

男性ホルモン軟膏は健康保険では使用できないが、OTC薬として市販されている。1回3gを1日1～2回陰囊皮膚に塗布（1回3mgテストステロン相当）で安定した血中テストステロン濃度が得られる<sup>7)</sup>。

#### 4. 男性ホルモン補充療法の副作用とその監視

男性ホルモン補充療法に際して考慮すべきリスクとして心血管系疾患、脂質代謝異常、多血症、体液貯留、前立腺肥大症、前立腺癌、肝毒性、睡眠時無呼吸症候群、女性化乳房、ざ瘡、精巣萎縮、不妊、行動・気分の変化が挙げられる。

治療開始後の血液検査は2～4週後、3ヵ月後、6ヵ月後、12ヵ月後、以後は1年毎に行い、検査値に基づいて治療の中止または適宜投与量の増減を行う。また、適宜専門医に患者の治療を依頼する。

## ● おわりに

LOH診療は始まったばかりであり、診療にあたっては注意深い配慮が必要である。また、今後推奨ランクの高いエビデンスを創出していく必要がある。そういった意味からも、「LOH症候群診療の手引き」を参考にさせていただいて診療を行っていただくことを希望する。それにより、今後診療結果の検証が可能になり、さらにグレードアップした「手引き」さらには文字通りの「ガイドライン」に近い将来作成されることを願っている。

## 文 献

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## Review Article

## Late-onset hypogonadism (LOH) and androgens: Validity of the measurement of free testosterone levels in the diagnostic criteria in Japan

Teruaki Iwamoto,<sup>1</sup> Toshihiko Yanase,<sup>2</sup> Hitoshi Horie,<sup>3</sup> Mikio Namiki<sup>4</sup> and Akihiko Okuyama<sup>5</sup>

<sup>1</sup>Center for Infertility and IVF, International University of Health and Welfare Hospital, Nasushiobara, Tochigi, <sup>2</sup>Department of Medicine and Bioregulatory Science, Division of Pathophysiological and Experimental Pathology, Graduate School of Medical Science, Kyushu University, Fukuoka, <sup>3</sup>IVD Department Diagnostics Testing Business Segment, Mitsubishi Chemical Medience Corporation, Tokyo, <sup>4</sup>Department of Urology, Kanazawa University School of Medicine, Kanazawa, Ishikawa, <sup>5</sup>Department of Urology, Osaka University School of Medicine, Suita, Osaka, Japan

**Abstract:** The basis of diagnosis of late-onset hypogonadism (LOH) is the measurement of androgen levels. Traditionally, total testosterone (total T) was also used as the primary indicator of gonadal function in Japan. In 1998, the International Society for the Study of the Aging Male was founded to conduct basic and clinical research on this issue internationally. As a result, it is said that bioavailable testosterone levels should be measured in the diagnosis of LOH. At present, however, there are a number of problems for bioavailable testosterone to become a routine diagnostic tool. Here, we will explain the various measurement indicators of androgens, measurement problems, standard values of total T, and free testosterone (free T) in Japan, and the diagnostic methods for LOH overseas. In Japan, the Japanese Urological Association and the Japanese Association of Men's Health recommend the measurement of free T levels in the diagnosis of LOH, for the following reasons: (i) It has been demonstrated that free T is more strongly correlated with calculated bioavailable testosterone, than total T, showing a statistically significant difference; (ii) The behavior of free T is highly consistent with that of bioavailable testosterone, with free T levels being markedly decreased due to aging; (iii) For the measurement of free T, a method that allows the measurement of free T only, without affecting the balance between free T and protein-bound testosterone in blood, is available; and 4) The mean total T by age range decreases only to 80% of the young adult mean even during presenile and senile periods, when LOH occurs most frequently, but the free T level shows a linear decrease with aging and drops to 50% of the young adult mean. For these reasons, we will describe the validity of the recommendation for the measurement of free T levels.

**Key words:** free testosterone, total testosterone, young adult mean (YAM).

### Introduction

In 2005, the International Society of Andrology (ISA), the International Society for the Study of the Aging Male (ISSAM), and the European Association of Urology (EAU) defined late-onset hypogonadism as 'a clinical and biochemical syndrome associated with advancing age and characterized by typical symptoms and a deficiency in serum testosterone levels. It may result in significant detriment in the quality of life and adversely affect the function of multiple organ systems'.<sup>1-3</sup> More specifically, it requires a diagnosis of hypogonadism requiring clinical treatment to be made in the presence of low androgen levels and signs/symptoms suggesting lack of androgens. The symptom most closely associated with hypogonadism is said to be low libido.<sup>4</sup> Other symptoms associated with hypogonadism include erectile dysfunction, decreased muscle mass and strength, increased body fat, decreased bone mineral density and osteoporosis, decreased vitality, and impaired mood. These symptoms are not necessarily specific to androgen deficiency but suggest lack of androgens. In other words, a diagnosis of late-onset hypogonadism (LOH) should be made in the presence of at least one of these symptoms with low androgen levels.<sup>5</sup>

Therefore, the basis of the diagnosis of LOH is the measurement of androgen levels. In Japan, as specified in the 'Clinical Practice Manual for Late-onset Hypogonadism Syndrome'<sup>6</sup> it has been decided to

recommend the measurement of free testosterone (free T) levels as a diagnostic test for LOH.

In this report, we explain problems in the measurement of androgen levels, methods for determination of normal ranges, diagnostic methods in various countries, and the validity of the reasons for the measurement of free T levels in the diagnosis of LOH in Japan.

