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Immunologic and mass-spectrometric estimates of SHBG concentrations in healthy women

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Abstract

Objective—Sex-hormone binding globulin (SHBG) concentrations across the adult female lifespan are not well defined. To address this knowledge gap, SHBG was quantified by both immunological and criterion methods, *viz*, mass spectrometry (MS).

Methods—Setting: Center for Translational Science Activities (CTSA). Participants: Healthy nonpregnant women (N = 120) ages 21 to 79 years. Outcomes: SHBG, testosterone (T), estradiol (E₂) and estrone (E₁) each determined by MS. Uni- and multivariate regression of SHBG concentrations on age, body mass index (BMI), total and visceral abdominal fat (TAF, AVF), albumin, glucose, insulin, sex steroids, selected cytokines, blood pressure, and lipids.

Results—By univariate regression, MS-estimated SHBG correlated negatively with BMI, TAF, AVF, insulin, free T and bioavailable T (bio T) (each $P < 10^{-4}$), but not with blood pressure or

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Author Contributions

Johannes D. Veldhuis: conception and design of research, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript and approved final version of manuscript.

Roy B. Dyer: performed experiments, analyzed data, and interpreted results of experiments, approved final version of manuscript.

Sergey A. Trushin: performed experiments, analyzed data, and interpreted results of experiments, approved final version of manuscript.

Olga P. Bondar: performed experiments, analyzed data, and interpreted results of experiments, approved final version of manuscript.

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George G. Klee: conception and design of research, interpreted results of experiments, edited and revised manuscript and approved final version of manuscript.

Disclosures

The authors have nothing to disclose.

lipids. By stepwise multivariate regression analysis, free and total T (both positive) and bio T (negative) were correlated with SHBG in all 4 assays (each $P < 10^{-15}$, $R^2 = 0.481$). In addition, TAF and BMI were negatively associated with SHBG ($P = 0.0066$) in 2 SHBG assays, and estrone and IL-8 with SHBG weakly ($P = 0.035$) in one SHBG assay each. When nonsignificant cytokines were excluded, SHBG was jointly associated with AVF, total T and HDL ($P < 10^{-9}$, $R^2 = 0.358$).

Conclusion—According to MS, three metabolic factors, T, AVF and HDL, together explain more than one-third of the interindividual variation in SHBG levels. We speculate that these measures reflect insulin action.

Keywords

Female; Sex steroid; Body composition; Obesity; Insulin

1. Introduction

Sex-hormone binding globulin (SHBG) concentrations tend to rise with age and decline with central obesity in men, increase with estrogen supplementation in both sexes, and decrease with testosterone (T) exposure [1–5]. SHBG levels may be further influenced by mutations, certain gene polymorphisms, ethnicity, diabetes mellitus, degree and distribution of adiposity, inflammation, adipocytokines, insulin, glucose and hyperlipidemia [1,6–11]. Investigations in healthy women and women with the metabolic syndrome or PCOS suggest that BMI, androgens, insulin, adiponectin and glucose intolerance predict SHBG concentrations [5,12–14]. Data in women as well as men are important, since SHBG seems to provide an index of cardiovascular risk in both genders [15].

A difficulty in establishing consistent clinical inferences is that immunological measurements of estradiol (E_2), estrone (E_1), and T are significantly discrepant, unless validated by physical methods like mass spectrometry (MS). This problem is especially marked when immunoassays are applied at low hormone concentrations [16]. Immunologically based assays of SHBG also exhibit significant inconsistencies [17,18]. Thus, the reproducibility of immunologically measured associations among sex steroids, SHBG and metabolic markers remains open to question.

Two recent reports, both methodological in focus, suggest the possibility of utilizing MS to quantify not only sex steroids but also SHBG [19,20]. The present study applies the MS strategy to both sex steroids and SHBG to better evaluate the clinical correlates of sex steroids and SHBG in healthy women. To better assess T correlations, total, free and bioavailable T levels were measured, respectively reflecting combined bound and unbound (total) T, unbound (free) T and SHBG-unbound (bioavailable) T moieties. The rationale was the speculation that these moieties may have distinguishable associations with SHBG. For robustness, four (2 immunological, 2 MS) assay methodologies were applied to quantify SHBG in 120 healthy women having a wide range of age, body composition, sex-steroid levels, inflammatory cytokines and metabolic surrogates.

