www.medscape.com

Measurement of Testosterone in the Diagnosis of Hypogonadism in the Ageing Male

M. J. Wheeler; S. C. Barnes

Posted: 12/30/2008; Clin Endocrinol. 2008;69(4):515-525. © 2008 Blackwell Publishing

Summary and Introduction

Summary

Many males in their old age demonstrate symptoms consistent with hypogonadism. With the introduction of new and more convenient methods of testosterone replacement treatment of these males is more practical. The diagnosis of hypogonadism in the older male has been controversial with some clinicians suggesting that symptoms should be treated without due reliance on testosterone concentrations. However, most professional bodies have proposed that a low testosterone concentration should be part of the diagnosis. This is, in turn, reliant on the testosterone measurement being reliable and read against an appropriate reference range. This review looks at the factors that can influence the interpretation of testosterone results for the ageing male.

Introduction

Statistics from the World Health Organization indicate that the life expectancy of populations in Europe and the USA has increased dramatically over the last 50 years and life expectancy at birth is estimated to increase by another 5 years between now and the year 2030 (Fig. 1). The proportion of the population that is over 65 years has almost doubled since 1950 and this is expected to increase by a further 10% by the year 2050. However, as life expectancy has increased so has the proportion of adult life spent either in a frail state or totally dependent on others.[1] This has a large cost implication for governments. Treatments that improve health and reduce the periods of frailty and dependency have benefits both to the individual and government finances. Testosterone (T) treatment may be one way to significantly improve the health of the ageing male[1,2] although some clinicians feel more studies are required to establish the benefits and define the risks.[3]

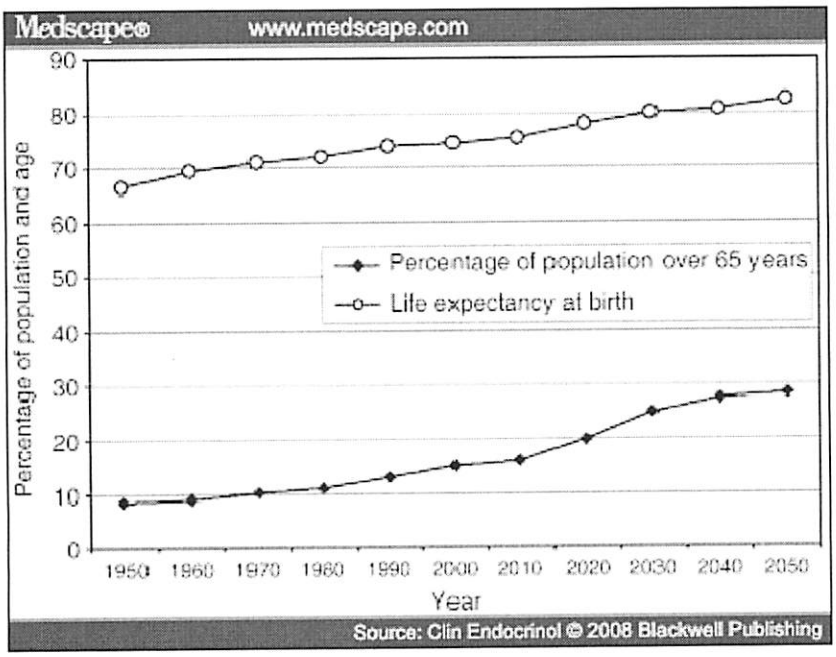


Figure 1. Trends in life expectancy and percentage of population over 65 years.

Vermeulen[4] highlights the similarities between the symptoms of hypogonadism in young males and the symptoms associated with ageing in the male but points out that the symptoms in the older male are multifactorial with concomitant decreases in GH and activity. Table 1 lists the clinical features of male hypogonadism and the commonly reported symptoms of the ageing male. There has been much debate and study of whether the symptoms in the ageing male are directly related to the declining levels of testosterone. Findings have been conflicting but as larger populations of subjects are studied more associations are being reported. For example more recent studies have shown a positive relation between bone density and bioavailable testosterone concentrations. Keles *et al.*[5] studied 174 males aged 22-76 years and found that log-free T was significantly associated with the bone mineral density (BMD) of the distal forearm, whereas the Minos Study[6] of 819 males aged 40-85 years found no association between T or calculated free T with bone morphology after adjustment of age, weight, height and lean body mass. Amory *et al.*[7] in their study of 70 males over the age of 65 years and with a T concentration of < 12·1 nmol/l, found that T replacement significantly increased BMD and Meier *et al.*[8] found testosterone to be independently associated with the risk of osteoporotic fracture in 609 males > 60 years. Behre *et al.*[9] also found that T replacement in hypogonadal males resulted in an increase in BMD, with the greatest increase occurring in the first year of therapy. However, one must not overlook the importance of the role of E_2 on BMD in males.[6] Cardiovascular disease is a major cause of death being approximately 50% of all deaths in developed countries with death rates increasing dramatically in old age.[10] Again studies of the relationship between cardiovascular disease and testosterone concentrations have been mixed.[11] There has been concern that exogenous androgen can in fact increase the risk of cardiovascular disease but this may be related, in part, to high doses of androgen replacement.[12] Haddard *et al.*[13] carried out a meta-analysis of randomized trials that assessed the effect of testosterone use on cardiovascular events. They concluded that the current evidence weakly supports the inference that testosterone use in males is not associated with important cardiovascular events. Rosano *et al.*[14] report lower testosterone was found in patients with coronary artery disease with testosterone concentrations being inversely related to the degree of coronary artery disease. Jankowska *et al.*[15] found that testosterone deficiency was an independent marker of poor prognosis in male patients with coronary heart failure. Shabsingh *et al.*[16] carried out a systematic literature search and concluded that there is insufficient evidence to support testosterone therapy in ageing males for the purpose of cardiovascular benefit. Nevertheless they do confirm that testosterone therapy improves insulin sensitivity, central obesity and lowers cholesterol and LDL. Similar findings were reported by Isidori *et al.*[17] from their meta-analysis of the effects of testosterone on body composition, lipid profiles and bone metabolism in middle aged males. An improvement in general well-being could also be an important benefit of testosterone replacement. There are several reports of testosterone therapy improving muscle strength and lean body mass as well as cognitive function and sexual improvement. Annewieke *et al.*[18] found a positive relationship between bioactive testosterone and muscle strength in 403 males aged 73-94 years and data from the Massachusetts Male Ageing Study[19] found that testosterone concentrations up to a critical level were positively correlated with muscle strength. More recently Schaap *et al.*[20] failed to find a correlation between total testosterone and free testosterone with muscle strength. Reports on the association between testosterone concentrations and cognitive function are also variable. In the Male Massachusetts Ageing Study[21] better cognitive functioning was associated with log free T concentrations, but this was lost in their adjusted model. Testosterone therapy for 3 months was found to improve spatial cognition but not in verbal and visual memory or mood.[22] Spatial and verbal memory was found to be improved with testosterone therapy in males aged 50-80 years in the study of Cherrier *et al.*[23]

Table 1. A Comparison of the Features of Male Hypogonadism and Presenting Symptoms in the Ageing Male

Medscape® www.medscape.com

Hypogonadism	Ageing male
Low libido	Decreased libido
Sexual dysfunction	Erectile dysfunction
Increased fat mass	Increased fat mass
Loss of muscle strength	Loss of muscle strength
Osteoporosis	Reduced bone mass
Reduced frequency of shaving	Depression
	Cognitive decline
	Increased cardiovascular disease
	Insulin resistance

Source: Clin Endocrinol © 2008 Blackwell Publishing

T replacement therapy is now much more acceptable to a wide range of males than in the past.[24] A few years ago the options were either injections of a testosterone ester every 1 or 2 weeks or implants of T pellets, often in the buttock. Alternative approaches now include T patches,[25] T gel[26] and buccal preparations.[27] There are also long acting injectable preparations of testosterone undecanoate that can give physiological concentrations of T for up to 3 months.[28,29] The newer preparations give a more consistent and physiological concentration of T without the large swings in concentration, seen with preparations such as testosterone enanthate, that in turn caused swings in mood and drive.[30,31] With more reports suggesting health benefits associated with T replacement and with the more convenient methods of T replacement treatment of hypogonadism in the elderly is likely to increase significantly. However, it should be noted that testosterone replacement is associated with a number of potential side-effects.[3] These include progression of prostate cancer and atherosclerotic heart disease, benign prostatic hyperplasia, sleep apnoea and breast enlargement. These side-effects are infrequent even when high doses of testosterone are given. The extent to which the newer replacement therapies, which maintain testosterone concentrations within the reference range, may be associated with these side-effects has not been fully investigated. In the future therefore it is likely there will be an increase in requests for testosterone measurement in older males, both to assess testosterone status in males complaining of sexual dysfunction, and in monitoring replacement.

