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Associations of Sex-Hormone-Binding Globulin (SHBG) with Non-SHBG-Bound Levels of Testosterone and Estradiol in Independently Living Men

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Results of *in vitro* experiments indicate that with increasing concentrations of SHBG, testosterone (T) is preferentially bound to SHBG in comparison with estradiol (E2). In these studies, the ratio of non-SHBG-bound E2 (non-SHBG-E2) to non-SHBG-T increased with increasing levels of SHBG. SHBG has consequently been regarded as an estrogen amplifier. In this cross-sectional study in 399 men aged between 40 and 80 yr we tested whether higher levels of SHBG are associated with a higher estrogen/androgen ratio *in vivo*. The mean T level of these men was in the eugonadal range [536 ± 152 ng/dl (18.6 ± 5.26 nmol/liter), mean ± SD]. With increasing SHBG levels the non-SHBG-bound fraction of T decreased from 80 to 36% and that of E2 from 89 to 53%. Higher levels of SHBG were associated with higher levels of both total T [regression coefficient (β) after adjustment for age and body mass index, 286 ± 15.8; $P < 0.001$] and total E2 ($\beta = 4.47 \pm 0.90$; $P < 0.001$). However, SHBG levels were negatively related with levels of non-SHBG-E2 ($\beta = -1.78 \pm 0.69$; $P < 0.001$), whereas there was a positive association between levels of SHBG and non-SHBG-T ($\beta = 32.0 \pm 9.78$; $P = 0.001$). Furthermore, we observed a negative relationship between SHBG levels and the E2/T ratio of either total ($\beta = -0.016 \pm 0.002$; $P < 0.001$) or non-SHBG-bound ($\beta = -0.011 \pm 0.002$; $P < 0.001$) hormone. Therefore, we conclude that in eugonadal men, higher SHBG levels are associated with lower levels of non-SHBG-E2 but slightly higher levels of non-SHBG-T. This means that SHBG cannot be regarded as an estrogen amplifier in eugonadal men. (*J Clin Endocrinol Metab* 90: 157–162, 2005)

SHBG, corticosteroid binding globulin, and albumin are important steroid hormone binding proteins in human plasma. Although recent evidence shows that SHBG can participate in signal transduction via its own membrane receptor (1), it is best known for its role as a binding protein of sex hormones in human plasma. In normal men and women, between 40 and 65% of circulating testosterone (T) and between 20 and 40% of circulating estradiol (E2) is bound to SHBG (2). Binding of T to SHBG decreases its metabolic clearance rate and its conversion rate to androstenedione (3). Binding to SHBG also prevents bound hormone from diffusing out of the bloodstream, thereby preventing hormone binding to the intracellular androgen or estrogen receptors. The non-SHBG-bound fraction of hormone is, therefore, considered to be bioactive (free hormone hypothesis as reviewed in Ref. 4).

T and E2 bind to the same binding site on SHBG, but the binding affinity for T is higher than that for E2 (5). *In vitro* experiments show that with increasing levels of SHBG and stable levels of T and E2 the ratio of unbound E2 to unbound T increases (6). On the basis of the relatively greater decrease in the bioavailability of T compared with that of E2, SHBG has been regarded as an estrogen amplifier. This might provide an explanation for the gynecomastia frequently observed in thyrotoxic men because thyrotoxicosis is associated with high concentrations of SHBG (7–9). An alternative explanation for this observation might be that levels of LH in these patients are increased, causing an increase in testicular E2 production (10), although others did not detect increased E2 production rates in hyperthyroidism (11, 12). In healthy males, there is a wide variation in SHBG concentrations. In cross-sectional studies, the plasma concentrations of T and SHBG are positively correlated (13). This correlation not only reflects the high binding affinity of SHBG for T, resulting in increased storage of the steroid, but may also be explained by the effect of SHBG levels on the bioavailability of T. Higher SHBG levels would then lead to lower levels of bioactive T, a decreased feedback signal on GnRH and thereby on LH secretion by the pituitary and a subsequent increase of T levels until a new set point is reached. This dependence of total T on variations in SHBG in men *in vivo* differs from the stable T levels in the *in vitro* experiments described above. It is, therefore, doubtful whether the conclusions drawn from these *in vitro* experiments apply to the *in vivo* situation. The aim of this study was to evaluate whether the relationships between T, E2, and SHBG in healthy men support the conclusions based on the *in vitro* experiments.

