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A multi-step, dynamic allosteric model of testosterone's binding to sex hormone binding globulin

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ABSTRACT

Purpose: Circulating free testosterone (FT) levels have been used widely in the diagnosis and treatment of hypogonadism in men. Due to experimental complexities in FT measurements, the Endocrine Society has recommended the use of calculated FT (cFT) as an appropriate approach for estimating FT. We show here that the prevailing model of testosterone's binding to SHBG, which assumes that each SHBG dimer binds two testosterone molecules and that the two binding sites on SHBG have similar binding affinity is erroneous and provides FT values that differ substantially from those obtained using equilibrium dialysis.

Methods: We characterized testosterone's binding to SHBG using binding isotherms, ligand depletion curves, and isothermal titration calorimetry (ITC). We derived a new model of testosterone's binding to SHBG from these experimental data and used this model to determine FT concentrations and compare these values with those derived from equilibrium dialysis.

Results: Experimental data on testosterone's association with SHBG generated using binding isotherms including equilibrium binding, ligand depletion experiments, and ITC provide evidence of a multi-step dynamic process, encompassing at least two inter-converting microstates in unliganded SHBG, readjustment of equilibria between unliganded states upon binding of the first ligand molecule, and allosteric interaction between two binding sites of SHBG dimer. FT concentrations in men determined using the new multistep dynamic model with complex allostery did not differ from those measured using equilibrium dialysis. Systematic error in calculated FT vales in females using Vermeulen's model was also significantly reduced. In European Male Aging Study, the men deemed to have low FT (<2.5th percentile) by the new model were at increased risk of sexual symptoms and elevated LH.

Conclusion: Testosterone's binding to SHBG is a multi-step dynamic process that involves complex allostery within SHBG dimer. FT values obtained using the new model have close correspondence with those measured using equilibrium dialysis.

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1. Introduction

Testosterone, the major androgen in humans, circulates in blood bound largely to sex hormone binding globulin (SHBG) and albumin (Bhasin et al., 2010; Hammond and Bocchinfuso, 1996; Mendel, 1989; Rosner, 1991; Rosner et al., 2007). Testosterone can also bind to orosomucoid and transcortin proteins, but the amount of testosterone bound to these proteins in human plasma is negligible. In many conditions that affect SHBG concentrations, such as obesity, diabetes, aging, hyperthyroidism, liver disease, and HIV-infection, total testosterone concentrations are altered because of changes in SHBG concentrations; in these conditions, expert panels have recommended the determination of free testosterone (FT) concentration to obtain an accurate assessment of androgen status (Bhasin et al., 2010; Hammond and Bocchinfuso, 1996; Mendel, 1989; Rosner, 1991; Rosner et al., 2007).

Reflecting the growing interest in men's health and the success of pharmaceutical advertising, testosterone sales have grown from 23 million dollars in 1993 to 70 million in 2000 to 1.7 billion dollars in 2012 (Spitzer et al., 2012). Testosterone is the second most frequently ordered test, next only to 25-hydroxyvitamin D. In 2012, nearly 4 million free testosterone tests were performed in the USA alone. A number of direct and indirect methods – equilibrium dialysis, ultrafiltration, tracer analog methods, and calculations based on simple law-of-mass action equations – have been developed for the determination of FT levels (Adachi et al., 1991; Mazer, 2009; Rosner, 1997; Rosner et al., 2007; Sinha-Hikim et al., 1998; Sodergard et al., 1982; Van Uytfanghe et al., 2004; Vermeulen et al., 1971, 1999; Winters et al., 1998). Expert panels have expressed concern about the accuracy and methodological complexity of the available assays for FT (Rosner et al., 2007; Sodergard et al., 1982; Vermeulen et al., 1971). Recognizing these methodological difficulties in the measurement of free testosterone, the Endocrine Society's Expert Panel suggested that "*the calculation of free testosterone from reliably measured total testosterone and SHBG using mass action equations provides the best approach for the estimation of free testosterone…*" (Rosner et al., 2007). Therefore, algorithms for calculating FT from total testosterone, SHBG and albumin concentrations using the extant linear binding model (also referred to as law-of-mass-action equations) (Adachi et al., 1991; Bhasin et al., 2011; Ly and Handelsman, 2005; Ly et al., 2010; Mazer, 2009; Morales et al., 2012; Morley et al., 2002; Rosner et al., 2007; Sartorius et al., 2009; Sodergard et al., 1982; Vermeulen et al., 1999) or empirically-derived equations (Ly and Handelsman, 2005; Ly et al., 2010; Nanjee and Wheeler, 1985; Sartorius et al., 2009) have been published and used widely (Adachi et al., 1991; Bhasin et al., 2011; Ly and Handelsman, 2005; Ly et al., 2010; Mazer, 2009; Morales et al., 2012; Morley et al., 2002; Rosner et al., 2007; Sartorius et al., 2009).

The current model of testosterone's binding to SHBG assumes that each SHBG dimer binds two testosterone molecules, and that each of the two binding sites on SHBG dimer has similar binding affinity irrespective of the occupancy of the adjacent binding site (no allostery). Equations to determine FT, using this model, have been proposed by Vermeulen, Sodergard, and Mazer (Mazer, 2009; Rosner et al., 2007; Sodergard et al., 1982; Vermeulen et al., 1971). In present work we characterized testosterone's binding to SHBG using equilibrium dialysis (varying ligand and SHBG concentrations) and isothermal titration calorimetry (ITC) to characterize testosterone's binding to SHBG. We considered several possible mechanistic models of molecular interactions, including the prevailing model of homogeneous binding of testosterone to SHBG as envisioned by Vermeulen (Vermeulen et al., 1999), Sodergard (Sodergard et al., 1982) and implemented in a spreadsheet by Mazer (Mazer, 2009), and various allosteric mechanisms, including positive and negative cooperativity (Koshland et al., 1966; Monod et al., 1965), and ensemble allostery (Freiburger et al., 2011; Hilser and Thompson, 2007) (Fig. 1). Based on our analyses of the experimental data of testosterone's binding to SHBG, we constructed a novel multistep binding model with complex intra-dimer allostery for the calculation of FT, which provided the best fit to the totality of experimental data. This new model was then utilized to determine FT concentrations in samples derived from randomized testosterone trials in men and women, and to compare the results with those obtained using equilibrium dialysis. Finally, we used the algorithm to examine the distribution of FT levels in community-dwelling men in the Framingham Heart Study (FHS) and related the deviations from the mean to the risk of sexual symptoms and elevated LH levels in an independent sample of men in the European Male Aging Study (EMAS).

2. Materials and methods

2.1. Biophysical characterization

Human SHBG purified from serum (Binding Site Group, Birmingham, UK, cat# BH089.X) was characterized by protein gel denaturation–renaturation experiments and by measuring its ability to bind testosterone. Testosterone concentration in the SHBG stock solution, measured using LC-MS/MS, was undetectable. Testosterone standard 1.0 mg/mL ± 2% (3.47 mM) was obtained from Cerilliant (Round Rock, TX).