Older males may present with erectile dysfunction and low libido that could be related to low T. The reliable investigation of T concentrations in these males depends on a number of different factors. In addition to the imprecision of the T measurement a number of other factors have to be taken into account. These include an accurate patient history,[31] time of sample collection, reliability of the collection and transport of the specimen, the accuracy and precision of the method used for T measurement and the appropriateness of the reference range. This review will discuss each of the technical parameters examining how they might affect the interpretation of the T result.

Diagnosis of Hypogonadism in the Older Male

There has been much debate about the diagnosis of hypogonadism in the older male and which males should be given T replacement therapy. This has led to the expert bodies defining the diagnosis of hypogonadism in the male but there are subtle differences between the groups. The Endocrine Society recommend that the diagnosis of androgen deficiency in males is made only in males with unequivocally low testosterone concentrations and have consistent symptoms and signs

associated with androgen deficiency. T replacement is only recommended in males with a low T concentration.[32] The American Society of Andrology also recommends T replacement in males only with a low T concentration. They include signs of testosterone deficiency in the diagnosis but allow that symptoms may be absent.[33] Both groups state that a testosterone concentration of < 10·4 nmol/l indicates androgen deficiency. The Canadian Society for the Study of the Ageing Male recognizes that there is a group of males that have hypogonadal symptoms that would benefit from testosterone therapy. They, however, state that there is no universal agreement on the criteria for diagnosis of hypogonadism in each case.[34] More controversially Carruthers[35] suggests that the symptoms are more important than the biochemistry suggesting that a male may be 'hypogonadal' at the tissue level while having a normal T level. Reduced arterial flow and pre-DHT receptor events are suggested.[36] Proper clinical trails are required to support these suggestions.

The main endocrine bodies, nevertheless, suggest that a testosterone result of < 10·4 nmol/l taken in the morning is suggestive of hypogonadism. This assumes that this is valid for all testosterone assays but quoted reference ranges can vary widely as will be discussed later. Testosterone methods have been criticized for their unreliability in both females[37] and males.[38] In their study of 124 young males, aged 21-35 years, Sikaris *et al.*[38] found significant differences between the results of different commercial assays for testosterone. The methods investigated showed poor agreement with gas chromatography mass spectrometry (GCMS) and there were large differences between the quoted reference ranges. They concluded that 'current commercial testosterone methods provide suboptimal assistance in confirming the clinical diagnosis of male reproductive disorders...' If the methods are suboptimal for the investigation of young males how less optimal are they when investigating androgen deficiency in older males? A single analytical result can be affected by physiology, concomitant drug therapies, the quality of the specimen and analytical effects. These factors will be discussed below. Another confounding factor is the SHBG concentration. This is elevated in the older male and can be increased in other clinical situations such as treatment with anticonvulsants and cirrhosis of the liver.[39,40] T concentrations therefore could be within the reference range, although the free testosterone could be low due to an elevated SHBG concentration. Although recommendations suggest that all low testosterone results should be confirmed with a repeat specimen they also suggest a repeat analysis on low normal results. However, patients on anticonvulsant therapy have T concentrations well within the normal range while the free T is low.[40]

Specimen Collection and Transport

Serum from clotted blood is acceptable for all methods for T measurement. Care must be taken when collecting into tubes containing anticoagulant. For example, Roche Diagnostics state that results from tubes containing sodium citrate need to be corrected by 10% and the Siemens Centaur assay (Siemens Medical Solutions Diagnostics) is only validated for serum. Having collected the blood in the correct tube, the specimen should be transported as quickly as possible to the laboratory. Delayed transport may lead to haemolysis that may make analysis impossible or unreliable. It has been reported that specimens left at room temperature for more than 24 h have an increased concentration of T.[41] This is a result of red cell enzymes converting androstenedione to T[42] and results from the author's laboratory showed that a delay of 48 h could increase results by up to 2 nmol/l.[43] An increase of that magnitude could cause a slightly subnormal result to become low normal. It was also shown that neither significant change in T concentration occurred over 6 h at room temperature nor in specimens stored for up to 48 h at 4 °C. This is line with the data in the Roche Diagnostics kit insert for T that states also that no change in result occurs over 48 h when the sample is stored at 4 °C.

Time of Collection

It is well established that T, like many steroids, shows a marked circadian rhythm in young males. It has often been reported that this is lost or very attenuated in older males.[44] More recently there have been reports that show that there is a clear T circadian rhythm in older males.[45,46] Figure 2 shows results from the author's laboratory for T concentration in specimens taken at 9·00-9·30 am and 4·00-4·30 pm for 10 young males aged 20-30 years and 10 older males aged 55-65 years. There is a clear circadian rhythm in all individuals. Figure 2 also demonstrates that at any age fit healthy males may have concentrations below the reference range (10·4-35 nmol/l) at 4·00 pm. The expert groups recommend that a sample for testosterone should be collected in the morning. This is not always practical as clinics may be in the afternoon. A male with a normal T concentration in an afternoon specimen usually may be considered to have normal T concentrations. If a

testosterone result for an afternoon specimen is below the reference range, a repeat analysis on an early morning specimen should always be carried out.

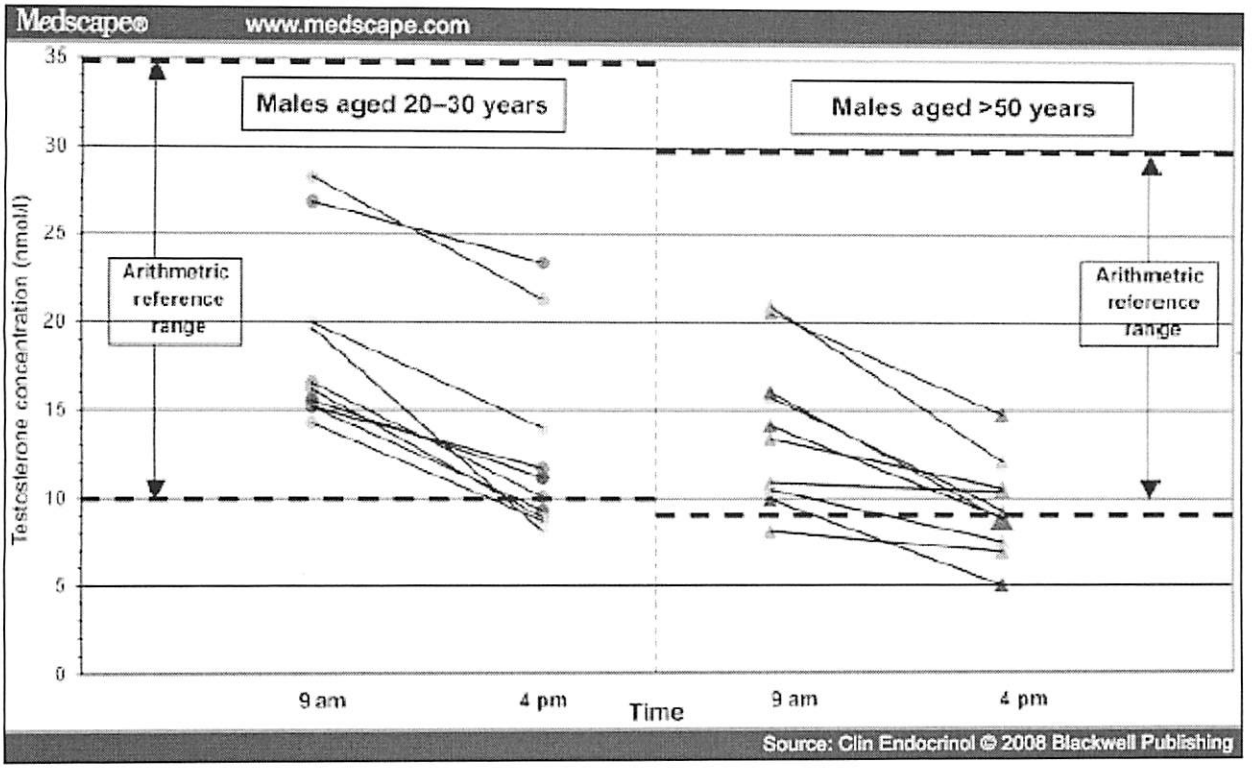


Figure 2. Testosterone concentrations at 9·00 am and 4·00 pm in young males aged 20-30 years and older males aged > 50 years.