### Measurement indicators of androgen, measurement problems, and standard values

#### Measurement indicators of androgens

Androgens include dihydrotestosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-S), testosterone (T), and dihydrotestosterone (DHT), which are biosynthesized from cholesterol in the adrenal glands and testes. About 98% of testosterone in the blood exists bound to sex hormone-binding globulin (SHBG) (SHBG-bound testosterone [SHBG-T]) and to albumin (albumin-bound testosterone [Alb-T]), and the remaining 2% exists as free T. Since the binding of Alb-T is weaker than that of SHBG-T, testosterone is easily separated from Alb-T. In addition, free T and Alb-T have the biological activity of androgen. Therefore, free-T and Alb-T comprise so-called bioavailable testosterone (BAT).<sup>7-9</sup> In other words, BAT is considered to be the sum of Alb-T and free T other than the SHBG-T fraction, which is precipitated by ammonium sulfate. In the guidelines by Nieschlag *et al.*,<sup>1-3</sup> total T is recognized worldwide as an indicator of androgen in the diagnosis of hypogonadism. Free T calculated from measured total T and SHBG values, or free T measured by reliable free

Correspondence: Teruaki Iwamoto, MD PhD, Center for Infertility and IVF, International University of Health and Welfare Hospital, 537-3, Iguchi, Nasushiobara-Shi, Tochigi-Ken 329-2763, Japan. Email: t4iwa@iuhw.ac.jp

Received 25 September 2008; accepted 1 October 2008.  
Online publication 27 November 2008

testosterone using a dialysis method, has gained a certain reputation as bioavailable testosterone.<sup>10,11</sup> In Japan as well, favorable correlations between BAT and calculated BAT and between calculated free T and free T have been reported,<sup>12,13</sup> and these findings are being further researched in some institutions. However, in Japan, reagents for measurement of SHBG have not been approved as *in vitro* diagnostics and are not covered by health insurance and are therefore not commonly used.

### Age-related changes in androgens

There are various reports on age-related changes in blood androgen concentrations in normal men. The cause of this inconsistency may vary depending on the study population consisting of men who are considered to be normal (men undergoing health checkups, outpatient men who have a disease but maintain high activity levels, or men selected randomly by an epidemiological survey), etc. In other words, there is no uniform definition for normal healthy men. Therefore, attention should be paid in the case of comparison of publications in terms of changes in androgens.

#### Age-related changes in total T

Findings from reports on age-related changes in total T vary: some reports have shown marked or moderate decrease, and others have shown no age-related decrease. Purifoy *et al.*<sup>14</sup> measured testosterone in 80 healthy active males aged 20 to 88 years, and showed a tendency toward age-related decreases ( $0.05 < P < 0.10$ ) in testosterone. Morley *et al.*<sup>15</sup> conducted a longitudinal study over a 15-year period in 77 very healthy men aged 61 to 87 years. This investigation demonstrated a statistically significant age-related decrease in testosterone, at a rate of 1.1 ng/mL per decade. Zmuda *et al.*<sup>16</sup> reported a 0.035 ng/mL per year decrease in total T over 13 years of observation in 66 men aged 41 to 61 years. In addition, Harman *et al.*<sup>17</sup> observed a decrease of 0.032 ng/mL (1%) per year in 890 generally healthy men in a cross-sectional study, or even in a longitudinal study. Feldman *et al.*<sup>18</sup> conducted the Massachusetts Male Aging Study (MMAS), which is a longitudinal study conducted over 7 to 10 years in more than 1100 men, and observed a slightly progressive decrease at a rate of 1.6% per year. However, the rate of decrease in a cross-sectional study was 0.8% per year. On the other hand, Jensen *et al.*<sup>19</sup> conducted a cross-sectional study in 924 randomly selected healthy men. They reported that there was no age-related decrease, with no differences in total T or in free androgen index ( $[\text{testosterone}/\text{SHBG}] \times 100$ ) between elderly people and non-elderly people. Also in Japan, there is a tendency toward age-related decreases in total T, but there is no statistical significance, as described later.

#### Age-related changes in free T

A consistent result showing age-related decreases in free T was obtained in a number of reports from cross-sectional studies. The percentage of age-related decrease is known to be significantly higher in free T than in total T. The cause of this may be that SHBG levels increase with age. A statistically significant decrease in free T was also observed in Japanese studies. Also in the longitudinal study by Harman *et al.*,<sup>17</sup> age-related decreases in free T index (a calculated value related to free or bioavailable T) were observed.

### Measurement problems

**Blood Sampling Time Points.** Androgens exhibit diurnal variation, beginning to decrease in the afternoon. In the guidelines by