2. Methods

2.1. Human subjects

One-hundred and twenty healthy independently living nonpregnant women were recruited from Olmsted County, MN, by local advertisements under Mayo IRB-approved consent. Subjects agreed to provide 50 mL (25 mL for serum, 25 mL EDTA-plasma) while blood fasting at 0800 h, and undergo a single-slice CT scan at L3–L4 to estimate abdominal fat. Inclusion criteria were age 21–80 years; mental competence; negative pregnancy test; $18 \text{ kg/m}^2 < \text{BMI} < 50 \text{ kg/m}^2$; $\text{Hb} > 13 \text{ g/dL}$ or hematocrit $> 36\%$; provision of written informed consent; and normal safety screening data. Exclusion criteria comprised fasting plasma glucose $> 126 \text{ mg/dL}$; anemia; any recent (5 biological half-lives) use of psycho- or neuroactive drugs or sex hormones; treated psychiatric illness (*e.g.*, psychosis, depression, mania); drug or alcohol abuse; acute or chronic metabolic, organ-level or systemic illness; hypothalamo-pituitary disease; recent transmeridian travel (exceeding 3 time zones within 10 days); acute weight loss or gain ($> 2 \text{ kg}$ change in 3 weeks); or failure to provide voluntary, written, witnessed IRB-approved informed consent. Women were studied within 8 days of menses onset, if menstruating. Menopause was defined by $\text{FSH} > 30 \text{ IU/L}$ with $\text{E}_2 < 50 \text{ pg/mL}$ and the clinical history. Patients with PCOS were excluded.

2.2. Total T, E_2 and E_1 assays by MS

Total T was determined in the Mayo Core Laboratory by MS, as described [21], using deuterated stable isotope (d_3 -testosterone) (CDN Isotopes, D-3793) as internal standard. Protein was precipitated from the mixture by the addition of acetonitrile. Testosterone and internal standard were recovered by on-line extraction (Phenomenex C18 4 mm L $\times$ 2 ID) utilizing high-throughput liquid chromatography (Thermo-Fisher Scientific Technologies TLX4 On-line Sample Preparation HTLC System). This was followed by analytical liquid chromatography (Supelcosil LC-C18 4.6 $\times$ 33 mm). Quantification was via an Applied Biosystems-MDS Sciex API 5000TM Tandem Mass Spectrometer equipped with a heated nebulizer ion source, atmospheric pressure ionization probe, and operated in the multiple-reaction monitoring positive mode. The calibration utilized a ten-point standard curve over a concentration range of 0–2000 ng/dL (Steraloids, A 6950). The following quadrupole 1 and 3 (Q1, Q3) mass pairs (Da) were monitored: Q1/Q3: 289.400/ 97.20; 289.400/109.200; 292.300/97.200. Sensitivity was 2.5 ng/dL. Intraassay CVs were 7.5%, 2.2%, 6.3%, and 28.8% for T levels of 8, 4, 2 and 1 ng/dL.

Total E_2 and E_1 were measured analogously by LC MS/MS, precisely as presented previously [22]. Sensitivities were 3.5 pg/mL and 2.8 pg/mL for E_2 and E_1 , respectively, with recoveries of 93%–110%. CVs were 2.3%, 6.1%, 14% and 13% for estrogen levels of 10, 5, 2.5, and 1.25 pg/mL.

2.3. Free and bioavailable T

Free T and bio T were calculated exactly as reported earlier [see Appendix of [23]]. The goal was to test possible differences in the relationships of total, free and bioavailable T with SHBG.

2.4. SHBG Assays

Four SHBG assays were used to measure SHBG in each sample.

