Journal of Steroid Biochemistry and Molecular Biology 190 (2019) 207–211

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Assessment of free testosterone concentration

Brian G. Keevil*, Jo Adaway

Department of Clinical Biochemistry, Manchester University Hospital NHS Trust, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK

ARTICLE INFO

Keywords:
Testosterone
Calculated free testosterone
Bio-available testosterone
Equilibrium dialysis
Free androgen index

ABSTRACT

Testosterone (T) is strongly bound to sex hormone binding globulin and measurement of free T may be more appropriate than measuring total serum T, according to the free hormone theory. This view remains controversial and it has its detractors who claim that little extra benefit is gained than simply measuring total T, but it is endorsed by recent clinical practice guidelines for investigation of androgen disorders in both men and women. Free T measurement is very challenging. The gold standard equilibrium dialysis methods are too complex for use in routine clinical laboratories, assays are not harmonized and consequently there are no common reference intervals to aid result interpretation. The algorithms derived for calculating free T are inaccurate because they were founded on faulty models of testosterone binding to SHBG, however they can still give clinically useful results. To negate the effects of differences in binding protein constants, some equations for free T have been derived from accurate measurement of testosterone in large population studies, however a criticism is that the equations may not hold true in different patient populations. The free androgen index is not recommended for use in men because of inaccuracy at extremes of SHBG concentration, and in women it can also give inaccurate results when SHBG concentrations are low. If the free hormone hypothesis is to be believed, then calculated free testosterone may offer the best way forward but better equations are needed to improve accuracy and these should be derived from detailed knowledge of testosterone binding to SHBG. There is still much work to be done to improve harmonization of T and SHBG assays between laboratories because these can have a profound effect on the equations used to calculate free testosterone.

1. Introduction

Testosterone (T) mainly circulates avidly bound to SHBG, weakly bound to albumin, CBG and orosomucoid and unbound as free T. The free hormone theory states that only unbound T is active and able to bind the androgen receptor of target tissues of the body [2]. The free hormone hypothesis has been questioned because T is weakly bound to albumin and an alternative hypothesis is that both free T and weakly bound T both contribute to androgen effects. The phrase 'Bioavailable T' denotes the sum of the free T and free weakly bound T and is supported by the belief that albumin-bound T can dissociate in capillaries and is particularly active in tissues with relatively long blood transit times. A third hypothesis suggests that T bound to SHBG is available by active transport to tissues such as prostate and testis [1,2]. The exact relationship and role in health between T, CBG and orosomucoid is poorly understood and will not be considered further.

The free hormone hypothesis has been recently supported by data from the European Male Aging Study data, which showed that men with low free T concentrations had physical and sexual symptoms consistent with T deficiency, even though their T concentrations may be normal. They also showed that, more obese men had low T but normal free T concentrations and had no physical or associated sexual symptoms [3].

The significance of a combined low T and low free T has also been confirmed in 5 year cross sectional and longitudinal health outcome studies in men [4]. In contrast to the EMAS study, Hsu showed that the total T was the main predictor of morbidity and mortality and that no useful independent data was provided by the free T measurement. This view is also supported by recent clinical guidelines for the assessment of male hypogonadism from the Australian Endocrine Society [5].

In men the latest clinical practice guidelines from the United States Endocrine Society propose that the initial assessment of androgen status is performed using total T and in those individuals who have a condition that alters SHBG or whose total T is near the lower limit of normal, it is recommended that free T concentration is obtained using either equilibrium dialysis or estimated using an accurate formula [27]. This view is at variance to the guidelines provided by the Australian Endocrine Society. The use of free T to assess hypogonadism in men is

* Corresponding author.
E-mail address: brian.keevil@uhsm.nhs.uk (B.G. Keevil).

https://doi.org/10.1016/j.jsbmb.2019.04.008
Received 1 April 2019; Received in revised form 5 April 2019; Accepted 6 April 2019
Available online 07 April 2019
0960-0760/

therefore controversial but there is little evidence to either support or reject its use in the assessment of hyperandrogenism in women.

In women current clinical practice guidelines for the diagnosis and treatment of Polycystic Ovary Syndrome and hirsutism recommend that hyperandrogenism is initially assessed with a high quality assay of total T.

However, when hirsutism with clinical evidence of endocrine disorder is present and the T is normal then it is recommended that free T should be calculated. A free T level calculated from T and SHBG is considered to be the single most-useful test of androgen excess in women [65].