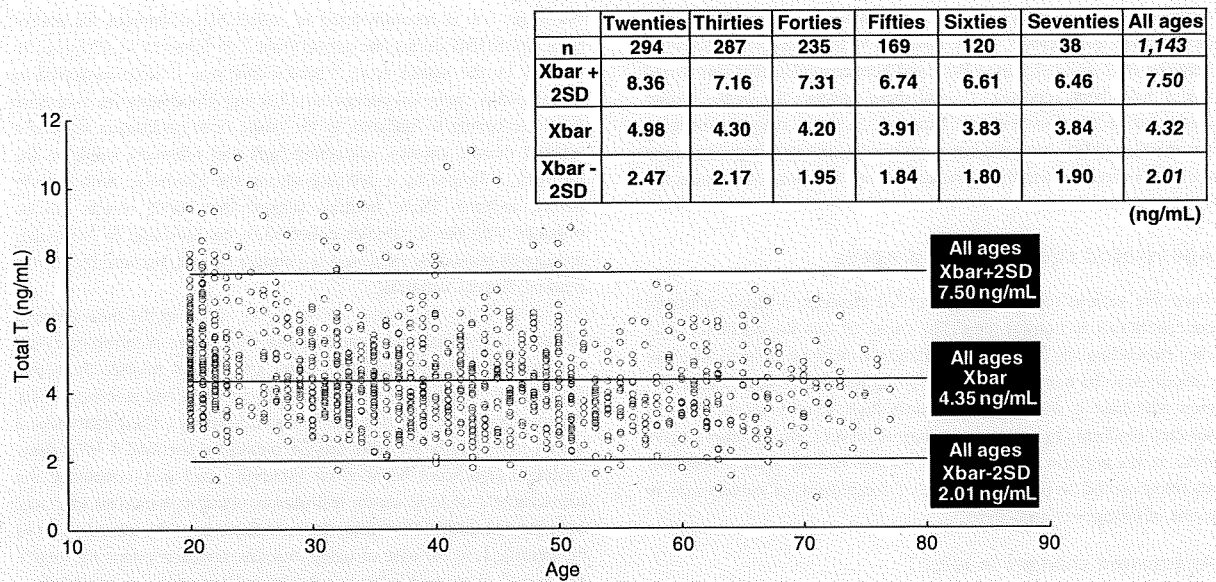
Nieshlag,<sup>1-3</sup> it is recommended to collect blood samples for androgens and SHBG during the period from 7.00 hours to 11.00 hours. In a study by the author *et al.*,<sup>20</sup> total T and free T were measured in three healthy male volunteers by collecting blood samples over time from awakening to bedtime. In the study, both parameters showed a marked diurnal rhythm, remaining high and relatively stable in the morning, followed by a decrease during the afternoon, with a nadir before bedtime. These findings suggest that stable results can be obtained with blood samples collected in the morning. In particular, precaution should be exercised in younger people, because they show marked diurnal variation.

**Measurement Differences Among Assay Kits.** In the measurement of total T, it should be kept in mind that values determined using various commercially available assay kits do not necessarily agree. Boots *et al.*<sup>21</sup> evaluated the between-kit variability and precision of six different commercially available testosterone assays. Their data showed most commercially available kits for measuring the total T level demonstrated significant between-kit variability, and they concluded the measurement of total serum testosterone using commercial kits may have limited utility. Therefore, the manufacturer of the kit used should be identified, in the case of using overseas standard values for total T and free T (described below) or the lower limit of the standard values for indication of androgen replacement therapy.

### Establishment of standard values of total T and free T in Japan

The normal ranges of total T and free T in Japanese males have been reported by many researchers. However, although the assay kits from DPC are used in many institutions, the normal ranges are not necessarily uniform. In addition, the normal range for free T, proposed by DPC (USA), has been changed three times over the past 10 years or so, even though the kit remains the same. As causes of this, the following were observed in the establishment of normal ranges: a small sample size, bias in age distribution, inconsistent blood sampling time points, inconsistent storage conditions of blood samples collected, and possible ethnic differences. In addition, there were concerns about the validity of not only the standard values but also measured individual values. The tracer in the free T assay kit from DPC is an iodinated testosterone derivative (iodinated 6-hydroxytestosterone-19-carboxymethyl ether histamine). Since it is a derivative, it does not replace testosterone that is bound to SHBG or Alb-Tumin, and only unbound (free) T in the sample and the iodinated testosterone derivative are competitively bound to the immobilized testosterone antibody. On the other hand, the immobilized testosterone antibody has lower affinity for testosterone than Alb-Tumin and SHBG. This prevents the binding of the immobilized testosterone antibody to bound T. Thus, the iodinated testosterone derivative is used<sup>22</sup> to provide a radioimmunoassay (RIA) system using a simple principle that allows only the recognition of free T in the sample. However, since it is a simple measurement system without stringent fractionation of bound and unbound forms, the system has not gained international recognition as a standard measurement system. Under this circumstance, a project for the establishment of standard values of total T and free T in Japanese adult men was launched by the Free T Review Committee, with the approval of the Scientific Committee of the Japanese Urological Association. In 2004, standard values were established and published in the *Journal of the Japanese Urological Association*. A summary is provided below.<sup>20</sup>

**Subjects and Method.** Among 1172 adult men who were living healthy lives, 1143 subjects aged 20 to 76 years with serum luteinizing hormone (LH) concentrations within its standard range (for 20 to



**Fig. 1** For total testosterone, statistical analysis was performed on the data from all subjects aged 20 to 77 years, and the standard range was defined as  $\bar{X} \pm 2SD$ . The upper limit ( $\bar{X} + 2SD$ ) and lower limit ( $\bar{X} - 2SD$ ) were 7.50 ng/mL and 2.01 ng/mL, respectively, with a mean ( $\bar{X}$ ) of 4.35 ng/mL.

70 years old: 1.1 to 25.9 mIU/mL) were selected. Blood samples were collected in the morning when total T and free T levels remained high and relatively stable. Separated serum samples were stored at  $-20^{\circ}\text{C}$  until assay. Both total T and free T were measured by RIA, using the DPC Testosterone Kit (Mitsubishi Kagaku Iatron) and DPC Free Testosterone Kit (Mitsubishi Kagaku Iatron), respectively. In consideration of the influence of aging, statistical analysis, for either total T or free T, was conducted on all data combined and on data by age group defined by 10-year intervals. The standard ranges were obtained from mean  $\pm 2$  standard deviation (2SD), after testing the distribution of measured values in each group and transforming the data into the optimal normal distribution on the histogram. The normality was examined using skewness, kurtosis, and histograms.

