Modulation of SHBG binding to testosterone and estradiol by sex and morbid obesity

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34   **ABSTRACT**

Objective: Sex hormone-binding globulin (SHBG) binds and transports testosterone and estradiol in
36   plasma. The possibility that SHBG is a mixture of transporting proteins has been postulated. We analyzed
in parallel the effects of obesity status on the levels and binding capacity of circulating SHBG and their
38   relationship with testosterone and estradiol.

Design: Anthropometric measures and plasma were obtained from apparently healthy young (i.e. 35 ± 7
40   years) premenopausal women (n=32) and men (n=30), with normal weight and obesity (BMI>30 kg·m^{-2}).

Methods: SHBG protein (Western blot), as well as the plasma levels of testosterone, estradiol, cortisol
42   and insulin (ELISA) were measured. Specific binding of estradiol and testosterone to plasma SHBG was
analyzed using tritium-labelled hormones.

44   **Results**: Significant differences in SHBG were observed within the obesity status and gender, with
discordant patterns of change in testosterone and estradiol. In men, testosterone occupied most of the
46   binding sites. Estrogen binding was much lower in all subjects. Lower SHBG of morbidly obese (BMI>40
kg·m^{-2}) subjects affected testosterone but not estradiol. The ratio of binding sites to SHBG protein levels,
48   was constant for testosterone, but not for estradiol. The impact of gender was maximal in morbid obesity,
with men showing the highest binding / SHBG ratios.

50   **Conclusions**: The results reported here are compatible with SHBG being a mixture of at least two
functionally different hormone-binding globulins, being affected by obesity and gender, and showing
52   different structure, affinities for testosterone and estradiol, and also different immunoreactivity.

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INTRODUCTION

56   Hormone-binding globulins are serpins, whose main role is to specifically bind and transport steroid and thyroid hormones in plasma. Sex hormone-binding globulin (SHBG) essentially binds /

58   transports testosterone and estradiol [1]. The finding of its possible use as marker of a number of metabolic [2,3] or psychological [4] disorders has contributed to enhance the study of SHBG role in the control of gene

60   expression [5] and sex hormone levels [6,7], as well as its more than probable implication in metabolic syndrome (MS) [8].

62   The function of SHBG is far from being fully understood. In fact, although it binds the hormone ligands in a high-affinity specific way, SHBG levels in plasma are low in comparison with albumin and

64   other plasma proteins. The latter bind non-specifically these same hormones with high capacity and low affinity. The equilibrium of free hormone levels, thus, is not solely dependent on SHBG binding ability or

66   levels, but also on its interaction with tissues and their complex and variably modulated compartmentation [9]. The absence of SHBG in rodents, in contrast with CBG, present in them and in humans, may hint at

68   additional functions for this serpin, but the presence of a specific testosterone-binding protein in rodent testicles [10], with a marked structural and gene parallelism with SHBG [11,12] suggests that at least for this

70   organ, a specific protein binding testosterone is required.

72   The high-affinity binding of sex hormones to SHBG competes with cell receptors, altering its direct availability, which prompts the question whether SHBG main role is to transport the hormones, or retain them to modulate their binding to cell membranes, including the specific binding of SHBG itself [13].

74   This sequestering role has been postulated for tissues, where membrane-related hormone-binding globulins may act barrier-like to limit or control the access of active hormones to cells, as is the case for

76   CBG [14]. However, hormone-binding globulins are required as vector for transfer of hormones to cell ligands [15], to elicit effects on gene expression and the activation of other cell functions [16,17].

78   SHBG is a glycoprotein [18], which appears in plasma as a dimer, containing two subunits, each one having a site able to bind testosterone, dihydrotestosterone or estradiol [19]. The affinity for

80   testosterone has been found to be higher than for estradiol [20] in line with the function of the precursor testosterone-binding protein of rodents compared with that of plasma and testicles in rabbits [21]. In human

82   plasma, SHBG has been found in several forms of variable molecular weight, distributed in markedly different proportions [22,23]; however, they shared the peptide size and immuno-reacting properties, albeit

84   only partially [22,24]. The difference in size has been largely attributed to the varying proportion of oligosaccharide chains [23,25]. SHBG hormone-binding affinity can be changed by molecular modulation of

86   the serpin structure, such as localized proteolysis [26], proteolytic cleavage resulting in increased free hormone levels because of loss of affinity of the protein to its ligand [2]. Other possible modifications of

88   SHBG structure / binding are the formation of O- or N-glycans [27,28]. An important factor of variability / regulation is the complex control [29,30] of the expression of the single SHBG gene described [12], able to

90   generate different products [12]. In addition, a number of genetic variants, showing different hormone-binding affinities, has been described [31,32].

92   Plasma concentrations of free testosterone and estradiol are related to the levels of SHBG [33]. Sex affects markedly the levels [34,35] and function of SHBG, essentially altering the levels of testosterone [36].

94   The important modulatory effects of estradiol and testosterone, confronting (or allied to) the effects of glucocorticoids [37] and insulin [38,39] have been clearly established to show sex-related differences [40,41]

96   especially under conditions of predominance of insulin resistance and glucocorticoid action as in

metabolic syndrome (MS) [37, 41]. SHBG has been associated to the risk of MS in men [38, 42], independently of testosterone levels [43], whereas the relation of this syndrome with total or free testosterone is –at most— weak [44, 45]. Women with MS showed increased testosterone and lower SHBG independently of menopausal status [46].