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Abbreviations: BMI, Body mass index; E2, estradiol; HPG, hypothalamo-pituitary-gonadal; non-SHBG-T, non-SHBG-bound T; T, testosterone.

Subjects and Methods

Subjects

The study is a cross-sectional, single-center study of 400 independently living men aged 40–80 yr. The study was originally designed to study the relationships between endogenous sex hormones and risk factors for, or manifestations of, chronic diseases. The subjects were recruited by asking female participants of other studies conducted by the department whether they knew any man who might be interested in volunteering for the study. Invitation letters were sent to 770 female participants. Eventually, 240 men volunteered for participation.

Subsequently, names and addresses of a randomly selected male population aged 40–80 yr were drawn from the municipal register of Utrecht, a large town in the middle part of The Netherlands. A total of 1230 invitation letters were sent. From this group, 390 men volunteered for participation.

From the 630 volunteers we excluded the subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently (n = 16). No additional health-related eligibility criteria, other than being physically and mentally able to visit the study center independently, were used. Of the remaining 614 men, 400 men were randomly selected to participate. To obtain equal numbers in each age decade, we sampled 100 men in each decade of age. One subject was excluded from analysis because of clear hypogonadism [total testosterone = 6.91 ng/dl (0.24 nmol/liter)]. Data collection took place between March 2001 and April 2002.

All participants gave written informed consent before enrollment, and the Institutional Review Board of the Utrecht University Medical Center approved the study.

Height and weight were measured in the standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Visceral and intraabdominal fat were assessed using ultrasound measurements (14, 15). Ultrasonography was performed with an HDI 3000 (Philips Medical Systems, Eindhoven, The Netherlands) using a C 4-2 transducer. The distances between the posterior edge of the abdominal muscles and the lumbar spine or psoas muscles were measured using electronic calipers. For all images, the transducer was placed on a straight line drawn between the left and right midpoint of lower rib and iliac crest. Distances were measured three times from three different angles: medial, left, and right for intraabdominal fat mass and medial for sc fat mass. Measurements were made at the end of quiet expiration, applying minimal pressure without displacement of intraabdominal contents as observed by ultrasound image. Visceral fat was measured as the distance between the skin and the linea alba and intraabdominal fat as the distance between the peritoneum and lumbar spine.

Details on lifestyle and health of the subjects have been published earlier (16).

Laboratory measurements

Fasting blood samples were obtained by venipuncture. Cell-free serum was immediately stored at –20 C. T was measured after diethyl extraction using an in-house RIA employing a polyclonal anti-T antibody (AZG 3290, a gift from Dr. J. J. Pratt, Groningen, The Netherlands). The lower limit of detection of the assay was 0.24 nmol/liter, and interassay variation was 6.0, 5.4, and 8.6% at 2.1, 5.6, and 23 nmol/liter, respectively. SHBG was measured using an immunometric technique on an Immulite analyzer (Diagnostic Products Corp., Los Angeles, CA). The lower limit of detection was 5 nmol/liter, and interassay variation was 6.1, 4.9, and 6.9% at 11.6, 36, and 93 nmol/liter, respectively. E2 was measured after diethylether extraction and Sephadex chromatography using an in-house RIA employing a polyclonal anti-E2 antibody. The lower limit of detection was 20 pmol/liter, and interassay variation was 10 and 3.1% at 81 and 660 pmol/liter, respectively.