Binding isotherms were established using equilibrium dialysis (varying either testosterone or SHBG concentrations) in 96-well dialysis plates containing dialysis chambers separated by membranes with 10 kDa cut-off (Harvard Apparatus, Holliston, MA). SHBG and testosterone were reconstituted in dialysis buffer (30 mM HEPES pH7.4, 90 mM NaCl, 1 mM $MgSO_4$, 187 μM $CaCl_2$), and mixed to a desired concentration. Two hundred microliters of SHBG-testosterone mixture was loaded on one side of the dialysis membrane and dialyzed overnight against equal volume of dialysis buffer (200 μL). The equilibrium was achieved by rotating the dialysis plate overnight at 22 °C. Preliminary experiments demonstrated that 16 hours were sufficient to achieve steady-state equilibrium. Each concentration/condition was tested in three different wells, and each titration was repeated at least two times.

Testosterone concentration was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) assay that has been certified by the Center for Disease Control and has a sensitivity of 2 ng/dL (Bhasin et al., 2011).

Isothermal calorimetry (ITC) was performed using automated Auto-ITC200 Calorimeter (MicroCal, Northampton, MA) at Biological Calorimetry Facility (Huck Institutes of Life Sciences, University Park, PA). SHBG was reconstituted in 30 mM HEPES buffer, pH7.4, to a final concentration of 5 μM. Testosterone standard was prepared in DMSO and diluted in protein buffer to 100 μM in 5% DMSO. DMSO was added to SHBG by weight to match DMSO content in testosterone solution. Samples were degassed prior to loading to the calorimeter. Testosterone was injected into protein solution in 15 equal steps 2 μL each. Total reaction volume was 203 μL. Isothermal titration calorimetry experiment was repeated twice. Heat produced by each injection was measured by the calorimeter. Interval between injections was set at 240 seconds so that the temperature could return to baseline. The heat generated after each injection (after subtracting the heat of dilution of ligand in buffer) was integrated to produce calorimetric isotherm depicting the relation of the total heat generated in the reaction to testosterone-to-SHBG molar ratio.

At time $t = 0$, we computed initial concentration of S_2 and S'_2 as defined by the SHBG concentration and K_{d11}. During the process of reaching chemical equilibria, the computer program recorded the total flux of SHBG molecules through each of the elementary reactions Φ_j. Total heat generated/consumed during the equilibration was calculated as $H_i = \sum_j \Phi_{ij} \Delta H_j$, for each injection number "i". Total

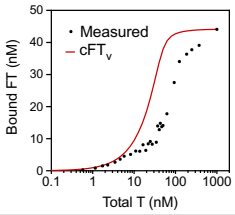
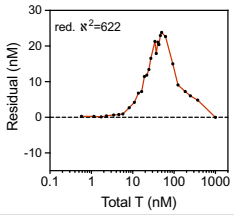
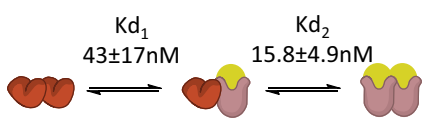
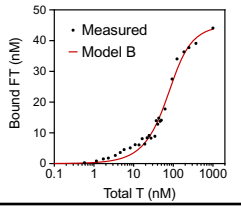
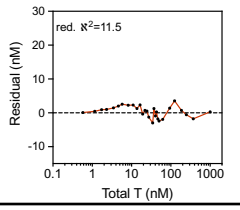
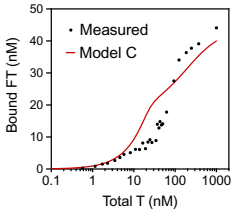
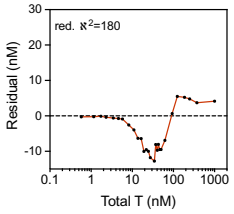
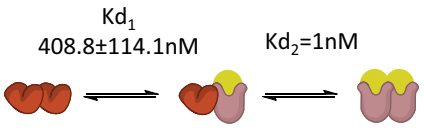
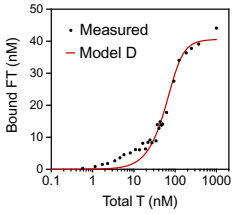
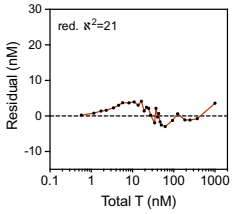
Model # and Description	Model Schematic	Fits for binding isotherms	Residuals for fits to binding isotherms
A) Vermeulen model Kd_1=Kd_2= 1nM			
B) Both Kds allowed to vary			
C) Negative cooperativity. Kd_1 =1nM; Kd_2 varied			
D) Positive cooperativity. Kd_2 =1 nM; Kd_1 varied			

Fig. 1. Schematic representations of the various models tested in this study to examine SHBG:T interaction. Column 1 describes the features of SHBG:T interaction. Column 2 displays the schematic and dissociation constants obtained from the fits (Column 3) to testosterone binding data (20 nM SHBG) from equilibrium dialysis. Different shapes represent distinct conformational states of SHBG monomers within the dimer. Column 4 shows the residuals for the fits to binding isotherms and corresponding reduced chi-square adjusted for the number of variables in the fit. The colors and shapes denote conformationally distinct states of monomers within the dimer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reaction throughput is a function of the reaction constants as well as the initial concentrations.

For each injection the starting total concentrations of T (T_i) and SHBG (S_i) were calculated as follows: $T_i = T_0\left(1-(1-\Delta V/V_0)^i\right)$, $S_i = S_0(1-\Delta V/V_0)^i$ (Velazquez-Campoy et al., 2004), where V_0 is the calorimeter cell volume, ΔV is injection volume. Therefore, for each injection "$\imath$" we obtained $H\imath$ as a function of reaction constants and enthalpies. For the fitting procedure, we placed a restriction on the enthalpy change values according to the Hess's law, such that the algebraic total of the enthalpies along the kinetic loop equals zero.

2.2. Numerical simulations of allostery

Various molecular models of testosterone's binding to SHBG were numerically tested using LabVIEW (National Instruments, Austin, TX) toolkit (Zakharov et al., 2012) (available at https://code.google.com/p/labview-biochemical-framework/). Parameter estimation for the models was performed using simulated annealing, as described previously (Zakharov et al., 2012). Numerical correction for the equilibrium dialysis was incorporated as a part of every simulation model. Since some of the models and equations (Nanjee and Wheeler, 1985; Sodergard et al., 1982; Vermeulen et al., 1999) were developed before the recognition of the two binding sites per SHBG dimer (Avvakumov et al., 2001), we adjusted SHBG concentration to account for its dimeric state.

The fits of both types of binding profiles and ITC to various models were compared by calculating the residuals and fit values for each model. For the models providing adequate visual fit to the binding profiles, reduced χ^2 values were computed.

2.3. Assessment of FT concentrations in clinical trials

We compared FT determined using the multi-step dynamic binding model (Fig. 1 Model G) with complex allostery developed in this study (cFT_{ZBJ}) and Vermeulen's equation (cFT_V) (as implemented by Mazer (2009)) with those measured using equilibrium dialysis in samples derived from randomized testosterone trials in men (Spitzer et al., 2012) and women (Huang et al., 2014). These samples had been collected in fasting state in the morning, stored at –80 °C, and never thawed.