If the free hormone hypothesis is to be believed, then SHBG-bound T is not biologically active, and therefore some estimate of the fraction not bound to SHBG may be a more suitable marker. Methods of determining free T include measurement of the non-protein bound T testosterone concentration by a variety of different assays. The older, direct analogue RIA methods have been discredited and are no longer recommended for use [6–8].

2. Bio-available T

Although more technically difficult than measurement of total T, the method of Tremblay and Dube [9] has been used to produce data on bioavailable T by several groups [10,11]. This technique uses saturated ammonium sulphate to precipitate SHBG, leaving the albumin-bound and the free fractions in the supernatant. The percentage of the total T found in the supernatant is measured using a radioactive testosterone tracer, and this represents the 'bioavailable' fraction. The concentration of total T is then used to calculate the bioavailable T. This measurement has been promoted as a legitimate marker of bioactive androgen and has been shown to correlate well with equilibrium dialysis methods of measuring free T, which have long been considered the 'gold standard'. Bioavailable T and calculated free T (cFT) have been shown to correlate strongly which may render the measurement of bioavailable T redundant unless measured at the extremes of albumin concentration [12]. Despite the good correlation, a proportional bias has been demonstrated between cFT and bio-available T [13]. Nevertheless, a major limitation of Bio-available T is the reported lack of agreement between laboratories because of variations in precipitation and assay methodology [6].

3. Equilibrium dialysis T

Equilibrium dialysis and ultrafiltration methods have been considered the reference methods for quantification of free T. Historically, dialysis- and ultrafiltration-based methods have mostly been indirect, using a labelled T tracer to determine the percentage free labelled T. Dialysis and ultrafiltration methods are analytically difficult with numerous technical issues and as such they are not suitable for use in routine clinical laboratories [12,14].

The classical way to determine the quantity of true free testosterone was to adopt a three stage approach. Unbound and bound T in undiluted serum were separated by equilibrium dialysis. T in dialysate was extracted and then column chromatography used to separate T from similar steroids which may cross-react in the sensitive radioimmunoassay used for T quantification. The radioimmunoassay detection limit was said to be acceptable and the detection limit of the overall method was found to be 6 pmol/L [15], although this is open to doubt. Whilst it is true that detection limits can be applied appropriately to the various LC–MS/MS or immunoassay assays used for detecting T the same cannot be said for the overall measurement of free T, because there are no free T reference standards or QCs available.

This method has been updated in recent years with the introduction of LC–MS/MS. Serum free T can now be determined by the direct dialysis of undiluted serum followed by LC–MS/MS assay of free T in the dialysate. Equilibrium dialysis for 24 h at 37 °C with protein free buffer was performed using relatively large volumes of serum, 500 µL or 1000 µL of serum for male and female samples respectively [16]. Free T concentrations are lower in females compared to males, but the percentages of free T are similar, with a range of 0.9–2.9% in men and 0.4–2.8% in women. As expected, free T in both women and men is positively associated with total T, and there is a strong negative association between percentage free T and serum SHBG. Observed ranges for free T measured by a state-of-the-art LC–MS/MS-based direct dialysis method are in full agreement with earlier findings obtained using the older indirect equilibrium dialysis [12,17–19] and ultra-filtration methods [20,21].

Equilibrium dialysis' is often named as the 'gold standard' method for the determination of free T but there are many technical difficulties with the method and it is therefore not surprising that there should be poor inter-laboratory agreement [22,23]. The poor agreement is caused by methodological differences in temperature, pH, or equilibrium shifts between free and bound T caused by sample dilution effects [12,24,25]. Indirect measurement of bioavailable T using a radioactive tracer [23], or using an LC–MS/MS method which has not been standardised against the gold standard can also increase total error. It is not difficult to see why these many technical challenges render equilibrium dialysis unsuitable for use in routine clinical laboratories, but as with many other areas of laboratory medicine there is still a need to standardise methods between reference laboratories. It has been suggested that this should probably be achieved using direct equilibrium dialysis (and/or ultrafiltration) methods [16], using high quality highly sensitive fully validated mass spectrometry-based assays for T [26]. It is also important that standardised reference ranges for free testosterone are used for the diagnosis of androgen disorders in women and men.

The latest endocrine society guidelines recommend that T should be measured using a CDC-certified assay or an assay which has been verified by an external quality control program using accuracy based target values [27]. If this approach is taken and assays have been calibrated to a reference measurement procedure, then harmonized male population reference ranges for testosterone can be used [28]. Although how this can be truly applied to immunoassays that demonstrate method specific bias remains to be seen. And crucially to aid result interpretation there are no harmonized reference intervals based on large population studies currently available for free T.