Non-SHBG-bound T and E2 were calculated using the method described by Sodergard *et al.* (17) using a fixed plasma albumin concentration of 40 g/liter. The equations for these calculations are given in Table 1. The association constants we used for the calculation of the binding of T (k_t) and E2 (k_e) to SHBG were 5.97×10^8 and 3.14×10^8, respectively (17). In the literature, various estimates for these binding affinities have been calculated on the basis of various methodologies. Values of 10×10^8 (18), 16×10^8 (2), or 19×10^8 (6) have also been reported for k_t, and values ranging from 3.14×10^8 (17) to 6.8×10^8 (2) have been reported for k_e. Changing these constants in the equations will obviously lead to changes in the calculated levels of unbound hormones and can influence the observed relationships between SHBG and the bioavailable levels of T and E2. Therefore, we repeated the analyses after introducing alternative values for k_t and k_e in the equations.

TABLE 1. Equations for the calculation of non-SHBG-T and non-SHBG-E2 according to Sodergard *et al.* (17)

	Equation
Non-SHBG-T (nmol/liter)	$\{(k_{at}*[albumin]*[FT])/(1 + k_{at}*[FT])\} + [FT]$
FT (nmol/liter)	$\{-b + \sqrt{(b^2 + 4a[TT])}\}/2a$, in which $a = k_{at} + k_t + (k_{at}*k_t)([SHBG] + [albumin] - [T])$ and $b = 1 + k_t[SHBG] + k_{at}[albumin] - (k_{at} + k_t)[T]$
Non-SHBG-E2 (pmol/liter)	$[E2] - \{k_e*[SHBG]*FE2/(1 + k_e*[FE2] + k_t*[FT])\}$
FE2 (pmol/liter)	$\{-b - \sqrt{(b2 - 4ac)}\}/2a$, in which $a = (k_{at}*[albumin] + 1)k_e$, $b = ([E2]*k_e) - (k_{ae}*[albumin] + 1)(1 + k_t*[FT]) - (k_e*[SHBG])$, and $c = [E2]*(1 + k_t*[FT])$

k_{at}, Association constant for binding of T to albumin ($4.06*10^4$; liter/mol); k_t, association constant for binding of T to SHBG ($5.97*10^8$; liter/mol); k_{ae}, association constant for binding of E2 to albumin ($4.21*10^4$; liter/mol); k_e, association constant for binding of E2 to SHBG ($3.14*10^8$; liter/mol); [FT], plasma concentration of free (non-albumin-non-SHBG bound) T (mol/liter); [FE2], plasma concentration of free (non-albumin-non-SHBG bound) estradiol (mol/liter); [T], plasma concentration of testosterone (mol/liter); [E2], plasma concentration of estradiol (mol/liter).

For conversion of (non-SHBG bound)T to ng/dl, multiply by 28.8; for conversion of (non-SHBG-bound) E2 to pg/ml, multiply by 0.27; for conversion of SHBG to μg/dl, multiply by 0.025.

Statistics

All calculations were performed using SPSS 11.0 software. Relations between SHBG and hormone levels were assessed using linear regression for continuous variables described as the linear regression coefficient (β) using SHBG as the independent variable before and after adjustment for age and BMI. Because site-specific differences in aromatase activity have been described (19), we tested whether adding visceral or abdominal fat mass to the regression analysis had any impact on the results. The linear regression coefficient β indicates the change of the dependent variable for every 1 nmol/liter change in SHBG. Adjustments for age and BMI were made by adding these parameters as independent variables to the regression model. Adjustments were made because both age and BMI have been shown to be associated with levels of SHBG, T, and E2 in men (13, 16).

