Reassessing Free-Testosterone Calculation by Liquid Chromatography–Tandem Mass Spectrometry Direct Equilibrium Dialysis

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Context: Assessment of free testosterone (FT) might help evaluate androgen status in patients with borderline total testosterone (T) and/or altered sex hormone–binding globulin (SHBG) levels. However, the validity of different methods to measure FT is debatable.

Methods: Serum from 183 women and 146 men was analyzed using equilibrium dialysis (ED), with FT directly measured by liquid chromatography–tandem mass spectrometry. FT calculation was re-evaluated for the mass action law–based equation according to Vermeulen (cFT-V), empirical equations according to Ly (cFT-L), and a proposed calculation based on a multistep, dynamic, allosteric model according to Zakharov (cFT-Z).

Results: FT level analyzed by ED [median,13 pmol/L (1.2% of T) in women; 248 pmol/L (1.5% of T) in men] was strongly inversely correlated to SHBG level, significantly to albumin level in women, and only weakly to SHBG level in men. The median [percentile (p) range, 2.5 to 97.5] ratios of calculated FT (cFT) over ED-FT (from European Male Aging Study samples) were 1.19 (0.9 to 1.47), 1.00 (0.69 to 1.42), and 2.05 (1.26 to 3.26) for cFT-V, cFT-L, and cFT-Z, respectively. The ratio for cFT-V was not significantly affected by SHBG, T, or albumin levels (ρ range, 0.17 to -0.01); ratios for cFT-L and cFT-Z were affected ($P < 0.05$ and $P < 0.001$, respectively) and strongly correlated with SHBG levels ($\rho = 0.72$ and 0.75, respectively). Rank correlations between cFT% and ED-FT% (for men) were 0.62, 0.74, and 0.89 for cFT-Z, cFT-L, and cFT-V, respectively.

Conclusion: FT results by direct ED confirm prior FT data from indirect ED and ultrafiltration methodologies. Calculations have inherent limitations, with clinically important differences among evaluated equations: cFT-V, although overestimating FT level, appears the most robust approximation, largely independent of SHBG, albumin, and T levels. **(*J Clin Endocrinol Metab* 103: 2167–2174, 2018)**

Testosterone (T) in the circulation is mostly bound to plasma proteins, in particular with high affinity to sex hormone–binding globulin (SHBG) and with lower affinity to albumin. Only a small fraction circulates as free hormone. A substantial part of between-subject variability of serum T levels results from differences in

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Abbreviations: cFT, calculated free testosterone; cFT-L, calculation of free testosterone empirical equations according to Ly; cFT-V, calculation of free testosterone by mass action law–based equation according to Vermeulen; cFT-Z, calculation of free testosterone based on a multistep, dynamic, allosteric model according to Zakharov; ED, equilibrium dialysis; EMAS, European Male Aging Study; FT, free testosterone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PCOS, polycystic ovary syndrome; SHBG, sex hormone–binding globulin; T, testosterone; UF, ultrafiltration.

SHBG concentrations. The latter are influenced by metabolic-, hormonal-, and disease-related factors; thus, they also are subject to variations within individuals. T tightly bound to SHBG is not directly available for diffusion into the tissues. Therefore, in situations with altered serum SHBG, free T (FT) levels might reflect tissue exposure more closely than total T levels (1–4). Indeed, the free hormone hypothesis (5) remains the physiological model that best explains modulation of tissue exposure to T by plasma-binding proteins. Scarce evidence for alternative mode of actions of T through cellular internalization of the T-SHBG complex or its binding to membrane receptors is mostly limited to data in cell lines without a documented major physiological role (3, 4, 6).

A proportion of T more loosely bound to albumin might dissociate in the capillary circulation and thus be available for diffusion into tissues to a variable extent depending on tissue blood flow. The small FT- and larger albumin-bound T fractions combined are often referred to as bioavailable T (4, 7). The relation between FT and bioavailable T appears to be rather constant, unless there are large deviations from normal plasma albumin levels (8). In this report, we focused on FT because there are much more data available.

