Estimation of Total, Bio-available and Free Testosterone levels in various age groups of men.

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ABSTRACT:

In recent years several studies recommended the estimation of total as well as bio-available and free testosterone levels to assess the variations provided by the measurements and thus developing the foundation for interpreting hormone status in all groups of men. Therefore present study documents the current testosterone status i.e. total, free and bio-available, including sex hormone binding globulin (SHBG) in variable age groups of men (n = 78) between 14 years to 65 years. They were grouped as male aged 14-24 yrs "young" (n = 24), 25-35 yrs "adult" (n = 20), 36-50 yrs "middle aged" (n = 29) and 51-65 yrs "older" (n = 15). Serum total testosterone and SHBG were measured by Electro Chemiluminescence's (ECL) technology whereas bio-available and free testosterones were calculated from pre-described calculation methods. Total testosterone levels are comparable to each other in adult and middle age groups, however significantly differ ($P < 0.001$) among older and younger group. Moreover, highest level of significant difference in free testosterone values were obtained for younger men in comparison with middle age group ($P< 0.001$) and moderate level of significance was noted when same was compared with adult and middle aged groups ($P < 0.05$). The assessment of data was gave similar outcome for bio-available testosterone as well; accept when older group was compared with middle aged men, which was found to be non-significant. In conclusion, the levels of total, free, bio-available testosterone and SHBG were compared with their normal ranges and noted to be match-able with their respective age groups accordingly.

INTRODUCTION:

Testosterone concentrations in men, primarily analyzed nearly half a century ago, are now measured as a routine test for initial indexing of androgen status[1]. Most clinical laboratories all around the world, especially at tertiary care centers, uses multichannel, fully automated analyzers for total testosterone estimation. Current evidence suggests that these analyzers are capable of satisfactorily quantifying the concentration of total plasma testosterone in men. It is documented and recommended that when concentrations of plasma testosterone are at the lower limit of normal (9.0 nmol/l), some value of bioactive testosterone group should be analyzed. This may be the free form (non-protein-bound) or bio-available form (free plus albumin-bound) of testosterone. The measurement of both can be done analytically, which is a laborious and time-consuming process or can be calculated using any one of a variety of available mathematical formula[1]. It is generally reported and clinically accepted that the concentrations of total, free and bio-available testosterone decline as men ages, the majority of elderly men have testosterone levels in the young adult range[1]. Therefore researchers, clinicians and endocrinologists are recommending the estimates of individual and intra-individual testosterone as well as that of bio-available and free testosterone levels to assess variations provided by the measurements and developing the foundation for interpreting hormone status in all groups. These measurements, they suggest will also be used as a reliability tool of one or two values of an individual's

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average hormone concentration[2].

The current view point of measuring androgens in individuals and groups also seems to be more relevant when a recent study correlated sex hormone levels with mortality over a median of 16 years of follow-up. The study included 1,114 US men during a period of 1988-1991 having no pervious history of cardiovascular disease or cancer at baseline[3]. The study reported that men with low free and bio-available testosterone levels may have a higher risk of mortality within 9 years of hormone measurement[3]. Furthermore, interestingly, another recent study showed that men with type 2 diabetes taking statins as lipid lowering medications exhibited low total testosterone levels. The results were supported by the fact that about 20% of men with metabolic syndrome, diabetes, and cardiovascular disease have testosterone levels below the normal range. Moreover, it is well documented that there are a further 20–25% with levels in the low normal range that may also be compatible with a diagnosis of hypogonadism, depending on clinical symptoms[4].

The present study in this regard, documents the current testosterone status i.e. total, free and bio-available, including sex hormone binding globulin (SHBG) in variable age groups of men, between 14 yrs to 65 years; assessing their noted levels and correlating it with normal reference values and their respective age groups.

MATERIALS AND METHODS:

Research Design: Males, aged between 14 to 65 years were recruited from outpatients' facility at Department of Biochemistry Laboratory services, Liaquat National Hospital and department of Pathology, Govt Lyari General Hospital, Karachi, during Jan 2007 to Dec 2009. Subjects were given verbal information regarding the study, and seventy eight gave their informed consent to take part. The study population comprised patients' routinely visiting medicine, endocrine and related specialty clinics. Demography, medical history, and drug histories were collected using a questionnaire. Clinical and biochemical assessments of androgens status were made inclusive of other measurements such as routine blood pressure, non-fasting lipid levels, height, weight, and waist circumference. Any additional information was obtained from our patients' database.