Obesity is known to lead to insulin resistance [47], increase the risk of MS [48], and it is also associated with hypercortisolism [49], hypoandrogenism [42] and increased estrogens [50]. In the present study we studied whether SHBG function is modified by obesity status. The key question was to determine whether the changes that SHBG experiences with obesity are a direct consequence of the existence of different molecular species of this protein.

SUBJECTS AND METHODS

Study population

A sample of 62 subjects (32 of them women) of Caucasian origin, aged between 18 and 50 y (i.e. 35 ± 7 y) and including patients at the extremes of the weight continuum [16 participants with severe obesity (BMI≥40 kg.m^{-2}), 19 obese subjects, and 28 age and gender-matched healthy-weight controls (BMI<25 kg·m^{-2})], was enrolled for hormonal and circulating assessment of target proteins. Six groups of subjects were established and studied comparatively: 1) women with normal weight: BMI<25 kg·m^{-2};, 2) obese women: 30≤BMI<40 kg·m^{-2}; 3) morbid obese women: BMI≥40 kg·m^{-2}; 4) men with normal weight: BMI<25 kg·m^{-2};, 5) obese men: 30≤BMI<40 kg·m^{-2}; 3) morbid obese men: BMI≥40 kg·m^{-2}. Inclusion criteria, in addition to the BMI ranges described above, were the absence of any systemic disease, and the absence of any infections in the previous month. None of the subjects was taking medication (including glucocorticoids or estrogens) or showed evidence of metabolic disease other than obesity. All subjects reported that their body weight had been stable for at least 3 months before the study. Liver disease and thyroid dysfunction were specifically excluded by biochemical workup. All women had regular menstrual cycles and were studied on days 3–8 of the cycle. All participants were in apparently good health, and free of cardiovascular pathologies, clinically defined diabetes or hypertension.

The protocol was approved by the Hospital Ethics Committee, and informed consent was obtained from each subject.

Baseline studies included a standardized questionnaire, physical examination and common laboratory tests. Height and weight were measured with the participant in light clothing and barefoot. BMI was calculated, and blood pressure was measured in the supine position, using a sphygmomanometer of appropriate cuff size; the first and fifth phases were recorded.

Analytical procedures

Blood samples were collected, after a 12-hour fast, into blood collection tubes with a serum separator. After 15 minutes, tubes were centrifuged at 1500x*g* for 10 min at room temperature. The serum glucose was measured in duplicate by a glucose oxidase/ peroxidase method (Glucose Analyzer 2; Beckman Coulter, Inc., Brea, CA USA). The coefficient of variation was 1.9%. Serum insulin levels were measured in duplicate by monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The lowest limit of detection was 28 pM. The intraassay coefficient of variation was 5.2% at a concentration 70 pM and 3.4% at a 900 pM. The interassay coefficients of variation were 6.9% and 4.5%

138 at 100 pM and 620 pM, respectively. Blood glycosylated hemoglobin (HbA1c) proportions were analyzed

by high pressure liquid chromatography (Merck Diagnostics, Darmstadt, Germany), with coefficients of

140 variation below 4%. The range for HbA1c in glucose-tolerant subjects was 3.8 – 5.4%. Total serum

cholesterol was measured through the reaction of cholesterol esterase/oxidase/ peroxidase, using a

142 BM/Hitachi 747 analyzer (Hitachi, Tokyo, Japan). HDL cholesterol was quantified after precipitation with

polyethylene glycol at room temperature. Total serum triacylglycerols were measured through lipolysis

144 and the analysis of glycerol evolved with glycerol-phosphate-oxidase/ peroxidase. Whole blood

hemoglobin levels (containing EDTA) were determined by a routine laboratory method (Coulter

146 Electronics, Hialeah, FL USA).

Solid-phase enzyme-linked immunosorbent assays (ELISA) based on the sandwich principle

148 were used for the quantitative determination of plasma estradiol (kit EIA-2693) and testosterone (kit EIA-

1559, both from DRG International; Marburg, Germany). Plasma cortisol was determined by microparticle

150 enzyme immunoassay (IMX system, Abbott Laboratories, North Chicago, IL USA), with intra and

interassay coefficients of variation less than 8% [51].

152

Western blot of SHBG

154 Plasma SHBG was estimated by Western blot using monoclonal anti-human SHBG (LS-

C198189; LifeSpan Biosciences, Seattle, WA USA) as primary antibody, and polyclonal against albumin

156 (sc-46293, Santa Cruz Biotechnology, Santa Cruz, CA USA) all of them raised against representative

peptides. Proteins were run by SDS-PAGE (100 $g \cdot L^{-1}$ resolving gel; 40 $g \cdot L^{-1}$ stacking gel). A protein MW

158 marker scale (kaleidoscope, BioRad, Berkeley, CA USA) was included in one of the wells. Samples of 1-2

µL of plasma were used. Proteins were transferred to PVDFImmobilon (Millipore, Billerica, MA USA)

160 membranes, which were visualized with Ponceau red. The membranes were blocked with TBST and 50

$g \cdot L^{-1}$ powdered skimmed milk; then they were incubated at 4 ºC overnight with the primary antibody

162 diluted 1/2000. The membranes were, then, washed four times and then exposed (for 1 h at room

temperature), diluted 1/3000, to the secondary antibody. After four washings, chemiluminiscence was

164 analyzed using ECL (Millipore) for 5 min. Then, densitometric measurements were done using the Total-

Lab program (Nonlinear USA, Durham, NC USA). This Western blot is an adaptation of that previously

166 developed by our group for the measurement of rat plasma CBG [52].