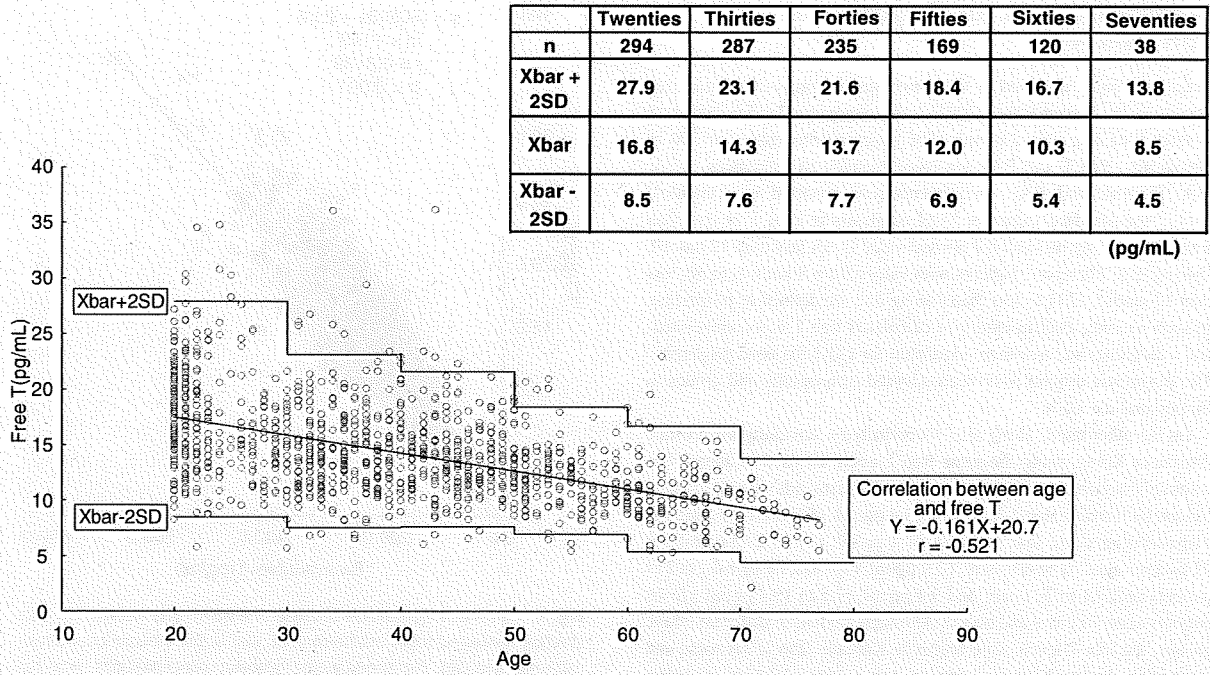
**Standard Total T Values.** Total T showed a tendency toward slight decrease [ $y = -0.027x + 5.5$  ( $r = -0.276$ )] with age, but remained consistent with little change in subjects aged 50 and above. Therefore, it was determined unnecessary to establish standard total T values for each age group, and statistical analysis was performed on all the measured values combined. As a result, the histogram of total T in subjects aged 20 to 70 years corresponded to a square-root distribution, and the standard range obtained from mean  $\pm 2SD$  was 2.01 to 7.50 ng/mL (mean: 4.32 ng/mL;  $n = 1143$ ) (Fig. 1).

**Standard Free T Values.** Free T decreased with age, showing a strong correlation with age. The correlation was expressed as  $y = -0.161x + 20.7$  ( $r = -0.521$ ), and the rate of decrease was  $-1.61$  pg/mL ( $-9.2\%$ ) per decade. Therefore, standard free T values were established for each age range by 10-year intervals. All the optimal histograms for the age groups corresponded to a square-root distribution. As a result, the normal ranges obtained from mean  $\pm 2SD$  were 8.5 to 27.9 pg/mL (mean: 16.8 pg/mL;  $n = 294$ ) in the 20s age group, 7.6 to 23.1 pg/mL (mean: 14.3 pg/mL;  $n = 287$ ) in the 30s age group, 7.7 to 21.6 pg/mL (mean: 13.7 pg/mL;  $n = 235$ ) in the 40s age group, 6.9 to 18.4 pg/mL (mean: 12.0 pg/mL;  $n = 169$ ) in the 50s age group, 5.4 to 16.7 pg/mL (mean: 10.3 pg/mL;  $n = 120$ ) in the 60s age group, and 4.5 to 13.8 pg/mL (mean: 8.5 pg/mL;  $n = 38$ ) in the 70s age group.