4. Calculated free T

As the equilibrium dialysis reference method is not practical for use in the routine clinical laboratory, several equations have been advocated to estimate free T. Most commonly applied are equilibrium binding equations derived from the law of mass action using estimates of the association constants for binding of T to SHBG and albumin respectively [12,29]. Equations have also been developed empirically, modelled on large sets of free T concentrations and free of assumptions about theoretical binding equilibria [30,31]. The weakness of these methods, apart from reliance on the accuracy of T and SHBG measurements, is that best-fit parameters in the test population may not be the same as in the patient population.

The calculation proposed by Vermeulen et al has been the most widely used but the main criticism of all these equations is that the model of binding of T to SHBG may not be accurate, and in addition the set of binding constants used may not be appropriate [14,32]. SHBG is a homodimeric glycoprotein with a molecular weight of approximately 90 kDa [33] and the distribution of testosterone bound to SHBG is different in males and females. When Estradiol is present, approximately 20% of SHBG binding sites are occupied by testosterone [34]. It was previously thought that the two binding sites on the SHBG molecule are equivalent, but using modern biophysical techniques it is now known that the binding sites are not equivalent, and they each bind SHBG with a different affinity [22]. As a result of this, alternative models of binding of T to SHBG have been advocated [14,22].

Calculated FT (cFT) estimates are based on the assumption that there is normal steady-state protein binding for T and the equations are dependent on reliability and accuracy of both the T and SHBG assays [6,35,36]. All of the equations will estimate free T incorrectly when the protein concentrations differ widely from physiological values, if there are large concentrations of competing steroids, or if the SHBG binding affinity is affected by a rare genetic variant [12,37]. Depending on the equation used, systematic differences between free T estimates have been reported compared with measured free T [18,21,30]. Discrepancies between free T measured using equilibrium dialysis and cFT are most likely caused by erroneous modelling of testosterone binding to SHBG.

Recent work shows that cFT-Vermeulen is strongly correlated to free T measured by the reference method (direct equilibrium dialysis), but free T is overestimated by 20–30%, thus agreeing with previous work [21,38,39]. However, the relationship between cFT-Vermeulen and measured free T was found to be linear and independent of serum T, albumin and SHBG concentrations. The lack of reliance on SHBG in the Vermeulen equation was thought to be strength of the cFT-Vermeulen since assessment of free T is especially important in patients at the extremes of the SHBG concentration range. The bias is probably due to imperfect estimations of the association constants for the binding of T to SHBG and albumin, as discussed above, and this would need to be allowed for when comparing different methods for cFT.

The cFT-Ly equation shows good performance in the mid-range of serum T and SHBG, but its accuracy depends on T and SHBG levels and this has clinical consequences: e.g. the underestimation of free T by the cFT-Ly equation at low SHBG concentrations could potentially mis-diagnose hyperandrogenism in women with PCOS or over diagnosis hypogonadism in obese men. CFT-Sakharov showed discrepant results relative to direct equilibrium dialysis and also compared to other published data obtained with equilibrium dialysis or ultra-filtration-based methods. It should be noted that the Zakharov equation is patented and not available for independent scrutiny. The many different versions of formulas and methods for calculating and measuring free T have been shown to cause problems with poor inter-laboratory agreement due to the use of different methods with different reference intervals [40,41].

A recent study in our laboratory (Adaway J unpublished data) has shown that commonly used assays can give different results for SHBG, even when traceable to the same international standard. Anonymised surplus male and female serum samples were analysed on four different immunoassay platforms (Abbott Architect, Roche, Beckman and Siemens). The results were used to calculate free testosterone using the Vermeulen equation, with a constant testosterone concentration of 10 nmol/L for males and 1.5 nmol/L for females, keeping the albumin concentration fixed at 40 g/L. The Abbott Architect and Siemens Advia Centaur assays were both traceable to the WHO 2nd international standard 08/266 but there was a mean difference of 4.6 nmol/L between the results. The Roche E170 and Beckman Access were both traceable to the WHO 1st International Standard 95/560 but there was a mean difference of 3.4 nmol/L between their results. In contrast, although the Beckman Access and Siemens Advia Centaur assays are traceable to different international standards, the mean difference between their results was only 1.59 nmol/L. Although these differences may seem small, the widespread use of universal reference ranges for cFT means that the SHBG assay used can cause the difference between male patients receiving or being denied testosterone replacement, or results supporting or being inconsistent with the diagnosis of PCOS in a female patient. The difference between the Abbott Architect and the Roche E170 results were the greatest, with the Roche E170 results being a mean 9.449 nmol/L higher than the Abbott Architect results. Moving between analysers, for instance if a patient was being followed up in Primary and Secondary care and the care providers use different laboratories, or if a laboratory changes immunoassay platform could cause diagnostic confusion if the same reference ranges for cFT were used. The differences in SHBG assay results are consistent with those found on the UKNEQAS scheme (Rachel Marrington-personal communication)