168 *Hormone-binding assays*

The basic procedure for hormone-binding assays was the same described by us previously for

170 CBG [52]. In short: samples of just-thawed plasma (10 µL) were mixed with 1 mL of PBS (phosphate buffer-

saline) pH7.4 containing 1 g/L gelatin. Total binding was estimated using 0.150 mL of diluted supernatant,

172 0.025 mL of PBS-gelatin, and 0.025 mL of ^{3}H-labelled estradiol (NET317: (2,4,6,7-^{3}H)-17β-estradiol,

Perkin-Elmer, Boston MA USA; specific activity 2.8 $GBq \cdot mmol^{-1}$) or testosterone (NET370: (1,2,6,7 ^{3}H)-

174 testosterone, Perkin-Elmer, specific activity 2.6 $GBq \cdot mmol^{-1}$). Hormones' final concentration was 30 nM,

with a specific radioactivity of 12 $kBq \cdot nmol^{-1}$). Non-specific binding was estimated under the same

176 conditions, but now adding 0.025 mL of non-labelled hormone in PBS-gelatin (final concentration 15 µM).

In both cases, the tubes were incubated under gentle shaking for 20 min at 37 ºC followed by 2 h at 4 ºC.

178 Then 0.200 mL of chilled dextran-charcoal PBS-gelatin buffer were added, and the tubes were vortexed

and kept 10 min in ice. Finally, the tubes were centrifuged 10 min at 4 ℃ and 2,000x*g*. Aliquots of the
180 supernatants were used to measure the radioactivity bound to protein through liquid scintillation counting.

182 *Statistical methods*

Statistical comparisons were done using two-way ANOVA analyses (sex, obesity), with the
184 Statgraphics Centurion XVI program package (Statpoint Technologies, Warrength VA USA).
Correlations were determined using the Prism 5 package (Graph-Pad Software Inc, La Jolla, CA USA).
186 Before statistical analysis, normal distribution and homogeneity of the variables were tested. Levels of
statistical significance were set at $P < 0.050$.

188

RESULTS

190 Table 1 shows the basic plasma anthropometric and metabolic values of the six experimental
groups. As expected, increased weight was concomitant with higher triacylglycerol levels in men and
192 women, as well as with increased glycaemia, insulin and HOMA-IR.

Table 2 presents the levels of plasma hormones and ratios between these levels. Estradiol
194 concentrations showed no significant differences between women (in the follicular phase) and men.
Morbid obesity was associated with increased estradiol levels, especially in men. As expected,
196 testosterone showed marked sex-related differences, increasing with the severity of obesity in women
and decreasing in men. No significant differences were observed regarding basal cortisol

198 The ratio of testosterone to estradiol concentrations showed significant differences according to
sex and obesity. Cortisol/ testosterone ratios were significantly different in obese women, but not in obese
200 men. In contrast, the cortisol/ estradiol ratio was significantly different in obese men. The estradiol/ insulin
ratios were similar according to sex and obesity status; however, the testosterone/ insulin ratios were
202 clearly different for sex and BMI, being the effects of obesity a consequence of the 6-fold difference
between morbidly obese men and controls.

204 Plasma SHBG binding capacity for estradiol and testosterone is depicted in Figure 1. Estradiol
binding showed no significant effect of sex, but obesity severity resulted in significant differences, solely
206 attributable to males, since the morbid obese had much higher and significantly different binding values
than those obese or with normal weight. The pattern for testosterone binding was quite different, with
208 significant effects of both sex and obesity, the latter attributable, again to the group of men, with morbidly
obese subjects showing the lowest testosterone binding values. These differences were magnified in the
210 estradiol *vs*. testosterone binding ratio. Both sex and obesity showed significant differences, with rather
uniform ratios for all groups except for the morbid obese men, with ratios several-fold higher than in all
212 other groups.

Figure 2 shows the levels of testosterone and estradiol as a proportion of SHBG binding capacity.
214 The only significant differences observed were for sex in the case of testosterone, since in men the levels
accounted for 60-80% of maximal binding capacity of SHBG against 5-20% for women. These data
216 contrast with the rather uniform 1-3 % values for estradiol levels *vs*. binding.

Figure 3 presents the amount of SHBG protein in plasma, determined through Western blot,
218 Normal-weight women had almost 2.5-fold higher levels of circulating SHBG than the corresponding
group of men; these differences were observed again in the morbid obese groups, but with all values

220   practically halved. These differences were statistically significant according to sex (lower values in men) and obesity status (decreasing values with increasing obesity).