Assessment of FT might improve precision in the diagnosis of hyperandrogenism in women and hypogonadism in men, in particular when total T levels are borderline and in situations known to alter SHBG levels (9, 10). Equilibrium dialysis (ED)– or ultrafiltration (UF)-based methods, considered the reference for determination of FT, were, in the past, mostly indirect, with addition of a labeled T tracer, determination of percentage of free labeled T and calculation of FT from the percentage of free labeled T and total T. ED and UF methods are technically challenging and have potential pitfalls; reliable implementation is labor intensive and poorly suited for high throughput (4, 8).

Therefore, there has been widespread use of easier-to-implement surrogate estimates of FT. The direct analog tracer-based immunoassays do not reliably reflect true FT and should not be used (9). Most frequently used in clinical and research settings are calculated estimates of serum FT levels based on serum total T and SHBG levels with or without the actual serum albumin concentrations (11). The simple FT index of total T over SHBG is less reliable and now generally abandoned in favor of alternative calculations (9, 12). Equations derived from the general law of mass action, with association constants for T binding to SHBG and albumin values derived from *in vitro* experiments (8, 13, 14), are frequently used in clinical practice. The version proposed by Vermeulen *et al.* (8) has been the most widely applied. Equations empirically developed by regression modeling on large sets of values for FT as determined with a reference method have also been used (11, 15). Although calculations seem to have performed well in many studies, acknowledging that they all have inherent limitations, there is ongoing controversy about the accuracy of calculated estimates of FT. It has been suggested that equations derived from the law of mass action are based on an inaccurate model of T binding to SHBG and/or that the chosen set of binding constants is not appropriate (4, 16). In this context, the alternative dynamic allosteric model of T binding to SHBG recently put forward (4, 17) may have far-reaching implications. If confirmed, this new model not only invalidates most of the methods commonly used for calculating FT levels, but also suggests that the percentage FT data obtained by most established methods including ED- or UF-based assays are incorrect.

All this translates into uncertainty as how to best calculate FT and its true measured value. In the current study, we used a state-of-the-art direct ED method to reassess FT in sets of representative serum samples. This method takes advantage of the ability of a highly sensitive and accurate measurement of T by liquid chromatography–tandem mass spectrometry (LC-MS/MS) to reliably measure the low FT concentration directly in the dialysate after ED. This more straightforward method avoids potential sources of inaccuracy in indirect ED, such as those resulting from tracer impurities or from measures to limit their impact (*e.g.*, sample dilution). We then used the measured FT results to re-evaluate some characteristics of two more established and a more recently proposed calculations for estimation of FT.

Material and Methods

Study populations

Serum samples from three different study populations were used in these experiments and were collected in accordance with local ethical committee guidelines. Subject characteristics are summarized in Table 1. Samples from 183 white women of reproductive age, consisting of 130 women with documented polycystic ovary syndrome (PCOS) according to the Rotterdam criteria (18) and 53 healthy control subjects from participants in the Verona PCOS Pathophysiology and Phenotype (Verona 3P) Study (19). Of the women with PCOS, 25% had SHBG levels <25 nmol/L and 13% had levels <20 nmol/L. Samples from 46 men were selected on the basis of a low to normal SHBG level (SHBG level in 50% of samples was <20 nmol/L) among participants in a study of healthy young men sampled in sibling pairs from the registries of semirural or suburban communities around Ghent Belgium (SIBLOS study) (20). Samples from 100 men were randomly selected among participants in the European Male Aging Study (EMAS) (21). One-third of the men in the EMAS cohort had SHBG concentrations in the upper tertile of the reference range (*i.e.*, 20 to 80 nmol/L).