Reference Ranges

The reliability of interpreting results also depends on the reliability of the reference range provided by the laboratory. Guidelines on the production of reference ranges have been published by the International Federation of Clinical Chemistry.[47-52] Laboratories may not find it practical to follow these guidelines. Collecting blood samples in the early morning to minimize the effects of circadian rhythm,[45,46] from 120 individuals,[51,53] and fulfilling all the exclusion criteria[48] is time consuming and the laboratory may not have the resources for such an exercise. On the other hand, a poorly derived reference range with wide limits will have low clinical sensitivity for the diagnosis of male hypogonadism.[51] It is also unclear what represents the normal reference range in older males. Vermeulen[4] suggests that the reference range for young males is valid for older males as he states there is insufficient data to suggest an altered testosterone requirement in older males. But his own research shows that testosterone, and particularly free testosterone concentrations fall with age. Figure 2 shows that of the 10 healthy elderly males two were below the reference range for young males with another two with borderline values. Would the investigation of androgen deficiency be justified in these males who have no clinical symptoms of hypogonadism? This could well be the conundrum if screening of older males is carried out.[31] Until more studies are carried out comparing the clinical use of reference ranges derived from both young and old males, the use of a reference range for young males must be seen as a convenience.

What are laboratories to do if they do not prepare their own reference ranges? Reference ranges supplied by manufacturers are only given as guidelines as stated in method inserts. Age and time of collection may not be supplied and exclusion criteria not given. The Siemens Coat-a-Count kit insert gives a T reference range split into males aged 20-49 and > 50 years. The data is collected from the Immulite and Immulite 2000 kit inserts and comprises 69 and 19 subjects, respectively. The company does not seem to have controlled for time of day. Roche Diagnostics quote a reference range for males, aged 18 years and over, of 9·9-27·8 nmol/l. Other studies reported in Roche's SHBG product information report a median total

testosterone of 14·1 nmol/l (95% range 4·51-28·7, *n* = 102) in males aged 17-40 years and a median total testosterone for older males of 13·2 nmol/l (95% range 4·69-20·5, *n* = 41). These males were apparently healthy with no signs of abnormal androgen status, no obesity or intake of steroid or thyroid drugs. No information was given about the time of sampling. Taieb *et al.*[54] compared the testosterone results for 116 samples (50 males, 55 females and 11 children) measured by 10 different commercial kits with results obtained by isotope dilution mass spectrometry (IDMS). They found that commercial assays usually underestimated the T concentration in males when compared to IDMS. In this paper they quoted the reference ranges provided by the manufacturers. Figure 3 has been prepared from these data and shows that there are large discrepancies between companies for the bottom and top of the reference range. Using the manufacturer's quoted data, the lowest concentration for the bottom of the male reference range is 2·9 nmol/l for the Vitros ECi method and the highest concentration is 59·98 nmol/l for the Immulite 2000 method. The mean for all the methods gives a reference range of 8·0-39·1 nmol/l while the IDMS method gave a reference range of 10·4-32·0 nmol/l. Considering the investigation of androgen deficiency a concentration of 8 nmol/l would be normal for four kits and low for six kits. This can, at best, make interpretation of data confusing and at worse misleading. The differences cannot be explained by bias as the Immulite and Vitros ECi have a similar bias to the ID-MS method.[54] Eight kits report normal values below the recommended cut-off value of 10·4 nmol/l. Laboratories should not use the reference range supplied with commercial methods.

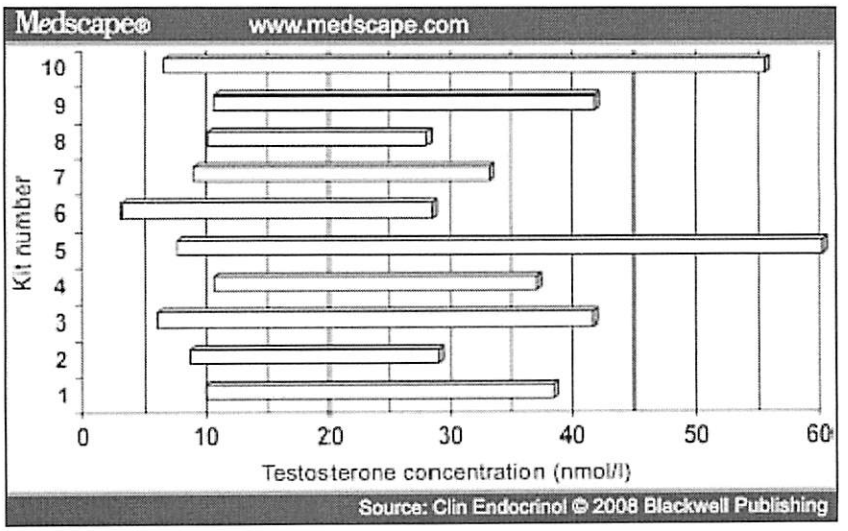


Figure 3. Reference ranges provided by kit manufacturers showing the great variation in quoted for normal testosterone *concentrations*. Adapted from Ref 54.

The situation is even more complicated. Few biological distributions are normally distributed and this is true of testosterone concentrations. Most reference ranges quoted by companies and laboratories are derived arithmetically assuming a normal distribution. Sikaris *et al.*[38] measured the T concentration in 124 normal males aged between 21 and 35 years by seven different commercial kits. They examined the changes of testosterone reference ranges when arithmetric, geometric and nonparametric statistics were applied to the data, the latter two parameters correcting for skewness of the data. Figure 4 has been prepared from their data to graphically illustrate the observations they made. These were (i) The arithmetic range from their data has a higher lower limit than each of the company's quoted range and (ii) this lower limit is even higher (by about 2 nmol/l, 0·58 ng/ml) if a geometric or nonparametric range is calculated. They found that the bottom of the reference range was 7·5-12·7 nmol/l (geometric) and 7·3-12·6 nmol/l (nonparametric) which compared closely to the GC/MS lower limit of 10·4 nmol/l. This suggests that not only is the quoted reference range in kit inserts inaccurate but that most reference ranges quoted by laboratories are also too low. This makes them less sensitive in detecting male hypogonadism.