222   When estradiol or testosterone binding were related to the amount of SHBG protein (Figure 4), the resulting binding / Western blot ratios showed the actual capacity to bind the hormones with respect

224   to the amount of protein present in plasma (i.e. the total binding capacity being a correlate of the amount of functional protein present). This value is ancillary to the number (or affinity) of the binding sites per

226   molecule of protein. Estradiol binding was markedly different among morbidly obese men. Thus, the ability to bind was a uniform correlate of the amount of SHBG available, but in morbid obese men the

228   ability to bind estradiol was much higher than that corresponding to the protein detected. In the case of testosterone, however, no significant differences for sex or obesity were found, with closely similar ratios

230   of binding/ SHBG protein ratios for all groups.

232   **DISCUSSION**

      SHBG binds both testosterone and estradiol, but not with the same affinity [1, 53] and may be deeply

234   modulated by sex [34, 35], insulin resistance [38, 39], proinflammatory cytokines [30] and other metabolic conditions or pathologies [54, 55]. The physiologically functional (circulating) protein appears as a dimer [19],

236   but the conditions used for Western blot do separate the individual monomers. The main consequence is the loss, under laboratory conditions, of possible fine regulation factors such as allosteric modulation [56]

238   and the discrimination of dimers and heterodimers [29]. However, the conditions used to determine the binding value (i.e. specific binding calculated after discounting unspecific binding) facilitate the direct

240   comparison of the functionality of the SHBG binding site (there is only one per glycoprotein monomer). That is, under the testing conditions, the affinity and capacity to bind a given hormone (testosterone or

242   estradiol) should remain unchanged. However, the binding / Western blot ratios, experimentally obtained, clearly were not. This result proves that in one of the groups of individuals (e.g. all the morbidly obese

244   men), the SHBG molecule was structurally different.

      The existence of varying molecular weight SHBG proteins [28], and the influence of the size of the

246   monomers on binding has been already described [53]. The changes in the SHBG molecule have been related to trimming of carbohydrate chains [23] and / or proteolytic shortening, in part attributed to regulatory

248   serpin proteases such as kallikrein [26]. But changes were observed also in subjects with MS [31, 32, 57, 58], hepatic cirrhosis [54] or just population variability [31, 32]. However, the marked change in SHBG ability to bind

250   estradiol in the group of morbidly obese men here described is outstanding.

      The binding/ Western ratio for testosterone, was similar in all groups, as expected, including the

252   morbidly obese men who also had the lowest levels of immunoreactive SHBG. The hormones' binding ratios did not show the expected uniform pattern, strongly deviated in morbid obesity instead; however

254   this deep change affected only men. Last, but not least, the relationship between the binding capacity of SHBG and the circulating hormone levels showed large differences for testosterone and estradiol (Figure

256   2), in addition to the marked difference due to sex, largely a correlate of the higher circulating testosterone levels of men. In any case, the percentage of hormone levels *vs.* binding in control women

258   was in the range of 1.5 % whereas that of testosterone was fivefold higher. These values were not within the expected range of work for SHBG to exert its transporting function under normal conditions; i.e. when

260   the maximal proportion of binding sites bound to estradiol represent only a small fraction of the maximal

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binding capacity of the protein, leaving no room for regulation. Consequently, the data on estradiol-related transporting capacity of SHBG did not match the currently accepted model of dual transport of androgen and estrogen for SHBG.

The results obtained do not support the assumption that SHBG is a protein indistinctly binding testosterone and estradiol as part of its physiological function. The data presented for males, even if only ascribed to the controls and non-extreme obesity (i.e. excluding the morbidly obese men), could not justify the transporting and regulatory function of SHBG for estradiol because of the sustained low level of binding site occupancy. This non-complying situation is magnified when compared with the SHBG-testosterone relationships, since all and every one of the anomalies described for estradiol were fulfilled as expected. The phylogenetic closeness of SHBG to the testicular testosterone-binding protein [10], and the data presented here suggest that SHBG acts, essentially, as a testosterone-binding globulin. The fact that SHBG also binds estradiol must be put in perspective [59], especially given the marked differences in low circulating levels of estradiol compared with testosterone. Competence for a single binding site per monomer could not justify a functional role of the protein for estradiol, at least in males: Their much higher levels of testosterone than women compounded by the higher affinity of SHBG for the androgen, may result in negligible estrogen binding as regulatory mechanism *in vivo* (at least in males and non-ovulating women). The critical role of estradiol for bone [60], energy homeostasis [61] and reproductive system in both females and males [62] has been clearly established, requiring a finer regulation of estrogen in males, playing functions not fully coincident with those of females. The kinetics of binding of testosterone and estradiol did not match their expected function (which nevertheless is exerted successfully), especially when we consider that SHBG is also a carrier for hormones that bind to specific membrane sites to transfer their charge [63]. In addition, most studies on SHBG function were centered on testosterone [64, 65]. As a comparison, CBG is devoted to the control of cortisol (or corticosterone in rodents), but also binds progesterone and a number of other ligands [66]; nnevertheless, no role of CBG in transport or control of progesterone has been definitively proven. There is thus a clear parallelism between both serpins.