Table 1. Summary Findings for FT Measured by the Direct ED Method, Expressed as Absolute Values and as Percentage of Total T Concentration

	Male			Female		
	Total	EMAS	SIBLOS[a]	Total	PCOS	Control
No.	146	100	46	183	130	53
Age, mean (range), y	51.0 (28.2–77.8)	56.8 (41.9–79.2)	41.0 (25.7–55.0)	24.0 (18.0–36.0)	23.0 (18.0–34.0)	25.5 (19.8–39.2)
BMI, mean (range), kg/m^2	27.2 (20.5–34.7)	27.9 (20.9–37.8)	26.0 (20.3–33.8)	23.2 (18.2–40.2)	24.9 (18.2–41.2)	20.5 (18.0–32.8)
Total T (p2.5–p97.5), median, nmol/L[b]	15.6 (8.58–30.6)	15.9 (9.20–29.4)	15.5 (8.54–32.6)	1.13 (0.48–2.53)	1.26 (0.57–2.83)	0.90 (0.47–1.79)
SHBG (p2.5 – p97.5), median, nmol/L	42.4 (15.6–78.5)	46.1 (20.3–78.8)	28.3 (14.1–78.7)	45.6 (13.6–112.3)	36.5 (13.5–99.0)	65.5 (28.8–128.6)
FT (p2.5–p97.5), median pmol/L[c]	248 (129–502)	212 (117–427)	312 (191–567)	13.0 (2.70–48.3)	16.3 (3.39–49.6)	8.3 (2.08–16.8)
FT as % total T (p2.5–p97.5), median	1.5 (0.9–2.8)	1.4 (0.9–2.3	2.1 (1.1–2.9)	1.2 (0.4–2.7)	1.4 (0.5–2.8)	0.9 (0.3–1.9)

[a]Samples from participants in SIBLOS selected on the basis of low to normal SHBG level.

[b]Values for total T can be converted from nmol/L to ng/dL by dividing by 0.0347.

[c]Values for FT can be converted from pmol/L to ng/dL by dividing by 34.7.

Assay procedures

Assay of serum SHBG levels was performed on an E170 Modular immunoassay analyzer and of albumin was performed on the Cobas 8000 (Roche Diagnostics; Indianapolis, IN). Assay of total T in serum and direct measurement of FT in dialysate after ED against serum as described later in Methods, was done by LC-MS/MS on an AB Sciex 5500 triple-quadrupole mass spectrometer (Toronto, ON, Canada), as previously described (22). This method was validated for T against an isotope dilution mass spectrometry candidate reference method (23) using a common serum panel, and it also has recently been compared with a reference method at the Centers for Disease Control and Prevention (Atlanta, GA) (24).

Serum FT level was determined by a direct dialysis method (*i.e.*, ED of undiluted serum against buffer with direct LC-MS/MS assay of FT in the dialysate). ED was performed using Fast Micro-Equilibrium dialyzer cartridges and regenerated cellulose 25 kDa membranes (Harvard Apparatus, Holliston, MA). Serum (men, 500 μL; women, 1000 μL) was dialyzed at 37°C for 24 hours at pH 7.28 using protein-free buffer prepared according to Yue *et al.* (25).

For assay of total T, a liquid–liquid extraction was performed on 100 μL serum samples. Interassay coefficient of variation for T is 6.5% at 3 ng/dL (104 pmol/L; n = 30) with a limit of quantitation of 1 ng/dL (35 pmol/L). For measurement of FT by direct ED, interassay coefficient of variability was 13.5% at 0.18 ng/dL (6.2 pmol/L) with a limit of quantitation of 0.07 ng/dL (2.4 pmol/L). Experiments on various buffer and serum volumes, nonspecific binding, and mass spectrometry ion suppression all yielded excellent target recoveries within 5% tolerance. Details are provided in a validation summary in the Supplemental Validation Summary.

Calculated estimations of FT

FT level was calculated from total T, SHBG and albumin serum levels according to the three following methods: (1) an equation based on the law of mass action as published by Vermeulen *et al.* (8) (cFT-V); (2) two empirically derived formulae (for men, the formula for T > 5 nM was used; for women, the formula <5 nM was used) as published by Ly and Handelsman (15) (cFT-L); and (3) according to a calculation based on a multistep, dynamic, allosteric model of testosterone binding to SHBG as published by Zakharov *et al.* (17) (cFT-Z). The values for cFT-Z from the EMAS samples (calculated with original T and SHBG from EMAS) were provided by Dr. R. Jasuja, Boston, MA, to the EMAS investigators. We had no direct access to the algorithm for cFT-Z; therefore, we were unable to present any data on cFT-Z for the samples from SIBLOS and from women.