Analytical Procedures: Patients were visiting usually in the morning to noon timings between 8:00 and 1:00 pm. No symptoms of hypogonadism were reported in the selected group and thus included in the study. Venous blood was taken; serum samples were produced by centrifugation and stored at -20°C for future analysis. Serum total testosterone and SHBG were measured by Electro ChemiLuminescence (ECL) technology on Roche Elecsys 2010 (Roche Diagnostics, Basil) immunoassay analyzer according to manufacturer's directions. Bio-available and free testosterone were calculated from total testosterone, SHBG and albumin concentration by a previously described calculation method[5,6]. These methods of assessing bio-available and free testosterone have been used in previous studies[1,4,6] and have determined to be reliable for the assessment of androgen status in men[7]. As recommended earlier[7] weight and height were recorded and used to derive BMI and waist circumference was measured midway between the lower costal margins and the iliac crests. Blood pressure was recorded using a manual sphygmomanometer. Serum albumin was analyzed by Hitachi 912 chemistry analyzer (Roche Diagnostics, Basil) using standard method.

Statistical analysis: Data were analyzed using the SPSS package ver 13 (SPSS, Chicago, IL). Testosterone and SHBG were assessed by using Student's t test for comparison of group means. Within smaller sub-groups, the normal distribution was assessed using Pearson's correlation testing. The two-way ANOVA was also used to compare groups when data did not fulfill the normal distribution. Results were considered statistically significant at $P < 0.05$.

RESULTS:

Total, free and bio-available testosterone levels in 78 patients from boys aged 14 to older men ages 65 were assessed in the present study. The patients were divided into four groups to evaluate a generalized androgen status of selected male population. The data of each group was assessed according to their age group and compared with other groups as well. Four groups includes male aged

14-24 yrs "young" (n = 24), 25-35 yrs "adult" (n = 20), 36-50 yrs "middle aged" (n = 29) and 51-65 yrs "older" (n = 15). Other baseline characteristics such as height and weight of the participants are provided in Fig 1-2. Except for slightly higher weight index in middle age and older group of men as compared to younger ones, all other parameters are in comparable range with each other.

The androgen hormone levels of the participants in the study span broad ranges of values (Table 1). Some of the low values in the table, such as the lower for total testosterone and free testosterone may indicate various factors related to progressing or older age. Another possibility of obtaining lower levels of total testosterone in older group is their self reported health status and medications. Therefore, some subjects may be taking unreported medications or had unreported conditions thus affecting hormone levels and thus this fact cannot be ruled out. However, the majority of values are within normal ranges. Total testosterone levels are comparable to each other in adult and middle age groups, however significantly differ ($P < 0.001$) among older and younger group; and with adult group and middle age group ($P < 0.05$). Similarly, variable levels of significant difference was noted when SHBG of older group was compared with younger men ($P < 0.01$) and adult with middle age men ($P < 0.001$). Moreover, highest level of significant difference in free testosterone values were obtained for younger men in comparison with middle age group ($P< 0.001$) and moderate level of significance was noted when same was compared with adult and middle aged groups ($P < 0.05$). The assessment of data was not very different for bio-available testosterone as well; accept when older group was compared with middle aged men, which was found to be non-significant. All measured levels of total, free, bioavailable testosterone and SHBG were compared with their normal ranges and noted to be match-able with their respective age groups accordingly.

DISCUSSION:

It is well ascertained that a number of measurements for testosterone are available from various clinical laboratories. However several reports suggest the importance to understand the differences among various measured analytes[7-12]. In this regard, generally the measurement of serum testosterone or "total" testosterone is usually performed by ELISA, MEIA and ECL assays and measures free plus protein bound testosterone. Furthermore, "Free" or dialyzable testosterone measurements are estimates of the fraction of testosterone in blood that is not bound to protein. These assays sometimes require determination of the percentage of unbound testosterone by a dialysis procedure, estimation of total testosterone, and the calculation of free testosterone. Free testosterone can also be calculated if total testosterone, SHBG, and albumin concentrations are known[9]. There is a third analyte of testosterone commonly made of "bio-available" or non-SHBG bound testosterone[11]. This analysis determines the amount of testosterone not bound to SHBG and includes that which is non-protein bound and weakly bound to albumin. This fraction is supposedly readily available to tissues and thus the name "bio-available" [7].