Results

The characteristics of the studied men are presented in Table 2. The mean T level was in the eugonadal range. Mean

TABLE 2. Characteristics of the 399 studied men

Characteristic	Mean ± SD
Age (yr)	60.2 ± 11.3
BMI (kg/m^2)	26.3 ± 3.48
SHBG (μg/dl)	1.01 ± 0.36 (40.6 ± 14.5)
T (ng/dl)	536 ± 152 (18.6 ± 5.26)
Non-SHBG-T (ng/dl)	300 ± 75.4 (10.4 ± 2.62)
E2 (pg/ml)	24.9 ± 6.15 (91 ± 23)
Non-SHBG-E2 (pg/ml)	17.9 ± 4.60 (66 ± 17)
E2 (pg/ml)/T (ng/dl)	0.05 ± 0.02 (5.20 ± 1.61)
Non-SHBG-E2 (pg/ml)/non-SHBG-T (ng/dl)	0.06 ± 0.02 (6.57 ± 1.94)

Systeme International units are given in *parentheses*.

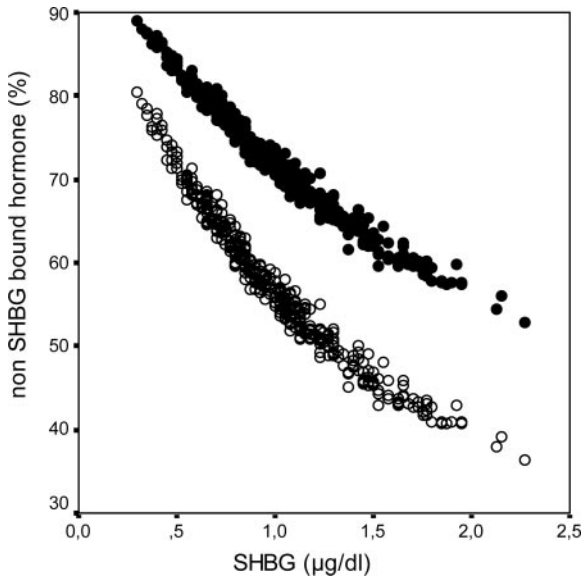

FIG. 1. SHBG *vs.* the percentages of non-SHBG-T (○) and non-SHBG-E2 (●) in 399 men. (For conversion of SHBG to nmol/liter, multiply by 40.)

values for abdominal and visceral fat mass as measured by ultrasound were 7.52 ± 2.23 and 2.65 ± 0.85 cm, respectively.

With increasing SHBG concentration, the percentages of hormone not bound to SHBG decreased from 80 to 36 for T and from 89 to 53 for E2 (Fig. 1).

The relationships between SHBG and T and non-SHBG-bound T (non-SHBG-T) before and after adjustment for age and BMI are presented in Table 3 and in Fig. 2. Higher levels of SHBG were strongly associated with higher levels of T (β = 286 ± 15.8; $P < 0.001$). SHBG and non-SHBG-T were related only after adjustment for age and BMI (β = 32.0 ± 9.78; $P = 0.001$).

The relationships between plasma levels of SHBG and E2 and non-SHBG-E2 are presented in Table 3 and in Fig. 3. High SHBG levels were associated with higher E2 levels (β = 4.47 ± 0.90; $P < 0.001$) but with lower concentrations of non-SHBG-E2 (β = –1.78 ± 0.69; $P = 0.008$).

Finally, the relationships between plasma levels of SHBG and the E2/T ratio and the non-SHBG-E2/non-SHBG-T ratio are presented in Table 3 and Fig. 4. SHBG levels were negatively related to both ratios (β = −0.016 ± 0.002; $P < 0.001$ and β = −0.011 ± 0.002; $P < 0.001$, respectively). Adding abdominal or visceral fat mass to the regression analyses did not change the results.

Introducing a k_t of 10×10^8 into the equations of Table 1 [according to the frequently used Vermeulen method (18)] did not essentially change the relationship between SHBG and non-SHBG-T (β = –2.56 ± 8.41; $P = 0.76$ after adjustment for age and BMI). After introducing a k_e of 6.8×10^8, the inverse relation between SHBG and non-SHBG-E2 was more pronounced (β = –3.76 ± 0.57; $P < 0.001$ after adjustment for age and BMI). Only after introducing the highest reported value for k_t [19×10^8 (6)] and the lowest reported value for k_e [3.14×10^8 (17)] did the relationship between SHBG and the ratio of non-SHBG-E2/non-SHBG-T appear to be slightly but significantly positive (β = 0.011 ± 0.004; $P = 0.01$ after adjustment for age and BMI). However, it is questionable whether it is allowed to combine these k values, as they were obtained under different circumstances.