5. Free androgen index

The free androgen index (FAI) uses the ratio of T to SHBG [42] and remains popular in routine Clinical Biochemistry laboratories because it is easy to calculate. The FAI is simply the inverse of SHBG especially in women where T concentration is much lower than in men. However, the FAI has been shown to overestimate cFT when the SHBG concentration is low [43,44]. Although the FAI is not used to assess T status in men it is widely used in the investigation of hyperandrogenism in women [45] and in clinical studies [46–48]. However, a recent study showed that the FAI/free T ratio also increases in women when SHBG is low, suggesting that care should also be taken when using the FAI in women [49]. A low SHBG is relatively common in women, the male and female reference ranges for SHBG overlap at low SHBG concentrations and low SHBG concentrations are found in many patients with hirsutism [50]. In addition, SHBG is lower in obesity [51], and it is also reduced in type 2 diabetes [52,53,51,52,50,51,49, 50,48,49,47,48] and in patients with metabolic syndrome [54].

The ramifications of a low SHBG concentration are also important in the assessment of hyperandrogenism. A recent study found that approximately 20% of women had a low SHBG which would give misleading information if the FAI was used to assess hyperandrogenism [55]. The authors concluded that the FAI may not be the best marker to evaluate hyperandrogenism in women and it would probably be better to use cFT or indeed an accurate T using LC–MS/MS.

6. Salivary T

Salivary T (Sal-T) may reflect the free testosterone available to target tissues and as such provide an index of bio-active testosterone, without having to resort to the more difficult equilibrium dialysis measurement of free T. However, testosterone in saliva is also technically challenging to measure because it is present at only < 2-3% of serum T.

Saliva contains only the non SHBG bound fraction of T which can freely diffuse across capillaries and salivary ducts and is known to be unaffected by saliva flow rates. Collection of saliva is non-invasive, well-accepted by patients and requires minimal training, thereby multiple samples can easily be collected to account for biological variability. Salivary hormone collection is well established [56], but there are technical issues with Sal-T including adsorption of T onto saliva collection devices and blood contamination of samples [57,58]. As a result, reliable results can only be obtained using passive drool collection techniques [38].

Immunoassays can be adapted to measure T in saliva, but these can be affected by bias caused by matrix-related problems and lack of specificity, similar to the problems seen with serum total T immunoassays [59,60]. A more recent study assessing the performance of three commercial immunoassays against LC–MS/MS found that all the assays were inaccurate especially at the lower concentrations found in women [61]. Despite the improvement in assay methodology with the introduction of LC–MS/MS it is unlikely that Sal-T will be used routinely for the investigation of hypogonadism in men.

High sensitivity assays capable of measuring Sal-T in the female range have been developed [16,49] that do not suffer the specificity issues of immunoassays. Keevil et al. found that salivary testosterone has significantly better correlation with serum cFT than serum total T in both males and females. Reference intervals for Sal-T were 5–46 pmol/L in females and 93–378 pmol/L in males. Fiers et al [38] compared another high sensitivity LC–MS/MS method for Sal-T with free T measured by equilibrium dialysis. They showed a good correlation between Sal-T and serum free T in women, but found a positive bias

with Sal-T compared to serum free T. The higher than expected Sal-T concentrations were explained by the binding of T to salivary proteins such as albumin and proline rich proteins. Protein binding of T in saliva was thought to affect the much lower Sal-T found in women but not in healthy men [38].

Another possible source of error in Sal-T measurement is the activity of 17β hydroxy steroid dehydrogenase in the salivary ducts, which is capable of converting testosterone to androstenedione [62,63]. This has been used to define additional metabolic risk in females with PCOS. Salivary testosterone androstenedione and the salivary T/A4 ratio were raised in women diagnosed with PCOS compared to healthy women. A high salivary T/A4 ratio was associated with an adverse metabolic phenotype, such as insulin and/or glucose intolerance, obesity, metabolic syndrome, or oligo/anovulation in only PCOS patients. In addition, significant correlations of the salivary T/A4 ratio and adverse hormonal and anthropometric parameters, as well as parameters of lipid and glucose metabolism were found [64].