Normally androgen status are reported to be the essential sex steroid acting on male physiological functions, and bio-available androgens, as mentioned earlier, comprise the free and albumin-bound fractions[13]. In present study we examined the age-related measurements in serum of total, free and bio-available testosterone levels comparing with each fraction of testosterone and its relationship with respective age group. It is noted that albumin-bound as well as bio-available testosterone levels declined with age, and their decrease was associated their respective age groups as well as with the increase of sex-hormone binding globulin (SHBG) level in fifties and sixties. Similar results were recognized in the level of all three fractions of testosterone, suggesting that SHBG and albumin levels plays an important role for maintaining bio-available sex steroid levels in males aged over sixty. Moreover, our study showed that SHBG levels associated inversely with bio-available sex steroid, in agreement with previous reported studies[1,4]. A study carried out for estrogens also showed similar pattern for albumin and SHBG bound fractions[13]. They postulated that the decrease of bio-available estradiol as well as testosterone is induced

by the decrease of albumin-bound fractions in combination with the increase of SHBG-bound fractions in males aged over sixty. In addition their physical characteristics of aging could be induced by the decrease of albumin-bound fractions caused by the decrease of serum albumin regardless of total sex steroid levels[13].

It is well documented that serum testosterone levels begin to decline in normal healthy men on no medications, in the mid- to late-thirties and this decline is linear into the nineties, at a rate of 0.4%/year. If men with chronic medical illnesses such as hypertension, heart disease, diabetes are evaluated, it was noted that the same age-associated decline in serum testosterone exists, but at a rate of 10–15% below that of healthy age-matched men. Moreover, in addition to this decline with age in total testosterone, there is an inversely proportional increase in sex hormone binding globulin (SHBG)[7,14-16] as seen in our study. Therefore, undoubtly, as man ages, the total testosterone level decreases, but the serum binding of testosterone increases. This increase in testosterone binding results in a "free" or bio-available testosterone level that decreases to a greater extent than total testosterone, a phenomenon observed in our present study as well. As a result, the availability of the free active form of testosterone in the serum is further reduced compared with total testosterone.

Arguably, the measurement of testosterone which depends on evaluation of protein-bound testosterone may not reflect of a deficiency of it, but may be the result of a change in the binding protein by a problem independent of the androgen state of the man such as seen in type 2 diabetes mellitus patients. As reported earlier, a decrease in SHBG occurs in type II diabetes mellitus as a result of an increase in insulin or insulin-like growth factor I levels[17,18]. This decline in SHBG level associated with decreased total androgen in diabetic patients, and unrelated to androgen deficiency, may be coincidentally related to impotence. However, this impotency is not because of any alterations in total, bio-available or free testosterone levels but due to the onset of diabetes mellitus and its concomitant physiological disturbances[7].

Previously it was argued that assessment of androgen status and the determination of age-associated hypogonadism (so called andropause) is not as clear in men as it is in women[7]. If it exists at all, it was agreed that, as an independent entity outside of a disease-associated decline in plasma concentrations of testosterone. As men age, there is a decline in serum total testosterone levels that begins about the age of 40 yr. In cross-sectional studies done earlier, the annual decline in total and free testosterone is 0.4% and 1.2%, respectively[19]. However, it was noted that these studies were cross-sectional, and although the serum testosterone level declines with age, the mean value for a group of disease-free men remains within the normal range for most laboratories, even into the ninth decade of life[14]. The reason available herewith is the fact that testosterone circulates in the blood 98% bound to protein. In men, approximately 40% of the binding is to the high affinity sex hormone binding globulin (SHBG), and approximately 60% bound weakly to albumin[6,7,20]. Several researchers also correlated other biochemical and physiological factors with altered or otherwise levels of androgens. Assessing association of total, free and bio-available testosterone with age groups as well with high or low levels of albumin or SHBG, mostly agreed upon, is another area worth exploring. In this regard, a recent study assessed the relationship of bio-available testosterone with immunoassayed total testosterone against a comprehensive panel of alternate serum measures[21]. In comparison with total testosterone, the component total haemoglobin, exhibited either a weaker or absence of correlation with the analyte in question. It is currently not clear whether these outcomes reflect a physiological association between bio-available testosterone and haemoglobin or whether trace amounts of haemoglobin in serum can influence bio-available testosterone assay measurement. The results of other assays utilizing diluted samples with long serum incubation times demonstrated that SHBG is capable of playing a significant role in androgen bioassay measures, as suggested, supported and mentioned earlier. An inverse relationship between serum SHBG concentration and percent of testosterone recovery has been observed and the release of serum testosterone from SHBG via diethyl ether extraction in another bioassay has been shown to