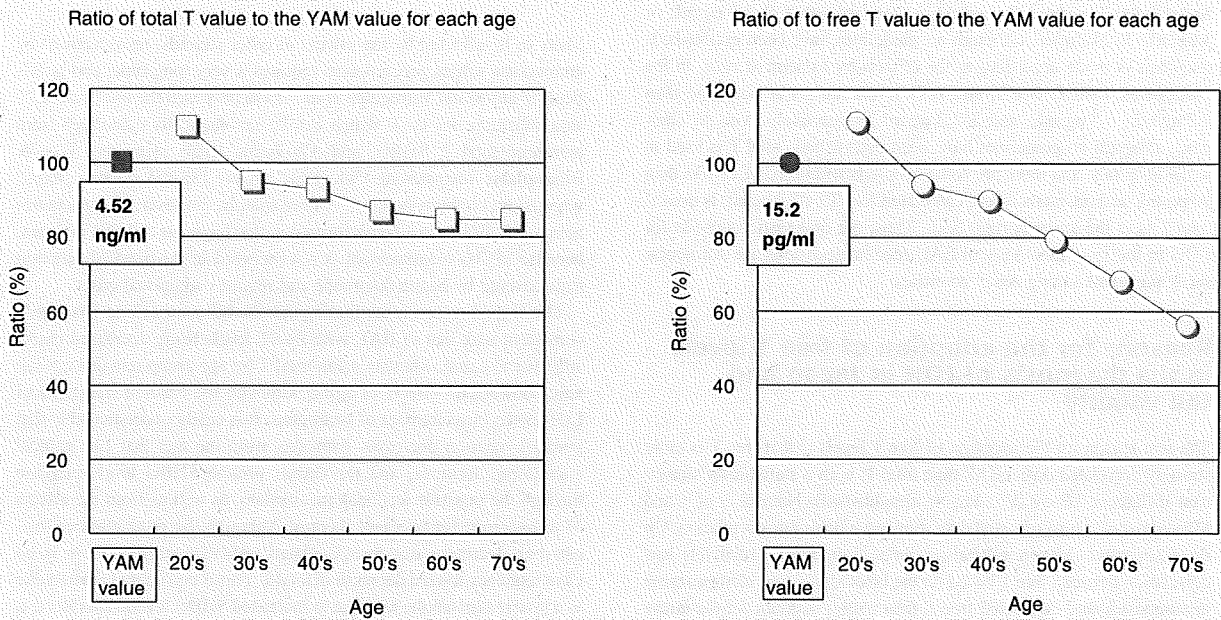
In this age range, the upper and lower limits of the standard range by age group changes from 27.9 to 13.8 pg/mL and from 8.5 to 4.5 pg/mL, respectively, showing that the rate of decrease is about 50% for both limits. However, when compared in absolute values, the upper limit of the standard range shows a large decrease of  $-14.1$  pg/mL over a period of 60 years, whereas the lower limit of the standard range shows a small decrease of  $-4.0$  pg/mL, with the mean lower limit of the standard values being 6.8 pg/mL (Fig. 2). The standard values established this time are in good agreement with those established by Ooi *et al.*<sup>23</sup> using the same assay kits from DPC. It is particularly interesting that the standard free T values in the respective age groups are in good agreement between Japanese and Westerners, although they have different physiques and lifestyles, such as dietary habits.

**Examination Based on the Young Adult Mean.** Sato *et al.*<sup>24</sup> have reported that, regarding the relationship between LOH and free T, abnormally low free T values are not necessarily consistent with clinical symptoms, even taking into consideration the standard values by age group. More specifically, they point out that patients with LOH do not necessarily have low testosterone levels. To resolve this issue, the Free-T Review Committee proposed an approach using the young adult mean (YAM) percentage as an indicator of the free T level currently maintained in comparison with the young adult level. As the effect of the age-related decreases in the standard values is more marked for free T than for total T, it is possible to detect an abnormal value with assessment using the YAM percentage, even within the standard ranges of total T and free T.

The YAM is already applied in routine clinical practice for the evaluation of bone mineral density in osteoporosis based on evidence-based medicine.<sup>25,26</sup> The YAM for total T and free T, calculated as means from young adults aged 20 to 39 years, were 4.53 ng/mL and 15.2 pg/mL, respectively. Using the YAM percentage obtained by dividing measured individual values by the YAM, changes in the standard values by age group were examined (Fig. 3). The results showed that the lower limit of the standard range of total T decreased to about 80% in men in their 50s, and then remained almost unchanged in older men up to their



**Fig. 2** For the analysis of free testosterone, all subjects aged 20 to 77 years were divided into age groups defined by 10-year intervals, and statistical analysis was conducted on data by age group. The standard range for each age group was defined as Xbar ± 2 standard deviation. A tendency toward gradual decrease with advancing age was observed. (Standard values by age group are displayed in the table within the figure.)



**Fig. 3** In the standard values (mean) by age group, the total testosterone percentage compared to the young adult mean decreased to about 80% in men in their 50s, and then remained almost unchanged thereafter, whereas an almost linear decrease to 56% in free testosterone was observed in men up to their 70s.

70s, whereas an almost linear decrease to 56% in free T was observed in men up to their 70s. Therefore, it is determined to adopt free T levels in the diagnosis of LOH, and the normal lower limit was set at a mean-2SD value of 8.5 pg/mL for men in their 20s. It has been pro-

posed that patients with values of not less than 8.5 pg/mL and less than 11.8 pg/mL, which is 70% of the mean value for men in their 20s, are to be indicated for androgen replacement therapy (ART) as LOH borderline cases.

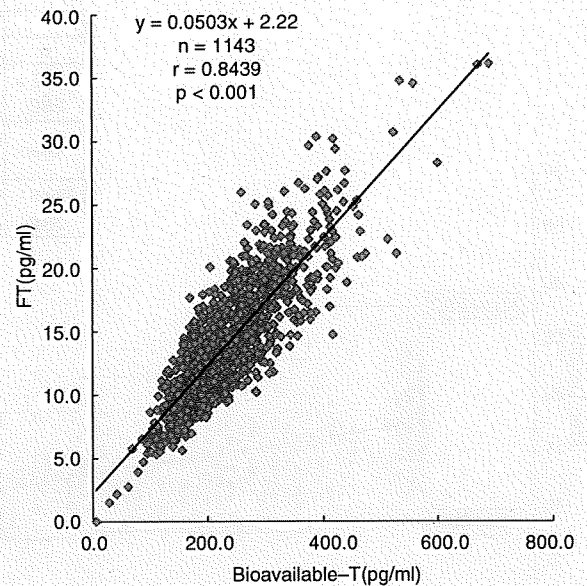
### Standard values for diagnosis of LOH overseas

In the guidelines approved by the consensus meeting of the Endocrine Society in the United States (2001),<sup>27</sup> the standard value for indication is total T of less than 2 ng/mL. For patients with values of 200 to 400 ng/mL, free T and BAT are recommended as references. In the ISA, ISSAM, and EAU recommendations reported by Lunenfeld *et al.*,<sup>28</sup> the standard value for LOH diagnosis is total T of less than 8 nmol/L (231 ng/dL) and the normal value is not less than 12 nmol/L (346 ng/dL). Therefore, patients with values from 8 nmol/L to not less than 12 nmol/L (231 to less than 346 ng/dL) are defined as borderline. In such patients, determination of calculated free T is recommended, and a diagnosis and treatment algorithm for LOH has been prepared.<sup>27</sup> The diagnostic criteria have been adopted in hospitals and clinics in Europe and the USA.