7. Conclusion

The estimation of free T in both men and women has always been based on the central dogma of the free hormone hypothesis. As discussed in this review, the hard evidence for this hypothesis is scant and the two most recent and largest studies in men provide contradictory evidence. Nevertheless, the Endocrine Society still recommends the measurement of free T for the investigation of hypogonadism in men although this view is not supported by the Australian Endocrine Society. The use of free T in women is also recommended by the Endocrine society and it is still widely measured in clinical laboratories to support clinical practice and research into hyperandrogenism, although the evidence for this is also weak. Measurement of free testosterone is fraught with difficulty. Equilibrium dialysis methods are too complex for routine clinical use; they lack harmonization and consequently common reference intervals. Equations for calculating FT are inaccurate because they were founded on faulty models of T binding to SHBG. Calculated FT methods offer are simple and inexpensive and may offer the best way forward, but more accurate equations are needed and these must be based on more detailed knowledge of the complicated binding testosterone to SHBG. There is also still much work to be done to improve harmonization of T and SHBG assays between laboratories because these can have a profound effect on the cFT equations. This calls for closer co-operation between regulatory bodies, EQA schemes and laboratories. Necessary requirements to improve the measurement of free T include the availability of commutable human serum based reference materials, internal standards for the calibration of assays and standardisation of sample preparation and chromatography techniques.

With the relatively weak evidence available to support the measurement of free T and the difficulty of its measurement, there still remains a question mark as to the usefulness of this test or indeed if accurate measurement of T using highly specific LC–MS/MS methods may not be more useful.