increase recovery of serum testosterone[22]. Consistent with the well-established positive relationship between serum SHBG and total testosterone[23,24] the authors found that the median serum total testosterone and bio-available testosterone levels were significantly decreased in serum samples within the lowest quartile of SHBG compared with the highest quartile, well supporting our findings. These findings are highly suggestive of a physiological rather than methodological relationship between the measures[21,25.26].

CONCLUSION:

In conclusion, total, free and bio-available testosterone levels in 78 patients including boys aged 14 to older men ages 65 divided in four age-matched groups were assessed in the present study. All measured levels of total, free, bio-available testosterone and SHBG were compared with their normal ranges and noted to be match-able with their respective age groups accordingly. It was argued that clinical situations, either of male andropause, impotency or otherwise, points out the difficulty in assessing androgen status when there is no good independent marker of androgen action that can be used in vivo. In addition, there is no single well designed clinical trial that have indicated that one method of testosterone measurement is better than any other. Therefore, assessing total serum testosterone in addition to free and bio-available, as demonstrated in present study, is less expensive to ascertain the general androgen status in men of varying age groups from young to old.

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REFERENCES:

1. Laboratory measurement of testosterone. 2009; 37:21-31.
2. Intra-individual variation in levels of serum testosterone and other reproductive and adrenal hormones in men. 2007; 67(6):853-62.
3. Sex steroid hormone concentrations and risk of death in US men. 2010; 171(5):583-92.
4. Statin therapy is associated with lower total but not bioavailable or free testosterone in men with type 2 diabetes. 2009; 32(4):541-6.
5. Vermeulen A, Stoica T, Verdonck L: The apparent free testosterone concentration, an index of androgenicity. J Clin Endocrinol Metab 1971; 33:759 –767.
6. van den Beld AW, de Jong FH, Grobbee, DE, Pols HA and Lamberts SW. Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. Journal of Clinical Endocrinology and Metabolism, 2000; 85: 3276–3282.
7. (a) Morris PD, Malkin CJ, Channer KS, Jones TH: A mathematical comparison of techniques to predict biologically available testosterone in a cohort of 1072 men. Eur J Endocrinol 2004; 151:241–249. (b) Plymate SR. Which Testosterone Assay Should Be Used In Older Men? J. Clin. Endocrinol. Metab. 1998; 83: 3436a-3438a
8. Hammond G, Nisker J, Jones L, Siiteri P. 1980 Estimation of the percentage of free steroid in undiluted serum by centrifugal ultrafiltration dialysis. J Biol Chem., 1980; 255: 5023–5026.
9. Sødergard R, Backstro¨m T, Shanbhag V, Carstensen H. 1982 Calculation of free and bound fractions of testosterone and estradiol-17b to human plasma proteins at body temperature. J Steroid Biochem., 1982; 16:801– 810.
10. Rosner W. Errors in measurement of plasma free testosterone. J Clin Endocrinol Metabol., 1997; 82:2014 –2015.
11. Nankin H, Calkins J. Decreased bioavailable testosterone in aging normal and impotent men. J Clin Endocrinol Metab., 1986; 63:1418 –1423.
12. Umstot E, Baxter J, Anderson R. A theoretically