Statistics

Spearman rank correlations (ρ) were used as a nonparametric measure of rank correlation. Passing Bablock regressions were used for linear nonparametric comparisons. Scatter plots, regression analysis, rank correlations, and significance levels were performed with MedCalc statistical software, version 15.6 (Ostend, Belgium).

Results

FT by direct ED

The results for FT measured by direct ED in the different sets of serum samples from women and men expressed both as absolute concentrations and as percentage of total T are summarized in Table 1.

In women, absolute FT and FT% were strongly inversely correlated with SHBG level (FT: $\rho = -0.64$; FT%: $\rho = -0.82$; Supplemental Fig. 1) and weakly inversely correlated with serum albumin (FT: $\rho = -0.20$, $P < 0.001$; FT%: $\rho = -0.12$, $P = 0.1$). FT (pmol/L), but not FT%, was directly correlated with total T ($\rho = 0.66$ and 0.05, respectively; Supplemental Fig. 1).

In men, FT% also was strongly inversely correlated with SHBG level ($\rho = -0.89$) (Supplemental Fig. 1), with an only weak inverse correlation between absolute FT and SHBG ($\rho = -0.23$). Weak inverse associations of FT and FT% with serum albumin ($\rho = -0.12$ and -0.17) did not reach significance. FT% was inversely correlated with total T ($\rho = -0.43$; Supplemental Fig. 1), whereas FT (pmol/L) was positively correlated ($\rho = 0.50$).

Calculated FT estimates vs FT by direct ED

Men

The results for FT (pmol/L) and FT% as measured by direct ED and estimated by three different calculation

modalities in 100 samples from the EMAS cohort are summarized in Table 2. In Fig. 1, the ratio of calculated FT (cFT) over FT measured by direct ED in individual serum samples is displayed according to SHBG and total T levels, respectively, for the three calculation methods. For each sample, a ratio >1 indicates a positive bias of cFT compared with measured FT; a ratio <1 indicates a negative bias. In Table 3, the ratios of cFT over measured FT are summarized. For cFT-V, an overestimation is noted with a median ratio of 1.19; the median ratio of cFT-L over dialysis is 1.0, whereas for cFT-Z the median ratio indicates an overestimation of FT by a factor of 2. Passing Bablock regression of calculated vs measured FT values shows linear relationships for cFT-V (Y = 40.2 + 1.0X), cFT-L (Y = −3.1 + 1.0X), and cFT-Z Y = –25.9 + 2.1X). When cFT-V and cFT-L were calculated with T and SHBG values from EMAS, as for cFT-Z, the observed trends remained similar. As seen in the graphs (Fig. 1) and reflected in the rank correlations (Table 3), the ratios over ED for both cFT-L and cFT-Z, but not for cFT-V, display a dependency on serum SHBG level. According to SHBG concentration, cFT-Z values range from being equal to the ED result to as much as triple the ED result at high SHBG levels. For cFT-L, the median ratio was 1.0; values range from 50% to 150% of the ED result from low to high SHBG levels. Additionally, ratios for cFT-Z, and especially cFT-L, also displayed a weaker but significant nonlinear correlation vs total T level; this was mainly apparent at lower T values. Weak correlations with serum albumin were observed for the ratios of cFT-L and cFT-Z over measured FT (Table 3; Supplemental Fig. 2). In contrast, the ratio of cFT-V over measured FT did not significantly vary with different SHBG, albumin, or T levels.

The different relationships between cFT and FT measured by direct ED according to the three calculations are also clearly apparent when looking at the FT%, as illustrated in Fig. 2, which displays FT% plotted against serum SHBG levels for all available results in men (samples are from the EMAS and SIBLOS cohorts). For EMAS samples, median (p2.5 to p97.5) FT% was 1.65% (1.13% to 2.64%) for cFT-V, 1.41% (1.08% to 1.69%) for cFT-L, and 3.04% (2.50% to 3.69%) for cFT-Z as compared with 1.39% (range, 0.90% to 2.29%) for direct ED-measured FT percentage level. Rank correlations for absolute cFT (or cFT%) vs ED absolute FT (or FT%) were 0.91 (0.89) for cFT-V, 0.76 (0.74) for cFT-L, and 0.73 (0.62) for cFT-Z.