(1) Immulite 2000	The Immulite 2000 SHBG method (L2SH2, Siemens Healthcare Diagnostics, Deerfield, IL) is a solid-phase, two-site chemiluminescent immunoassay. A polystyrene bead coated with a monoclonal antibody specific for SHBG binds to SHBG in the serum sample, then alkaline phosphatase-conjugated anti-SHBG (polyclonal) is added to the antibody sandwich complex. After washing, a chemiluminescent substrate (phosphate ester of adamantyl dioxetane) is added and hydrolysis produces emission of light. The photon output is proportional to the concentration of SHBG in the sample. The measurement range is from 2.0 to 180 nmol/L. This method is standardized against the 1st International Standard for SHBG from the National Institute for Biological Standards and Control (NIBSC) code 95/560. Intraassay CVs are 2.7% and 3.1% at 5.5 and 95.9 nmol/L respectively. Interassay CVs are 4.0% at 5.4 nmol/L and 5.9% at 74 nmol/L.
(2) COBAS e411	The COBAS e411 SHBG assay (Roche Diagnostics, Indianapolis, IN) is an automated immunoassay based on the sandwich principle. A biotinylated monoclonal SHBG-specific antibody and a monoclonal SHBG-specific antibody labeled with a ruthenium complex form a sandwich complex with SHBG in the serum sample. Thereafter, streptavidin-coated microparticles are added, and magnetically captured on the surface of the electrode. Application of voltage induces chemiluminescent emission, which is measured by a photomultiplier. This method is standardized against the 1st International Standard for SHBG from the National Institute for Biological Standards and Control (NIBSC) code 95/560. Measuring range is from 0.800 to 200 nmol/L. Intraassay CVs are 2.1%, 2.4% and 2.7% at 14.1, 44.2 and 204 nmol/L respectively. Interassay CVs are 2.7% at 14.1 nmol/L and 5.6% at 204 nmol/L.
(3) MS-IAL assay	The MS-IAL assay involves immunoextraction of SHBG from EDTA plasma, trypsin digestion into peptides and MS quantitation of the IALGGLFPASNLR (IAL) peptide. Extraction is performed by using 20 µl of serum mixed with 8 µg of anti-human SHBG monoclonal antibody (GenWay, San Diego, CA) conjugated to 100 µl of magnetic beads. A stable isotope of the peptide is added to each reaction to adjust for recovery. Three transitions are monitored by MS (721.9 ²⁺ /657.4, 721.9 ²⁺ /804.4, 721.9 ²⁺ /917.5). The assay is standardized to the WHO 95/560 International Standard Sex Hormone Binding Globulin, using 7 calibrators prepared by spiking purified SHBG into depleted serum. Quantification is performed by LC-MS/MS using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The assay has CVs of 9.0%, 9.5% and 5.5% at control levels of 10.7, 56.2, and 147.6 nmol/L.
(4) MS-SISCAPA assay	The MS-SISCAPA assay involves trypsin digestion before immunoextraction of IAL peptide, and MS quantitation of IAL peptide. First, 20 µL of EDTA plasma is trypsin digested. Next, IAL peptide is immune-extracted using a polyclonal rabbit anti-SHBG peptide antisera conjugated to protein G magnetic beads using an automated Thermo KingFisher Flex Magnetic Particle Processor. The assay is standardized exactly as described for SHBG quantification by MSIAL (above). CVs are 15.3%, 5.3%, and 3.1% at SHBG levels of 10.9, 55.7, and 141 nmol/L.

2.5. Proinflammatory Cytokine Multiplex Assay

The Human Proinflammatory 7-Plex Ultrasensitive Kit from Mesoscale Discovery (K15008C, Gaithersburg, MD) comprises a 96-well microtiter plate with spatially spotted capture antibodies against 7 analytes: total adiponectin, TNF alpha, IL-1beta, IL-6, IL-8, IL-10, and IL-12. Captured analytes are detected by a cocktail of specific antibodies to each analyte forming an Ab-analyte-Ab sandwich. Detection antibodies are coupled to an electrochemiluminescent compound, which is activated by an electrical charge applied to each well of the plate. The light signal generated is captured by a cooled coupled detector and interrogated against a standard curve. Quality controls at 3 different levels were purchased from R & D Systems (QC-01, Minneapolis, MN), and included with each plate (n = 7). CVs ranged from 4% to 20% in these assays.

2.6. Clinical assays

Albumin, fasting plasma glucose, serum insulin, total cholesterol, triglycerides (TG) and HDL-C concentrations were assayed by Mayo Clinical Laboratories, as described [24].

2.7. AVF and TAF

A single-slice CT scan at L3–L4 was used to estimate abdominal visceral fat (AVF) and total (AVF plus subcutaneous) abdominal fat (TAF), as defined previously [25].

2.8. Statistical methods

The Matlab advanced statistical toolbox (Mathworks, Natick, MA, 2012 version) was used to perform linear regressions of SHBG estimates on age, sex steroids, BMI, AVF, TAF and metabolic and cytokine parameters. Univariate regression was performed first. Higher-order (nonlinear) models were not significantly superior by the Akaike information criterion. Logarithmic transformation of the independent variable was used where indicated. The sample size calculated to give a power of 90% was 100 women to detect an 18% contribution of key variables to the regression on SHBG at $P < 0.05$.

Significance was construed at $P < 0.01$ to protect against false positives due to multiple comparisons [26]. Therein, $0.01 < P < 0.05$ was considered a trend. Slope coefficients (beta) and 95% confidence intervals of slopes were estimated along with R^2 values (square of correlation coefficient). Stepwise backward-elimination multivariate regression analysis with correction for collinearity comprised the final model, which was protected at experiment-wise overall $P < 0.001$. Only model variables with absolute t values greater than 3.0 and $(1-R^2)$ [tolerance] greater than 0.10 were used here. Subset models in the different tables provide crossvalidation of the key outcomes.

