

Epidemiological Assessment of Testosterone Levels in Women Population: A Factorial Analysis of Cell Proliferation

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Abstract

In women, testosterone is produced in the ovaries, adrenal glands, and fat cells. Women may produce much or little of testosterone. Excess Testosterones and deficiencies are among the common hormonal disorders in women. Testosterone in women plays a role in the hormonal cascade that kick-starts puberty and stimulates hair growth. In adult women, testosterone is necessary for estrogen synthesis and have played a key role in the prevention of bone loss, sexual desire, and satisfaction. Testosterone can affect the breast and it is well implicated in testosterone therapy for women. The need to bring about shreds of evidence linking specific human health effect due to testosterone imbalance in women for the assessment of reproduction insufficiency was the purpose of this investigation. Results from clinical observations and epidemiological studies implicate testosterone imbalance as a significant treatment of public health. A total of 186 women of Eket Community in Akwa Ibom State, Nigeria, were purposively and disproportionately selected, using simplified sample size formula. Blood serums were prepared using the blood obtained from donors. The *in vitro* assay of the testosterone and sex hormone binding globulin was achieved using enzyme-linked immunosorbent assay technique. The ratio of sex hormone binding globulin to testosterone was calculated and free testosterone was obtained. The *in vitro* hormonal quantification showed that 38 (20%) of women had testosterone level above the reference range - 4.42-32.02 ng/ml/mmol/L, while 148 (80%) had low testosterone level. This showed that a fraction of women in Eket community with high levels of a form of testosterone called "free" testosterone may have polycystic ovary syndrome (PCOS), characterized by irregular or absent of menstrual periods, infertility, blood sugar disorders (pre-diabetes and type 2 diabetes), and, in some cases, symptoms such as acne and excess hair growth may set in.

Keywords: Androgen; Women; Testosterone; Free testosterone; Sex hormone binding globulin epidemiology

Introduction

The prevalence of reproductive abnormality in women is due to elevated testosterone, an antagonist of estrogen dominance and sometimes elevated steroid sex hormone binding globulin (SHBG), which makes it possible for estrogen to dominate in the cells to exert epithelia cells proliferation. The balance between stimulatory effects of the estrogens and inhibitory effects of the testosterone is the critical factor that regulates mammary cell proliferation both in normal and in cancer tissues [1]. Several epidemiological studies have examined the correlation between circulating testosterone and the risk for breast cancer. Testosterones play a role in normal breast physiology. Androgen receptor (AR) signaling is recognized as an important therapy of breast carcinogenesis. The frequency of AR expression in breast cancer makes it an attractive therapeutic target, while deregulation of this hormone poses a health risk. A major limitation was that the androgen assays used were developed primarily to measure the higher levels found in men and lack reliability in the low ranges found in normal women [2]. Testosterone levels revealed considerable daily variability, though some epidemiological data are based on a single blood sample assay that is collected at non-standard times. Another concern is using serum testosterone levels to determine androgenic effects at the tissue level where circulating testosterone is firmly bound to sex hormone binding globulin (SHBG), while the free hormone is bioactive [3]. SHBG and total testosterone levels differ

greatly based on genetic or epigenetic grounds, metabolic and endocrine influences [3]. It has been accepted that measuring free or bio-available testosterone predicts androgenic effects more accurately than total testosterone levels [2]. It is purveyed that most androgenic activity in women originates from the peripheral conversion of precursors such as estrogens into androgens within the cells of target tissues, and this activity cannot be detected by measurement of circulating androgens only [4]. In a recent study about the levels of testosterone in saliva, they were significantly fewer patients with breast cancer compared to controls and these differences were profound in postmenopausal women [5]. Though breast cancer patients, when compared to controls, revealed androgen insufficiency, while a relative imbalance of sex steroid hormones favors estrogens.

