect of shift work on the night-time secretory patterns of melatonin, prolactin, cortisol and testosterone. *Eur J Appl Physiol Occup Physiol* 1990; **60**: 288-92
- 58 Touitou Y, Benoit O, Foret J, *et al.* Effects of a two-hour early awakening and of bright light exposure on plasma patterns of cortisol, melatonin, prolactin and testosterone in man. *Acta Endocrinol* 1992; **126**: 201-5
- 59 Axelsson J, Akerstedt T, Kecklund G, Lindqvist A, Attefors R. Hormonal changes in satisfied and dissatisfied shift workers across a shift cycle. *J Appl Physiol* 2003; **95**: 2099-105
- 60 Fox CA, Ismail AAA, Love DN, Kirkham KE, Loraine JA. Studies on the relationship between plasma testosterone levels and human sexual activity. *J Endocrinol* 1972; **52**: 51-8
- 61 Vermeulen A, Verdonck G. Representative of a single point plasma testosterone level for long-term hormonal milieu. *J Clin Endocrinol Metab* 1992; **74**: 939-42
- 62 Morley JE, Patrick P, Perry III HM. Evaluation of assays to measure free testosterone. *Metabolism* 2002; **51**: 554-9
- 63 Glass AR, Swerdloff RS, Bray GA, Dahms W, Atkinson RL. Low serum testosterone and sex hormone binding globulin in massively obese men. *J Clin Endocrinol Metab* 1977; **45**: 1211-9
- 64 Stanik S, Dornfield LP, Maxwell MH, Viosca SP, Korenman SG. The effect of weight loss on reproductive hormones. *J Clin Endocrinol Metab* 1981; **53**: 828-31
- 65 Vermeulen A, Kaufman JM, Deslypere JP, Thomas G. Attenuated LH pulse amplitude but normal LH pulse frequency and its relation to plasma androgens in hypogonadism of obese men. *J Clin Endocrinol Metab* 1993; **76**: 1140-6
- 66 Giagulli VA, Kaufman JM, Vermeulen A. Pathogenesis of the decreased androgen levels in obese men. *J Clin Endocrinol Metab* 1994; **79**: 997-1000
- 67 Svartberg J, Midtby M, Bonaa KH, Sundsfjord J, Joakimsen RM, Jorde R. The association of age, lifestyle factors and chronic disease with testosterone in men: the Tromso study. *Eur J Endocrinol* 2003; **149**: 145-52
- 68 Semple CG, Robertson WR, Mitchell R, *et al.* Mechanisms leading to hypogonadism in men with burns injuries. *BMJ* 1987; **295**: 403-7
- 69 Wheeler MJ, Crisp AH, Hsu LKG, Chen CN. Reproductive hormone changes during weight gain in male anorectics. *Clin Endocrinol* 1983; **18**: 423-9
- 70 Wabitch M, Ballauff A, Hull R, *et al.* Serum leptin, gonadotrophins, and testosterone concentrations in male patients with anorexia nervosa during weight gain. *J Clin Endocrinol Metab* 2001; **86**: 2982-8
- 71 Semple CG, Gray CE, Beastall GH. Male hypogonadism-A non-specific consequence of illness. *Quart J Med* 1987; **64**: 601-7
- 72 Turner HE, Wass JAH. Gonadal function in men with chronic illness. *Clin Endocrinol* 1997; **47**: 379-403
- 73 Diver MJ, Manning JT, Gage AR, Scutt D, Cross N, Fraser WD. Temporal correlation of luteinising hormone levels with indices of androgen status in normal men. *J Endocrinol* 1999; **160**(Suppl.): P49
- 74 Gray A, Feldman HA, McKinlay JB, Longcope C. Age, disease and changing sex hormone levels in middle-aged men: results of the Massachusetts male aging study. *J Clin Endocrinol Metab* 1991; **73**: 1016-25
- 75 Rosner W. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocr Rev* 1990; **11**: 80-91
- 76 Rosner W. Plasma steroid binding proteins. *Endocrinol Metab Clin N Am* 1991; **20**: 697-720