3. Results

Epidemiological (age, BMI), metabolic (AVF, TAF, albumin, glucose, insulin, adiponectin), endocrine (SHBG by each of 4 assays; E_1 , E_2 , total, free and bio T by MS), and cytokine (IL-10, IL-12, IL-1 beta, TNF-alpha, IL-6, IL-8) and epidemiological (BP and lipids) data in all 120 women are summarized in Table 1A. Values include the mean, median, SD, minimum and maximum (absolute range). In conformation with the *a priori* recruitment criteria, age range was 21 to 79 years, BMI 19 to 47 kg/m², fasting plasma glucose 74–118 mg/dL and insulin 1.1 to 14 mU/L. Albumin, E_2 , E_1 , total T, free T, and bio T concentrations were normal for women. Each of total T, E_2 and E_1 declined significantly with age: Supplemental Fig. 1. Age explained 7%–16% of the variance in these 3 sex steroids, while AVF, BMI and insulin accounted for 6%–17% of the variance in bio T and free T concentrations: Supplemental Fig. 2. Other covariates of the sex steroids measured in these 120 women are given in Supplemental Table 1. Cytokine BP and lipid measurements exhibited an expected wide degree of variation. Table 1B shows (expected) differences based upon pre- and postmenopausal status (see *Methods*).

SHBG concentrations measured in the 4 assays in all 120 women manifested an absolute range of 13 to 406 nmol/L [Table 1A and 1B (*bottom*)]. Mean SHBG did not differ among

the 4 assay types ($P > 0.45$ trend). All six pairwise correlations among the 4 SHBG assays were highly significant (each $P = 10^{-68}$); Table 2. Regression slopes were 0.918 to 1.079 (absolute range). Ninety-five percent confidence intervals of slopes overlapped 1.00 for two of the six pairs (MS-SISCAPA/ Cobas, and MS-IAL/Immulin), indicating near identity of the assay pairs. The square of the correlation coefficient (R^2) ranged from 0.928 for the MS-SISCAPA/Immulin assay pair to 0.973 for the two immunologic (Immulin/Cobas) assays. The two MS (IAL and SISCAPA) assays were correlated at $P < 10^{-79}$ with $R^2 = 0.954$ and slope = 0.929.

Univariate relationships were assessed between log SHBG concentrations and epidemiologic, metabolic, endocrine and cytokine measurements in the 120 women. Correlation outcomes for all 4 SHBG assays are summarized in Table 3. Protected $P = 0.01$ was construed as significant. Each of BMI ($P < 10^{-4}$), AVF ($P < 10^{-4}$), TAF ($P < 10^{-4}$), insulin ($P < 10^{-3}$), free T ($P < 10^{-4}$), and bio T ($P < 10^{-4}$) was a negative predictor of SHBG in all 4 assays. The negative effects of AVF, TAF and BMI on the simple arithmetic mean (of 4) SHBG value(s) are represented in Supplemental Fig. 3, wherein $0.020 \leq R^2 \leq 0.23$. Age, albumin, glucose, total T, TNF-alpha and IL-8 were trends and/or less consistent among the 4 assay systems. The strong negative univariate effects (justified by the Akaike information criterion) of free and bio T (both $P < 10^{-4}$) contrast with the positive effects of total T ($P < 0.05$) as shown in the top, middle and bottom rows for all 4 SHBG assays in Fig. 1. Matching log-linear regressions are tabulated for comparison in Table 3. Outliers were tested at the level of $P < 0.001$, via F ratio assessment, and none was identifiable.

Experiment-wise overall protected $P < 0.01$ was employed in multivariate stepwise backward-elimination regression *exploring all independent variables, including sex steroids*: Table 4. Because Fig. 1 suggested heteroscedasticity, independent variables were log transformed before further regression (Table 3). Under these conditions, three measures of sex steroids jointly yielded statistical significance $P < 10^{-15}$ in each of the 4 SHBG assays: (1) total T (positive correlate), (2) free T (positive correlate), and (3) bio T (negative correlate). Thus both linear (Fig. 1) and log-linear (Tables 3 and 4) models pertained. Less consistently, BMI correlated negatively with SHBG in the two MS assays. TAF (negatively, $P < 0.01$) and TNF-alpha (positively, trend only) predicted SHBG in the two immunoassays. IL-8 and E_1 were weak positive trend-level predictors of SHBG in 1 assay each. Note that insulin dropped out of the multivariate models. Overall multivariate R^2 values were 0.481 to 0.613, thereby indicating that approximately 48%–61% of the SHBG variance among subjects was explained by the multivariate model. In each case, the 3 predominant variables were measures of T (total, bio and free) availability. This raised the question of what associations exist with SHBG when sex steroids are omitted from the multivariate model.

Supplemental Table 2 shows condensed multivariate correlation outcomes (each overall $P < 10^{-6}$) obtained after omitting sex steroids as independent variables. In this parsimonious framework, albumin and AVF (both negatively) accounted for significant variance in SHBG concentrations in all 4 SHBG assays: Fig. 2. The AVF effect ($P < 10^{-5}$) was stronger than that of albumin ($P < 0.05$). The joint correlation of AVF and albumin with SHBG in each of the 4 assays was $P < 10^{-6}$. IL-10 was also a significantly positive, albeit weaker, covariate

of SHBG in both MS assays, wherein overall P was $<10^{-8}$ and joint R^2 was 0.316 (SISCAPA) and 0.297 (IAL).