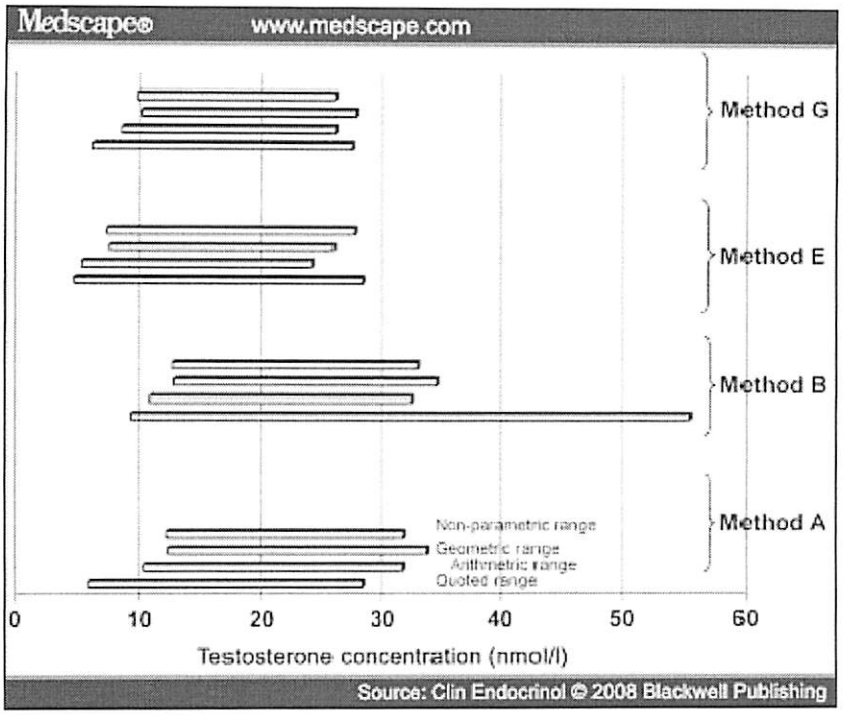


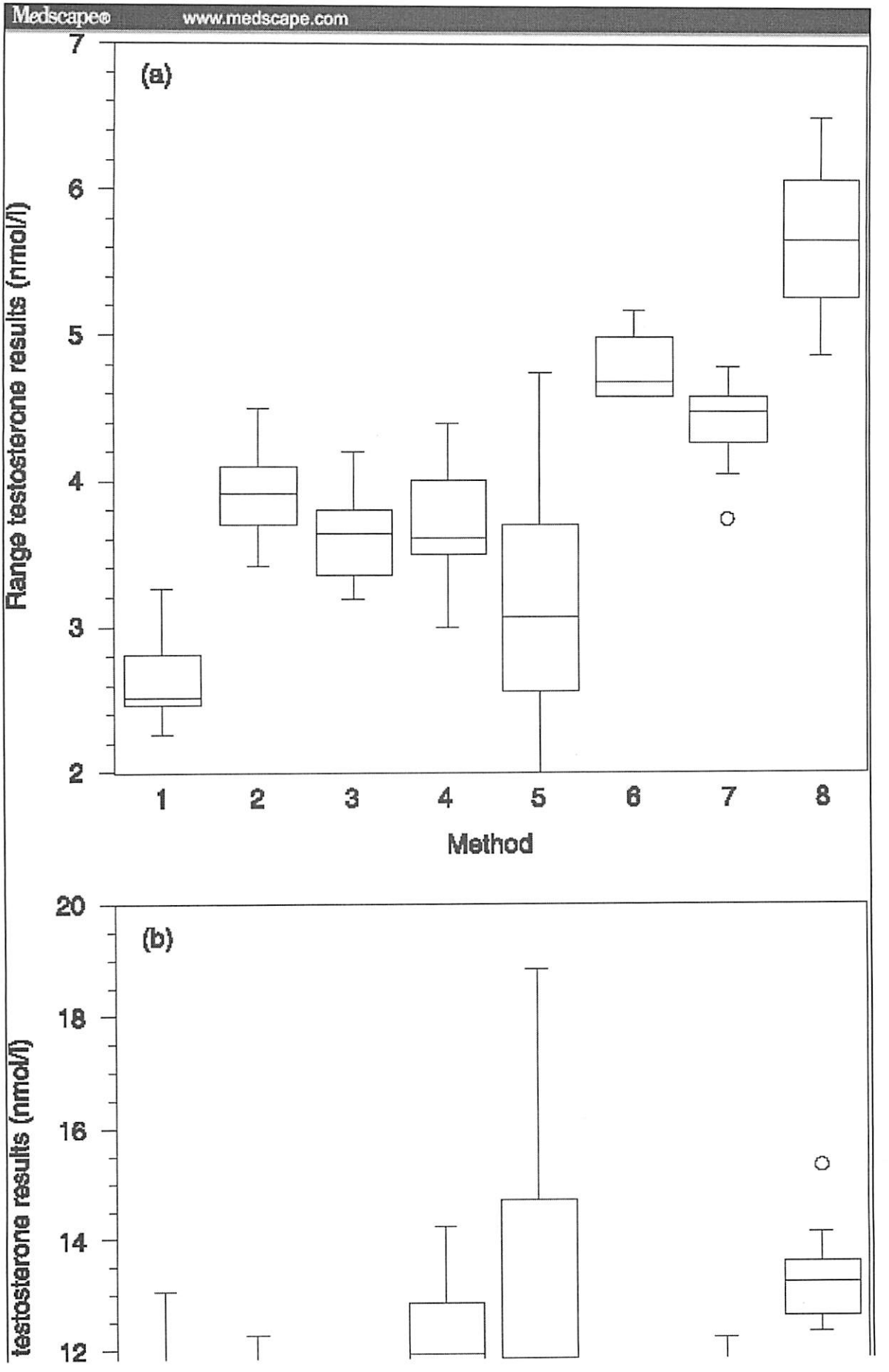
Figure 4. The effect of using different statistical approaches in establishing reference ranges. Adapted from Ref 55.

The reference range in the author's laboratory, calculated as the mean ± 2SD, was 10-35 nmol/l (2·88-10·1 ng/ml) for younger males and for older males 9-30 nmol/l (2·59-8·64 ng/ml). In Fig. 2 one elderly male is below the age-related reference but using the range for young males, two further older males are borderline normal. The data of Sikaris *et al.*[38] suggests that, if the data had been log transformed the lower limit of our reference ranges would be about 2 nmol/l (0·58 ng/ml) higher, that is, 12 nmol/l for younger males. Now four older males would be below the normal range. These data show that, using the appropriate statistics, more older males are diagnosed as hypogonadal. Also more older males will be diagnosed with hypogonadism if the reference range for young males is used. This might go someway to explaining the confusion over the diagnosis of hypogonadism in the older male. Boyce *et al.*,[55] however, suggest that published ranges are in fact too high. They used a commercial assay to measure T and carried out a power transformation of their data to give a normal distribution. They found a lower limit of normal of 10·07 nmol/l for males < 40 years and 7·41 nmol/l for males > 40 years. We suggest that an age-related reference range should be used for older males if the reference range is prepared from aged males, that serum is collected in the early morning and the data is corrected for skewness.

Bias and Precision of Methods

Wang *et al.*,[56] using five commercial kits, measured total testosterone in 62 eugonadal and 60 hypogonadal males. Results were compared with those from liquid chromatography tandem mass spectrometry (LC-MSMS). They suggested only three of these methods were sufficiently reliable to be used for the investigation of hypogonadism in the male. Their data are similar to those reported by Taieb *et al.*[54] Recent comparative studies are based on regression analysis rather than examining individual results. Recently an evaluation has been carried out of all the testosterone kits available in the UK. This work was carried out for the Procurement and Supplies Agency of the Department of Health and may be found on the PASA website URL: http://www.pasa.nhs.uk/evaluation/publications/per/clinical_biochemistry.asp.[57,58] Four serum specimens were sent out to two laboratories for each commercial T method. The laboratories were chosen by the diagnostic company. The four serum specimens were analysed four times over 5 days according to CLSI protocol EP15-A.[59] Figure 5 shows the results for the two pools with concentrations at and slightly below the bottom of the male reference range. The data show that there is approximately a 3 nmol/l (0·86 ng/ml) and 4 nmol/l (1·15 ng/ml) difference between the medians across the kits for the two pools, respectively. Also some kits produced results that were below or in the male reference range across the course of the study. The earlier DPC (now Siemens Medical Solutions Diagnostics) kit had particularly poor performance but

the current kit has a much improved performance. The result of the new configuration resulted in an increased concentration of the median by about 2 nmol/l (0·58 ng/ml). This has not been reflected in the reference range data in the method insert nor is the bias of one kit against another reflected in the reference ranges provided for each kit. Over the course of the study the variation of results for these two pools by each method was at least 2 nmol/l (0·58 ng/ml) and often 4 nmol/l (1·15 ng/ml). In any assay therefore a result for a sample could be 1-2 nmol/l (0·29-0·58 ng/ml) below the reference range on one day but within the range by the same amount on another day. This is the amount of variation due to assay variation and does not take into account the additional variation between samples in the same individual due to the episodic secretion of T. Episodic secretion of T has been reported as causing a change in concentration in the male of between 10% and 20%[60,61] so that a single sample will always have this amount of uncertainty. It is advisable therefore, as recommended by the Endocrine Society[31] to repeat any sample that is either below the reference range or is borderline.


Medscape®
www.medscape.com
(a)
Range testosterone results (nmol/l)
7
6
5
4
3
2
1
2
3
4
5
6
7
8
Method
(b)
testosterone results (nmol/l)
20
18
16
14
12

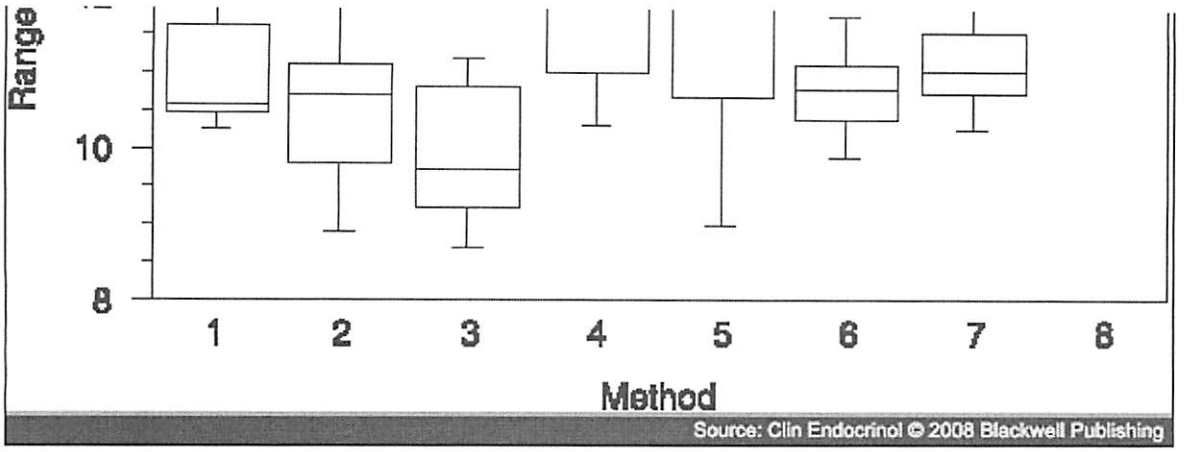


Figure 5. Median and range of results, using NCCLS protocol EP 15-A, of serum samples sent to two laboratories for each of eight different automated testosterone methods: (a) concentration of testosterone between normal female and male concentrations (hypogonadal male range) and (b) testosterone concentrations at lower normal male concentration. (See ref 58 for details; reprinted with permission).