- 77 Sakiyama R, Pardridge WM, Musto NA. Influx of testosterone-binding globulin (TeBG) and TeBG-bound sex steroid hormones into rat testis and prostate. *J Clin Endocrinol Metab* 1988; **67**: 98-103
- 78 Plymate SR, Tenover JS, Bremner WJ. Circadian variation in testosterone, sex hormone-binding globulin and calculated non sex hormone-binding globulin bound testosterone in healthy young and elderly men. *J Androl* 1989; **10**: 366-71
- 79 Wang C, Catlin DH, Starcevic B, *et al.* Testosterone metabolic clearance and production rates determined by stable isotope dilution/tandem mass spectrometry in normal men: influence of ethnicity and age. *J Clin Endocrinol Metab* 2004; **89**: 2936-41
- 80 Rhoden EL, Morgentaler A. Risks of testosterone-replacement therapy and recommendations for monitoring. *New Engl J Med* 2004; **350**: 482-92
- 81 Anderson DC. Sex hormone binding globulin. *Clin Endocrinol* 1974; **3**: 69-96
- 82 Nanjee MN, Wheeler MJ. Plasma free testosterone - is an index sufficient? *Ann Clin Biochem* 1985; **22**: 387-90

- 83 Carter GD, Holland SM, Alagband-Zadeh J, Rayman G, Dorrington-Ward P, Wise PH. Investigation of hirsutism: testosterone is not enough. *Ann Clin Biochem* 1983; **20**: 262-3
- 84 Mathur RS, Moody LO, Landgrebe S, Williamson HO. Plasma androgens and sex hormone-binding globulin in the evaluation of hirsute females. *Fertil Steril* 1981; **35**: 29-35
- 85 Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 1999; **84**: 3666-72
- 86 Kapoor P, Luttrell, Williams D. The free androgen index is not valid for adult males. *J Steroid Biochem Mol Biol* 1993; **45**: 325-6
- 87 McCann DS, Kirkish LS. Evaluation of free testosterone in serum. *J Clin Immunoassay* 1985; **8**: 234-6
- 88 Sinha-Hikim I, Arver S, Beall G, *et al.* The use of a sensitive equilibrium dialysis method for the measurement of free testosterone levels in healthy cycling women and in human immunodeficient virus infected women. *J Clin Endocrinol Metab* 1998; **83**: 1312-8
- 89 Vermeulen A, Stoica T, Verdonck L. The apparent free testosterone concentration, an index of androgenicity. *J Clin Endocrinol Metab* 1971; **33**: 759-67
- 90 Kley HK, Bartman E, Kruskemer HL. A simple and rapid method to measure non protein-bound fractions of cortisol, testosterone and estradiol by equilibrium dialysis: comparison with centrifugal filtrations. *Acta Endocrinol* 1997; **85**: 209-19
- 91 Hammond GL, Niskier JA, Jones LA, Siiteri PK. Estimation of the percentage of free steroid in undiluted serum by centrifugal ultrafiltration-dialysis. *J Biol Chem* 1980; **255**: 5023-6
- 92 Rosner W. Errors in the measurement of plasma free testosterone. *J Clin Endocrinol Metab* 1997; **82**: 2014-5
- 93 Winders SJ, Kelley DE, Goodpaster B. The analog free testosterone assay: are the results in men clinically useful? *Clin Chem* 1986; **44**: 2178-82
- 94 Cheng RW, Reed MJ, James VHT. Plasma free testosterone: equilibrium dialysis vs. direct radioimmunoassay. *Clin Chem* 1986; **32**: 1411
- 95 Wilke TJ, Utley DJ. Total testosterone, free-androgen index, calculated free testosterone and free testosterone by analog RIA compared in hirsute women and in otherwise-normal women with altered binding of sex-hormone-binding globulin. *Clin Chem* 1987; **33**: 1372-5
- 96 Sodergard R, Backstrom T, Shanbag V, Cartensen H. Calculation of free and bound fractions of testosterone and estradiol 17 β to human plasma protein at body temperature. *J Steroid Biochem* 1982; **26**: 801-10