sound and practicable equilibrium dialysis method for measuring percentage of free testosterone. J Steroid Biochem., 1985; 22:639–648.
13. Association of bioavailable estradiol levels and testosterone levels with serum albumin levels in elderly men. 2008; 11(2):63-70.
14. Vermeulen A, Deslypere J. Testicular endocrine function in the ageing male. Maturitas 1985; 7:273–279.
15. Plymate SR, Tenover JS, Bremner WJ. Circadian variation in testosterone, sex hormone binding globulin, and calculated non-sex hormone binding globulin bound testosterone in healthy young and elderly men. J Androl., 1989; 10:366 –371.
16. Tenover J, Matsumoto A, Plymate S, Bremner W. The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: Response to clomiphene citrate. J Clin Endocrinol Metab., 1987; 65:1118 –1126.
17. Peiris A, Stagner J, Plymate S, Vogel R, Heck M, Samols E. Sex hormone binding globulin levels in normal men: role of pulsatile insulin secretion. J Clin Endocrinol Metab., 1993; 76:279 –282.
18. Plymate S, Matej L, Jones R, Friedl K. Inhibition of sex hormone binding globulin (SHBG) production in the human hepatoma (Hep G2) cell line by insulin and prolactin. J Clin Endocrinol Metab. 1988; 67:460–464.
19. Gray A, Feldman HA, McKinley JB, Longcope C. 1991 Age, disease, and changing sex hormone levels in middle-aged men: results of the Massachusetts Male Aging Study. J Clin Endocrinol Metab., 1981; 73:1016 –1025.
20. Pardridge W. Selective delivery of sex steroid hormones to tissues in vivo by albumin and by sex hormone-binding globulin. Ann NY Acad Sci., 1988; 538:173–192.
21. Need, E. F., O'Loughlin, P. D., Armstrong, D. T ... Aging Study, Wittert, G. A. and Buchanan, G. Serum testosterone bioassay evaluation in a large male cohort. Clinical Endocrinology 2010; 71: 87–98
22. Chen J, Sowers MR, Moran FM, McConnell DS, Gee NA, Greendale GA, Whitehead C, Kasim-Karakas SE, Lasley BL. Circulating bioactive androgens in midlife women. Journal of Clinical Endocrinology and Metabolism, 2006; 91: 4387–4394.
23. de Ronde W, van der Schouw YT, Muller M, Grobbee DE, Gooren LJG, Pols HAP, de Jong FH. Associations of sex-hormone-binding globulin (SHBG) with non-SHBG-bound levels of testosterone and estradiol in independently living men. Journal of Clinical Endocrinology and Metabolism, 2005; 90, 157–162.
24. Vermeulen, A., Kaufman, J. & Giagulli, V. Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males. Journal of Clinical Endocrinology and Metabolism, 1996; 81, 1821–1826.
25. Roy P, Franks S, Read M, Huhtaniemi IT. Determination of androgen bioactivity in human serum samples using a recombinant cell based in vitro bioassay. The Journal of Steroid Biochemistry and Molecular Biology 2006; 101: 68–77.
26. Raivio T, Palvimo JJ, Dunkel L, Wickman S, Jänne OA. Novel assay for determination of androgen bioactivity in human serum. The Journal of Clinical Endocrinology and Metabolism, 2001; 86: 1539–1544.

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Table 1: Determination of total, free and bio-available testosterone and sex hormone binding globulin in various age groups of men.

Men Age groups	T.Testosterone nmol/L	SHBG nmol/L	Free-Testo nmol/L	Bio-Testo nmol/L
14-24 yrs	17.56 ± 2.76^{a}	47.83 ± 8.80^{b}	0.41 ± 0.13^{a}	7.80 ± 1.23^{a}
25-35 yrs	15.35 ± 2.10^{b}	39.26 ± 7.84^{a}	0.38 ± 0.10^{b}	7.10 ± 2.10^{c}
36-50 yrs	14.28 ± 1.89^{b}	38.12 ± 8.77^{d}	0.34 ± 0.009^{b}	6.01 ± 1.10^{d}
51-65 yrs	$6.83 \pm 1.58^{a,b}$	$69.56 \pm 6.43^{a,b}$	$0.17 \pm 0.008^{a,b}$	$5.10 \pm 1.90^{a,c,d}$

Normal ranges: Total Testosterone (nmol/L) = Children = < 0.69, Males = 9.9-52.3; SHBG (Sex hormone binding globulin-nmol/L) = Children = 10-80, Males = 14-48; Free Testosterone (Free-T-nmol/L) = Males= 0.31-1.041; Bio-available testosterone (Bio-Testo-nmol/L) = Young Males = 2.88-8.90, Adult Males = 2.49-8.15, middle-aged males = 2.11-6.59, old aged-males = 1.388-5.82).

Results are expressed as mean ± SD; [a]Significant difference < 0.001, [b]Significant difference < 0.01, [c]Significant difference < 0.05, [d]non-significant.