To further test the negative association of AVF and positive association of T with SHBG, correlations were performed separately in 3 groups: all 120 women, 60 pre- and 60 postmenopausal women: Table 5. BP and lipids were included in the starting model, along with sex steroids. Cytokines were excluded, given no consistent significant effects under any conditions. Regression in both pre- and postmenopausal women (individually as well as together) yielded strong effects of T (positive) and AVF (negative) on SHBG measured by Mayo MS-IAL. Similar findings were obtained for the other 3 SHBG assays (not shown). Overall P values for the multivariate model were <0.001 with R^2 values of 0.243–0.414. Albumin was an additional determinant in the combined ($N = 120$) group as was HDL in the premenopausal ($N = 60$) group.

4. Conclusions

Novel aspects of the paper comprise: (1) the first use of a criterion method like mass-spectrometry for precise SHBG estimates in women; (2) evidence of good agreement between (certain) RIA's and mass spectrometry, making either acceptable for clinical screening; (3) significant joint influences of T (positive) and AVF (negative) on criterion-method SHBG measures in both pre- and postmenopausal women; and (4) the individual association of HDL (positive) with SHBG, but only in premenopausal women. Collectively, these data suggest strongly that accurately measured SHBG constitutes a useful marker of surrogates of insulin action (T concentration, AVF and HDL) with different implications in pre- and postmenopausal women.

By employing mass-spectrometry platforms for SHBG and sex-steroid quantification, the present analyses identify individual ($P < 10^{-3}$ each) negative effects of BMI, AVF, TAF, insulin, free T and bio T on SHBG concentrations in 120 healthy women. The same correlations were observable in 2 immunochemiluminescence SHBG assays, suggesting robust associations. In contrast to these well reproduced outcomes, univariate age, albumin, total T, glucose, TNF-alpha and IL-8 were either nonsignificantly (trend level $0.01 < P < 0.05$) associated with SHBG or associated in one, two or at most 3 of the SHBG assays. Multivariate regression further demonstrated that the three T moieties (free, bio and total T) correlate jointly with SHBG in all four assays. BMI was a strong covariate of MS-quantified SHBG and TAF of immunologic SHBG. Logarithmic T measures and body composition surrogates together explained 48%–61% of the variance in SHBG concentrations in nonpregnant women.

SHBG has been proposed as biomarker of risk factors associated with cardiovascular disease, hyperlipidemia, visceral adiposity, osteopenia, diabetes mellitus, syndrome X (metabolic syndrome) and (in women) hyperandrogenism with or without concomitant PCOS [3,27,28] [Introduction]. An unexpected finding was the markedly positive multivariate associations of free and total T with SHBG, and the negative multivariate association of bioavailable T with SHBG in all 4 measurement systems. These distinctions could suggest at least partially independent mechanisms. A possible explanation is that

bioavailable T (albumin-bound T), total T and free T may have unequal access to certain target tissues [29–31]. It is perhaps relevant that the strongly negative univariate correlations between SHBG and insulin (each $P < 10^{-3}$), as well as between SHBG and AVF or BMI or TAF (each $P < 10^{-4}$), were fully explained by a multivariate model containing the T moieties along with only BMI (both MS SHBG assays) or TAF (both immunologic SHBG assays). Although the exact reason for these associations is not known, E_2 derived from T can induce [5] whereas T or DHT can either stimulate [32,33] or inhibit [4] SHBG production. A corollary possibility is that T or its derivatives interact with body composition, insulin/IGF-I secretion or insulinomimetic-peptide repression of the SHBG gene [34]. In addition, high AVF and plasma insulin predict low SHBG and high T in women, while predicting low SHBG and low T in men and low HDL in both sexes [5,10]. In obese adults, a negative relationship exists between fat-cell size (not captured directly in BMI, AVF or TAF) and SHBG, whereas a positive relationship exists between fat-cell size and insulin resistance [35]. In principle, the insulin potentiator, adiponectin, produced in adipocytes and putatively regulated by T could further integrate effects of T, insulin, HDL, BMI, AVF or TAF on SHBG [1,5,33,36]. Nonetheless, unlike data in men, older women and children, the present analysis in women ages 21–79 did not identify adiponectin as a significant determinant of SHBG levels in any of the 4 assay systems.

Inflammatory cytokines, as evaluated here, were not correlated with SHBG univariately in any SHBG assay. However, in a multivariate context, IL-8 and TNF-alpha as well as IL-10 were weakly (trend-level) associated with SHBG in 1 or 2 of the 4 SHBG assays. In postmenopausal women, CRP, soluble IL-6 receptor, and IL-6 are negative predictors of SHBG in certain immunoassays [1,37]. Further studies are therefore needed on this point.