Discussion

In this study, the relationship between SHBG levels and the E2/T ratio was studied in eugonadal healthy men. The concept of SHBG as an estrogen amplifier (6) is based on the observation that with stable T and E2 levels, an increase of SHBG will decrease unbound T more than unbound E2, resulting in an increase of the non-SHBG-E2/non-SHBG-T ratio. However, in eugonadal men, this theory does not apply because the hypothalamo-pituitary-gonadal (HPG) axis will respond to a decreasing level of non-SHBG-T with an increase in LH and T, assuming that non-SHBG-T is driving the feedback inhibition of the male HPG axis. The validity of this hypothesis is supported by our observation that increased levels of SHBG are associated with increased levels of total T but are barely associated with the level of non-SHBG-T.

Levels of SHBG show only a modest positive association with total levels of E2 but are negatively related with those of non-SHBG-E2. As a result, a high concentration of SHBG is associated with a lower (non-SHBG-bound) estrogen/androgen ratio and vice versa. Endogenous E2 can also have an effect on LH release by the pituitary (20, 21). However, in contrast to T, E2 levels are not directly regulated by HPG axis activity. When bioavailable E2 levels decrease, this might lead to increased LH release by the pituitary with a resulting increase in testicular T production. Total E2 levels will be increased only if T is subsequently aromatized, the extent of which is influenced by parameters such as age and BMI. The regulation of peripheral E2 levels by the HPG axis is indirect and therefore probably not as tight compared with T levels.

The fact that an intact HPG axis appears to prevent the non-SHBG-T concentration to fall with increasing SHBG lev-

TABLE 3. Linear regression coefficients (β) for the relationships of SHBG with T, non-SHBG-T, E2, non-SHBG-E2, and estrogen/androgen ratios before and after adjustment for age and BMI

	Unadjusted		Adjusted for age and BMI	
	β ± SE	P	β ± SE	P
T (ng/dl)	252 ± 16.8	<0.001	286 ± 15.8	<0.001
Non-SHBG-T (ng/dl)	10.2 ± 10.4	0.33	32.0 ± 9.78	0.001
E2 (pg/ml)	3.22 ± 0.84	<0.001	4.47 ± 0.90	<0.001
Non-SHBG-E2 (pg/ml)	−2.77 ± 0.62	<0.001	−1.78 ± 0.69	0.008
E2 (pg/ml)/T (ng/dl)	−0.016 ± 0.002	<0.001	−0.016 ± 0.002	<0.001
Non-SHBG-E2 (pg/ml)/non-SHBG-T (ng/dl)	−0.011 ± 0.002	<0.001	−0.011 ± 0.002	<0.001

For β, the result given indicates change of the dependent variable for every 1 µg/dl change in SHBG. For conversion of (non-SHBG-bound) T to nmol/liter, multiply by 0.0347; for conversion of (non-SHBG-bound) E2 to pmol/liter, multiply by 3.67.