Interference

It has been known for many years that some patient samples give spuriously high results when measured by some direct commercial assays.[62] Identification of a cross-reacting substance has been long in coming although, because solvent extraction of such samples can significantly lower the testosterone result, the interference appears to come from a water soluble substance such as steroid conjugate. The only substance to date that has been identified as a cross-reactant is dehydroepiandrosterone sulphate[63,64] but at physiological concentrations this does not appear to affect results for male serum. There has, however, been a lot of focus on the use of DHEA as a dietary supplement.[65] In some countries, such as the USA, DHEA is readily available from health shops as a dietary supplement. In a study of 140 elderly males a dose of 25 mg was shown to increase DHEAS concentrations to 9·73 ± 5·44 μmol/l.[66] Doses of 50 and 100 mg DHEA resulted in mean increases of approximately 15 and 20 μmol/l.[65] There is no recommended dose so that 50 mg and even 100 mg may be taken as supplement. Even at 100 mg dose DHEA has no significant effect on normal testosterone concentrations[67] but the resulting increase in DHEAS concentrations may be sufficient to increase a slightly subnormal testosterone concentration into the normal range due to the cross-reaction of the antiserum in the testosterone assay. It is recommended that any patient who has been taking DHEA as a supplement should stop taking their capsules before testosterone is estimated.

Bioavailable Testosterone

T circulates in blood bound to SHBG and albumin. The nonprotein fraction of T is usually known as free T. The proportions reported vary between papers according to the methods used. Swinkel *et al.*[68] reported free testosterone in males as1·7 ± 0·52% when using both equilibrium dialysis and ultrafiltration with a total testosterone RIA employing a prepurification by chromatography. Using a commercial automated T method, ammonium precipitation to measure non-SHBG bound T and calculated free T, Diver *et al.*[46] report mesor values for free T and non-SHBG T of 393·47 ± 44·62 pmol/(equivalent to 2·51 ± 0·28%) and 3·66 ± 0·19 (equivalent to 23·3 ± 1·2%) in elderly males, respectively. Methods to measure free testosterone include direct measurement, by using a formula or ratio or by using a commercial kit. There has been discussion over a number of years whether the free testosterone is the main portion available to the tissues.[69-71] It has been proposed that the testosterone bound to albumin is also available to tissues as binding is of low avidity.[69,72] Simple biochemical methods have been developed to measure this non-SHBG fraction of T.[18,73] This fraction can also be determined by calculation.[74-76] Although some use the term bioavailable or bio-active for non-SHBG bound T[45] to save confusion it is best used as a generic term for all of the above[18] and free T, calculated free T and non-SHBG bound T for the different fractions.[77] All, but the direct measurement by commercial assay of free T, require total T for their determination and therefore are affected by the errors of T measurement.

Free Testosterone

Because the SHBG concentration increases with age a low normal T may be associated with a free T below the reference range in older males. Free T may be measured directly using equilibrium dialysis,[78] ultrafiltration[79] or steady state gel filtration.[80,81] All these methods are lengthy, require a high degree of skill and are unsuited to routine use and hence to the routine investigation of hypogonadism in the elderly. The direct measurement by equilibrium dialysis has been used as the reference method in studies of the different methods for bioactive T.[82]

Calculated Free T

Indirect mathematical methods, using routine methods for T, SHBG and albumin in particular, have been introduced to provide an estimate of the free T concentration. More complex calculations, that take into consideration the binding affinities of the binding proteins for T, have been produced[82,83] and are the most commonly used. These methods require the measurement of albumin and, in some formulae cortisol-binding globulin, as well as T and SHBG and they depend on the relevant association constants being known. However, reported association constants of SHBG for testosterone vary.[82-84] Vermeulen *et al.*[82] examined the effect of albumin concentrations of 40, 45 and 50 g/l on the calculation of testosterone when using an association constant for albumin of $3{\cdot}6 \times 10^{[4]}$ l/mol. They found small unimportant changes in the free calculated free testosterone result. They subsequently used a constant albumin concentration of 43 g/l in their calculation. Others have used the relationship between measured free T, using equilibrium dialysis or steady state gel filtration, and SHBG to derive much simpler calculations.[84-87]

Free Androgen Index (FAI)

Others have examined the clinical efficacy of a simple ratio of testosterone and SHBG concentrations (commonly called the free androgen index) as an indicator of the free testosterone level[85,88] and have compared this measurement with other measures of bio-available T.[18] Vermeulen et al.[82] have shown that this method has a high correlation with equilibrium dialysis ($r = 0{\cdot}848$) but the relationship is variable and they concluded that the FAI is an unreliable index of bioavailable T.

Commercial Kits

Commercial kits are also available for the measurement of free T. These are direct analogue methods that do not rely on the measurement of T. Vermeulen *et al.*[82] compared the results of the Coat-a-Count (Siemens Medical Solutions Diagnostics) analogue RIA kit with equilibrium dialysis and found that although the methods had a high correlation of $r = 0{\cdot}937$ the kit gave about 20% the result from equilibrium dialysis. Others have found similar findings[89] and its use in research has been severely criticized.[90] More recently Fritz *et al.*[91] have further examined this kit using equilibrium dialysis and in preparations of altered protein and SHBG concentrations. They concluded that the results from this kit were related to T concentrations and not to free T. Martinez-Jabaloyas *et al.*[92] compared the enzyme analogue immunoassay for direct free testosterone measurement from Diagnostic Systems Laboratories, TX, with calculated free testosterone using the method of Vermeulen *et al.*[82] In a study of 133 males they found poor correlation between the two methods with the EIA giving about 10% the value of the calculated free testosterone.

Non-SHBG Bound T

As more research suggested that the T bound to albumin might be available to tissues simple methods have been developed to measure this fraction of T in serum.[72,93] Current methods are based on these earlier methods with minor modifications although the method of Dechaud *et al.*[94] used ether extraction and Celite purification steps. In outline after incubation of serum with a tracer amount of tritiated T, SHBG-bound T is precipitated with cold saturated ammonium sulphate in a 1 : 1 ratio. The radioactivity is counted in the supernatant to determine the amount of T not bound to SHBG. Although the method is simple it is less suited for routine clinical investigations. It requires setting up a separate nonautomated assay that requires β scintillation counting; only the larger teaching hospitals are likely to have liquid scintillation equipment. Vermeulen *et al.*,[82] in their comparison of T methods, found good correlation between the non-SHBG T and FT determined by equilibrium dialysis with $r = 0{\cdot}974$. The non-SHBG result was about 20 times the FT result.

Therefore, from the studies of Vermeulen *et al.*[82] the FAI and the DPC commercial kit direct T method are poor indicators of bioavailable T. Both non-SHBG bound testosterone and calculated free testosterone methods have good agreement with equilibrium dialysis and are simpler measures of bioavailable T than measuring free T by the direct methods such as equilibrium dialysis. As the non-SHBG methods are nonautomated, calculated free T would appear to be the most convenient method as a measure of bioavailable T for the routine clinical laboratory as it requires only T and SHBG measurement; both are widely available on automated immunoassay analysers. The Vermeulen calculation is readily available on the website of the International Society for the Study of the Ageing Male (ISSAM).

The general availability of this calculation means that it could be used without consideration of the methods used. However, as has been shown previously, the different testosterone methods can differ by 20-25%. No study, similar to the one carried out by the GMEC evaluation study, has been carried out for SHBG. A comparison of the method means for SHBG on the UK NEQAS shows about a 10% difference between methods. Using the Vermeulen calculation[82] a 10% variation in the SHBG result at 10 nmol/l results in a 5·5% variation of the calculated free testosterone; at 50 nmol/l it is 10·47%. On the other hand, a 10% variation in the testosterone result at 10 nmol/l results in a 21·8% variation in the free calculated free testosterone. Therefore the measurement of T is the most critical in the calculation of free T and one can expect more than a 25% difference across the different methods. This calculation should be used to calculate free T concentration in clinical samples only after a reference range has been set up for normal subjects with samples collected in the early morning.

In addition different methods have been used to derive the different formulae for free T calculation and it would be not surprising to find that there are significant differences between results when using different calculations. Ho *et al.*[95] compared four calculation methods for free T with the FAI. They found that mean biases from the Vermeulen calculation on the ISSAM website ranged from 5·8-56·0%. Ronde *et al.*[96] report similar findings although they found larger differences between the Vermeulen and Sodergard calculations. They concluded that without in-house validation, the indiscriminate use of algorithms is highly questionable.