On the other hand, Hong *et al.*<sup>29</sup> conducted a screening for prostate disease in Korea, using the same free T assay kit used in Japan. The results showed that, in 403 patients (42 to 78 years old) without prostate cancer, the median free T level was 19.4 pg/mL (8.8 to 31.0 pg/mL) in 41 patients aged less than 50 years, 17.1 pg/mL (1.9 to 29.5 pg/mL) in 97 patients aged 51 to 60 years, 12.9 pg/mL (1.9 to 27.8 pg/mL) in 219 patients aged 61 to 70 years, and 9.4 pg/mL (1.5 to 19.7 pg/mL) in 76 patients aged 70 years and above. Although studied in different ethnic groups, the free T levels were higher in Koreans than in Japanese and Canadians. In the Second Japanese-Asian Men's Health & Aging Conference held in Japan in 2007, the diagnostic method for LOH was investigated in participating physicians from Asian countries. It was found that, in China, total T is mainly used, although there are some hospitals where calculated bioavailable testosterone (cBAT) is used. In Korea, total T is used, by setting a standard value of 2.8 ng/mL or 3 ng/mL. In Malaysia, although no guidelines have been established, diagnosis is made according to the criteria by Lunenfeld *et al.* In the Philippines, total T, free T, and BAT levels are set at not more than 2.7 ng/mL, 5.5 pg/mL, and 281.3 pg/mL, respectively. Also in Thailand, although no guidelines have been established, total T is used in many hospitals, and there are some hospitals where free T of less than 90 pg/mL and hospitals where calculated free T of 7.2 ng/dL is used if total T is at the lower normal limit. Thus, the diagnostic methods for LOH in the ISA, ISSAM, and EAU recommendations are not necessarily prevalent among Asian countries.

### Reasons for the adoption of free T levels in the diagnosis of LOH in Japan and the validity

The first reason for the adoption of free T levels is that free T is more strongly correlated with cBAT than total T, with a statistically significant difference ( $P < 0.005$ ; Fig. 4; unpublished). Namiki *et al.*<sup>12</sup> and Matsuda *et al.*<sup>13</sup> also reported data showing a favorable correlation. On the other hand, Martinez-Jabaloyas *et al.*<sup>30</sup> reported a relatively low correlation between free T based on the labeled analogs, using enzyme immunoassay and calculated free T, thus casting a doubt for the utility of free T. Although we don't know the exact reason for such difference, free T at least measured using the DPC kit seems to be clinically useful. Internationally, the measurement of BAT and cBAT has been considered. However, routine measurement of BAT is not possible for the following reasons: a complicated high-performance liquid chromatography technique has to be used for the measurement of BAT; although the measurement of BAT by treating SHBG-T with ammonium sulfate precipitation is considered to be the most reliable method that is less



**Fig. 4** Calculated bioavailable testosterone (cBAT) was obtained by substituting measured total testosterone as well as the albumin-bound testosterone and sex hormone-binding globulin concentrations into the equation in the literature<sup>11</sup> shown below. The finding of a favorable correlation between BAT and free testosterone means that free testosterone can be used as an indicator to predict BAT levels.

likely to be affected by the effects of other steroids, measurement by ammonium sulfate precipitation requires a very long time, and is difficult to automate. Vermeulen *et al.*<sup>11</sup> have proposed the use of cBAT<sup>4,5</sup> as a substitute for BAT, which can be automatically calculated from measured total T, SHBG, and Alb-Tumin levels using the 'Free & Bioavailable Testosterone Calculator' on the ISSAM website (<http://www.issam.ch/freetesto.htm>). Unfortunately, however, the measurement of SHBG cannot be performed in the diagnosis of LOH in Japan, because SHBG measurement is not included in the gonadal function tests covered by health insurance and requires additional costs.

The second reason is the finding that the behavior of free T is highly consistent with that of BAT, with free T levels being decreased markedly due to aging, whereas mean total T by age group remains consistent, showing no effects of aging, although the basis of diagnosis of LOH is the measurement of androgens. It is easily understandable that gonadal function decreases with age, from the fact that LH, follicle stimulating hormone, and the carrier protein SHBG levels increase through the negative feedback mechanism, as well as from the effects of testosterone replacement therapy. Although the production of testosterone decreases due to age-related testicular atrophy, there is no clear tendency toward decrease in total T. This is considered attributable to a lowered metabolic rate due to increased SHBG levels. Therefore, it is considered that the decrease in total T occurs at a late stage where LOH has advanced, whereas the decrease in free T occurs at a relatively early stage. From this point of view, free T is considered to be a potentially more sensitive indicator of LOH than total T. In addition, practically, under the current circumstances in Japan, either one of total T or free T has to be chosen as the test covered by health insurance.