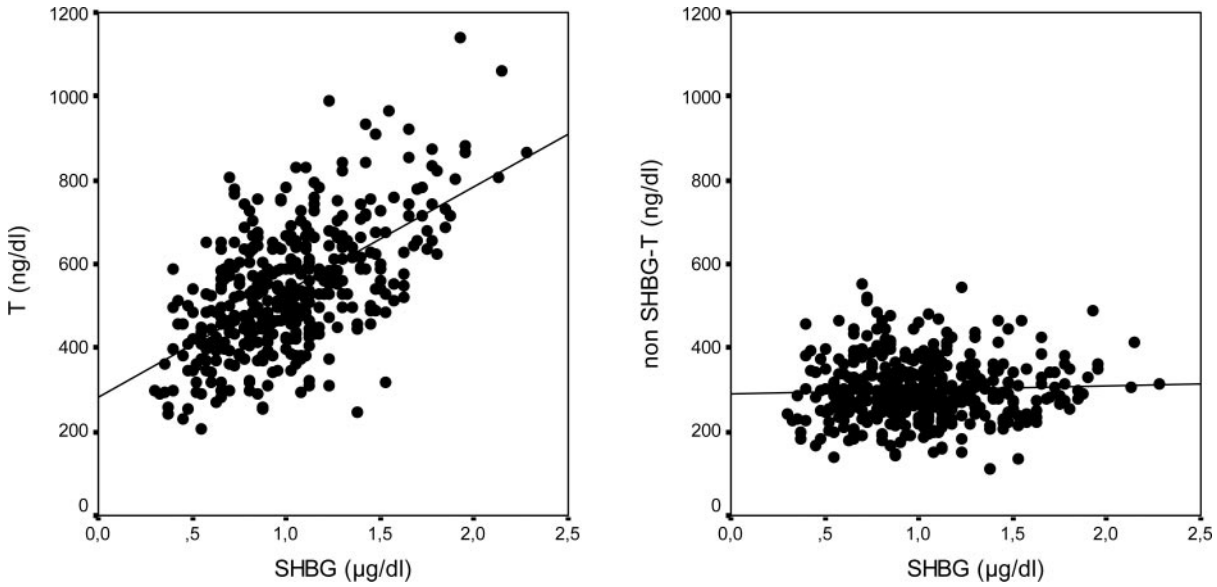

FIG. 2. SHBG *vs.* T (*left*) and non-SHBG-T (*right*) in 399 men. The relationship between SHBG and T is given by the formula [T] = 280 + 252[SHBG] and that between SHBG and non-SHBG-T by [non-SHBG-T] = 290 + 10.2[SHBG]. [For conversion of (non-SHBG-bound) T to nmol/liter, multiply by 0.0347; for conversion of SHBG to nmol/liter, multiply by 40.]

els makes the *in vivo* situation in eugonadal men totally different from the *in vitro* situation where changes in hormone binding to SHBG do not evoke adaptations in the HPG axis. This lack of similarity between *in vivo* and *in vitro* conditions was already alluded to by Rosner (22) but, to our knowledge, was never formally tested.

Our findings in healthy men seem to conflict with conditions associated with high SHBG levels in men such as advanced age, liver disease, hyperthyroidism, and estrogen administration (22). These conditions are associated with increased estrogen/androgen ratios and gynecomastia (23, 24), and they seem to confirm the concept of SHBG as an estrogen amplifier. However, besides the altered SHBG levels, these conditions are also associated with altered gonadal function. Hypogonadism is frequently observed in liver cirrhosis patients (25, 26). In hyperthyroid men, lower levels of non-SHBG-T are frequently (7–9) but not always (27, 28) reported, which suggests that the HPG axis in these men is not always able to fully compensate for the rise in SHBG concentration. Moreover, the increased estrogen/androgen ratio in hyperthyroid subjects might be caused by increased androgen aromatization (10, 29). The age-associated increase in SHBG is not associated with an increase in T levels (30), which suggests that the HPG axis of older men is not capable of responding to a fall in T levels. Therefore, it is likely that the relative hypogonadism and not the increased SHBG per