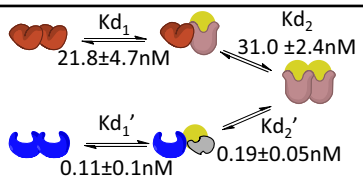
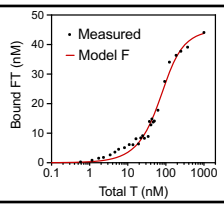
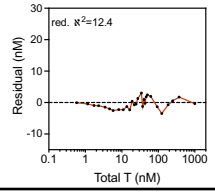
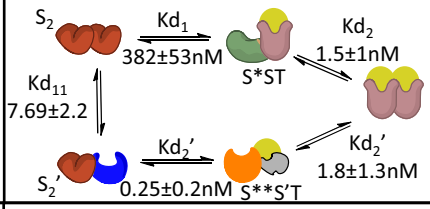
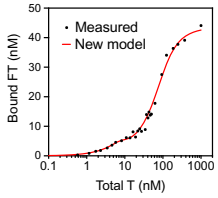
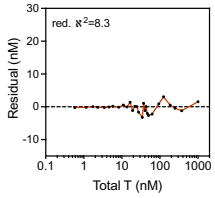
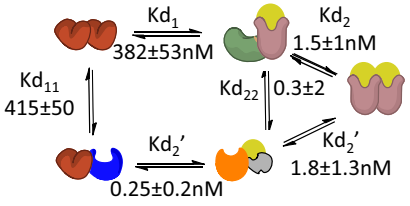
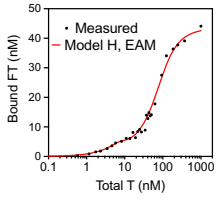
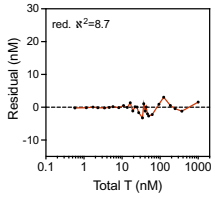
Model # and Description	Model Schematic	Fits for binding isotherms	Residuals for fits to binding isotherms
E) Two static populations; no inter-conversion in un-liganded state			
F) New Model Developed in this study			
G) Ensemble allostery model (generalized EAM)			

Fig. 1. (*continued*)

Testosterone in Men with Erectile Dysfunction (TED) Trial, whose results have been published (Spitzer et al., 2012), was a randomized trial to determine whether addition of testosterone to an optimized regimen of sildenafil citrate is superior to placebo in improving erectile function in men with erectile dysfunction (ED) and low testosterone. At baseline and after 12-weeks of testosterone or placebo administration, total testosterone concentrations were measured using LC-MS/MS and SHBG concentrations using a two-site immunofluorometric assay (DELFIA®, Perkin-Elmer, Waltham, MA) (21). FT was measured in the same samples by equilibrium dialysis (Bhasin et al., 2012).

Testosterone Dose-Response in Surgically-Menopausal Women (TDSM) Trial was a placebo-controlled, randomized trial in which surgically menopausal women, 21–60 years, who had undergone hysterectomy with or without partial or total oophorectomy, and had total testosterone <31 ng/dL or FT<3.5 pg/mL were administered a stable regimen of transdermal estradiol patch (50-μg Alora, Watson Pharmaceuticals) for a 12-week run-in phase and then randomized to receive weekly IM injections of placebo or 3.0, 6.25, 12.5 or 25.0 mg testosterone enanthate for 24 weeks (Spitzer et al., 2012). At baseline and after 6-months of intervention, total testosterone and SHBG levels were measured, and FT concentrations were measured by equilibrium dialysis as well as calculated using the new model (cFT_{ZBJ}) and Vermeulen's method (cFT_V) as implemented by Mazer (2009).

2.4. Distribution of free testosterone in community-dwelling men in the FHS and EMAS

Epidemiologic data were derived from Generation 2, Examination 7 and Generation 3, Examination 1 of the FHS (Bhasin et al., 2011; Krasnoff et al., 2010; Splansky et al., 2007), as well as the EMAS (Lee et al., 2009; Wu et al., 2010). We examined the distribution of FT in a reference sample of healthy young men (19–40 years; $N = 434$) in FHS Generation 3, who were free of cancer, CVD, diabetes mellitus, obesity, hypertension, hypercholesterolemia and smoking, as recommended by the International Federation of Clinical Chemists (Elveback, 1973; Solberg, 1987), and as described previously (Bhasin et al., 2011). Because the wild type SHBG protein was used in experiments, we included only men with the wild type SHBG (CC for rs6258 SNP) genotype, who constitute nearly 98% of the population (Ohlsson et al., 2011). The relation of age to FT was elucidated in a combined sample of men from FHS Generations 2 and 3 (FHS broad sample).

In EMAS, we determined whether deviations in FT levels from the mean were associated with increased risk of sexual symptoms that have been found to be associated with hypogonadism in men. The simultaneous presence of three sexual symptoms (Wu et al., 2010) was taken as an indicator of decreased sexual function. As elevated LH levels are indicative of primary testicular dysfunction, we also determined whether low FT levels were associated with increased risk of elevated LH (LH >9.4 U/L) (Tajar et al., 2010).

2.5. Statistical analysis

The fits of data to various models were compared using the reduced χ^2 values for each model, accounting for a number of varying parameters. For clinical trials data, the distributions of measured and calculated FT were derived for each of the relevant samples. Calculated FT levels were plotted separately for men and women against values obtained by equilibrium dialysis over the relevant physiologic ranges. Agreement between measured and calculated FT values was estimated using Deming (orthogonal) Regression, and Bland–Altman style plots were used to assess the difference between calculated and measured concentrations as a function of the measured concentration. Age-specific estimates were derived for each decade of age in the FHS broad sample. Graphical depictions of association between FT, total testosterone, and SHBG were generated, with scatter plot smoothing using Generalized Additive Models with tensor product smooths (Wood, 2006). The odds of expression of sexual dysfunction or elevated LH were estimated as a function of FT using logistic regression with adjustment for age.

3. Results

3.1. Prevailing model of SHBG:T interaction is erroneous and results in significant deviation in calculated free T

We conducted systematic evaluation of calculated free T values using the extant SHBG:T interaction model (cFT_v) in two, placebo controlled, randomized clinical trials in men and women receiving testosterone supplementation (TED and TEAM). Preliminary studies revealed that cFT values obtained using the Vermeulen's equation in samples derived from the TED Trials were systematically lower than those measured by equilibrium dialysis. To determine the molecular basis of this discrepancy, we used three experimental approaches to characterize testosterone's binding to SHBG: binding isotherms by varying testosterone concentrations at a fixed SHBG concentration, ligand depletion curves by varying SHBG concentrations at a fixed testosterone concentration, and isothermal titration calorimetry (ITC).