The third reason is that, in the measurement of free T by the assay kit from DPC, we employed a method<sup>28</sup> allowing the measurement of free

T alone without affecting the balance between free T and protein-bound T in blood, and carried out measurement in consideration of the blood sampling time points and the stability of serum samples during storage. As mentioned above, this measurement system was designed to prevent the binding of labeled-T to SHBG or Alb-Tumin. In particular, Alb-Tumin has a hydrophobic cavity in the structure, and serves as a non-specific carrier of many hydrophobic compounds (lipids, steroids, drugs). Thus, as a compound (e.g. sulfobromophthalein) that easily binds to the hydrophobic cavity of Alb-Tumin is added in advance in the measurement system, this method can prevent the influence of Alb-Tumin on the measurement, leading to an improvement in the precision of detection of free T.

The fourth reason is that, as mentioned earlier, the mean total T by age range decreases only to 80% of the YAM even during presenile and senile periods, when LOH occurs most frequently, but the free T level shows a linear decrease with aging and drops to 50% of the YAM. In addition, the rate of age-related decrease in free T varied greatly in the upper and lower limits of the standard range. More specifically, over a period of 50 years from the 20s to 70s, the upper limit showed a decrease by 14.1 pg/mL from 27.9 to 13.8 pg/mL, while the lower limit showed a small decrease by 4.0 pg/mL from 8.5 to 4.5 pg/mL. This identifies the fact that, in free T, both the upper limit of the standard range and the mean value decrease greatly with age, but the lower limit is almost unchanged. It means that, for the establishment of a standard value for diagnosis of LOH, it is more reasonable to adopt the approach assessing what magnitude of decrease in the upper limit of the standard range of free T from the peak value in the 20s induces LOH than the approach simply focusing on the lower limit of the standard range for each age group.

For these reasons, free T has been used as an essential test in the diagnosis of LOH in Japan. ISSAM<sup>28</sup> defines LOH as 'a biochemical syndrome associated with advancing age and characterized by a deficiency in blood testosterone levels.' Assuming that free T carries out its role as a bioavailable androgen, a decrease in this hormone means the induction of so-called andropause. Interestingly, from this point of view, our finding that the lower limit of the standard range of free T remains relatively constant regardless of age can be taken as the minimum blood concentration for the maintenance of 'manliness.'

## Summary

Determination of androgen levels is essential to the diagnosis of LOH. However, under the current situation, there is no consensus regarding which androgens should be measured for the diagnosis of LOH. The ISA, ISSAM, and EAU recommend measurement of total T levels, which do not necessarily decrease significantly with age, in the diagnosis of LOH. One reason for this may be the difficulty in directly measuring active androgens. Even BAT and calculated BAT are not yet available for routine clinical use due to technical or precision problems. For the reasons described above, it is determined to adopt free T levels in the diagnosis of LOH in Japan. The standard value for diagnosis of LOH is set at a mean-2SD value of 8.5 pg/mL, by calculating the mean value in young adults in their 20s (the YAM). In addition, it has been proposed that patients with values of not less than 8.5 pg/mL and less than 11.8 pg/mL, which is 70% of the mean value for men in their 20s, are to be indicated for ART as LOH borderline cases.

The Japanese Urological Association and the Japanese Association of Men's Health have started verification of the appropriateness of free T as a biological marker of LOH, in consideration of the various clinical symptoms of LOH. In Japan, the use of the YAM of free T in the standard diagnostic criteria for LOH has been proposed. However, it

should be verified that free T is clinically applicable as a criterion for determining the LOH cases to be indicated for ART. In addition, it is required to clarify whether normalization of androgen levels by ART can lead to improvement of symptoms.

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Members of Working Committee of the Japanese Urological Association for free testosterone to reestablish the standard values of total T and free T in the serum in adult Japanese males

Katsuyuki Baba MD  
Mikihiko Honda MD  
Nobuhisa Ishii MD  
Tadaichi Kitamura MD  
Yukihiro Kondo MD  
Ken Marumo MD  
Tsuneharu Miki MD  
Koichi Nagao MD  
Hiroshi Okada MD  
Yoshikazu Sato MD  
Jintetsu Sou MD  
Ryuji Tomiyama, Basci  
Suguru Usui MD

Shinichi Hisasue MD  
Shigeo Horie MD  
Naoki Itoh MD  
Eitetsu Koh MD  
Yoshiaki Kumamoto MD  
Tadashi Matsuda MD  
Kouei Muguruma MD  
Hajime Nawata MD  
Haruaki Sasaki MD  
Taro Shuin MD  
Masanobu Tanimura MD  
Akira Tsujimura MD  
Hiromi Yokoyama MD



## ORIGINAL ARTICLE

**The relationship of serum and salivary cortisol levels to male sexual dysfunction as measured by the International Index of Erectile Function**Y Kobori<sup>1</sup>, E Koh<sup>1</sup>, K Sugimoto<sup>1</sup>, K Izumi<sup>1</sup>, K Narimoto<sup>1</sup>, Y Maeda<sup>1</sup>, H Konaka<sup>1</sup>, A Mizokami<sup>1</sup>, T Matsushita<sup>2</sup>, T Iwamoto<sup>3</sup> and M Namiki<sup>1</sup><sup>1</sup>Department of Integrative Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan; <sup>2</sup>Ofuna Chuo Hospital, Ofuna, Kanagawa, Japan and <sup>3</sup>IUHW Hospital, Nasushiobara, Tochigi, Japan