Funding

This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] W. Rosner, Plasma steroid-binding proteins, Endocrinol. Metab. Clin. North Am. 20 (4) (1991) 697–720.
[2] R. Sakiyama, W.M. Pardridge, N.A. Musto, Influx of testosterone-binding globulin (TeBG) and TeBG-bound sex steroid hormones into rat testis and prostate, J. Clin. Endocrinol. Metab. 67 (1) (1988) 98–103.
[3] L. Antonio, F.C. Wu, T.W. O'Neill, S.R. Pye, T.B. Ahern, M.R. Laurent, et al., Low free testosterone is associated with hypogonadal signs and symptoms in men with normal total testosterone, J. Clin. Endocrinol. Metab. 101 (7) (2016) 2647–2657.
[4] B. Hsu, R.G. Cumming, F.M. Blyth, V. Naganathan, L.M. Waite, D.G. Le Couteur, et al., Evaluating calculated free testosterone as a predictor of morbidity and mortality independent of testosterone for cross-sectional and 5-year longitudinal health outcomes in older men: the concord health and ageing in men project. The journals of gerontology series A, Biol. Sci. Med. Sci. 73 (6) (2018) 729–736.
[5] B.B. Yeap, M. Grossmann, R.I. McLachlan, D.J. Handelsman, G.A. Wittert, A.J. Conway, et al., Endocrine Society of Australia position statement on male hypogonadism (part 1): assessment and indications for testosterone therapy, Med. J. Aust. 205 (4) (2016) 173–178.
[6] W. Rosner, R.J. Auchus, R. Azziz, P.M. Sluss, H. Raff, Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement, J. Clin. Endocrinol. Metab. 92 (2) (2007) 405–413.
[7] K.S. Fritz, A.J. McKean, J.C. Nelson, R.B. Wilcox, Analog-based free testosterone test results linked to total testosterone concentrations, not free testosterone concentrations, Clin. Chem. 54 (3) (2008) 512–516.
[8] R.S. Swerdloff, C. Wang, Free testosterone measurement by the analog displacement direct assay: old concerns and new evidence, Clin. Chem. 54 (3) (2008) 458–460.
[9] R.R. Tremblay, J.Y. Dube, Plasma concentrations of free and non-TeBG bound testosterone in women on oral contraceptives, Contraception 10 (6) (1974) 599–605.
[10] R.R. Cooke, J.E. McIntosh, R.P. McIntosh, Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model, Clin. Endocrinol. 39 (2) (1993) 163–171.
[11] R.R. Cooke, R.P. McIntosh, J.G. McIntosh, J.W. Delahunt, Serum forms of testosterone in men after an hCG stimulation: relative increase in non-protein bound forms, Clin. Endocrinol. 32 (2) (1990) 165–175.
[12] A. Vermeulen, L. Verdonck, J.M. Kaufman, A critical evaluation of simple methods for the estimation of free testosterone in serum, J. Clin. Endocrinol. Metab. 84 (10) (1999) 3666–3672.
[13] F. Giton, J. Guechot, J. Fiet, Comparative determinations of non SHBG-bound serum testosterone, using ammonium sulfate precipitation, Concanavalin A binding or calculation in men, Steroids 77 (12) (2012) 1306–1311.
[14] A.L. Goldman, S. Bhasin, F.C.W. Wu, M. Krishna, A.M. Matsumoto, R. Jasuja, A reappraisal of testosterone's binding in circulation: physiological and clinical implications, Endocr. Rev. 38 (4) (2017) 302–324.
[15] A. Torma, T.A. Jaatinen, H.L. Kaihola, P. Koskinen, K. Irjala, A method for measurement of free testosterone in premenopausal women involving equilibrium dialysis, chromatography, and radioimmunoassay, Steroids 60 (3) (1995) 285–289.
[16] T. Fiers, F. Wu, P. Moghetti, D. Vanderschueren, B. Lapauw, J.M. Kaufman, Reassessing free-testosterone calculation by liquid chromatography-tandem mass spectrometry direct equilibrium dialysis, J. Clin. Endocrinol. Metab. 103 (6) (2018) 2167–2174.
[17] A. Vermeulen, T. Stoica, L. Verdonck, The apparent free testosterone concentration, an index of androgenicity, J. Clin. Endocrinol. Metab. 33 (5) (1971) 759–767.
[18] K.K. Miller, W. Rosner, H. Lee, J. Hier, G. Sesmilo, D. Schoenfeld, et al., Measurement of free testosterone in normal women and women with androgen deficiency: comparison of methods, J. Clin. Endocrinol. Metab. 89 (2) (2004) 525–533.
[19] L.M. Swinkels, H.A. Ross, T.J. Benraad, A symmetric dialysis method for the determination of free testosterone in human plasma, Clin. Chim. Acta 165 (2-3) (1987) 341–349.
[20] G.L. Hammond, Access of reproductive steroids to target tissues, Obstet. Gynecol. Clin. North Am. 29 (3) (2002) 411–423.
[21] L.P. Ly, G. Sartorius, L. Hull, A. Leung, R.S. Swerdloff, C. Wang, et al., Accuracy of calculated free testosterone formulae in men, Clin. Endocrinol. 73 (3) (2010) 382–388.
[22] M.N. Zakharov, S. Bhasin, T.G. Travison, R. Xue, J. Ulloor, R.S. Vasan, et al., A multi-step, dynamic allosteric model of testosterone's binding to sex hormone binding globulin, Mol. Cell. Endocrinol. 399 (2015) 190–200.
[23] J.S. Hackbarth, J.B. Hoyne, S.K. Grebe, R.J. Singh, Accuracy of calculated free testosterone differs between equations and depends on gender and SHBG concentration, Steroids 76 (1-2) (2011) 48–55.
[24] Y. Chen, M. Yazdanpanah, X.Y. Wang, B.R. Hoffman, E.P. Diamandis, P.Y. Wong, Direct measurement of serum free testosterone by ultrafiltration followed by liquid chromatography tandem mass spectrometry, Clin. Biochem. 43 (4-5) (2010) 490–496.
[25] W. Rosner, Errors in the measurement of plasma free testosterone, J. Clin. Endocrinol. Metab. 82 (6) (1997) 2014–2015.
[26] H.W. Vesper, J.C. Botelho, Standardization of testosterone measurements in humans, J. Steroid Biochem. Mol. Biol. 121 (3-5) (2010) 513–519.
[27] S. Bhasin, J.P. Brito, G.R. Cunningham, F.J. Hayes, H.N. Hodis, A.M. Matsumoto, et al., Testosterone therapy in men with hypogonadism: an endocrine society clinical practice guideline, J. Clin. Endocrinol. Metab. 103 (5) (2018) 1715–1744.
[28] T.G. Travison, H.W. Vesper, E. Orwoll, F. Wu, J.M. Kaufman, Y. Wang, et al., Harmonized reference ranges for circulating testosterone levels in men of four cohort studies in the United States and Europe, J. Clin. Endocrinol. Metab. 102 (4) (2017) 1161–1173.
[29] R. Sodergard, T. Backstrom, V. Shanbhag, H. Carstensen, Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature, J. Steroid Biochem. 16 (6) (1982) 801–810.
[30] W. de Ronde, Y.T. van der Schouw, H.A. Pols, L.J. Gooren, M. Muller, D.E. Grobbee, et al., Calculation of bioavailable and free testosterone in men: a comparison of 5 published algorithms, Clin. Chem. 52 (9) (2006) 1777–1784.
[31] L.P. Ly, D.J. Handelsman, Empirical estimation of free testosterone from testosterone and sex hormone-binding globulin immunoassays, Eur. J. Endocrinol. 152 (3) (2005) 471–478.