Table 2. Summary Findings for FT by Direct ED Compared With FT by Calculations for the EMAS Sample Set (n = 100)

	FT (p2.5–p97.5), Median, pmol/L	FT (p2.5–p97.5), Median, %
Direct ED	212 (117–427)	1.4 (0.9–2.3)
cFT-V	263 (155–432)	1.7 (1.1–2.6)
cFT-L	225 (110–413)	1.4 (1.1–1.7)
cFT-Z	459 (254–843)	3.0 (2.5–3.7)

Women

Data for samples from women were available only for cFT-V and cFT-L. The observations for the ratios of cFT-V and cFT-L over FT by ED, using for cFT-L the formula intended for T < 5 nM/L, were similar to those for the samples from men (Supplemental Fig. 3). The median ratio (range) was 1.33 (0.75 to 2.76) for cFT-V and 0.91 (0.38 to 2.24) for cFT-L; the ratio for cFT-L was significantly dependent on serum SHBG level ($\rho = 0.67$; $P < 0.0001$) and serum total T level ($\rho = 0.28$; $P < 0.0001$), whereas this was not seen for cFT-V vs either SHBG level ($\rho = 0.09$; $P = 0.24$) or serum total T level ($\rho = 0.06$; $P = 0.39$).

Median FT% (p2.5 to p97.5) was 1.5% (0.7% to 3.0%) for cFT-V and 1.1% (0.7% to 1.5%) for cFT-L, compared with 1.2% (0.4% to 2.7%) according to ED. Compared with FT% by ED, FT% by cFT-V and cFT-L followed clearly distinct patterns (Supplemental Fig. 4). Rank correlations for FTC (FT%) vs ED FT(FT%) were 0.90 (0.81) for cFT-V and 0.77 (0.50) for cFT-L.

Discussion

In this study, we reassessed FT with a state-of-the-art direct ED assay. The findings support and further validate the basic tenets of FT in men and women as previously established with traditional indirect ED and UF methodologies. They further highlight important differences in commonly used or recently proposed algorithms for deriving calculated FT values from serum total T and SHBG (and albumin) levels compared with FT measurement by direct ED.

FT level by direct ED

Although FT concentrations were ~20-fold lower in women compared with men, values for FT% were similar and ranged between 0.9% and 2.9%, with a median of 1.5% in men and between 0.4% and 2.8% with a median of 1.2% in women. As expected, in both men and women, absolute FT was positively associated with total T level and FT% as strongly negatively associated with serum SHBG. The associations for men might be affected by the fact that some older men in the EMAS cohort do not have a fully functional hypothalamic-pituitary-testicular axis (26).

The ranges we observed for FT and FT% are in full agreement with earlier findings obtained with the older indirect ED (1, 8, 12, 27) and UF (2, 28) methods. Our