A univariate trend ($0.01 < P < 0.05$) toward a negative effect of age on SHBG in women was inferable. The trend was not significant at protected $P < 0.01$. In contrast, in healthy men, age is a strong and uniformly positive predictor of immunologically quantified SHBG [2,38]. The markedly negative associations between SHBG and each of AVF, TAF, BMI and insulin, all four of which typically increase with age, may have contributed to the tendency of SHBG to fall with age in the women studied here.

Strengths, weaknesses and caveats include the use of mass spectrometry, and the need to corroborate the present insights in population-based cohorts; investigate basic mechanisms coregulating SHBG, albumin, insulin, androgens and body composition; assess possible impaired fasting glycemia or oral glucose tolerance; clarify the basis for opposite multivariate effects of free vs bio T on SHBG inferred independently of assay type; examine a wider repertoire of cytokines; and assess the roles of genetic polymorphisms, ethnicity and race in influencing SHBG levels in women [6–8,39].

In summary, by employing MS measurements of sex steroids and SHBG, and by including key potential variables [body compositional surrogates (BMI, AVF, TAF), age, albumin, glucose, insulin, sex steroids, BP, lipids, and cytokines], robust multivariate correlates emerge between T and SHBG (positive) and between AVF and SHBG (negative), accounting for more than one-third of interindividual variability in MS-quantified sex-steroid and SHBG concentrations in healthy women ages 21–79 years. In premenopausal

women, HDL was an additional positive correlate of SHBG by MS. From a translational perspective, the strong correlations between SHBG measurements by MS and immunological assays, as reported here, suggest that accuracy of the latter may suffice in most clinical contexts. Altogether, the present data suggest the need for enhanced concepts of integrative metabolism, which more explicitly link biological function and regulation of SHBG, AVF, T, HDL and insulin in women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DK	Diabetes and Kidney Disease
NIH	National Institutes of Health
AG	NIH Aging
SHBG	sex-hormone binding globulin
T	testosterone
E₂	estradiol
E₁	estrone
DHT	5alpha-dehydrotestosterone
MS	mass spectrometry
BMI	body mass index
CT	computerized tomography
Hb	hemoglobin
TG	triglyceride
BP	blood pressure
AVF	abdominal visceral fat
TAF	total abdominal fat.

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Fig. 1.

Impact of free T (*top row*), bio T (*middle row*) and total T (*bottom row*) on (log) SHBG concentrations in each of 4 indicated SHBG assays in 120 healthy women. Univariate P values were $<10^{-4}$ (free T), $<10^{-4}$ (bio T) and <0.05 (total T). For comparison, Table 3 gives the log-linear transformed regressions for each T moiety.

AVF and Albumin Predict Log SHBG in Four Assays in Women (N=120)

Fig. 2.

Joint (two-variable) control of (log) SHBG quantified in 4 assays, as indicated, by albumin and AVF in 120 healthy women. P and R² are for the multivariate model. Individual partial P values are given in brackets on the corresponding X and y axes.

Table 1

A - Epidemiologic, Metabolic Endocrine and Cytokine Data in all 120 Women.					
	Mean	Median	SD	Min	Max
Age (year)	49	49	17	21	79
BMI (kg/m ²)	26	25	4.8	19	47
AVF (cm ²)	82	60	62	7.1	297
TAF (cm ²)	307	282	158	52.9	883
Albumin (g/dL)	4.4	4.4	0.20	4.0	5.1
Glucose (mg/dL)	90	89	7.2	74	118
Insulin (mU/L)	4.9	4.3	2.5	1.1	13.8
E ₁ (pg/mL)	50	35	45	12	258
E ₂ (pg/mL)	53	16	87	3	670
T (ng/dL)	24	22	12	8.4	73
Free T (ng/dL) ^a	0.2	0.2	0.2	0.04	1.5
Bio T (ng/dL) ^a	2.7	2.1	2.3	0.5	17.5
IL-10 (pg/mL)	34.6	1.1	230	0.3	1800
IL-12 (pg/mL)	136	0.4	962	0.04	8640
IL-1-beta (pg/mL)	0.9	0.2	0.9	0.06	2.5
TNF-alpha (pg/mL)	3.5	2.6	3.8	1.0	38
IL-6 (pg/mL)	1.2	0.6	2.4	0.1	16
IL-8 (pg/mL)	3.9	2.9	5.6	1	60.1
Adiponectin (mg/L)	12.1	11.8	5.7	2.6	28.7
SHBG-Immulinite (nmol/L)	82	66	62	17	406
SHBG-Cobas (nmol/L)	82	65	52	18	370
SHBG-MS IAL (nmol/L)	80	63	58	15	400
SHBG-MS-SISCAPA (nmol/L)	76	58	60	13	363
Systolic BP (mm Hg)	119	118	16	88	160
Diastolic BP (mm Hg)	69	69	11	37	95
Cholesterol (mg/dL)	193	188	30	114	297
Triglycerides (mg/dL)	101	90	36	39	232
HDL (mg/dL)	52	52	19	5.2	99