[32] S. Bhasin, A perspective on the evolving landscape in male reproductive medicine, J. Clin. Endocrinol. Metab. 101 (3) (2016) 827–836.
[33] S. Khosla, Editorial: sex hormone binding globulin: inhibitor or facilitator (or both) of sex steroid action? J. Clin. Endocrinol. Metab. 91 (12) (2006) 4764–4766.
[34] J.F. Dunn, B.C. Nisula, D. Rodbard, Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma, J. Clin. Endocrinol. Metab. 53 (1) (1981) 58–68.
[35] A.M. Matsumoto, W.J. Bremner, Serum testosterone assays–accuracy matters, J. Clin. Endocrinol. Metab. 89 (2) (2004) 520–524.
[36] F. Tosi, T. Fiers, J.M. Kaufman, M. Dall'Alda, R. Moretta, V.A. Giagulli, et al., Implications of androgen assay accuracy in the phenotyping of women with polycystic ovary syndrome, J. Clin. Endocrinol. Metab. 101 (2) (2016) 610–618.
[37] G.L. Hammond, Plasma steroid-binding proteins: primary gatekeepers of steroid hormone action, J. Endocrinol. 230 (1) (2016) R13–25.
[38] T. Fiers, J. Delanghe, G. T'Sjoen, E. Van Caenegem, K. Wierckx, J.M. Kaufman, A critical evaluation of salivary testosterone as a method for the assessment of serum testosterone, Steroids 86 (2014) 5–9.
[39] K. Van Uytfanghe, D. Stockl, J.M. Kaufman, T. Fiers, H.A. Ross, A.P. De Leenheer, et al., Evaluation of a candidate reference measurement procedure for serum free testosterone based on ultrafiltration and isotope dilution-gas chromatography-mass spectrometry, Clin. Chem. 50 (11) (2004) 2101–2110.
[40] M. Le, D. Flores, D. May, E. Gourley, A.K. Nangia, Current practices of measuring and reference range reporting of free and total testosterone in the United States, J. Urol. 195 (5) (2016) 1556–1561.
[41] S. Lazarou, L. Reyes-Vallejo, A. Morgentaler, Wide variability in laboratory reference values for serum testosterone, J. Sex. Med. 3 (6) (2006) 1085–1089.
[42] R.S. Mathur, L.O. Moody, S. Landgrebe, H.O. Williamson, Plasma androgens and sex hormone-binding globulin in the evaluation of hirsute females, Fertil. Steril. 35 (1) (1981) 29–35.
[43] A. Vermeulen, Reflections concerning biochemical parameters of androgenicity, Aging Male Off. J. Int. Soc. Study Aging Male 7 (4) (2004) 280–289.
[44] C.K. Ho, M. Stoddart, M. Walton, R.A. Anderson, G.J. Beckett, Calculated free testosterone in men: comparison of four equations and with free androgen index, Ann. Clin. Biochem. 43 (Pt 5) (2006) 389–397.
[45] J.H. Barth, H.P. Field, E. Yasmin, A.H. Balen, Defining hyperandrogenism in polycystic ovary syndrome: measurement of testosterone and androstenedione by liquid chromatography-tandem mass spectrometry and analysis by receiver operator characteristic plots, Eur. J. Endocrinol. 162 (3) (2010) 611–615.
[46] L. Engmann, S. Jin, F. Sun, R.S. Legro, A.J. Polotsky, K.R. Hansen, et al., Racial and ethnic differences in the polycystic ovary syndrome metabolic phenotype, Am. J. Obstet. Gynecol. 216 (5) (2017) 493 e1-.e13.
[47] L. Jaspers, K. Dhana, T. Muka, C. Meun, J.C. Kiefte-de Jong, A. Hofman, et al., Sex steroids, sex hormone-binding globulin and cardiovascular health in men and postmenopausal women: the rotterdam study, J. Clin. Endocrinol. Metab. 101 (7) (2016) 2844–2852.
[48] D. Macut, K. Tziomalos, I. Bozic-Antic, J. Bjekic-Macut, I. Katsikis, E. Papadakis, et al., Non-alcoholic fatty liver disease is associated with insulin resistance and lipid accumulation product in women with polycystic ovary syndrome, Hum. Reprod. 31 (6) (2016) 1347–1353.