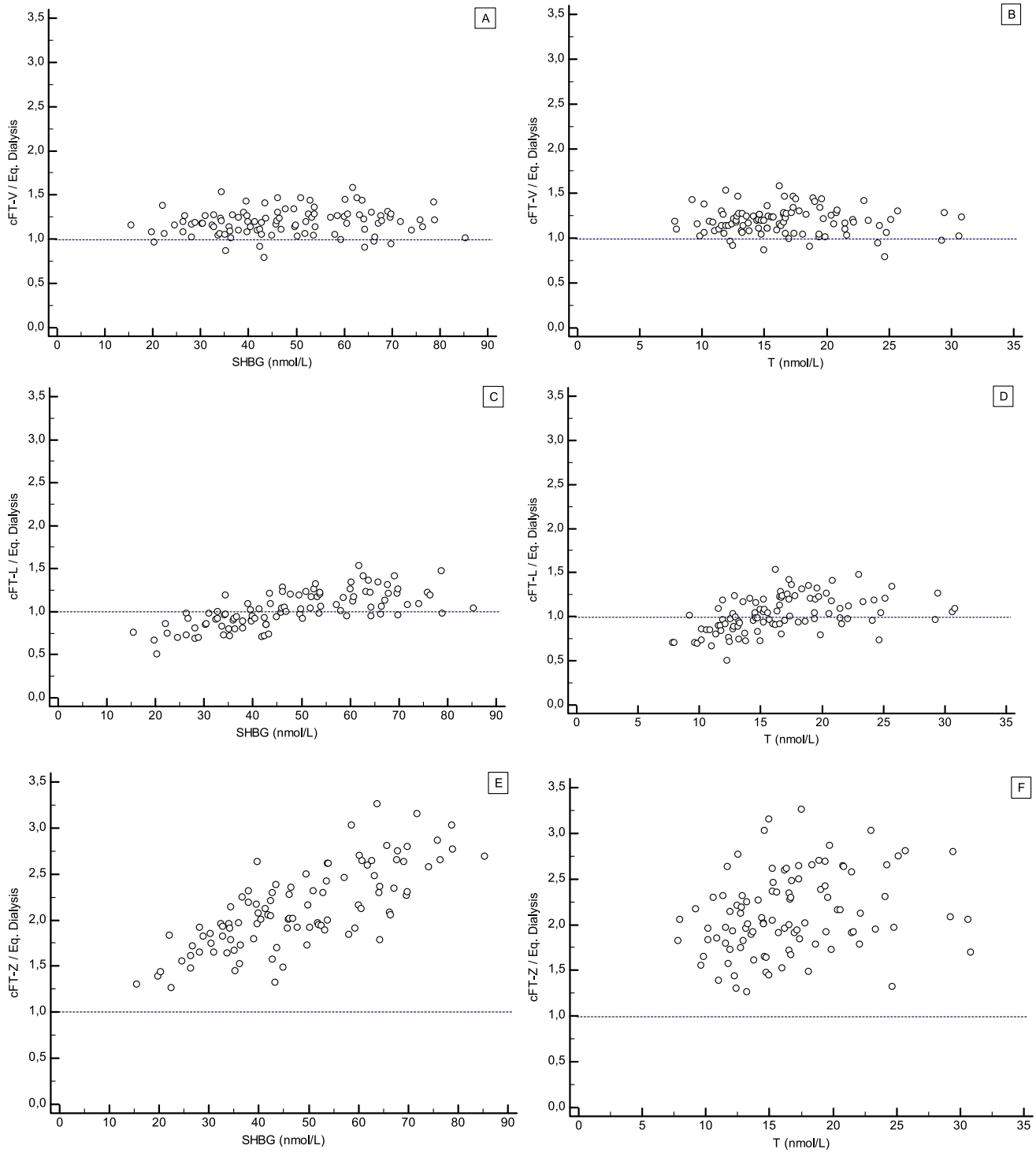

Figure 1. Ratio of the estimated absolute FT value by calculation over the measured FT by direct ED for 100 samples from the EMAS cohort plotted against (A, C, E) SHBG levels and (B, D, F) total T levels, respectively, using three different calculation modalities: (A, B) cFT-V, (C, D) cFT-L, and (E, F) cFT-Z.

results are also concordant with the limited data on FT for men from direct measurement by isotope dilution-gas chromatography–mass spectrometry after UF (29) and by LC-MS/MS after UF or ED (30). For healthy cycling women, Törmä *et al.* (31) reported levels similar to those we report in the current study by a direct ED method with use of a ^{3}H-testosterone–based radioimmunoassay, and Sinha-Hikim *et al.* (32) reported comparable FT% values by a direct ED method with use of a ^{125}I-testosterone–based radioimmunoassay. Taken together, it would appear that the range of FT% of 1% to 3% in men and 0.5% to 3% in women, based on the literature and hereby confirmed with a state-of-the-art mass spectrometry–based direct ED method, can be considered

Table 3. Ratio of Calculated FT Over FT Measured by Direct ED According to Three Different Calculation Modalities

	cFT-V/Dialysis	cFT-L/Dialysis	cFT-Z/Dialysis
Ratio (p2.5–p97.5), median	1.19 (0.90–1.47)	1.00 (0.69–1.42)	2.05 (1.26–3.26)[a]
ρ vs SHBG	0.17	0.72[b]	0.75[b]
ρ vs T	0.02	0.55[b]	0.31[c]
ρ vs albumin	−0.01	0.23[c]	−0.24[c]

[a]cFT-Z/dialysis calculated with T and SHBG values obtained in EMAS. Median ratios when calculated with these values were 1.33 and 1.05 for cFT-V/dialysis and cFT-L/dialysis, respectively.