3.2. Different models of testosterone's binding to SHBG

We examined several possible models (Fig. 1) of ligand binding using the experimental data obtained in this study. Consistent with the reported crystal structure of liganded SHBG (Avvakumov et al., 2002; Grishkovskaya et al., 1999, 2000, 2002), all models were constrained to eventually converge to a single double-liganded conformational state of SHBG dimer. The simplest linear interaction model, proposed by Vermeulen, assumes that each binding site interacts with testosterone with the same Kd of 1 nM, regardless of the occupancy of the other binding site (Fig. 1, model A). Second model incorporates non-interacting monomers that have different binding affinities (Fig. 1, model B). Here, association constants of both steps are allowed to vary while fitting the binding data. In the third and the fourth models, positive and negative cooperativity was included (Koshland et al., 1966); i.e., the binding of the first testosterone molecule either facilitates (Fig. 1, model C) or suppresses (Fig. 1, model D) binding of the second testosterone molecule. In these models, we tested the presence of intra-dimer cooperativity by fixing one of the Kds to the consensus value of 1 nM with an additional constraint of $Kd_1 < Kd_2$ (negative cooperativity) or $Kd_1 > Kd_2$ (positive cooperativity).

Because none of the simpler models of cooperativity fit the data, we considered the possibility that SHBG monomers have conformational heterogeneity. Model E assumes two distinct populations of SHBG and an allosteric interaction between the monomers. As is evident from columns 2 and 3 of Fig. 1, none of the above models A–E captured the distinct features of SHBG:T binding isotherms. Thus, we arrived at model F: a multi-step interaction between SHBG and T where the conformational heterogeneity in at least two interconverting states exits in unliganded SHBG dimers. Both states are capable of binding to T with distinct affinity. After associating with the first testosterone molecule, the monomers within the dimer exhibit allosteric rearrangement and subsequently converge to the same conformational state of fully occupied SHBG dimer. For completeness of analysis we tested an even more complex, ensemble allostery model (EAM; Fig. 1 Model G) which has an additional equilibrium amongst the intermediate states as described in theoretical terms by Hilser and Thompson (2007) and reported for aminoglycoside *N*-(6′)-acetyltransferase by Freiburger et al. (2011).

General Approach for the Evaluation of the Various Molecular Models: We applied a three-step approach to evaluate these molecular models of testosterone's binding to SHBG. First, we examined the data fits of the binding isotherms for each of the models for relative goodness of fit. We analyzed the fits statistically using analyses of residuals as well as a reduced chi square statistic, a measure of the goodness of fit. Second, we evaluated whether the models captured the key characteristic features of the binding isotherms (two distinct saturation plateaus, including an apparent plateau at lower testosterone concentrations, and the asymmetry of the isotherm around the EC_{50} value). We posited that if a model does not capture these unique characteristics of the binding isotherm, then the model is not useful regardless of the number of parameters. As is apparent from Fig. 1, certain fits have similar chi-square values but miss the key features of the binding isotherms. Finally, for the most parsimonious model that accurately captured the features of equilibrium binding data, we examined the predicted free T values and compared them with those measured directly using equilibrium dialysis.

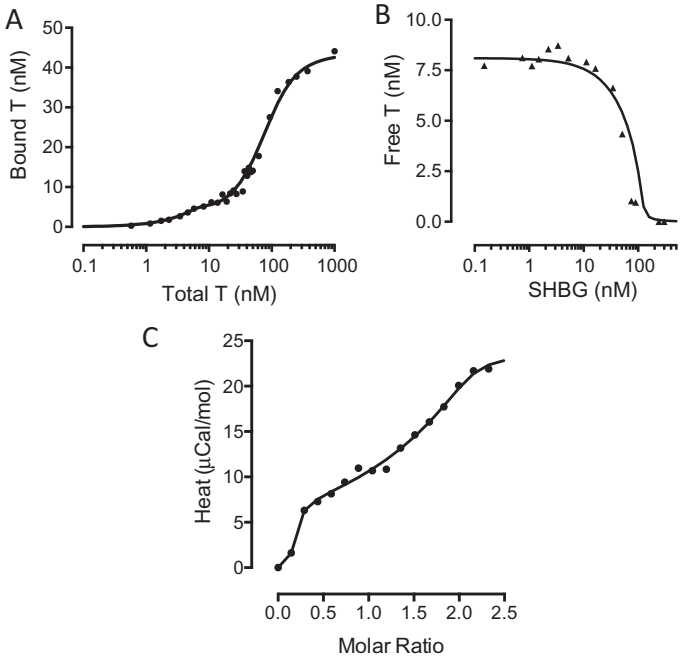


Fig. 2. Biophysical characterization of SHBG:T interaction (equilibrium binding). (Panel A) Graded concentrations of testosterone were incubated overnight with 20 nM SHBG and the amount of bound testosterone was plotted against SHBG concentration. The fit curve represents the fit of data to the new model (Model F). (Panel B) Ligand (Testosterone) depletion. A constant concentration of testosterone (8.7 nM) was incubated with increasing SHBG concentrations. Free testosterone concentration is plotted against SHBG concentration. The depletion of free testosterone by increasing SHBG concentrations is best described by the new model (solid line represents the fit of data to the new model). (Panel C) Isothermal titration calorimetry. The integrated ITC curve was generated after subtracting the buffer heats. SHBG concentration is 5 μM and heat is plotted as a function of [T]/[SHBG] molar ratio. Experimental points are overlaid with the fit of data to the new model shown by a solid line.

3.3. Biophysical characterization of testosterone's binding to SHBG reveals evidence of complex homo-allostery within SHBG dimer

3.3.1. Equilibrium binding

To generate the binding isotherms, 20 nM SHBG (dimer) was incubated with graded concentrations of testosterone (0–400 nM) at 22 °C, as described in the methods section. When bound testosterone concentration was plotted against total testosterone concentration (Fig. 2A), the binding isotherm displayed several characteristic features: two distinct saturation plateaus (including an apparent plateau at lower testosterone concentrations), and asymmetry of the isotherm around the EC_{50} value. Data were also generated for 5 and 10 nM SHBG and exhibited similar binding profile (not shown). The relation of bound testosterone to total testosterone could not be adequately explained by Vermeulen's model. Only a new multi-step model with complex allostery fit the binding

isotherm optimally with the lowest reduced χ^2 (Fig. 1, Model F) and explained the observed saturation plateaus, including the plateau at lower testosterone concentrations, and the asymmetry of binding isotherm around EC_{50}.

3.3.2. Testosterone depletion curves

As an independent assessment of testosterone's binding to SHBG, we incubated various amounts of SHBG (0.1–0.5 μM) with a fixed concentration of testosterone (8.7 nM), and analyzed the depletion of unbound testosterone when increasing concentrations of SHBG were added (Fig. 2B). The relation of FT to SHBG concentration in depletion experiments was again best fit using the new model. The analysis of residuals (Fig. 1) revealed that the optimal fit was consistently provided by the new multistep dynamic model with complex allostery.

3.3.3. Isothermal titration calorimetry (ITC)

To validate the new model further and to evaluate the thermodynamic parameters associated with testosterone's binding to SHBG, we measured the heat produced as progressively larger amounts of testosterone bind to SHBG. The ITC data elicit a characteristic shoulder (Fig. 2C) and cannot be described as a simple sigmoidal curve predicted by Vermeulen's model. Using the computational framework developed in LabVIEW (Zakharov et al., 2012), we generated the fits of the ITC data (mathematical treatment is detailed in Section 2, Materials and methods) (Freiburger et al., 2011). The shape of ITC curve can be explained as a convoluted result of testosterone's binding and multiple conformational rearrangements defined by the comprehensive model incorporating allostery. Model constants obtained as a result of linked fit in Figs 1A and 1B were used as a starting point for the fit; enthalpies and reaction constants computed from the fit ITC data are presented in Fig. 3. While we used an independent enthalpy parameter for each reaction in the model, they are not simultaneously identifiable.