The apparent increase of estradiol-binding "capacity" in morbidly obese men should be explained in terms of increased SHBG protein levels, but they were not. Furthermore, testosterone levels were unaffected, and the ratios for testosterone binding / Western blot were uniform for all men (and women) groups, a situation completely different to that of estradiol. A possible explanation of this inconsistency must lie on the existence of more than one type of SHBG protein, with at least one specifically binding estradiol, and the other to testosterone. Thus justifying the data observed in this study, despite testosterone alone responding as expected. This interpretation is coincident with previous reports that found different molecular species of SHBG binding either androgen or estradiol, but not both (in cirrhotic patients) [54], as well as the importance and common occurrence of SHBG alleles [22].

The extreme change in estradiol binding of morbidly obese men in comparison with the other groups could not be explained by the measured (Western) SHBG protein. But testosterone complies under the same conditions. Since estradiol levels were much lower, a plausible Estradiol-Binding Globulin should be present in the morbidly obese men plasma in lower levels than the "current" or "testosterone-prone" SHBG. This situation mimics that described for cirrhosis [54]. The "estradiol-prone" SHBG, structurally close to "testosterone-prone" SHBG should be formed by differential post-translational selective cleavage [26] of the SHBG transcript [22], thus preserving the similarity and similar (albeit probably not identical) immunoreactive properties [24] of both SHBG molecular species. The protein measured in this

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study had immunoreactive properties obviously dependent of the affinity and specificity of the antibody
used, decanted towards one or other of the isoforms postulated, a situation parallel to that described by
our group in rat CBG [52]. Thus, in fact, we were not measuring SHBG (under the one molecule-for-all-
hormones concept) but either a composite (with unknown proportions of both isoforms) or essentially one
of these isoforms, that closer to bind / regulate testosterone. Anyway, a change in proportions of both
forms of SHBG or changes in molar specific immunoreactivity may uncover the existence of more than
one molecular species of circulating SHBG [54]. The speculative ascription of different molecular species to
the roles of transport and binding of estradiol and testosterone may help in understanding the complex
conundrum of SHBG in relation with the control of sex hormone transport, function and their relationships
under conditions of pathological alterations of energy homoeostasis (30). Sex and age intensify the
negative effects of MS, as observed in Koreans [67]. Insulin is known to modulate the levels of testosterone
through SHBG [30, 68, 69], but what about estradiol? Binding of estradiol or testosterone to SHBG monomers
(and probably dimers) changes their shape [53], thus affecting the conditions of binding to the cell
membrane SHBG receptor sites.

An additional clue to the importance of the antagonistic gender-related function of cortisol and
testosterone [37] can be directly derived from the analysis of their concentration ratios, markedly decreased
with obesity in women and less affected in men. These differences are difficult to explain with the
assumption of a unique SHBG regulating both testosterone and estradiol, since the cortisol / estradiol
concentration ratios were affected by obesity but not by sex. A similar picture can be drawn from the
ratios of both sex hormones versus insulin: no effects were observed for estradiol, but men (albeit not
women) showed marked effects of obesity, depending on its severity. These data hint at a much more
significant (and gender-related) implication of testosterone in obesity [70]. This is in agreement with the sex-
related incidence of the development of MS with age [71], but also with the known relationship of SHBG
(probably the isoform controlling testosterone availability) and the incidence of MS [58, 72] and the
development of gender-related [73] increased cardiovascular risk. These effects may be largely
independent of the protective anti-inflammatory effect of estrogen [74]. The lack of clear discrimination of
SHBGs and their separate control of estrogen and androgen, has obscured the implication of
testosterone (or the lack of it) in contrast with the surge of glucocorticoids in the development of
cardiovascular risk in women and old men [75] in parallel to increased frailty [76]. Since the fall in testosterone
levels, at least in men, tend to be related to age [69], and this occurs in parallel to a fall in SHBG levels [65],
we can assume that the results obtained in the present study should be extended to a larger population,
with a wide age range. The important amount of data available on the dynamics of testosterone action
and protein deposition [77] _ENREF_37 in conjunction with the main anabolic hormone, insulin, countered
by corticosteroids [37] show that testosterone is a critical factor in the aging process [78]. The discriminating
role of the postulated dual SHBGs may help understand better the gender-related process of aging by
establishing a clear mechanism of regulation of the plasma kinetics and transport of testosterone, but will
also allow a deeper study of estrogens without interference.

In conclusion, our data point to SHBG as a globulin mainly centered in the control of testosterone
levels, with estradiol regulation taking a markedly second place; or, simply, estradiol being controlled by a
different SHBG variant. These data confirm the existence of different SHBG molecular species to
discriminately bind estradiol and testosterone, previously reported in cirrhotic subjects [54]. The sex-related
differences in the relationships of binding versus levels of hormones and SHBG could not be explained by

346   assuming that a single dimeric protein may experience the dramatic changes in affinity for testosterone and estradiol (differently for both, in fact) observed in women and men, and the marked differential effects of obesity on the ability of the same protein to bind testosterone and estradiol (Box 1).

348   Thus, we postulate that SHBG protein must be present in at least two different molecular species, or isoforms, to explain its differential function for both hormones, specially affected by sex and obesity.