- 97 Ivison FM, Robinson A, Diver MJ. Concordance of results for two methods of calculating free testosterone concentrations: are the results valid? Proceedings of the ACB National Meeting, 2003: 83 (Abstract 10)
- 98 Manni A, Pardridge WM, Cefalu W, *et al.* Bioavailability of albumin bound testosterone. *J Clin Endocrinol Metab* 1985; **61**: 705-10
- 99 Pardridge WM. Serum bioavailability of sex steroid hormones. *Clin Endocrinol Metab* 1986; **15**: 259-78
- 100 Pardridge WM. Selective delivery of sex steroids to tissue *in vivo* by albumin and by sex hormone binding globulin. *Ann NY Acad Sci* 1998; **538**: 173-92
- 101 Cumming DC, Wall SR. Non sex hormone-binding globulin-bound testosterone as a marker for hyperandrogenism. *J Clin Endocrinol Metab* 1985; **61**: 873-6
- 102 Garden GA, Hannan CJ, Spencer CA, Plymate SR. The role of non sex hormone-binding globulin bound testosterone on luteinising hormone in hyperthyroid men. *Steroids* 1988; **52**: 385-6
- 103 Cooke RR, McIntosh RP, McIntosh JGA, Delahunt JW. Serum forms of testosterone in men after an HCG stimulation: relative increase in non protein bound forms. *Clin Endocrinol* 1990; **32**: 165-75
- 104 Korenman SG, Morley JE, Mooradian AD, *et al.* Secondary hypogonadism in older men: its relation to impotence. *J Clin Endocrinol Metab* 1990; **71**: 963-9
- 105 Kaiser FE, Morley JE. Gonadotrophins, testosterone and the aging male. *Neurobiol Aging* 1994; **15**: 559-63
- 106 Bhasin S, Bagatell CJ, Bremner WJ, *et al.* Issues in testosterone replacement in older men. *J Clin Endocrinol Metab* 1998; **83**: 3435-47
- 107 Tremblay RR, Dube JY. Plasma concentration of free and non TEBG bound testosterone of women on oral contraceptives. *Contraception* 1974; **10**: 599-605
- 108 Barrett-Connor E, Von Muhlen DG, Kritz-Silverstein D. Bioavailable testosterone and depressed mood in older men: the Rancho Bernardo Study. *J Clin Endocrinol Metab* 1999; **84**: 573-7
- 109 Morales A, Heaton JPW, Carson CC. Andropause: a misnomer for a true clinical entity. *J Urology* 2000; **163**: 705-12
- 110 Van den Beld AW, De Jong FH, Grobbee DE, Pols HAP, Lamberts SWJ. Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density and body composition in elderly men. *J Clin Endocrinol Metab* 2000; **85**: 3276-82
- 111 Khosla S, Melton J, Atkinson EJ, O'Fallon WM. Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab* 2001; **86**: 3555-61
- 112 Szulc P, Munoz F, Claustat B, *et al.* Bioavailable estradiol may be an important determinant of osteoporosis in men: the MINOS study. *J Clin Endocrinol Metab* 2001; **86**: 192-9
- 113 Lejeune H, Dechaud H, Pugeat M. Contribution of bioavailable testosterone assay for the diagnosis of androgen deficiency in elderly men. *Ann Endocrinol* 2003; **64**: 117-25
- 114 Mendel CM. The free hormone hypothesis: a physiological based mathematical mode. *Endocr Rev* 1989; **10**: 232-74
- 115 Ekens RP, Edwards PR. Plasma protein-mediated transport of steroid and thyroid hormones: a critique. *Am J Physiol* 1988; **255**: E403-8
- 116 Liu PY, Swerdloff RS, Veldhuis JD. The rationale, efficacy and safety of androgen therapy in older men: future research and current practice recommendations. *J Clin Endocrinol Metab* 2004; **89**: 4789-96

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