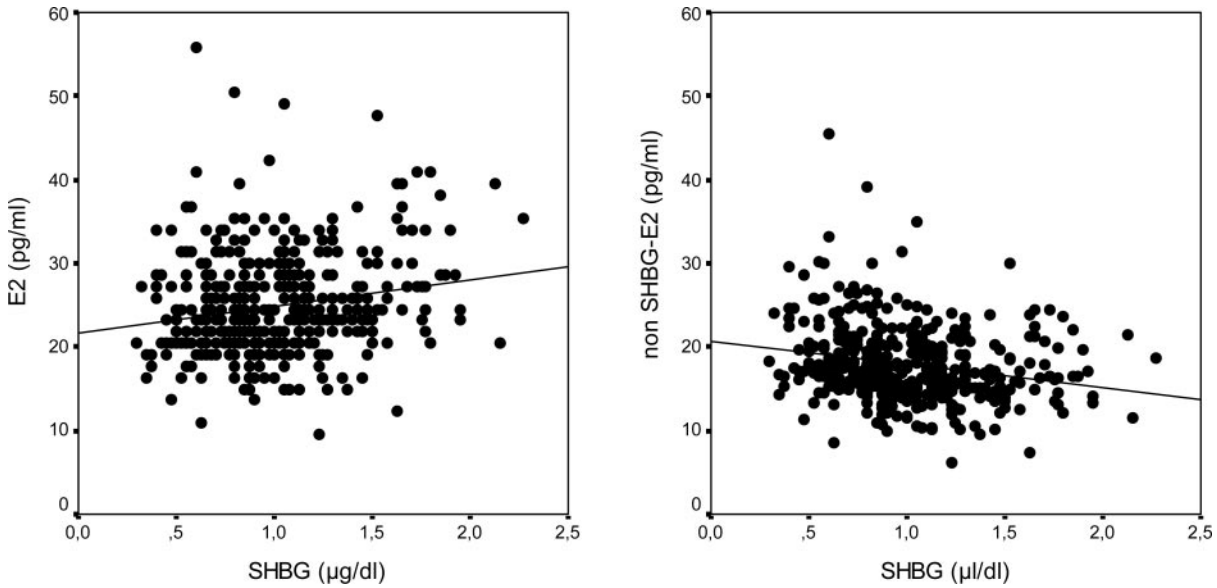

FIG. 3. SHBG *vs.* E2 (*left*) and non-SHBG-E2 (*right*) in 399 men The relationship between SHBG and E2 is given by the formula [E2] = 21.6 + 3.22[SHBG] and that between SHBG and non-SHBG-E2 by [non-SHBG-E2] = 20.7 –2.77[SHBG]. [For conversion of (non-SHBG-bound) E2 to pmol/liter, multiply by 3.67; for conversion of SHBG to nmol/liter, multiply by 40.]