350   These conclusions suggest a much needed establishment of analytical procedures to identify these physiologically distinct forms of SHBG, since the sole use of antibody-based measurements could not

352   discriminate between them.

354   **DECLARATION OF INTERESTS**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the

356   research reported.

AUTHOR CONTRIBUTIONS

366   JMFR, JMMN and FJO selected the volunteers, obtained the samples and established the basal metabolic conditions of the subjects, including insulin. RA, NC, ME, JAFL, MMG, JG and LM did the

368   SHBG-related laboratory work. ME, JAFL, MMG, and XR supervised the laboratory work, organized the results and did the final statistical analyses. MA conceived the study and wrote the paper. All senior

370   Authors participated in the discussion of the results and in shaping the final text. At the time this investigation took place, RA, NC and LM were undergraduate students.

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598

600   **FIGURE LEGENDS**

602   Figure 1   Estradiol and testosterone binding, as well as their ratio, for women and men of normal
                         weight, obese and morbidly obese

604   The data are the mean ± sem for each group. White: women, grey: men; WC = women of normal weight;
       WO = women obese; WMO = women morbidly obese; MC = men of normal weight; MO = men obese;

606   MMO = men morbidly obese. Statistical analysis of the differences between groups: Two-way ANOVA,
       (data within the Figure) was carried out for the parameters "sex" (S) and "obesity" (O). NS represents P

608   values higher than 0.050. One-way ANOVA for obesity in each sex group (data on top of the Figure) O-W
       factor "obesity" in women; O-M factor "obesity" in men. Post-hoc Duncan test for one-way ANOVAs

610   (obesity): different letters over the columns indicate a significant (P<0.05) difference between them; the
       absence of letters reflects the absence of significant differences between the groups.

612

       Figure 2   Relationship between the levels of estradiol or testosterone in plasma with respect to the
614   binding ability of this same plasma, expressed as percentages.

       Figure conventions are the same as in Figure 1.

616

       Figure 3   SHBG Western blot of women and men of normal weight, obese and morbidly obese

618   Figure conventions are the same as in Figure 1; a.u. = arbitrary units (the MC mean value was attributed
       the arbitrary value of 100).

620

       Figure 4   Ratios of estradiol and testosterone binding versus the SHBG Western blot data, for
622   women and men of normal weight, obese and morbidly obese

       Figure conventions are the same as in Figure 1.

624

       Box 1   The SHBG dual hormone-binding conundrum

626   Summary of the available reasons and experimental data proving the unsustainability of the SHBG dual
       role controlling / transporting estradiol and testosterone

628   T = testosterone, E2 = estradiol

Table 1   Basic characteristics of the human subject groups used in the present study

parameters	units	women			men			P		
		normal weight	obese	morbid obese	normal weight	obese	morbid obese	sex	obesity	interaction
number of individuals		14	10	8	13	9	8	--	--	--
age	years	36 ± 2	32 ± 2	32 ± 1	35 ± 2	41 ± 3	36 ± 2	NS	NS	NS
weight	kg	61 ± 1	90 ± 3	121 ± 5	69 ± 2	101 ± 4	141 ± 10	0.0003	<0.0001	NS
height	m	1.66 ± 0.01	1.64 ± 0.02	1.64 ± 0.02	1.74 ± 0.02	1.75 ± 0.03	1.76 ± 0.03	<0.0001	NS	NS
BMI	kg/m^2	22.2 ± 0.4	33.4 ± 1.0	45.0 ± 1.3	22.7 ± 0.3	33.2 ± 0.8	45.4 ± 2.2	NS	<0.0001	NS
fasting plasma glucose	mM	4.73 ± 0.13	4.76 ± 0.12	4.97 ± 0.15	5.32 ± 0.15	5.52 ± 0.16	6.13 ± 0.21	<0.0001	0.0040	NS
HbA$_{1C}$	%	4.6 ± 0.1	4.9 ± 0.1	4.8 ± 0.2	4.8 ± 0.1	4.7 ± 0.1	5.4 ± 0.2	NS	0.0030	0.0270
HOMA-IR		1.25 ± 0.18	2.43 ± 0.36	2.44 ± 0.33	1.40 ± 0.23	3.19 ± 0.64	3.96 ± 0.42	0.0094	<0.0001	NS
total plasma cholesterol	mM	4.53 ± 0.13	5.00 ± 0.29	5.23 ± 0.27	5.23 ± 0.21	5.72 ± 0.19	5.39 ± 0.41	0.0128	NS	NS
HDL-cholesterol	mM	1.51 ± 0.11	1.30 ± 0.08	1.31 ± 0.11	1.32 ± 0.11	1.27 ± 0.11	1.04 ± 0.08	NS	NS	NS
LDL-cholesterol	mM	2.69 0.17	3.09 ± 0.24	3.18 ± 0.29	3.45 ± 0.21	3.74 ± 0.17	3.39 ± 0.42	0.0107	NS	NS
plasma triacylglycerols	mM	0.779 ± 0.054	1.37 ± 0.30	1.66 ± 0.19	1.13 ± 0.18	1.67 ± 0.33	2.14 ± 0.26	0.0422	0.0002	NS

The values are presented as mean ± sem for each group. Statistical analysis of the differences between groups (2-way ANOVA) was carried out for the parameters "sex", "obesity" and their interaction. NS represents P values higher than 0.050.