Several studies have been carried out in this area, but none has disconnected the risk associated with increased estradiol levels from the testosterone, and since testosterone are the obligate precursors for estradiol synthesis, a major confounding factor in assessing the role of testosterone independently of known cancer-promoting estrogens effect is important. In line with these observations, a recent study by Schmitt et al. [6] concluded that increased breast cancer risk with increasing body mass index among postmenopausal women is due to increase in estrogens. The association of testosterone with breast cancer risk did not persist after adjustment of estrone [7]. This probably gave further proof that estrogens are strongly associated with the risk of cancer and infertility. Some authors conclude that conversion of testosterone to estrogens by aromatase particularly estradiol is required to exert a mitogenic response [8]. These results suggest that the contribution of testosterone to breast cancer risk is

largely through their role as substrates for estrogen production. Other studies have found no association between testosterone and breast cancer [9]. Observations expressed the difficulty in separating direct effects of circulating testosterone from its potential to be aromatized into estradiol. Testosterones are indeed protective against estrogen-induced mammary proliferation at certain levels. Additionally, testosterone treatment significantly reduced mammary epithelial estrogen receptor expression, thus suggesting a potential mechanism for the growth inhibitory effect [10]. Some researchers have found that treatment of intact cycling monkeys with the AR antagonist (flutamide) resulted in a significant increase in mammary epithelial proliferation [11], this added to the evidence that endogenous testosterone can limit mammary proliferation and cancer risk. One study on primates also suggests that inclusion of testosterone with estrogen/progesterone may counteract breast cell proliferation [12].

This research was set out to establish that free testosterone level in women is a potent causation of mammary cell proliferation since testosterone is aromatized to estrogens.

Materials and Methods

Ethical approval

Ethical approval was obtained from the Ministry of Health, Akwa Ibom State, Nigeria. A letter of approval was granted 18 September 2015 and issued 28 September 2015.

Collection of blood serum

Blood samples from individuals of Eket community were based on 186 samples, using a simplified sample size formula, (equation 1 in Online sample Size Calculator by Raosoft, Inc.) [12]. The confidence level was 95%, the margin of error or confidence interval was 5%, total population size was 172, 856 and percentage population coverage 50%.

Collection of blood samples

A convenient vein was located on the left of the volunteer's hand; a tourniquet was tied to the hand above the elbow. The point of venipuncture was swooped with a wet cotton wool impregnated with methylated spirit. The needle was assembled with the syringes after wearing hand gloves. The vein was penetrated to draw 2 ml of blood, and a dry cotton wool was applied at the open point to inhibit bleeding. The collected blood was emptied into plain sample bottles and allows for about 1-hour clotting. The used needle was discarded into a disposable box. After blood clotting, it was centrifuged at 2000 g rpm for 10 minutes. The serum was collected as supernatant using disposable Pasteur pipette and refrigerated at -20°C before taking with an ice pack for hormonal assay.

Preparation of reagents

All reagents were allowed to reach room temperature (18-25°C) before use. Reagents were mixed by gentle inversion or swirling prior to use and foaming was avoided. Samples with expected testosterone concentrations over 18 ng/ml were quantitated by dilution with the diluent provided by the Diagnostic Automation Inc.

Test principle for sex hormone binding globulin and testosterone

The enzyme-linked immunosorbent assay (ELISA) is based on the sandwich principle. The microtiter wells are coated with a monoclonal [mouse] antibody directed towards a unique antigenic site of the SHBG and TT (testosterone) molecule. An aliquot of a sample containing endogenous SHBG or TT is incubated in the coated well. After a washing step, enzyme conjugate is added, which is a monoclonal anti-SHBG or TT antibody conjugated with horseradish peroxidase. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase is proportional to the concentration of SHBG or TT in the sample.

Chemicals

All chemicals used for this investigation are reagents of grade or higher, purchased from Reporter Assay Systems (AccuDiag™ ELISA) Diagnostic Automation/Cortez diagnostics Inc. 23961 Craftsman Road, Suite D/E/F Calabasas, CA 91302, USA. Venipuncture was carried out by licensed Phlebotomist.