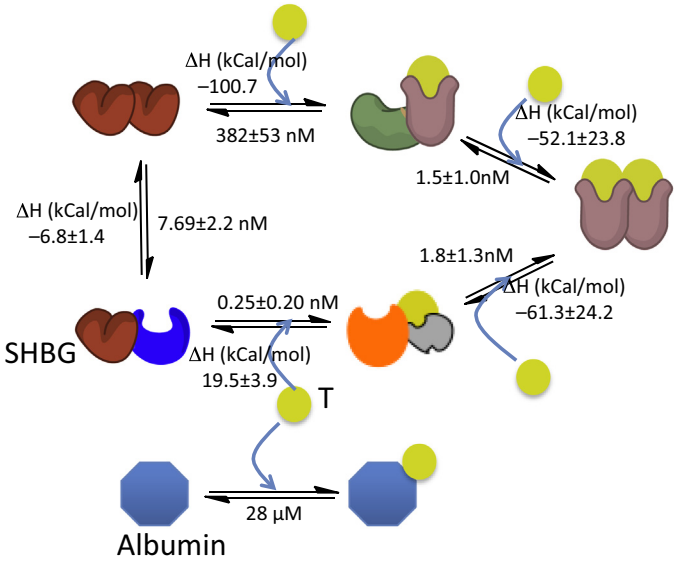


Fig. 3. The new binding model depicts a multi-step dynamic process, encompassing at least two inter-converting microstates in unliganded SHBG, readjustment of equilibria between unliganded states upon binding of the first ligand molecule, and allosteric interaction between two binding sites of SHBG dimer. All dissociation constants are listed in nM. Thermodynamic parameters associated with testosterone's binding to SHBG derived from the fit of binding isotherms and ITC data to the new model developed in this study are shown in the figure. These parameters together describe the binding isotherms, depletion curves and ITC data to the new model, and were utilized to obtain FT values (cFT_{ZBJ}) in samples obtained in clinical trials (TED, TDSM) and community dwelling populations (FHS and EMAS).

3.3.4. Effects of estradiol and dihydrotestosterone (DHT)

Addition of estradiol 17β in concentrations ranging from 10 to 500 pg/mL had no significant effect on percent free testosterone. Similarly, free testosterone concentrations in men treated with graded doses of testosterone enanthate plus placebo whose DHT concentrations extended from physiologic to supraphysiologic range did not differ from those treated with testosterone enanthate plus dutasteride whose DHT concentrations were very low (Bhasin et al., 2012), indicating that DHT over the range of concentrations relevant in male and female physiology has little effect on percent free testosterone.

3.4. Application of new model to clinical trials data

SHBG and albumin are predominantly the two proteins that bind testosterone with significant affinity; the binding affinities of transcortin and orosomucoid for testosterone are extremely low. Accordingly, we included testosterone's interaction with albumin in the equilibria describing its binding to SHBG to determine FT (FT_{ZBJ}) in serum samples from two randomized trials: the Testosterone in Erectile Dysfunction (TED) (Spitzer et al., 2012, Fig. 4A) and the Testosterone Dose Response in Menopausal Women (TDSM) (Huang et al., 2014, Fig. 4B) trials. The comprehensive model was implemented in the LabVIEW framework (Zakharov et al., 2012).

cFT_V significantly underestimated FT levels relative to equilibrium dialysis in men participating in the TED trial and in women participating in the TDSM trial. In contrast, new model provided values that were not statistically different from those measured by equilibrium dialysis in men (slope 1.01 ± 0.01) and women (slope 0.97 ± 0.05). The Bland–Altman plots (Fig. 4, middle and right panels) found no significant difference between the cFT_{ZBJ} and those obtained using equilibrium dialysis in either men or women; the relative deviation of values calculated using the new model from those measured using equilibrium dialysis was evenly distributed around 0, likely reflecting multiple sources of measurement error in testosterone assay, SHBG assay, and equilibrium dialysis. The Deming regression was used to compare the values derived using the new model and cFT_v with those obtained using equilibrium dialysis. These comparisons (Fig. 4) reaffirm the substantial bias of cFT_v from values derived using equilibrium dialysis and the substantially better correspondence between cFT_{ZBJ} and equilibrium dialysis in both men and women.

3.5. Relation between percent FT with total testosterone and SHBG

Inter-subunit allostery in the new model suggests that SHBG can regulate FT fraction over a wide range of total testosterone concentrations without getting saturated. Indeed, we found that percent FT calculated using the new model changed very modestly over a wide range of total testosterone concentrations (Fig. 5). In contrast, the Vermeulen's equation suggests a negative relation between percent FT and total testosterone. Furthermore, as SHBG concentrations increase, percent FT calculated using our new model shows only a modest decline in contrast to the marked decline in percent FT calculated using Vermeulen's equation.

3.6. Application of the new model incorporating complex allostery to community-dwelling men in the Framingham Heart Study (FHS) (Krasnoff et al., 2010; Splansky et al., 2007) and European Male Aging Study (EMAS) (Lee et al., 2009; Wu et al., 2010)

Table 1 summarizes the characteristics of the reference sample of healthy men, ≤ 40 years. The men in FHS broad sample and EMAS were on average older and had higher average age, body mass index, systolic blood pressure, blood glucose, and cholesterol than the reference sample (Krasnoff et al., 2010; Splansky et al., 2007). The mean