B - Baseline Subject Data by Menopausal Status.			
	Pre-	Post-	T-test
	(N = 60)	(N = 60)	(P value)
Age (year)	34 ± 1.2	64 ± 1.1	<10 ⁻⁵
BMI (kg/m ²)	25 ± 0.9	27 ± 6.8	0.001
T (ng/dL)	28 ± 1.6	19 ± 1.1	<0.001
Albumin (g/dL)	4.4 ± 0.028	4.4 ± 0.024	0.30
Glucose (mg/dL)	88 ± 0.68	92 ± 1.1	0.003
Insulin (μU/mL)	4.7 ± 0.26	5.0 ± 3.8	0.45
E ₁ (pg/mL)	65 ± 6.4	35 ± 4.7	<0.001
E ₂ (pg/mL)	88 ± 14	16 ± 4.2	<10⁻⁵
SHBG-I (nmol/L)	86 ± 8.1	61 ± 4.6	0.010
SHBG-C (nmol/L)	85 ± 7.4	64 ± 4.5	0.016
SHBG-IAL-MS (nmol/L)	83 ± 8.1	60 ± 4.9	0.017
SHBG-SISCAPA-MS (nmol/L)	78 ± 7.6	59 ± 4.8	0.037
DHT (pg/mL)	96 ± 8.1	12 ± 3.8	<10⁻⁵
AVF (cm ²)	55 ± 4.9	109 ± 9.2	<10⁻⁵
TBF (cm ²)	254 ± 17	360 ± 22	0.002
Systolic BP (mmHg)	112 ± 1.7	127 ± 2.0	<10⁻⁵
Diastolic BP (mmHg)	68 ± 1.3	69 ± 1.3	0.30
Cholesterol (mg/dL)	187 ± 4.2	204 ± 3.6	<0.001
Triglycerides (mg/dL)	94 ± 5.0	109 ± 4.9	0.043
HDL (mg/dL)	51 ± 2.1	52 ± 2.8	0.83

^aCalculated.

Boldface denotes P < 0.05. Data are mean ± SEM.

SHBG-I and SHBG-C denote Immulite and Cobas assays, respectively.

Table 2

SHBG Inter-Assay Correlation Table in Women.

y	x	R ²	P	slope (95% C.I.)
MS-1	MS-2	0.954	<10 ⁻⁷⁹	0.929 (0.892–0.967)
MS-1	Immulite	0.928	<10 ⁻⁶⁸	0.918 (0.872–0.965)
MS-1	Cobas	0.936	<10⁻⁷¹	1.00 (0.958–1.053)
MS-2	Immulite	0.953	<10⁻⁷⁹	0.977 (0.938–1.017)
MS-2	Cobas	0.956	<10 ⁻⁸⁰	1.067 (1.025–1.109)
Immulite	Cobas	0.973	<10 ⁻⁹³	1.079 (1.050–1.108)

MS-1 SISCAPA Mayo (mass spectrometry).

MS-2 IAL (Mayo mass spectrometry).

Data are R² (square of correlation coefficient), P value, linear-regression slope of y regressed on x with 95% confidence intervals (C.I.).

N = 120 women.

Boldface denotes that regression slopes overlap unity, indicating nearly complete agreement between the two (paired) measurements of SHBG.

Table 3

Univariate Log SHBG Correlations in Women: predominant impact of fat mass androgens.

	SISCAPA	IAL-Mass Spec	Immulite	Cobas
Age	<u>0.039</u> (–0.006)	<u>0.043</u> (–0.007)	<u>0.030</u> (–0.007)	0.058 (–0.006)
BMI	<10^{–4} (–0.059)	<10^{–4} (–0.060)	<10^{–4} (–0.052)	<10^{–4} (–0.050)
AVF	<10^{–4} (–4.8 × 10 ^{–3})	<10^{–4} (–4.9 × 10 ^{–3})	<10^{–4} (–4.1 × 10 ^{–3})	<10^{–4} (–4.2 × 10 ^{–3})
TAF	<10^{–4} (–1.8 × 10 ^{–3})	<10^{–4} (–1.9 × 10 ^{–3})	<10^{–4} (–1.7 × 10 ^{–3})	<10^{–4} (–1.6 × 10 ^{–3})
Albumin	<u>0.039</u> (–0.56)	0.073	0.78	0.10
Glucose	<u>0.030</u> (–0.016)	<u>0.026</u> (–0.018)	0.061	<u>0.033</u> (–0.015)
Insulin	<0.001 (–0.084)	<0.001 (–0.088)	<0.001 (–0.072)	<0.001 (–0.079)
Estrone (E ₁)	0.39	0.49	0.13	0.49
Estradiol (E ₂)	0.54	0.59	0.39	0.67
Total T	<u>0.028</u> (0.010)	<u>0.019</u> (0.011)	<u>0.025</u> (0.010)	<u>0.023</u> (0.010)
Free T calc	<10^{–4} (–1.5)	<10^{–4} (–1.6)	<10^{–4} (–1.3)	<10^{–4} (–1.3)
Bio T calc	<10^{–4} (–0.13)	<10^{–4} (–0.14)	<10^{–4} (–0.11)	<10^{–4} (–0.11)
IL-10	0.14	0.13	0.29	0.23
IL-12	0.12	0.11	0.26	0.19
IL-1-beta	0.72	0.99	0.89	0.99
TNF-alpha	0.053	0.093	<u>0.047</u>	<u>0.047</u>
IL-6	0.45	0.67	0.47	0.64
IL-8	<u>0.040</u>	0.068	0.06	0.067
Adiponectin	0.68	0.57	0.54	0.47