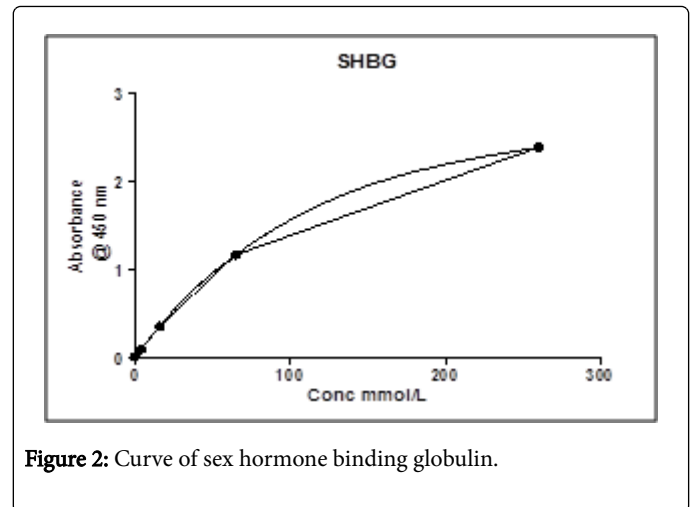
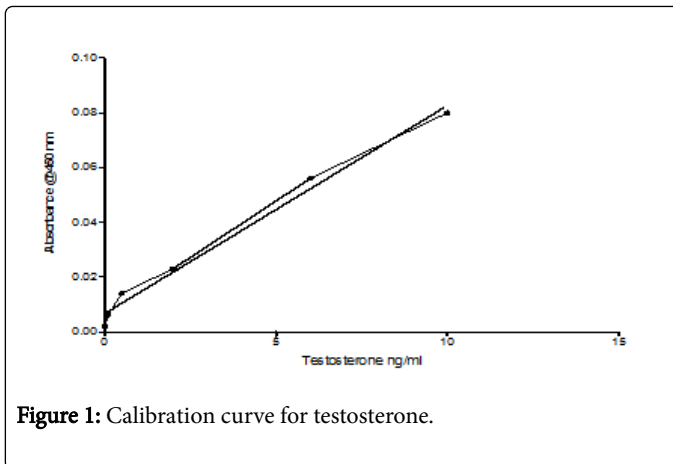
Statistical analysis

Investigations on testosterone and sex hormone binding globulin levels were carried out using enzyme-linked immunosorbent assay and concentrations of testosterone and SHBG was interpolated by Spline and Lowess method using the Spline Curve via graph prism version 6.5.

Results

Concentrations of testosterone were interpolated using linear regression. Graph-Pad prism version 6.02 was used. The absorbance was read with respect to their optical density at 450 nm in a microplate reader. The plot depicts a linear correlation of the absorbance. The mean absorbance value (optical density 450 nm) for each set of reference standards, controls and samples were calculated. The standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on linear-linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis. By using the mean absorbance value for each sample, the corresponding concentration of testosterone ng/ml from the standard curve was determined. Values obtained for diluted samples were further converted by applying the appropriate dilution factor in the calculations sees the standard curve in Figures 1 and 2.

The average absorbance values for each of standards, controls, and samples were calculated. By using semi-logarithmic graph paper, a standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. Using the mean absorbance value for each sample, the corresponding concentration from the standard curve was determined. The 1-hour of the samples was read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as >260 nmol/l. For the calculation of the concentrations, this dilution factor was taken into account.



Lab No.	Age	T. concentration ng/mL	SHBG mmol/L	Free. T	Lab. No.	Age	T. concentration ng/mL	SHBG Mmol/L	Free.T. mmol// ng/ml
1	36	2.82	30.2	10.71*	41	28	8.99	59.4	6.6
2	38	2.29	110.5	48.25**	42	37	2.6	11	4.23
3	28	1.63	43	26.38**	43	45	1.91	16.3	8.53
4	33	2.16	12.1	5.6	44	60	12.55	134	10.67**
5	30	2.95	4	1.36	45	45	9.85	78.2	7.93
6	28	1.76	34.9	19.83**	46	66	13.9	110	7.91
7	26	2.68	38.1	14.22**	47	29	7.99	45.9	5.74
8	30	2.03	29.5	14.53**	48	56	15.67	153.4	9.79
9	33	1.9	25.6	13.47**	49	74	9.42	97.8	10.38**
10	29	2.55	11.3	4.43	50	45	5.82	30.5	5.24
11	44	2.42	30.6	12.64	51	39	12.56	78.9	6.28
12	28	2.16	153	70.83**	52	38	13.85	107.2	7.74
13	31	1.9	11	5.79	53	39	22	88.5	4.02
14	34	1.76	23.5	13.35**	54	27	7.17	30.4	4.23
15	40	2.16	15.6	7.22	55	44	11.9	98.9	8.31
16	30	22.34	162.1	7.25	56	49	9.66	69.9	7.23
17	34	2.16	34.7	16.06**	57	39	1.9	23.9	12.57**
18	37	7.72	67.9	8.79	58	35	1.55	9.3	6
19	30	3.1	15.9	5.12	59	60	4.18	15.5	3.71
20	36	10.9	79.8	7.32	60	26	12.67	133	10.49**
21	45	1.8	11.9	6.61	61	39	13.34	80	5.99
22	44	5.11	12.5	2.44	62	26	8.1	98.9	12.20**
23	33	4.97	38.9	7.82	63	27	9.11	67.3	7.39