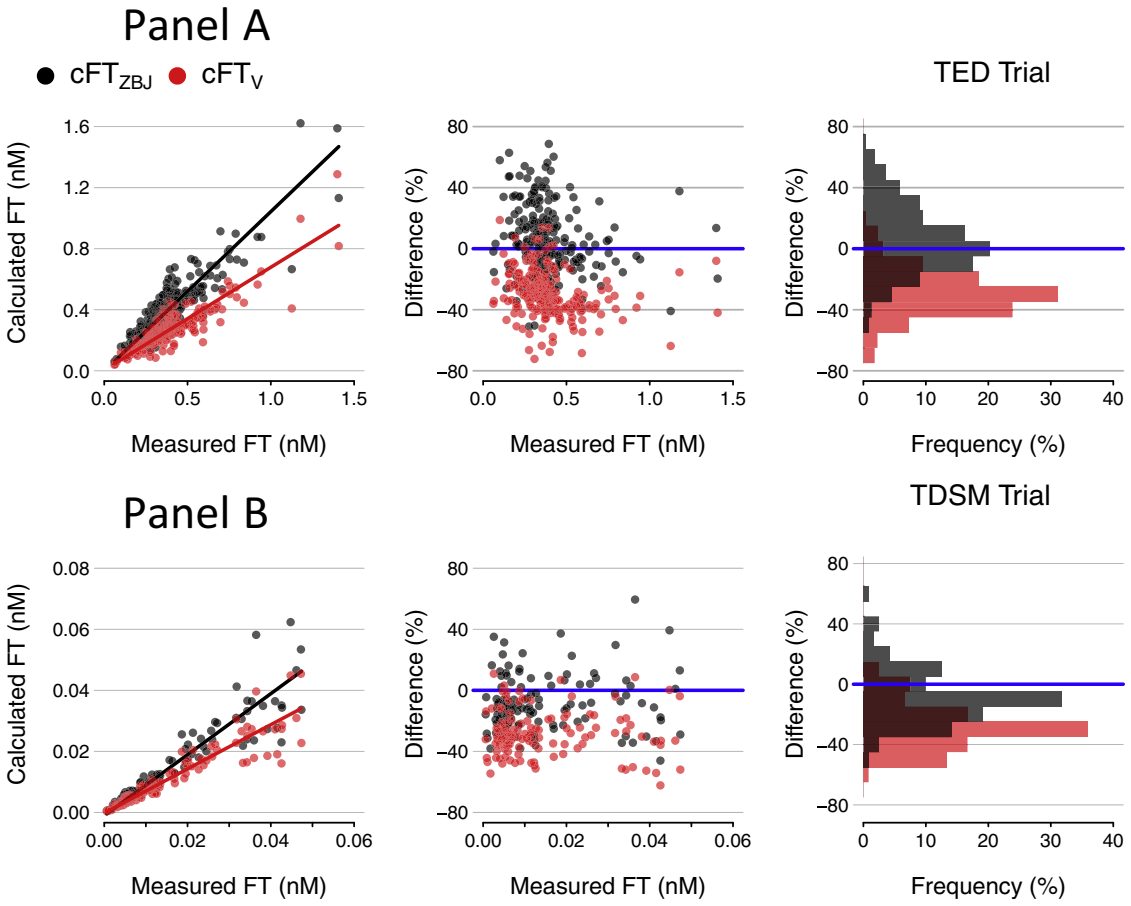


Fig. 4. Comparison of the free testosterone concentrations derived using the Vermeulen equation (cFT_V) or the new model (cFT_{ZBJ}) with those measured using the equilibrium dialysis in samples from randomized testosterone trials. At left: The comparison of the free testosterone concentration calculated by the Vermeulen's equation (cFT_V) and the new model (cFT_{ZBJ}) to that measured by equilibrium dialysis in samples from a randomized testosterone trial in men (Panel A) or from a randomized testosterone trial in women (Panel B). Solid lines are derived using Deming (orthogonal) regression; the black line represents fit for cFT_{ZBJ}, and the red line for cFT_V. The middle panels show Bland–Altman-style plots of cFT_{ZBJ} (black) and cFT_V (red) versus measured concentrations. The right: Panel show histograms displaying the relative deviations from the measured values. These are distributed symmetrically around zero for cFT_{ZBJ} but exhibit systematic deviation from zero for cFT_V for male subjects. cFT_{ZBJ} values for female subjects in TDSM trial also substantially reduce the deviation observed in cFT_V. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(median) free testosterone in the reference sample was 237.8 (229.5) pg/mL, and the 2.5th percentile value was 114.6 pg/mL (Table 2); the corresponding values in FHS broad sample and EMAS were lower than those in the reference sample, as expected from their older age and comorbidities. Fig. 6 (upper panel) shows the age-specific distribution of FT in the FHS Broad sample and the expected cross-sectional decrease in FT concentrations with increasing age.

We then determined whether deviations in FT from the mean (in standard deviation units or T-scores) were associated with the risk of sexual symptoms or elevated LH levels in EMAS (Lee et al., 2009; Wu et al., 2010). The men with FT levels more than 2 SDs below the mean of the reference sample (T score <–2) were at increased risk for having sexual symptoms and elevated LH (Fig. 6, lower panel).

4. Discussion

Several lines of evidence presented here indicate that the current linear model of testosterone's binding to SHBG (single binding site or two identical, non-interacting binding sites on SHBG) that has formed the basis of Vermeulen, Sodegard, and Mazer's equations to estimate free testosterone concentrations does not accurately explain the experimental data from binding isotherms, ligand depletion experiments, and ITC (after correcting for two binding sites per dimer stoichiometry). While the discrepancy between testosterone concentrations estimated using the above-mentioned equations and those measured using equilibrium dialysis has been recognized (Ly et al., 2010), our data provide a mechanistic explanation for this discrepancy. Simple models of homotropic allostery with positive or negative cooperativity within a dimer also did not adequately explain the experimental data. Only the dynamic model that incorporates allostery optimally fits the experimental data derived from three independent methods. Furthermore, FT concentrations calculated using the new model incorporating multistep interaction were not significantly different from those measured by equilibrium dialysis in samples derived from men and women in two separate clinical trials. Men deemed to have low cFT_{ZBJ} (<2 SD below the mean) were at higher risk of having sexual symptoms or elevated LH.

Our analysis of the steady state experimental binding data indicates that in the absence of testosterone, SHBG molecule can assume one of at least two inter-converting microstates in dynamic equilibrium. The binding of testosterone to one of the monomers of the SHBG dimer in a given microstate affects the interaction of testosterone with the unoccupied second binding site on the SHBG dimer. The model suggests a dynamic re-adjustment of populations of intermediate species as testosterone concentration is altered.