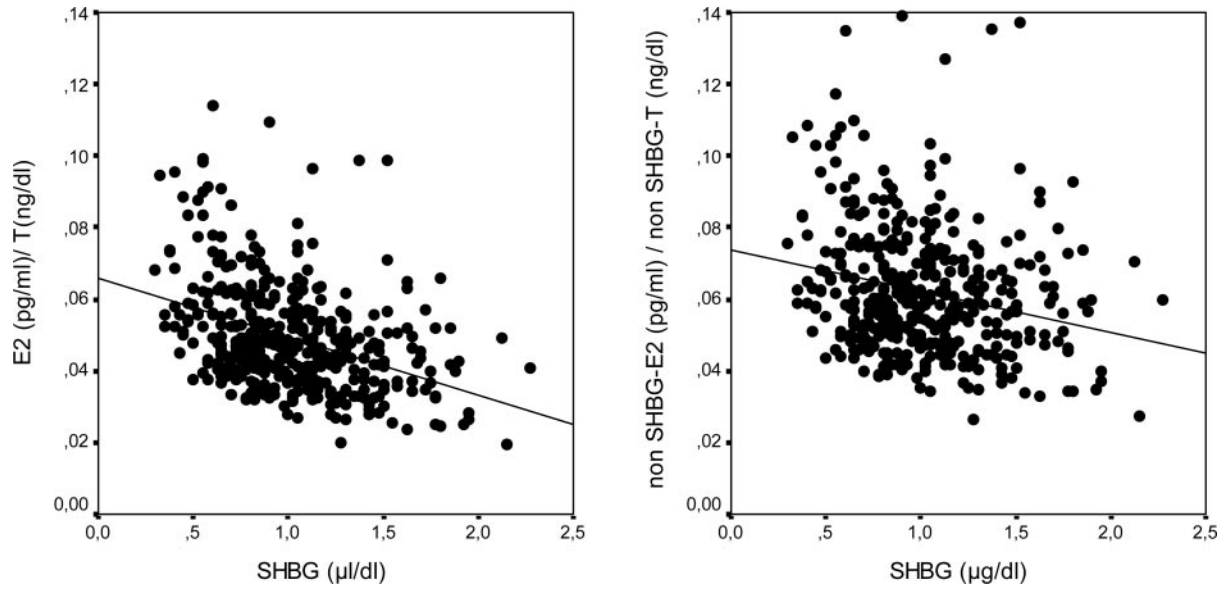

FIG. 4. SHBG *vs.* the ratio of E2 over T (*left*) and the ratio of non-SHBG-E2 over non-SHBG-T (*right*) in 399 men. The relationship between SHBG and E2/T ratio is given by the formula E2/T = 0.066 −0.016[SHBG] and that between SHBG and non-SHBG-E2 by non-SHBG-E2/non-SHBG-T = 0.074 −0.011[SHBG]. (For conversion of T to nmol/liter, multiply by 0.0347; for conversion of E2 to pmol/liter, multiply by 3.67; for conversion of SHBG to nmol/liter, multiply by 40.)

se may explain the high estrogen/androgen ratio in these men.

The question of the clinical relevance of our observation arises. In the pathogenesis of gynecomastia, a high estrogen/androgen balance seems to be of importance (23, 24). According to our results, men with low levels of SHBG and a resulting high estrogen/androgen ratio would have a higher risk of developing gynecomastia, although this association has not been reported in the literature. Probably the changes in the estrogen/androgen ratio brought about by SHBG in eugonadal men are too subtle to cause gynecomastia.

Our results show that high levels of SHBG are associated with lower levels of non-SHBG-E2 but normal or even slightly higher levels of non-SHBG-T. The decreased feedback inhibition of non-SHBG-E2 on the release of LH by the pituitary probably explains the slightly positive relationship between levels of non-SHBG-T and SHBG.

It is well known that lower levels of non-SHBG-E2 in men are associated with lower bone mineral density (31, 32). Apparently, even in eugonadal men, elevated SHBG levels might contribute to estrogen deficiency and to conditions such as osteoporosis.

One might speculate that while passing through capillaries, a proportion of the bound hormone dissociates from SHBG and in fact becomes bioavailable. In that case, the amount of bioavailable hormone might be underestimated when using the described equations for the calculation of the bioavailable fractions. Consequently, the amount of bioavailable E2 would be underestimated more in comparison with the amount of bioavailable T because of the weaker binding of E2 to SHBG. However, the validity of this hypothesis remains to be determined.

For the calculation of the levels of non-SHBG-E2 and non-SHBG-T we used the equations as described by Sodergard *et al.* (Table 1) (17) in which the association constants for the binding of T (k_t) and E2 (k_e) to SHBG are 5.97×10^8 and 3.14×10^8, respectively. In the literature, alternative estimates for these binding affinities are reported (2, 6, 18). Use of a higher association constant in the equation will tilt the slope of the regression lines shown in Figs. 2 and 3 (*right panels*) slightly down and vice versa. Theoretically, combining a high k_t with a low k_e in the equations of Table 1 can result in a positive relation between SHBG and the non-SHBG-E2/non-SHBG-T ratio. However, when the combination of values as reported by Dunn *et al.* (2) and Burke and Anderson (6) were used, this was not the case.

Although the subjects we studied were prone to health selection bias, this does not undermine the conclusions of this study. In fact, it contributed to the uniformity of the analyses because there were only a few hypogonadal subjects (based on T and non-SHBG-T levels) in this group of men. On the other hand, it prevented us from doing separate analyses on data from eugonadal and hypogonadal men.

The conclusion of our study is that in eugonadal men, higher SHBG levels are associated with lower levels of non-SHBG-E2 but unaltered or even slightly higher levels of non-SHBG-T. This means that SHBG cannot be regarded as an estrogen amplifier in eugonadal men.

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