24	34	2.7	16.6	6.14	64	39	12.77	110	8.61
25	32	1.9	6.9	3.63	65	56	5.81	23	3.95
26	23	10	37.6	3.76	66	58	1.33	9.9	7.44
27	34	9.55	40.5	4.24	67	35	6.88	25.5	3.7
28	23	2.36	15.5	6.56	68	47	2.15	13	6.04
29	26	1.72	12.2	7.09	69	46	10.89	67.8	6.22
30	29	3.89	37.9	9.74**	70	38	10.98	76.8	6.99
31	30	4.43	18.4	4.15	71	37	8.23	56.2	6.82
32	40	9.11	97.6	10.71**	72	31	4.35	12.9	2.96
33	44	2.42	13.7	5.66	73	37	2.38	18.9	7.94
34	58	1.23	9.9	8.04	74	41	9.9	45.9	4.63
35	36	2.89	17.1	5.91	75	38	10.78	156	14.47**
36	46	7.19	35.9	4.99	76	50	12.45	165	13.25**
37	37	12.8	76.5	5.97	77	38	9.67	67	6.92
38	33	2.16	23.5	10.87**	78	59	10.45	98	9.38**
39	23	1.24	8	6.45	79	53	13.32	56.9	4.27
40	22	10.23	37.3	3.64	80	56	10.89	78.9	7.24

Table 1A: Testosterone and SHBG concentration in 186 women.

Lab. No	Age	T. concentration ng/mL	SHBG mmol/L	Free T.	Lab. No	Age	T. concentration ng/mL	SHBG Mmol/L	Free T. mmol//ng/ml
81	45	5.43	38.4	7.07	121	44	1.92	18	9.37**
82	44	2.44	18	7.37	122	39	2.89	12.5	4.32
83	39	1.67	12.5	7.48	123	38	2.68	19.8	7.38
84	38	1.55	19.8	12.77**	124	28	4.64	27.8	5.99
85	28	6.78	27.8	4.1	125	49	8.98	79.9	8.89
86	49	10.89	79.9	7.33	126	67	2.76	19.2	6.95
87	67	2.98	19.2	6.44	127	45	1.2	9.2	7.66
88	45	1.78	9.2	5.16	128	44	12	73.8	6.15
89	44	9.98	73.8	7.39	129	51	7.86	46.9	5.96
90	51	5.55	46.9	8.45	130	34	10.02	68.6	6.84
91	34	12.61	68.6	5.44	131	35	1.4	8.5	6.07
92	35	2.81	8.5	3.02	132	55	3.8	59.9	15.76**
93	55	7.9	59.9	7.58	133	63	7.8	38.9	4.39
94	63	5.76	38.9	6.75	134	39	4.5	17	3.77
95	39	1.7	17	10**	135	39	4.9	17	3.46
96	63	3.87	16.9	4.36	136	63	4.7	16.9	3.59

97	57	14.34	155.9	10.87**	137	57	8.34	155.9	18.69**
98	27	10.39	167.1	16.08**	138	27	13.64	167.1	12.25**
99	38	9.91	37.4	3.77	139	38	9.89	37.4	3.74
100	44	9.14	79.9	10.71	140	44	5.5	79.9	14.52**
101	50	9.99	89.9	8.99	141	50	8.92	89.9	10.07
102	28	8.5	66.8	7.85	142	28	9.22	66.8	7.24
103	46	5.89	24.8	4.21	143	46	4.6	24.8	5.39
104	56	10.2	50.9	4.99	144	56	8.99	50.9	5.66
105	44	2.33	13.8	5.92	145	44	3.1	13.8	4.45
106	55	2.6	17.9	6.88	146	55	2.67	17.9	6.7
107	27	19.8	99.2	5.01	147	27	14.4	99.2	6.88
108	26	2.12	33.9	15.99	148	26	4.67	33.9	7.25
109	40	4.91	23.8	4.84	149	54	3.93	23.8	6.05
110	47	10.98	89	8.1	150	65	2.12	24.7	11.65**
111	33	21.15	86.5	4.08	151	45	2.88	19.7	6.84
112	28	2.17	12	5.52	152	23	3.55	23.9	6.73
113	33	12.67	115	9.07**	153	54	5.55	36.9	6.64
114	25	2.17	13.3	6.12	154	43	4.45	39.8	8.94
115	39	19.09	98.9	5.18	155	61	12	56.1	4.67
116	35	1.18	13.55	11.48**	156	45	2.77	27.97	10.09**
117	45	7.67	56.5	7.36	157	44	4.16	34.9	8.38
118	26	6.09	34	5.58	158	65	1.4	6.8	4.85
119	54	4.27	45.5	10.65**	159	27	5.58	50.9	9.12**
120	32	7.67	46.7	6.08	160	33	6.56	34.6	5.27