Conclusion

Although Sikaris *et al.*[38] felt that current commercial T assays were suboptimal for the investigation of male reproductive disorders this is the main biochemical measurement that clinicians use in the investigation of hypogonadism. Imprecision and inaccuracy of assays has to be taken into account when interpreting results but these are out of the control of the clinician and to some extent the laboratory. There are some factors that are within the control of the clinician that will add to the reliability of a T result. These include collection of the specimen into the correct blood tube, quick transfer of the specimen to the laboratory[41,43] and collection of a morning sample.[45,46] It is advisable to ask the patient to stop any dietary supplements particularly DHEA[64,65] and to be aware of the affect of anticonvulsant therapy on SHBG levels.[40] Interpretation of the final result will depend on having a reliable reference range against which to compare the result. This is the responsibility of the laboratory which in turn may need the help of clinicians in collecting appropriate samples. Reference ranges will vary because of the differences that exist between methods[38,56-58] and this makes the suggestion of a fixed concentration such as 10·4 nmol/l,[32] less helpful. It is suggested that a reference range for young males should be used for the older male.[4] It is unclear what this suggestion is based on. Because older males without symptoms of hypogonadism have a lower reference range a proportion of these males would be found to have a low T level when compared to the reference range for young males. Therefore in any screening programme of the older male or any health checks males may be unnecessarily investigated for hypogonadism. Whatever reference range the clinician uses a normal T result on a single morning sample suggests normal gonadal function as long as liver function is normal and the patient is not on anticonvulsant therapy. If a sample is taken in the afternoon and a low T result is obtained the patient should be retested with a morning sample. Results just below the reference range, borderline results and all patients on anticonvulsant therapy should have T measured with SHBG so that a free or non-SHBG T can be determined. Calculation of free T using Vermeulen's formula,[82] as it is readily available on the web and is widely used, seems the most convenient method for the laboratory. The FAI nor the commercial kits for direct T measurement should be used based on current research.[82,89-91] No matter which formula is used the reference range for the methods used in the laboratory should be established.[94,95] Although establishing a reliable reference range for any analyte is difficult combining normal data with a laboratory using the same methods can increase the database. This is a feasible option these days with fewer automated analysers being used across laboratories. Table 2 provides

guidelines for controlling factors of sample and patient preparation and interpretation of results, so that the clinician can have more confidence in making their diagnosis. The correct reference range to use in elderly males is contentious and requires more research into the relationship between T concentrations, symptoms of hypogonadism and response to therapy in the older male.

Table 2. Suggested Steps in Helping to Provide a Testosterone Result That is as Reliable as Possible for the Diagnosis of Hypogonadism in the Older Male

Medscape®	www.medscape.com
Patient preparation	Stop any DHEA therapy
Pre-analytical steps	If the patient is on anticonvulsants (other than valproate) measure SHBG as well as T so that free T can be calculated. Check liver function as well as any other biochemistries as examination suggests. Collect blood into a 'serum' blood tube. If a blood tube containing anticoagulant is used check with the laboratory that their r have been validated for this specimen. Collect blood between 8 am and 10 am if possible. Send specimen to lab as soon as possible. If delayed keep in fridge until sent.
Laboratory	Be clear which blood specimens have been validated for the methods used. Use only in-house established reference ranges for the methods used. It is suggested 120 samples should be used. Collaborat other labs using same methods to increase database. Check with clinicians to se if they wish to use a reference range for young men or older men. Establish reference range for free T or non-SHBG T as preferred by clinician.
Result interpretation	A result well into the normal range suggests normal gonadal function. Repeat a low afternoon sample using a sample at 9 am. Repeat borderline results, T with SHBG for free T calculation.
	Source: Clin Endocrinol © 2008 Blackwell P

References

1. Lunenfeld, B. (1999) Hormone replacement therapy in the aging male. *The Aging Male*, 2, 1-5.
2. Lunenfeld, B., Gooren, L.J.G., Morales, A. & Morley, J.E., (eds) (2007) *Textbook of Men's Health and Aging*. Informa Healthcare UK Ltd, Andover, UK.
3. Bhasin, S. & Buckwalter, J.G. (2001) Testosterone supplementation in older men: a rational idea whose time has not yet come. *Journal of Andrology*, 22, 718-731.
4. Vermeulen, A. (2001) Androgen replacement therapy in the aging male - a critical evaluation. *Journal of Clinical Endocrinology and Metabolism*, 86, 2380-2390.
5. Keles, K., Aydin, G., Basar, M.M., Hayran, M., Atalar, E., Orkun, S. & Batislam, E. (2006) Endogenous sex steroids and bone mineral density in healthy men. *Joint, Bone, Spine*, 73, 80-85.
6. Szulc, P., Uusi-Rasi, K., Claustrat, B., Marchand, F., Beck, T.J. & Delmas, P.D. (2004) Role of sex steroids in the regulation of bone morphology in men. The MINOS study. *Osteoporosis Internal*, 15, 909-917.
7. Amory, J.K., Watts, N.B., Easley, K.A., Sutton, P.R., Anawalt, B.D., Matsumoto, A.M., Bremner, W.J. & Tenover, J.L. (2004) Exogenous testosterone or testosterone with finasteride increases bone mineral density in older men with low serum testosterone. *Journal of Clinical Endocrinology and Metabolism*, 89, 503-510.
8. Meier, C., Nguyen, T.V., Handelsman, D.J., Schindler, C., Kushnir, M.M., Rockwood, A.L., Meikle, A.W., Center, J.R., Eisman, J.A. & Seibel, M.J. (2008) Endogenous sex hormones and incident fracture risk in older men: The Dubbo Osteoporosis Epidemiology Study. *Archives of Internal Medicine*, 168, 47-54.
9. Behre, H.M., Kliesch, S., Leifke, E., Link, T.M. & Nieschlag, E. (1997) Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. *Journal of Clinical Endocrinology and Metabolism*, 82, 2386-2390.

10. Lunenfeld, B. (2007) Aging men-the challenge ahead. In: B. Lunenfeld, L.J.G. Gooren, A. Morales & J. E. Morley eds. *Textbook of Men's Health and Aging*. Informa Healthcare, Andover, UK, pp. 465-471.
11. von Eckardstein, A. & Wu, F.C.W. (2003) Testosterone and atherosclerosis. *Growth Hormone and IGF Research*, 13, S72-S84.
12. Crook, D. (2007) Atherosclerotic risk assessment of androgen therapy in aging men. In: B. Lunenfeld, L.J.G. Gooren, A. Morales & J. E. Morley eds. *Textbook of Men's Health and Aging*. Informa Healthcare, Andover, UK, pp. 465-471.
13. Haddard, R.M., Kennedy, C.C., Caples, S.M., Tracz, M.J., Bolona, E.R., Sideras, K., Uraga, M.V., Erwin, P.J. & Montori, V.M. (2007) Testosterone and cardiovascular risk in men: a systematic review and meta-analysis of randomized placbo-controlled trials. *Mayo Clinic Proceedings*, 82, 29-39.
14. Rosano, G.M.C., Sheiban, I., Massaro, R., Pagnotta, P., Marazzi, G., Vitale, C., Mercuro, G., Volterrani, M., Aversa, A. & Fini, M. (2007) Low testosterone levels are associated with coronary artery disease in male patients with angina. *International Journal of Impotence Research*, 19, 176-182.
15. Jankowska, E.A., Biel, B., Majda, J., Szklarska, A., Lopuszanska, M., Medras, M., Anker, S.D., Banasiak, W., Poole-Wilson, P.A. & Ponikowski, P. (2006) Anabolic deficiency in men with chronic heart failure: Prevalence and detrimental impact on survival. *Circulation*, 114, 1829-1837.
16. Shabsingh, R., Katz, M., Yan, G. & Makhsida, N. (2005) Cardiovascular issues in hypogonadism and testosterone therapy. *American Journal of Cardiology*, 96 (Suppl.) 67M-72M.
17. Isidori, A.M., Giannetta, E., Gianfrilli, D., Greco, E.A., Bonifacio, V., Aversa, A., Isidori, A., Fabbri, A. & Lenzi, A. (2005) Effects of testosterone on sexual function in men: results of a meta-analysis. *Clinical Endocrinology*, 63, 381-394.
18. Annewieke, W., van den Beld, A.W., de Jong, F.H., Grobbee, D.E., Pols, H.A.P. & Lamberts, S.W.J. (2000) Measures of bioavailable serum testosterone and estradiol and their relationship with muscle strength, bone density, and body composition in elderly men. *Journal of Clinical Endocrinology and Metabolism*, 85, 3276-3282.
19. O'Donnell, A.B., Travison, T.G., Harris, S.S., Tenover, J.L. & McKinlay, J.B. (2006) Testosterone, dehydroepiandrosterone, and physical performance in older men: Results from the Massachusetts Male Aging Study. *Journal of Clinical Endocrinology and Metabolism*, 91, 425-431.
20. Schaap, L.A., Pluijm, S.M.F., Deeg, D.J.H., Penninx, B.W., Nicklas, B.J., Lips, P., Harris, T.B., Newman, A.B., Kritchevsky, S.B., Cauley, J.A., Goodpaster, B.H., Tylavsky, F.A., Yaffe, K. & Visser, M. (2008) Low testosterone bevels and decline in physical performance and muscle strength in older men: findings from two prospective cohort studies. *Clinical Endocrinology*, 68, 42-50.
21. Fonda, S.J., Bertrand, R., O'Donnell, A., Longcope, C. & McKinlay, J.B. (2005) Age, hormones, and cognitive functioning among middle-aged and elderly men: Cross-sectional evidence from the Massachusetts male aging study. *Journals of Gerontology Series A - Biological Sciences and Medical Sciences*, 60, 385-390.
22. Janowsky, J.S., Oviatt, S.K. & Orwoll, E.S. (1994) Testosterone influences spatial cognition in older men. *Behavioural Neuroscience*, 108, 325-332.
23. Cherrier, M.M., Asthana, S., Plymate, S., Baker, L., Matsumoto, A.M., Peskind, E., Raskind, M.A., Brodkin, K., Bremner, W., Petrova, A., LaTendresse, S. & Craft, S. (2001) Testosterone supplementation improves spatial and verbal memory in healthy older men. *Neurology*, 57, 80-88.
24. Nieschlag, E. (2006) Testosterone treatment comes of age: new options for hypogonadal men. *Clinical Endocrinology*, 65, 275-281.
25. Dobs, A.S., Meikle, A.W., Arver, S., Sanders, S.W., Caramelli, K.E. & Mazer, N.A. (1999) Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. *Journal of Clinical Endocrinology and Metabolism*, 84, 3469-3478.
26. Swerdloff, R.S., Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A.M., Snyder, P.J., Weber, T.M., Lonstreth, J. & Berman, N. (2000) Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. *Journal of Clinical Endocrinology and Metabolism*, 85, 4500-4510.
27. Salehian, B., Wang, C., Cunningham, G., Davidson, T., Mcdonald, V., Berman, N., Dudley, R.E., Ziel, F. & Swerdloff, R.S. (1995) Pharmacokinetics, bioefficiency, and safety of sublingual testosterone cyclodextrin in hypogonadal men:

comparison to testosterone enanthate: a clinical research centre trial. *Journal of Clinical Endocrinology and Metabolism*, 80, 3567-3575.

28. von Eckardstein, S. & Nieschlag, E. (2002) Treatment of male hypogonadism with testosterone undecanoate injected at extended intervals of 12 weeks: a phase II study. *Journal of Andrology*, 23, 419-425.
29. Schubert, T., Minneman, T., Hubler, D., Rouskova, D., Christoph, A., Oettel, M., Ernst, M., Mellinger, U., Krone, W. & Jockenhovel, F. (2004) Intramuscular testosterone undecanoate: pharmacokinetic aspects of a novel long-term treatment of men with hypogonadism. *Journal of Clinical Endocrinology and Metabolism*, 89, 5429-5434.
30. Findlay, J.C., Place, V. & Snyder, P.J. (1989) Treatment of primary hypogonadism in men by the transdermal administration of testosterone. *Journal of Clinical Endocrinology and Metabolism*, 68, 369-373.
31. Gooren, L.J.G., Morales, A. & Lunenfeld, B. (2007) Screening of the aging male. In: B. Lunenfeld, L.J.G. Gooren, A. Morales, & J.E. Morley eds. *Textbook of Men's Health and Aging*. Informa Healthcare UK Ltd, Andover, UK, pp. 63-95.
32. Bhasin, S., Cunningham, G.R., Hayes, F.J., Matsumoto, A.M., Snyder, P.J., Swerdloff, R.S. & Montori, V.M. (2006) Testosterone therapy in adult men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism*, 91, 1995-2010.
33. Anonymous (2006) Testosterone replacement therapy for male aging: ASA position statement. *Journal of Andrology*, 27, 133-134.
34. Bain, J., Brock, G. & Kuzmarov, I. (2007) Canadian Society for the Study of the Aging Male: response to Health Canada's position paper on testosterone treatment. *Journal of Sexual Medicine*, 4, 558-566.
35. Carruthers, M. (2004) *Androgen Deficiency in the Adult Male: Causes, Diagnosis and Treatment*. Taylor & Francis, London.
36. Carruthers, M. (2008) The paradox dividing testosterone deficiency symptoms and androgen assays: a closer look at the cellular and molecular mechanisms of androgen action. *Journal of Sexual Medicine*, 5, 998-1012.
37. Herold, D.A. & Fitzgerald, R.L. (2003) Immunoassays for testosterone in women: better than a guess? *Clinical Chemistry*, 49, 1250-1251.
38. Sikaris, K., McLachlin, R.I., Kazlauskas, R., de Kretser, D., Holden, C.A., Handelsman, D.J., (2005) Reproductive hormone reference intervals for healthy fertile young men: Evaluation of automated platform assays. *Journal of Clinical Endocrinology and Metabolism*, 90, 5928-5936.
39. Anderson, D.C. (1974) Sex-hormone binding globulin. *Clinical Endocrinology*, 3, 69-96.
40. Toone, B.K., Wheeler, M. & Fenwick, P.B.C. (1980) Sex hormone changes in male epileptics. *Clinical Endocrinology*, 12, 391-395.
41. Hammer, E.J. & Astley, J.P. (1985) Increase in serum testosterone following contact with blood-cells. *Annals of Clinical Biochemistry*, 22, 539-540.
42. van der Molen, H.J. (1968) Interconversion of progesterone and 20-alpha-dihydroprogesterone and of androstenedione and testosterone *in vitro* by blood and erythrocytes. *Acta Endocrinologica*, 58, 419-444.
43. Wheeler, M.J. (2003) Factors that effect the interpretation of testosterone results. *CPD Clinical Biochemistry*, 2003, 80-85.
44. Bremner, W.J., Vitiello, M.V. & Prinz, P.N. (1983) Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. *Journal of Clinical Endocrinology and Metabolism*, 56, 1278-1281.
45. Plymate, S.R., Tenover, J.S. & Bremner, W.J. (1989) Circadian variation in testosterone, sex hormone-binding globulin and calculated non-sex hormone-binding globulin bound testosterone in healthy young and elderly men. *Journal of Andrology*, 10, 366-371.
46. Diver, M.J., Imtiaz, K.E., Ahmad, A.M., Vora, J.P. & Fraser, W.D. (2003) Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clinical Endocrinology*, 58, 710-717.
47. Solberg, H.E. (1986) Approved recommendation (1986) on the theory of reference values. 1. The concept of reference values. *Clinica Chimica Acta*, 165, 111-118.
48. Petitclerc, C. & Solberg, H.E. (1987) Approved recommendation (1987) on the theory of reference values. 2. Selection of individuals for the production of reference values. *Clinica Chimica Acta*, 170, S1-S11.

49. Solberg, H.E. & Petitclerc, C. (1988) Approved recommendation (1988) on the theory of reference values. 3. Preparation of individuals and collection of specimens for the production of reference values. *Clinica Chimica Acta*, 177, S3-S11.
50. Solberg, H.E. & Stamm, D. (1991) Approved recommendation on the theory of reference values. 4. Control of analytical variation in production, transfer and application of reference values. *Clinica Chimica Acta*, 29, 531-535.
51. Solberg, H.E. (1987) Approved recommendation (1987) on the theory of reference values. 5. Statistical treatment of collected reference values - determination of reference limits. *Clinica Chimica Acta*, 170, S13-S32.
52. Dybkaer, R. & Solberg, H.E. (1987) Approved recommendation (1987) on the theory of reference values. 6. Presentation of observed values related to reference values. *Clinica Chimica Acta*, 170, S33-S41.
53. Reed, A.H., Henry, R.J. & Mason, W.B. (1971) Influence of statistical method used on the resulting estimate of reference range. *Clinical Chemistry*, 17, 275-284.
54. Taieb, J., Mathian, B., Millot, F., Patricot, M., Mathieu, E., Queyrel, N., Lacroix, I., Somma-Delpero, C. & Boudou, P. (2003) Testosterone measurement by 10 immunoassays and isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women and children. *Clinical Chemistry*, 49, 1381-1395.
55. Boyce, M.J., Baisley, K.J., Clark, E.V. & Warrington, S.J. (2004) Are published normal ranges of serum testosterone too high? Results of a cross-sectional survey of serum testosterone and luteinizing hormone in elderly men. *British Journal of Urology International*, 94, 881-885.
56. Wang, C., Catlin, D.H., Demers, L.M., Starcevic, B. & Swerdloff, R.S. (2004) Measurement of total serum testosterone in adult men: Comparison of current laboratory methods *versus* liquid chromatography-tandem mass spectrometry. *Journal of Clinical Endocrinology and Metabolism*, 89, 534-543.
57. Lamph, S., Wheeler, M. & Halloran, S. (2003) Eight testosterone assays. *MHRA Evaluation Report*, MHRA 03127.
58. Lamph, S., Wheeler, M. & Halloran, S. (2004) DPC Immulite and Immulite 2000. *MHRA Evaluation Report*, MHRA 04027.
59. Carey, R.N. (2006) User verification of performance for precision and trueness: approved guideline, EP15-A2. Clinical Laboratory Standards Institute, Pennsylvania, USA.
60. Velduis, J.D., Kolp, L.A., Rogol, A.D. & Johnson, M.L. (1987) Physiological attributes of episodic testosterone secretion. *Annals of the New York Academy of Sciences*, 513, 507-508.
61. Mulligan, T., Iranmanesh, A., Gheorghiu, S., Godschalk, T. & Veldhuis, J.D. (1995) Amplified nocturnal luteinizing hormone (LH) secretory burst frequency with selective attenuation of pulsatile (but not basal) testosterone secretion in healthy aged man: possible Leydig cell desensitization of endogenous LH signalling: a clinical research centre study. *Journal of Clinical Endocrinology and Metabolism*, 80, 3025-3031.
62. Wheeler, M.J. & Lowy, C. (1987) Warning on serum testosterone measurement. *Lancet*, ii, 514-515.
63. Heald, A.H., Butterworth, A., Kane, J.W., Borzomato, J., Taylor, N.F., Layton, T., Kilpatrick, E.S., & Rudenski A. (2006) Investigation into possible causes of interference in serum testosterone measurement in women. *Annals of Clinical Biochemistry*, 43, 189-195.
64. Warner, M.H., Kane, J.W., Atkin, S.L. & Kilpatrick, E.S. (2006) Dehydroepiandrosterone sulphate interferes with the Abbott Architect direct immunoassay for testosterone. *Annals of Clinical Biochemistry*, 43, 196-199.
65. Arlt, W. (2004) Concensus document on substitution therapy with DHEA in the elderly. *Seminars in Reproductive Medicine*, 22, 379-388.
66. Percheron, G., Hogrel, J.Y., Denot-Ledunois, S., Fayet, G., Forette, F., Baulieu, E.-E., Fardeau, F. & Marini, J.-F. (2003) Effect of 1-year oral administration of dehydroepiandrosterone to 60- to 80-year-old individuals on muscle function and cross-sectional area: a double-blind placebo-controlled trial. *Archives of Internal Medicine*, 163, 720-727.
67. Yen, S.S.C., Morales, A.J. & Khorram, O. (1995) Replacement of DHEA in aging men and women. *Annals of the New York Academy of Sciences*, 774, 128-142.
68. Swinkels, L.M.J.K., Ross, H.A. & Benraad, T.J. (1978) A symmetric dialysis method for the determination of free testosterone in human plasma. *Clinica Chimica Acta*, 165, 341-349.
69. Pardridge, W.M. (1986) Receptor-mediated peptide transport through the blood-brain barrier. *Endocrine Reviews*, 7, 314-330.
70. Meikel, C.M. (1989) The free hormone hypothesis. *Endocrine Reviews*, 10, 232-274.
71. Ekins, R. (1990) Measurement of free hormones in blood. *Endocrine Reviews*, 11, 5-46.