1

Table 2   Hormone levels in serum of women and men with normal weight, obese or morbidly obese.

plasma hormones and hormone ratios	units	sex	normal weight	obese	morbid obese	1-way anova P-obesity	P-sex	2-way anova P-obesity	P-interaction
estradiol	pM	women	162 ± 29	173 ± 25	256 ± 48	NS	NS	0.0043	NS
		men	134 ± 16 ^{A}	124 ± 13 ^{A}	215 ± 22 ^{B}	0.0044			
testosterone	nM	women	1.19 ± 0.18 ^{A}	1.59 ± 0.23 ^{AB}	2.31 ± 0.36 ^{B}	0.0125	<0.0001	0.0040	0.0002
		men	19.6 ± 1.4 ^{A}	17.4 ± 1.8 ^{A}	11.1 ± 1.1 ^{B}	0.0020			
cortisol	nM	women	407 ± 38	374 ± 64	277 ± 64	NS	NS	NS	NS
		men	354 ± 27	382 ± 34	333 ± 74	NS			
insulin	pM	women	42.2 ± 5.4 ^{A}	82.5 ± 12.1 ^{B}	77.4 ± 10.9 ^{B}	0.0034	NS	<0.0001	NS
		men	41.4 ± 6.2 ^{A}	93.1 ± 17.7 ^{B}	103 ± 10 ^{B}	0.0004			
testosterone/ estradiol ratio		women	6.73 ± 1.10 ^{A}	8.19 ± 0.80 ^{AB}	10.3 ± 1.4 ^{B}	NS	<0.0001	0.0078	0.0046
		men	176 ± 27 ^{A}	155 ± 19 ^{A}	57.9 ± 11.5 ^{B}	0.0084			
cortisol/ testosterone ratio		women	441 ± 65 ^{A}	277 ± 59 ^{AB}	136 ± 37 ^{B}	0.0074	<0.0001	0.0063	0.0045
		men	19.1 ± 2.1	23.0 ± 2.3	24.2 ± 5.4	NS			
cortisol/ estradiol ratio	x10^{-3}	women	3.23 ± 0.67	2.27 ± 0.53	1.49 ± 0.61	NS	NS	0.0087	NS
		men	3.16 ± 0.47 ^{A}	3.25 ± 0.40 ^{A}	1.47 ± 0.33 ^{B}	0.0280			
estradiol/ insulin ratio		women	3.66 ± 0.68	4.43 ± 1.50	3.82 ± 0.88	NS	NS	NS	NS
		men	4.49 ± 0.98 ^{A}	1.54 ± 0.20 ^{B}	2.26 ± 0.39 ^{AB}	NS			
testosterone/ insulin ratio		women	31.5 ± 7.2	33.6 ± 12.6	33.6 ± 6.2	NS	<0.0001	0.0006	0.0005
		men	681 ± 142 ^{A}	232 ± 32 ^{B}	112 ± 13 ^{B}	0.0020			

The values are presented as mean ± sem for each group. Statistical analysis of the differences between groups (1- and 2-way ANOVA) was carried out for the parameters "sex", "obesity" and their interaction. NS represents P values higher than 0.050. Post-hoc Duncan test for 1-way anovas: different superscript letters represent significant differences between the corresponding groups
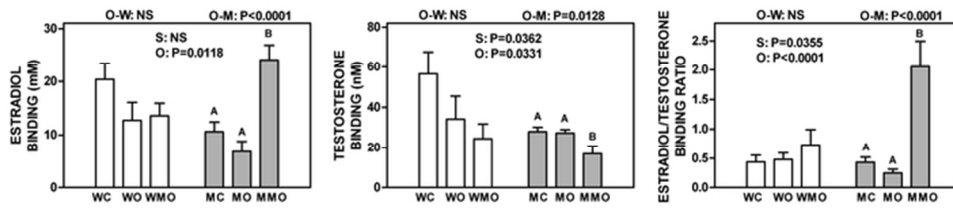
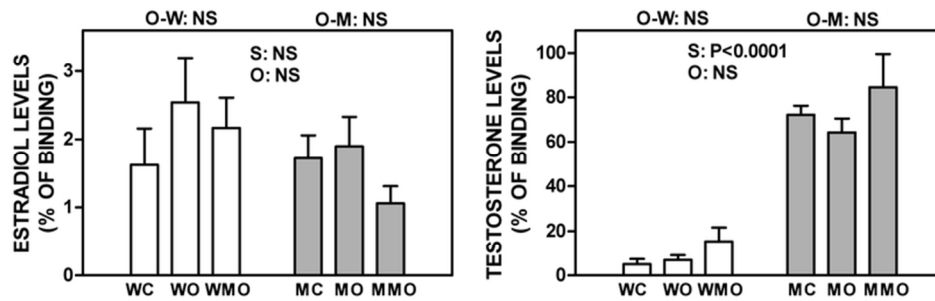Figure 1
Figure 1
62x15mm (300 x 300 DPI)