Table 1B: Testosterone and SHBG concentration in 186 women.

Lab. No.	Age	T. concentration (ng/ml)	SHBG (mmol/l)	Free T. (mmol/l/ ng/ml)
161	34	1.9	2.89	1.52
162	43	3.3	13.5	4.09
163	65	3.7	7.9	2.13
164	56	8.3	27.8	3.34
165	67	2.3	16.9	7.34
166	60	1.2	19.3	16.08**
167	65	0.3	1.4	4.67
168	54	2.1	3.1	1.47
169	33	0.3	1.6	5.33

170	24	0.8	1.8	2.25
171	31	9.2	28.9	9.2**
172	30	9.3	29.3	3.15
173	45	2.2	12	5.45
174	55	2.9	21	7.24
175	47	4.7	12	2.55
176	49	8.4	19.4	2.3
177	50	6.7	20	2.98
178	50	8.2	14	1.71
179	54	0.9	2.6	2.89
180	44	2.7	15.9	5.89
181	54	4.8	21	4.37
182	45	5.3	14.6	2.75
183	36	4.7	18.44	3.92
184	28	8.6	28.9	3.36
185	36	2	8	4
186	81	3.9	16.3	4.17
Control-1		2.03	65	32.02
Control-2		15.17	67.1	4.42

N=186; T. concentration=Testosterone concentration; SHBG=Sex Hormone Binding Globulin; free T.=free Testosterone
 **represent values above normal range indicating testosterone elevation in women

Table 1C: Testosterone and SHBG concentration in 186 women.

Tables 1A-1C shows results of total and free testosterone of 186 women in Eket community of Akwa Ibom state.

Discussion

In trying to interpret the laboratory results on serum testosterone, free testosterone, and sex hormone binding globulin concentration, it was observed that most laboratories establish its reference ranges that compensate for calibration errors in equipment or for their methods. What seems frustrating is that, instead of adjusting the results and rendering them in some standardized fashion, each laboratory, establishes an adjusted reference range. This makes direct comparisons of results from one laboratory with another impossible, while exemplified results are shown in Figures 1 and 2. Thus, this result will be interpreted without necessarily comparing with existing data. Testosterone is an excellent target for cancer therapy because it has been shown to be an effective target for breast and prostate cancer treatment due to its expression in the majority of breast cancer [13]. A clearer understanding of the mechanism of testosterone signaling in the breast is yet to be revealed, but with continued research efforts, there is hope that viable breast cancer therapies will be exploited through testosterone signaling. Excess amounts of testosterone can pose a problem, resulting in such "virilizing effects" as acne, hirsutism (excess hair growth in "inappropriate" places, like the chin or upper lip)

and thinning of hair on the head (balding). About 10 percent of women with high levels of free testosterone have polycystic ovary syndrome (PCOS), characterized by irregular or absence of menstrual periods, infertility, blood sugar disorders (pre-diabetes and type 2 diabetes) [14]. Most women with PCOS are overweight or obese, though small percentages have a normal body weight. When such women are left untreated, high levels of testosterone, regardless of whether a woman has PCOS or not, are associated with serious health consequences, such as insulin resistance and diabetes, high cholesterol, high blood pressure and heart disease [15]. In addition to PCOS, other causes of high testosterone levels - hyperandrogenism include congenital adrenal hyperplasia (a genetic disorder affecting the adrenal gland that afflicts about one in 10,000 to one in 18,000 individuals, where about half are women) and other adrenal abnormalities, and ovarian or adrenal tumours [16]. Medications such as anabolic steroids, occasionally abused by bodybuilders and other athletes for performance enhancement, can also cause hyperandrogenic symptoms [16].