[49] B.G. Keevil, P. MacDonald, W. Macdowall, D.M. Lee, F.C. Wu, Salivary testosterone measurement by liquid chromatography tandem mass spectrometry in adult males and females, Ann. Clin. Biochem. 51 (Pt 3) (2014) 368–378.
[50] M. Pugeat, J.C. Crave, J. Tourniaire, M.G. Forest, Clinical utility of sex hormone-binding globulin measurement, Horm. Res. 45 (3-5) (1996) 148–155.
[51] D.S. Guzick, R. Wing, D. Smith, S.L. Berga, S.J. Winters, Endocrine consequences of weight loss in obese, hyperandrogenic, anovulatory women, Fertil. Steril. 61 (4) (1994) 598–604.
[52] K.I. Birkeland, K.F. Hanssen, P.A. Torjesen, S. Vaaler, Level of sex hormone-binding globulin is positively correlated with insulin sensitivity in men with type 2 diabetes, J. Clin. Endocrinol. Metab. 76 (2) (1993) 275–278.
[53] D. Goodman-Gruen, E. Barrett-Connor, Sex hormone-binding globulin and glucose tolerance in postmenopausal women. The Rancho Bernardo Study, Diabetes Care 20 (4) (1997) 645–649.
[54] D.E. Laaksonen, L. Niskanen, K. Punnonen, K. Nyyssonen, T.P. Tuomainen, R. Salonen, et al., Sex hormones, inflammation and the metabolic syndrome: a population-based study, Eur. J. Endocrinol. 149 (6) (2003) 601–608.
[55] B.G. Keevil, J. Adaway, T. Fiers, P. Moghetti, J.M. Kaufman, The free androgen index is inaccurate in women when the SHBG concentration is low, Clin. Endocrinol. 88 (5) (2018) 706–710.
[56] M. Groschl, Current status of salivary hormone analysis, Clin. Chem. 54 (11) (2008) 1759–1769.
[57] M. Groschl, M. Rauh, Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis, Steroids 71 (13-14) (2006) 1097–1100.
[58] E.A. Shirtcliff, D.A. Granger, E. Schwartz, M.J. Curran, Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results, Psychoneuroendocrinology 26 (2) (2001) 165–173.
[59] J.S. Mitchell, T.E. Lowe, Matrix effects on an antigen immobilized format for competitive enzyme immunoassay of salivary testosterone, J. Immunol. Methods 349 (1-2) (2009) 61–66.
[60] C. Wang, D.H. Catlin, L.M. Demers, B. Starcevic, R.S. Swerdloff, Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry, J. Clin. Endocrinol. Metab. 89 (2) (2004) 534–543.
[61] K.M. Welker, B. Lassetter, C.M. Brandes, S. Prasad, D.R. Koop, P.H. Mehta, A comparison of salivary testosterone measurement using immunoassays and tandem mass spectrometry, Psychoneuroendocrinology 71 (2016) 180–188.
[62] T. Blom, A. Ojanotko-Harri, M. Laine, I. Huhtaniemi, Metabolism of progesterone and testosterone in human parotid and submandibular salivary glands in vitro, J. Steroid Biochem. Mol. Biol. 44 (1) (1993) 69–76.
[63] D. Szydlarska, W. Grzesiuk, A. Kondracka, Z. Bartoszewicz, E. Bar-Andziak, Measuring salivary androgens as a useful tool in the diagnosis of polycystic ovary syndrome, Endokrynol. Pol. 63 (3) (2012) 183–190.
[64] J. Munzker, L. Lindheim, J. Adaway, C. Trummer, E. Lerchbaum, T.R. Pieber, et al., High salivary testosterone-to-androstenedione ratio and adverse metabolic phenotypes in women with polycystic ovary syndrome, Clin. Endocrinol. 86 (4) (2017) 567–575.
[65] R.S. Legro, S.A. Arslanian, D.A. Ehrmann, K.M. Hoeger, M.H. Murad, R. Pasquali, et al., Diagnosis and treatment of polycystic ovary syndrome: an Endocrine Society clinical practice guideline, J. Clin. Endocrinol. Metab. 98 (12) (2013) 4565–4592.