72. Cumming, D.C. & Wall, S.R. (1985) Non-sex hormone-binding globulin-bound testosterone as a marker of hyperandrogenism. *Journal of Clinical Endocrinology and Metabolism*, 61, 873-876.
73. Kapoor, D., Clarke, S., Channer, K.S. & Jones, T.H. (2007) Erectile dysfunction is associated with low bioactive testosterone levels and visceral adiposity in men with type 2 diabetes. *International Journal of Andrology*, 30, 500-507.
74. Emadi-Konjin, P., Bain, J. & Bromberg, I.L. (2003) Evaluation of an algorithm for calculation of serum 'bioavailable' testosterone (BAT). *Clinical Biochemistry*, 36, 591-596.
75. Giton, F., Urien, S., Born, C., Tichet, J., Guechot, J., Callebert, J., Bronsard, F., Raynaud, J.P. & Fiet, J. (2007) Determination of bioavailable testosterone [non-sex hormone-binding globulin (SHBG)-bound testosterone] in a population of healthy French men: influence of androstenediol on testosterone binding to SHBG. *Clinical Chemistry*, 53, 2160-2168.
76. Kratzik, C.W., Schatzl, G., Lackner, J.E., Lunglmayr, G., Brandstatter, N., Rucklinger, E. & Huber, J. (2007) Mood changes, body mass index and bioavailable testosterone in healthy men: results of the Androx Vienna Municipality Study. *British Journal of Urology International*, 100, 614-618.
77. Sandberg, A.A., Slaunwhite, W.R. & Antoniades, H.N. (1957) The binding of steroids and steroid conjugates to human plasma proteins. *Recent Progress in Hormone Research*, 13, 209-267.
78. Forest, M.G., Rivarola, M.A. & Migeon, C.J. (1968) Percentage binding of testosterone, androstenedione and dehydroepiandrosterone in plasma. *Steroids*, 12, 323-343.
79. Viahos, I., MacMahon, W., Sgoutas, D., Bowers, W., Thompson, J. & Trawick, W. (1982) An improved ultrafiltraton method for determining free testosterone in serum. *Clinical Chemistry*, 28, 2286-2291.
80. Fisher, R.A., Anderson, D.C. & Burke, C.W. (1974) Simultaneous measurement of unbound testosterone and estradiol fractions in undiluted plasma at 37 °C by steady-state gel filtration. *Steroids*, 24, 809-824.
81. Wheeler, M.J. & Nanjee, M.N. (1985) A steady-state gel filtration method on micro-columns for percentage free testosterone in serum. *Annals of Clinical Biochemistry*, 22, 185-189.
82. Vermeulen, A., Verdonck, L. & Kaufman, J.M. (1999) A critical evaluation of simple methods for the estimation of free testosterone in serum. *Journal of Clinical Endocrinology and Metabolism*, 84, 3666-3672.
83. Sodergard, R., Backstrom, T., Shanbhag, V. & Carstensen, H. (1982) Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. *Journal of Steroid Biochemistry*, 16, 801-810.
84. Dunn, J.F., Nisula, B.C. & Rodbard (1981) Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *Journal of Clinical Endocrinology and Metabolism*, 53, 58-68.
85. Nanjee, M.N. & Wheeler, M.J. (1985) Plasma free testosterone: is an index sufficient? *Annals of Clinical Biochemistry*, 22, 387-390.
86. Morris, P.D., Malkin, C.J., Channer, K.S. & Jones, T.H. (2004) A mathematical comparison of techniques to predict biologically available testosterone in a cohort of 1072 men. *European Journal of Endocrinology*, 151, 241-249.
87. Ly, L.P. & Handelsman, D.J. (2005) Empirical estimation of free testosterone from testosterone and sex-hormone binding globulin immunoassays. *European Journal of Endocrinology*, 105, 471-478.
88. Blight, L.F., Judd, S.J., White, G.H. (1989) Relative diagnostic value of serum non-SHBG testosterone, free androgen index and free testosterone in the assessment of mild to moderate hirsutism. *Annals of Clinical Biochemistry*, 26, 311-316.
89. Winters, S.J., Kelley, D.E. & Goodpaster, B. (1998) The analog free testosterone assay: are the results clinically useful? *Clinical Chemistry*, 44, 2178-2182.
90. Rosner, W. (2001) An extraordinarily inaccurate assay for free testosterone is still with us. *Journal of Clinical Endocrinology and Metabolism*, 86, 2903.
91. Fritz, K.S., McKean, A.J.S., Nelson, J.C. & Wilcox, R.B. (2008) Analog-based free testosterone test results linked to total testosterone concentrations, not free testosterone concentrations. *Clinical Chemistry*, 54, 512-516.
92. Martinez-Jabaloyas, J.M., Quelpo-Zaraoza, A., Gil-Salom, M. & Chuan-Nuez, P. (2006) Evaluation of an immunoassay kit to measure free testosterone. *Actas Urologicas Espanolas*, 30, 598-601.

93. Manni, A., Pardridge, W.M., Cefalu, W., Nisula, B.C., Bardin, C.W., Santer, S.J. & Santen, R.J. (1985) Bioavailability of albumin-bound testosterone. *Journal of Clinical Endocrinology and Metabolism*, 61, 705-710.
94. Déchaud, H., Lejeune, H., Garoscio-Cholet, M., Mallein, R. & Pugeat, M. (1989) Radioimmunoassay of testosterone not bound to sex-steroid protein in plasma. *Clinical Chemistry*, 35, 1609-1614.
95. Ho, C.K.M., Stoddart, M., Walton, M., Anderson, R.A. & Beckett, G.J. (2006) Calculated free testosterone in men: comparison of four equations and with free androgen index. *Annals of Clinical Biochemistry*, 43, 389-397.
96. De Ronde, W., van der Schouw, Y.T., Pols, H.A.P., Gooren, L.J.G., Muller, M., Grobbee, D.E. & de Jong, F.H. (2006) Calculation of bioavailable and free testosterone in men: a comparison of 5 published algorithms. *Clinical Chemistry*, 52, 1777-1784.

Reprint Address

Dr M. J. Wheeler, The Old Dairy, Old Bystock Drive, Bystock, Exmouth, EX8 5EQ, UK. Tel./Fax: +44 (0) 1395 263513; E-mail: mjw@the2wheelers.plus.com ; mike.wheeler@kcl.ac.uk

Clin Endocrinol. 2008;69(4):515-525.