Tables 1A-1C present the testosterone concentration in women. The result showed that 38 women have elevated free testosterone concentration above the normal range (2.03-15.17 ng/ml). This accounted for 20% of 38/186 women population with testosterone elevation. Investigations have shown that excessive amount of male

hormone production by the ovary is the main feature of the polycystic ovarian syndrome (PCOS), which leads to many of the findings that women with PCOS experience [17]. According to the Center for Young Women's Health, PCOS results in many tiny cysts or bumps inside the ovaries hyperprolactinemia can result in abdominal pain and irregular periods. PCOS is caused by hormones in the brain and ovaries, which act as chemical messengers that inform the body when to ovulate, menstruate and grow hair among other activities. The Center also reported that skin cells and hair follicles are extremely sensitive to the slightest increase in female testosterone levels, and this can cause acne and facial hair [18]. A history of preeclampsia an average of 17 years earlier appears to be associated with elevated levels of testosterone, which may contribute to the increased risk of vascular morbidity in such women [19].

The concentration of testosterone and SHBG were interpolated by Spline and Lowess method using the Spline Curve as provided in Figures 1 and 2. Also, other factors that may influence this investigation include sampling time, which is an important consideration when interpreting serum testosterone. The result showed that intra-individual variation in testosterone levels of approximately 10% is observed when samples are collected from the same individual at the same time of the day over several days. Conditions that increase SHBG include aging, hyperthyroidism and hepatic cirrhosis, or that which decrease SHBG include obesity, diabetes mellitus, and glucocorticoid usage can affect the bioavailability of testosterone.

Low testosterone levels can as well be a problem, producing effects such as low libido, fatigue, decreased the sense of well-being and increased susceptibility to bone loss, osteoporosis, and fractures [18]. Symptoms such as flagging desire and general malaise have different causes, and testosterone deficiency - hyperandrogenism is usually not undiagnosed. Low androgen levels may affect women at any age but occur during the transition to menopause, or perimenopause. Testosterone levels start dropping in a woman's above twenty years, and by the time she reaches menopause, testosterone has declined 50%. Further declines in ten years following menopause indicate ever-decreasing ovarian function [15]. For many women, the effects of this further testosterone decline include aggravation of hot flashes and accelerated bone loss. These effects may not become obvious until the women are in their late 50s or early 60s.

The increase in SHBG with age means that older women may have normal total testosterone levels, even if they are hypogonadal, as they have low levels of free or bio-available testosterone (TT) [20]. Conversely, obesity decreases SHBG and TT, even when the bio-available fraction may be normal [21]. Low free and bioavailable testosterone concentrations in the normal range are associated with diabetes, independent of adiposity [22]. Low concentrations are found in myxoedema, hyperprolactinemia, and syndromes of excessive androgen activity. Measurement of SHBG is useful in evaluating mild disorders of testosterone metabolism and enables identification of women with hirsutism, who are likely to respond to estrogen therapy. The ratio of SHBG to testosterone is also known as the Free Androgen Index (FAI) or the Free Testosterone Index (FTI). This ratio correlates with both measured and calculated values of free testosterone and helps to discriminate subjects with excessive testosterone activity from normal individuals [23]. Metabolic clearance of SHBG is biphasic, with a fast initial distribution from vascular compartment into extracellular space (half-life of a few hours), followed by a slower degradation phase (half-life of several days). SHBG binds sex steroids with high affinity (KD approximately 10^{10} M), dihydrotestosterone (DHT) \geq

testosterone (T) \geq estrone/estradiol (E) NCCLS (2002). Although each monomeric subunit contains 1 steroid binding site, the dimer tends to bind only a single sex-steroid molecule. The main function of SHBG is sex-steroid transport within the bloodstream and to extravascular target tissues [24]. SHBG also plays a key role in regulating bio-available sex-steroid concentrations through the competition of sex steroids for available binding sites and fluctuations in SHBG concentrations [25]. Because of the higher affinity of SHBG for DHT and TT, SHBG have profound effects on the balance between bio-available testosterone and estrogens.

Conclusion

The evidence for adverse reproductive outcomes (infertility, cancers, and malformations) from hormonal imbalances is strong, and there is mounting evidence for effects on other endocrine markers, including thyroid, neuroendocrine, obesity and metabolism, and insulin and glucose homeostasis. The precautionary principle is to enhancing hormonal and reproductive health and should be used to inform decisions about health status and risk from, potential hormonal imbalances.

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