[b]$P < 0.001$.

[c]$P < 0.05$.

as robustly established. Nevertheless, a minority of laboratories reported different values (17, 33), some of which may reflect technical issues arising from the use of variants of the (mostly indirect) ED- and UF-based methods, which have not been well validated. To minimize disparities in FT results from different laboratories methods in the future, efforts toward interlaboratory standardization of FT measurement, preferably using direct ED or UF methods, should be encouraged.

Calculated estimates of FT

Calculated FT estimates are critically dependent on reliability and calibration of the T and SHBG assays (9, 34, 35). Furthermore, they are based on the assumption of a normal steady-state protein-binding characteristic for T, which is not the case in every individual. Any equation will incorrectly estimate FT in situations such as presence of large concentrations of competing steroids, large deviations from physiological protein concentrations, or rare genetic variants of SHBG affecting T binding affinity (3, 8). Besides these occasional problems, more systematic differences between FT estimates depending on the used equation and as compared with measured FT have been reported (11, 12, 28).

Our results show that cFT-V is strongly correlated to FT measured by direct ED but systematically overestimates FT by 20% to 30%. This confirms our prior findings (22, 23) and those of others (28). The relation between cFT-V and measured FT is linear and independent of serum T, SHBG, and albumin levels. This is a strength of the cFT-V approach for clinical use, because assessment of FT is most relevant in patients with high or low SHBG levels. This also indicates that the equation derived from the mass action law predicts the binding behavior of T to serum proteins quite well. The systematic positive bias observed for cFT-V has to be taken into account when comparing FT levels across different methods. This bias likely reflects that the *in vitro* determined association constants used in the equation are imperfect approximations of the actual *in vivo* association constants for binding of T to SHBG and albumin. FT measured with our prior in house indirect ED, which involves a correction for serum dilution effect with use of the same basic equation and set of association constants as in cFT-V (1, 8), shows a similar positive bias compared with FT measured by direct ED (data not shown). This explains our prior findings of correlation without bias between cFT-V and FT by indirect ED (8, 35).

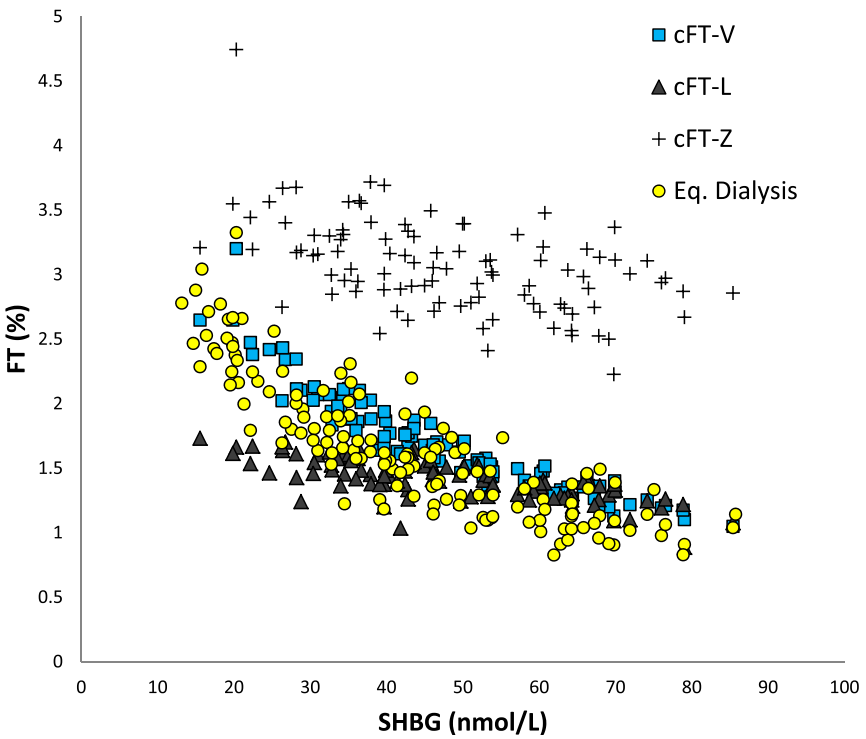

Figure 2. FT% as estimated by cFT-V, cFT-L, and cFT-Z and as measured by direct equilibrium (Eq.) dialysis (EMAS, n = 100), plotted against SHBG concentration (nmol/L).