Figure 2 Relationship between the levels of estradiol or testosterone in plasma with respect to the binding ability of this same plasma, expressed as percentages.
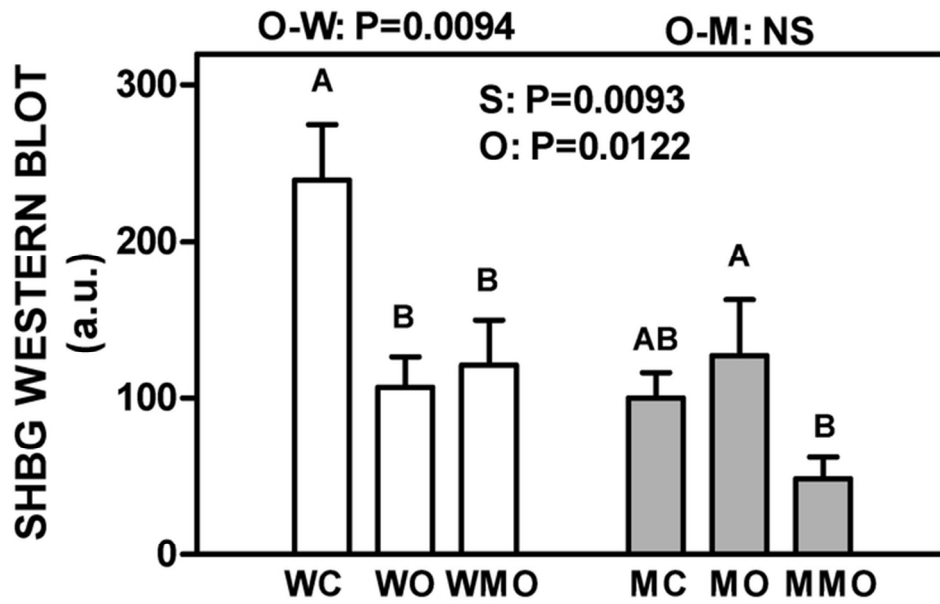Figure 2
72x26mm (300 x 300 DPI)

Figure 3 SHBG Western blot of women and men of normal weight, obese and morbidly obese
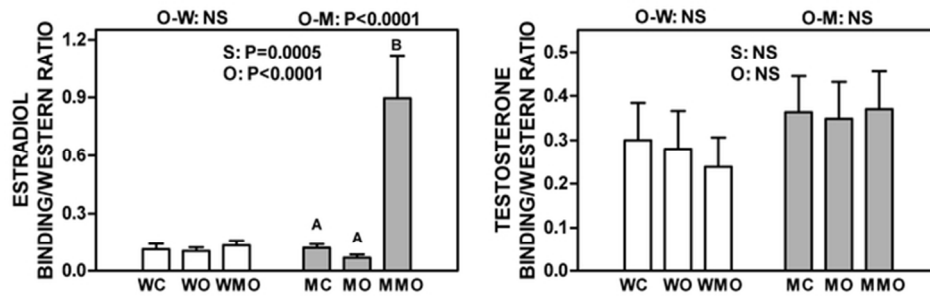Figure 3
66x44mm (300 x 300 DPI)

Figure 4 Ratios of estradiol and testosterone binding versus the SHBG Western blot data, for women and men of normal weight, obese and morbidly obese

Figure 4

62x22mm (300 x 300 DPI)

The SHBG dual hormone binding conundrum

Current accepted knowledge:

 A unique SHBG binds, transports and regulates the levels and availability of T and E2

Already known data that so not support the "single SHBG" current theory

- M and W show different (and varying) levels of T and E2 with similar SHBG protein concentrations
- Widely different T and E2 levels in plasma apparently do not compete or interfere on SHBG function despite using a single binding site for which they compete as ligands
- Different SHBG forms have been found in humans with structural and binding affinity differences for T and E2)
- Many animals do not have SHBG, but most possess non-circulating T-binding proteins; i.e. T may be regulated independently of E2. In any case T and E2 levels are regulated in plasma despite the eventual absence of SHBG

Additional proof, presented here, against the "single SHBG" current theory

- Circulating E2 (lower than T) at most may cover 3% of the potential binding sites of concurrent plasma SHBG, which is not compatible with T occupying 65-90 % of the sites. These data anr not compatible with a regulation role of SHBG for E2
- Obesity affects T (but not E2) binding. This is incompatible with both hormones binding (competing) for the same SHBG binding site if the protein remains unchanged
- The ratio of hormone binding vs. protein level (Western blot) should be constant unless the protein affinity is changed (by changing the protein itself). The ratio for T is unaltered by sex and obesity, but that for E2 is affected by both

The conclusions are far reaching:

- Cannot be explained by the single type of SHBG, which brings us to assume that, at least there are two SHBGs, one regulating T and the other for E2
- Most of the methods used for the measurement of SHBG rely on their immunoreactivity, but they may detect/ measure a T- or E2-prone SHBG molecular species of a mixture of both in different proportions
- Consequently, there are no reliable methods for SHBG measurement available, in the sense that SHBG levels could not be related to a specific protein carrying out a specific function. This, largely invalidates many studies on SHBG function
- Specific discriminative methods for the SHBGs may help clarify the intuited role of estrogens and androgens on metabolic syndrome-related pathologic states.