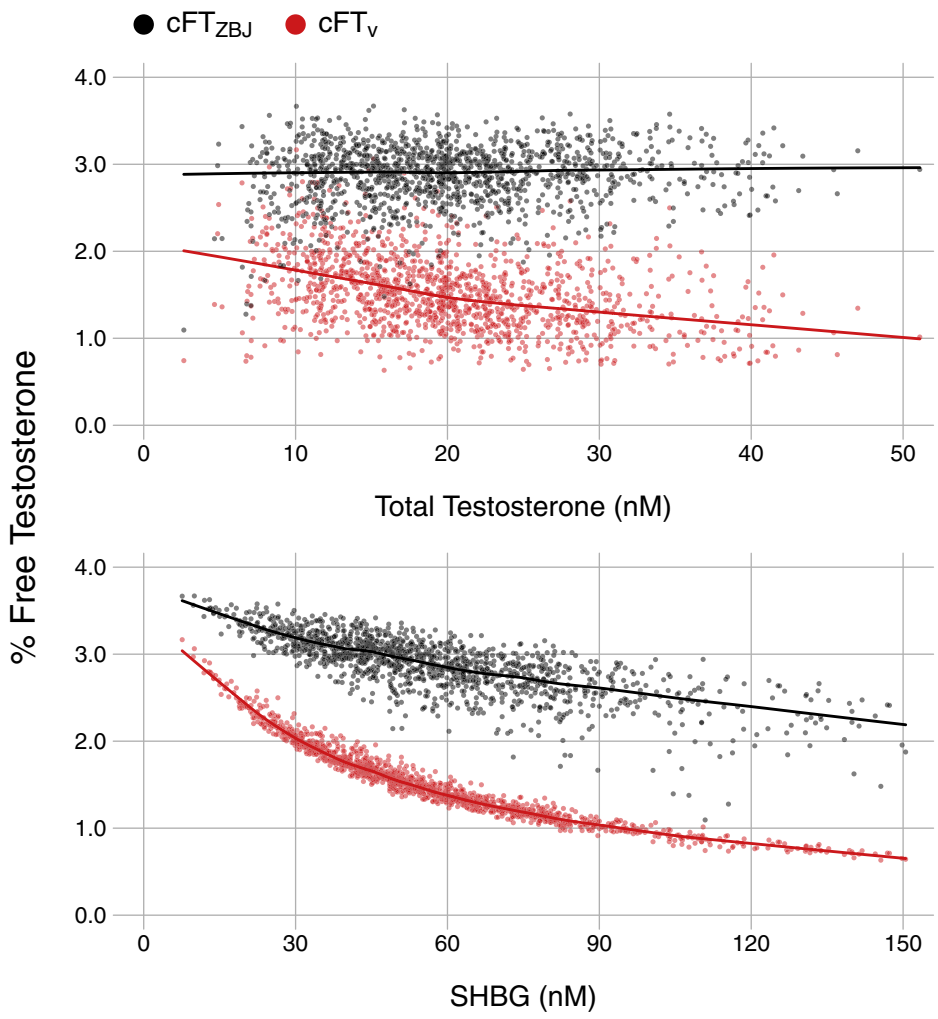


Fig. 5. Relation between %cFT with total T and SHBG. The free fraction (%) of testosterone, computed by the new model, is displayed as a function of total testosterone (upper panel) and SHBG (lower panel) within the FHS broad sample as calculated by the two models.

Because of the dynamic nature of these processes, all parameters of the model cannot be uniquely determined. Thus, testosterone's binding to SHBG is not a single homogenous reaction but rather a series of inter-related molecular processes that are shown in Fig. 3. The fits of the data to the new model that incorporates complex allostery display a dynamic re-adjustment of populations of intermediate species as testosterone concentration changes. Because of the multiple equilibria and dynamic and allosteric nature of these processes, testosterone's binding to SHBG cannot be described as a simple linear equation of ligand binding equilibrium. Accordingly, we implemented the multi-species allostery model in LabVIEW framework (Zakharov et al., 2012). Optimal fit parameters for the ITC and equilibrium dialysis data (Sections 3.1.1, 3.1.2, 3.1.3) were obtained by the simulated annealing optimization, using global fit approach similar to that described (Freiburger et al., 2011). This set of parameters that can be used to compute free testosterone is shown in Figs 3 and 2e. Similar fit could also be obtained using the generalized Ensemble Allostery Model (Fig. 1 Model G) proposed by Hilser and Thompson (2007) and Freiburger et al. (2011) but our data suggest that a simpler interaction schema (Model F) adequately captures the complex interaction of testosterone binding to SHBG. More importantly, the free testosterone values determined using model F closely match those determined using the equilibrium dialysis without the systematic deviation observed in cFT$_v$ values obtained from the Vermeulen's model.

The new dynamic model (Model F) leads us to reconsider several dogmas related to testosterone's binding to SHBG and has important physiologic and clinical implications. First, the fraction of circulating testosterone, which is free is substantially greater ($2.9 \pm 0.4\%$) than has been generally assumed (%cFT$_V$ $1.5 \pm 0.4\%$). Second, percent FT is not significantly related to total testosterone over a wide range of total testosterone concentrations. However, the percent FT declines as SHBG concentrations increase, although it does not decline as precipitously as predicted by the Vermeulen's model. Due to the allostery between the two binding sites, SHBG is able to regulate FT levels in a much larger dynamic range.

Several factors may have contributed to the formulation of the prevailing hypothesis that monomers within SHBG dimer display identical binding affinity without any dynamic interaction between the subunits. The extant linear ligand binding equations were formulated in an era that preceded the appreciation of the dimeric nature of circulating SHBG. However, the Mazer's implementation of the Vermeulen's model, as applied in these analyses used the correct stoichiometry – two molecules of testosterone binding to each SHBG dimer. Therefore, the discrepancy between cFT$_v$ and the reference method cannot be explained solely on the basis of incorrect stoichiometry. Furthermore, the range of testosterone and SHBG concentrations used in binding experiments and Scatchard plots were limited and did not generally extend into the high range (Dunn et al., 1981; Hauptmann et al., 2003; Metzger et al., 2003; Petra et al., 1986),

Table 1
Characteristics of population-based samples.

Variable	Gen 3 reference sample ≤40 years ($n = 434$)	Broad sample ($n = 3145$)	EMAS ($n = 3329$)
Age, years	32.6 (5.7)	49.3 (13.8)	60.0 (11.0)
Age, decades			
<30	120 (28%)	217 (7%)	–
30–39	281 (65%)	621 (20%)	–
40–49	33 (8%)	811 (26%)	792 (24%)
50–59	–	736 (23.4%)	890 (27%)
60–69	–	465 (15%)	832 (25%)
70–79	–	270 (9%)	797 (24%)
80–90	–	22 (0.7%)	18 (1%)
Total testosterone, ng/dL	726 (220)	621 (227)	475 (174)
SHBG, nmol/L	40.6 (17.0)	48.0 (23.7)	43.0 (19.8)
Free testosterone (cFT_{ZBJ}), pg/mL	237.8 (75.0)	191.5 (75.2)	143.5 (52.0)
Luteinizing hormone, IU/L	N/A	N/A	6.2 (3.4)
Systolic BP, mmHg	115.6 (8.7)	124.0 (15.3)	146 (21)
Diastolic BP, mmHg	74.2 (7.5)	77.3 (9.5)	87 (12)
Hypertension treatment	–	683 (22%)	975 (29%)
Total cholesterol, mg/dL	179.2 (30.4)	192.3 (36.3)	214 (48)
LDL cholesterol, mg/dL	111.0 (27.6)	119.2 (31.4)	133 (44)
HDL cholesterol, mg/dL	47.7 (11.8)	46.3 (12.6)	54 (14)
Triglycerides, mg/dL	104.9 (73.5)	140 (108)	139 (103)
Glucose, mg/dL	93.1 (6.8)	102.8 (23.2)	102 (25)
Body mass index, kg/m^2	25.6 (2.7)	28.3 (4.6)	28 (4)
Prevalent CVD	–	287 (9%)	551 (17%)
Hypertension	–	1050 (33%)	960 (29%)
Smoker	–	504 (16%)	705 (21%)
Diabetes	–	236 (8%)	254 (8%)
Sexual symptoms	N/A	N/A	368 (12%)

Broad sample is all men in FHS Gen 2 and Gen 3.
EMAS, European Male Aging Study.

which may have prevented appreciation of the second binding site. Also, a single crystal structure of the ligand-bound SHBG may have further contributed to the erroneous impression that the binding events associated with testosterone's binding to two binding sites on SHBG dimer are identical. The inability to resolve the unliganded SHBG structure (Avvakumov et al., 2010) as well as the increased stability of SHBG upon ligand binding (Avvakumov et al., 2000) may be related to significant rearrangement of SHBG molecule upon binding of the first ligand, as predicted by the conformational heterogeneity in complex allostery. The additional energy barrier that SHBG has to overcome may result in altered affinity for binding of the second ligand molecule.