Our results for cFT-L show that median cFT-L approximates closely median FT by direct ED in men and women, as calculated with the equations intended for high and low T levels, respectively (16). However, agreement between cFT-L and measured FT was found to be strongly dependent on SHBG and T levels. Thus, cFT-L performs differently depending on serum T and SHBG levels and increasingly underestimates FT at low SHBG or low T levels. This may limit accuracy of cFT-L in hypogonadal men with low T levels and in obese men or women with PCOS with low SHBG levels.

The cFT-Z values reported here have been supplied by the authors who reported data for cFT-Z in the EMAS cohort in the publication describing their multistep, dynamic, allosteric model to calculate FT (17). We requested access to the cFT-Z algorithm from the research group that developed this allosteric model algorithm. However, at the time of completion of this work, we had not been able to gain direct access to the algorithm. Therefore, it was not possible to make comparisons with cFT-Z for all three cohorts. This is a limitation of the current study that is beyond our control. We felt it important to evaluate cFT-Z in the current study because the results obtained by the authors according to their allosteric model to replicate the dimeric binding of T to SHBG differed substantially from the model based on the law of mass action (4, 17). Using the allosteric model, they reported higher FT% in men of 3% to 5% and that cFT-V substantially underestimated FT compared with their findings for FT by dialysis (17). Our results for the EMAS samples, indeed, do reproduce their finding that cFT-Z values are markedly higher than cFT-V values. Similarly, cFT-Z values are much higher compared with cFT-L. However, in contrast to their findings, our results also show that cFT-Z is markedly higher (about double) compared with FT measured by direct ED. Moreover, accuracy of cFT-Z as reflected in the ratio of cFT-Z over measured FT was strongly dependent on serum SHBG levels and, to a lesser degree, on T and albumin levels. At present, it is unclear what underlies the apparent discrepancy between the results reported by Zakharov *et al.* (17) and the findings in the current study performed on a same set of samples. A factor involved may be differences in ED methods between laboratories giving discrepant measured FT results. The descriptive nature of this study does not allow us to address possible merits or demerits of basic assumptions on which the allosteric model is based.

In summary, for none of the three evaluated equations does calculated FT perfectly match FT measured with a state-of-the-art direct ED assay. However, there are distinct differences in how the respective equations behave. cFT-Z appears far off target relative to the results of direct ED in this study as well as compared with a substantial body of published data obtained with a variety of ED- or UF-based methods. Although cFT-L performs well in the midrange levels of serum T and SHBG, the dependence of its accuracy on T and SHBG levels has clinical implications (*e.g.*, underestimation by cFT-L of FT at low SHBG concentrations could impair the ability to detect hyperandrogenism in PCOS or lead to overdiagnosis of hypogonadism in obese men). Although a systematic positive bias affects the external comparability of cFT-V with other methods, the consistency of its performance compared with directly measured FT, independent of serum T and SHBG levels, ensures strong internal validity. This is an important asset for clinical applications of FT assessments in patients with widely different T and SHBG levels. The cFT-V equation admittedly is a simplified representation of the binding of T to its binding proteins. Moreover, there is room for refinement of the association constants implemented in the equation. Nevertheless, contrary to what has been suggested (4, 17, 28, 36), our results do confirm that cFT-V is based on a valid model of T binding to SHBG.

In conclusion, calculated estimates of FT have inherent limitations with distinct and clinically important differences in the performance of different algorithms. Of the three methods we evaluated in this study, cFT-V, albeit systematically overestimating FT, most robustly approximated directly measured FT in samples representative of a broad range of T and SHBG levels. There is a need for collaborative efforts to further validate and harmonize methods to measure and calculate FT levels.

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