Data are P values (slope).

Boldface are significant at P < 0.01 protected.

Underscored are trends (0.01 < P < 0.05).

SHBG was expressed as its natural logarithm.

Due to insufficient serum in 7 samples, DHT values were not included in the above regressions.

None of univariate blood pressure or lipid measures given in Table 1A and B correlated (all P > 0.10) with any of the 4 SHBG measurements.

Table 4

Results of Stepwise Multivariate Log SHBG Correlations in 120 Women.

	MS-SISCAPA	MS-IAL	Immulite	Cobas
<i>Overall P^a</i>	5.1×10^{-22}	6.9×10^{-22}	7.8×10^{-16}	6.4×10^{-16}
<i>Overall R²</i>	0.613	0.583	0.515	0.481
TAF	NS	NS	0.0063 (−0.00074)	0.0066 (−0.0018)
Free T	8.4×10^{-8} (12.4)	1.2×10^{-6} (11.7)	5.6×10^{-4} (8.3)	6.6×10^{-4} (8.1)
Bio T	2.0×10^{-9} (−1.2)	2.9×10^{-8} (−1.1)	6.1×10^{-5} (−0.81)	5.6×10^{-5} (−0.80)
BMI	0.00028 (−0.030)	0.00039 (−0.030)	NS	NS
Estrone (E ₁)	NS	NS	0.035 (−0.0020)	NS
Total T	9.6×10^{-10} (0.023)	5.3×10^{-10} (0.024)	4.7×10^{-8} (0.023)	1.2×10^{-7} (0.021)
TNF-alpha	NS	NS	0.035 (0.022)	0.032 (0.022)
IL-8	0.033 (0.014)	NS	NS	NS
Insulin	NS	NS	NS	NS
Pre- vs postmenopausal	P < 0.01	P < 0.01	P < 0.01	P < 0.01

Data in parentheses are slopes of the linear multivariate regression. All variables from Table 1A and 1B (except BP and lipids) were allowable.

Boldface denotes P < 0.01 for the individually contributing variable.

The insulin contribution is shown to highlight its loss of significance (each P > 0.10) under multivariate collinearity correction.

^a Overall and individual P protected at experiment-wise P < 0.01.

Table 5

Multivariate Regression of Mayo Mass-Spectrometry SHBG Levels on Epidemiological Data in Pre- and Postmenopausal Women.

	All Women (N = 120)	Premenopausal (N = 60)	Postmenopausal (N = 60)
Age (year)	NS	NS	NS
BMI (kg/m ²)	NS	NS	NS
T (ng/dL)	P = 0.003 (slope = 1.24)	P = 0.045 (slope = +1.34)	P = 0.030 (slope = +1.18)
Albumin (g/dL)	P = 0.025 (slope = -53.6)	NS	NS
Glucose (mg/dL)	NS	NS	NS
Insulin (μU/mL)	NS	NS	NS
E ₁ (pg/mL)	NS	NS	NS
E ₂ (pg/mL)	NS	NS	NS
DHT (pg/mL)	NS	NS	NS
AVF (cm ²)	P < 0.001 (slope = -0.48)	P < 0.001 (slope = -0.65)	P = 0.002 (slope = -0.048)
TBF (cm ²)	NS	NS	NS
Systolic BP (mmHg)	NS	NS	NS
Diastolic BP (mmHg)	NS	NS	NS
Cholesterol (mg/dL)	NS	NS	NS
Triglycerides (mg/dL)	NS	NS	NS
HDL (mg/dL)	P = 0.003 (slope = 0.44)	P = 0.001 (slope = 0.69)	NS
Overall P value	8.06 × 10⁻¹⁰	5.12 × 10⁻⁶	3.53 × 10⁻⁴
R ²	0.358	0.414	0.243

NS denoted P > 0.05.

Boldface denotes P < 0.05.

Starting variables were those of Table 1B.