While the new algorithm developed in this study accurately determines FT in healthy men and women, large variations in the circulating concentrations of other interacting hormones or SHBG (e.g., during pregnancy and acute illnesses) could potentially alter the regulation of testosterone's bioavailability. In a previous study (Bhasin et al., 2012), we found that free testosterone concentrations in men treated with graded doses of testosterone enanthate plus placebo whose DHT concentrations extended from physiologic to supraphysiologic range did not differ from those treated with testosterone enanthate plus dutasteride whose DHT concentrations were very low, indicating that DHT over the range of concentrations relevant in male and female physiology has little effect on percent free testosterone. Similarly, over a wide range of estradiol concentrations prevalent in men and women, cFT_{ZBJ} concentrations were similar to those measured using equilibrium dialysis. We do not know whether very high estrogen concentrations, such as those observed during pregnancy, or very high DHT concentrations may affect testosterone's binding to SHBG.

Transcortin and orosomucoid display very low affinity for SHBG; their role in regulating free testosterone was not assessed in this investigation and needs further clarity; it is remarkable that FT concentrations derived using the new dynamic model were not significantly different from those determined by equilibrium dialysis in randomized trials even though inter-individual differences in transcortin, and orosomucoid were not considered, consistent with the view that these proteins play a minor role in regulating free testosterone in healthy men and women.

The current algorithm and the experimental data were generated using wild type SHBG which is present in nearly 98% of Caucasians. Genome wide association studies have revealed several SHBG polymorphisms, two of which affect testosterone's binding to SHBG (Ohlsson et al., 2011). Therefore, in the future, the algorithm may include a term for SHBG genotype. Additional research is needed to extend the model to incorporate SHBG polymorphisms that affect testosterone's binding to SHBG.

The FHS population is predominantly white (Krasnoff et al., 2010; Lee et al., 2009); the distribution of FT in multi-ethnic cohorts should be studied. Also, the distribution of normative ranges generated here in a community dwelling, healthy young men, as recommended by the International Federation of Clinical Chemists (IFCC) (Elveback, 1973; Solberg, 1987), needs further validation in clinical populations and in randomized trials to determine clinical usefulness. Our approach of generating the reference range in healthy young men is similar to the use of T-scores for bone mineral density. However, for some analytes, it may be appropriate to generate age-adjusted reference ranges; accordingly, we have presented age-adjusted distributions as well.

Table 2
Distribution of free testosterone (pg/mL) in community dwelling men of the Framingham Heart Study (FHS) and European Male Aging Study (EMAS).

	Gen 3 reference sample ≤40 years ($n = 434$)	Offspring cohort ($n = 1367$)	Broad sample ($n = 3145$)	EMAS ($n = 3329$)
Mean	238	169	192	144
SD	75	68	75	52
99th percentile	444	359	398	287
97.5th percentile	402	328	362	263
95th percentile	370	296	331	239
75th percentile	285	204	237	173
Median	229	161	181	137
25th percentile	182	118	138	107
5th percentile	130	73	84	71
2.5th percentile	115	61	70	58
1st percentile	90	46	54	36

5. Conclusions

In summary, experimental data generated using several independent methods provide evidence of an allosteric mechanism of testosterone binding to SHBG dimer. FT concentrations derived using the new dynamic model incorporating multistep interaction with allostery does not differ significantly from those measured using equilibrium dialysis in men and significantly reduces the systematic deviation in cFT values in women. The application of the new model to clinical trials data has revealed new insights into the percent of circulating testosterone that is free, the relation between percent FT and total testosterone and SHBG. The use of additional experimental models, including dimerization-deficient SHBG mutants, would allow further insights into molecular mechanisms of testosterone's interaction with SHBG. The extension of the new dynamic model incorporating allostery should also be further explored in clinical populations as its availability on desktops and mobile devices can provide a convenient and accurate approach for determining FT at the point-of-care, and facilitating the diagnosis and treatment of men and women with androgen disorders.

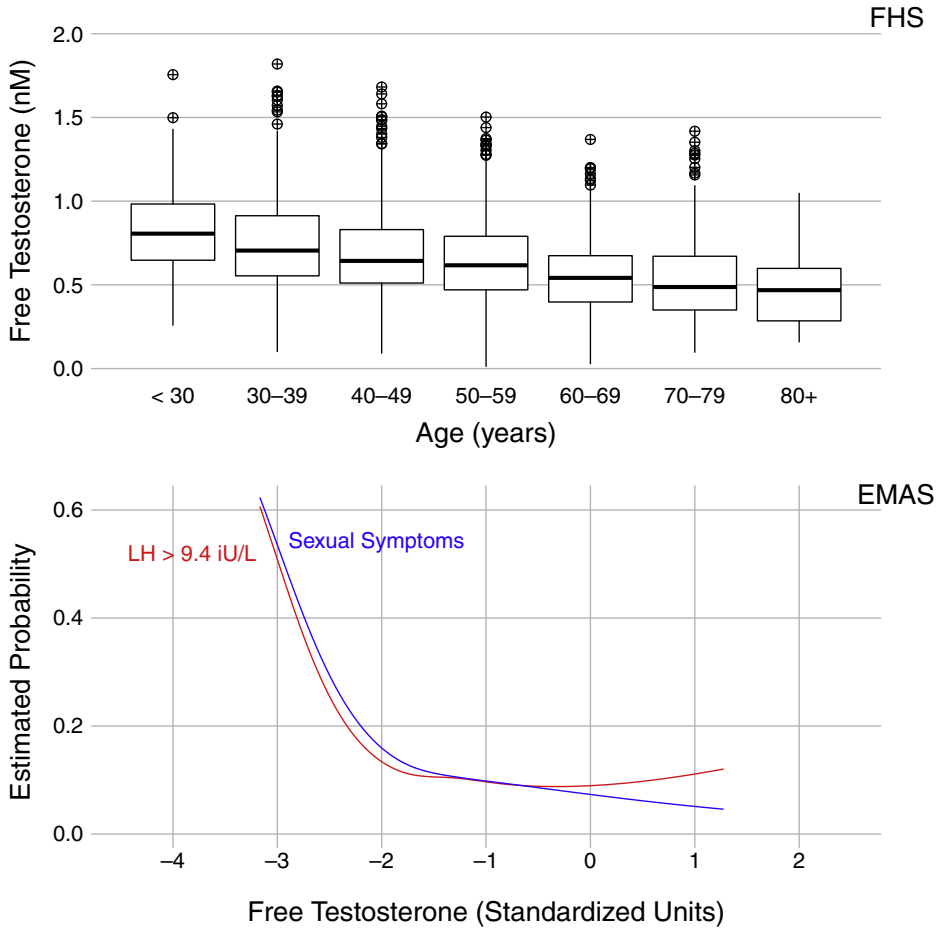


Fig. 6. Upper panel shows the distribution of free testosterone by decades of age in the FHS. The box-and-whisker plots depict age-specific free testosterone by the new model developed in this study in the broad FHS sample. The lower panel shows the age-adjusted probability of having sexual symptoms in elevated LH (>9.4 U/L) in relation to standard deviation from the mean free testosterone concentration of the reference sample in the European Male Aging Study.

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