To evaluate the biomarkers of sexual function, we investigated the relationship between questionnaire responses and biological hormones such as testosterone (T) and cortisol (F) in serum and saliva. The study population included 105 men aged 30–72 years (mean: 49 ± 4.5, median: 49). Levels of all serum hormones (Total-T, Free-T, Bioavailable-T, Total-F and Bioavailable-F) and salivary hormones (Saliva-T and Saliva-F) were measured directly by liquid chromatography/tandem mass spectrometry. The International Index of Erectile Function (IIEF) was used as a questionnaire to evaluate sexual dysfunction. Free-T and Bioavailable-T showed significant inverse correlations with age ( $P < 0.01$ ). In the group not taking antidepressants, the levels of Bioavailable-F and Saliva-F showed significant inverse correlations with a portion of the IIEF score ( $P < 0.05$ ). However, reductions in Bioavailable-T and Saliva-T showed no association with the IIEF score. In the group taking antidepressants, these hormone levels showed no correlation with IIEF. *International Journal of Impotence Research* (2009) 21, 207–212; doi:10.1038/ijir.2009.14; published online 7 May 2009

**Keywords:** testosterone; cortisol; erectile dysfunction; liquid chromatography/tandem mass spectrometry

**Introduction**

Male sexual hormones such as androgens are generally considered to be associated with sexual function. Among the androgens, testosterone (T) is a useful marker that is frequently used to evaluate male hypogonadism. Androgen production decreases with aging, and decreased testosterone is thought to induce late-onset hypogonadism, one of the most common symptoms of which is erectile dysfunction (ED).<sup>1</sup> Late-onset hypogonadism is defined as a biochemical syndrome associated with advancing age and characterized by a deficiency in serum androgen levels with or without decreased genomic sensitivity to androgens. It may result in significant alterations in the quality of life and

adversely affect the function of multiple organ systems.<sup>2</sup> In Japan, measurement of free testosterone is recommended to diagnose late-onset hypogonadism.

Serum total testosterone (Total-T) consists of testosterone that binds strongly to sex hormone-binding globulin (SHBG), the testosterone that loosely binds to albumin and free testosterone (Free-T). The latter two are designated collectively as bioavailable-T (Bio-T), and its measurement is considered to be an index of androgen activity.<sup>3</sup> In addition, the T of saliva (Sa-T) is regarded as free testosterone, and its measurement is thought to reflect blood Free-T and Bio-T.<sup>4</sup> It has been reported that Total-T does not vary, but Free-T decreases gradually with aging.<sup>5</sup>

Cortisol (F), which is an adrenal cortical hormone, does not show changes in level with aging, but is increased by stress.<sup>6</sup> Cortisol remains constant after increasing during the period of sexual maturation at the same time as T. Cortisol is known to raise blood pressure and blood sugar levels as well as cause sterility and immune dysfunction. When we feel stress, fear or tension, sympathetic nervous activity

Correspondence: Dr Y Kobori, Department of Integrative Cancer Therapy and Urology, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan.  
E-mail: bori2555@mail.goo.ne.jp

Received 7 November 2008; revised 2 April 2009; accepted 2 April 2009; published online 7 May 2009

is increased relative to parasympathetic nervous activity, and the penis is contracted. Blood cortisol levels are known to increase in the hypothalamic-pituitary-adrenocortical system at the same time as that in which blood norepinephrine levels increase in the sympathetic nervous-adrenal medullary system when sympathetic nervous activity is dominant. Thus, ED may occur when cortisol levels are high. Although the cortisol level may reflect sexual function, there have been no earlier investigations of the relationships between these two parameters. Similar to Bio-T, bioavailable cortisol (Bio-F) is an active form that does not bind to globulin, and saliva cortisol (Sa-F) is secreted in the saliva as the free type.<sup>7</sup> Here, we developed methods to measure testosterone and cortisol levels in the blood and saliva by liquid chromatography/tandem mass spectrometry (LC/MS-MS).<sup>8</sup> We measured various hormones directly using this method and investigated the correlations of these hormone levels and sexual function on the basis of questionnaire responses from 105 patients.

### Materials and methods

#### Patients

The study population included 103 men aged 32–72 years (mean: 49 ± 4.5, median: 49). All patients visited our hospital for treatment of urological symptoms, including late-onset hypogonadism, with or without ED. Thirteen patients were taking antihypertensive agents and two patients were taking antidiabetic drugs. None of the patients were taking hormone medication (supplementation or deprivation) or phosphodiesterase type 5 (PDE5) inhibitors. All patients gave full informed consent to participate in this study. Saliva and serum samples

were collected in the morning (between 0900 and 1100 hours). In addition, we investigated their sexual function and examined whether the patients had taken any psychotropic drugs. Each patient also completed validated questionnaires exploring anxiety and depression (Hospital Anxiety and Depression Scale [HAD scale]). This study was approved by the Internal Review Board of Kanazawa University, and was supported by a medical research fund from Kanazawa University Hospital (Kanazawa, Japan).

#### LC-MS/MS

Levels of all serum hormones (Total-T, Free-T, Bio-T, Total-F and Bio-F) and salivary hormones (Sa-T and Sa-F) were measured directly by LC/MS-MS. LC/MS-MS is effective for determining fixed quantities of very small amounts of material in biological samples.<sup>9</sup> The LC/MS-MS apparatus used was an API4000 (Applied Biosystems, Kanazawa, Ishikawa, Japan) mass spectrometer and an HP1100 (Hewlett-Packard, Kanazawa, Ishikawa, Japan) liquid chromatograph. Bioavailable T (Bio-T) and F (Bio-F) were determined after separation of the SHBG-bound steroid.

Conventionally, Bio-T was calculated on the basis of albumin, SHBG and Total-T, using the method proposed by the International Society for the Study of the Aging Male. However, Bio-T and Bio-F were measured directly as levels of non-SHBG-bound T and F using LC/MS-MS in our study.

#### Definition of erectile function

To evaluate sexual dysfunction, the International Index of Erectile Function (IIEF) was used as a questionnaire study.<sup>10</sup> IIEF is divided into five domains (erectile function, orgasmic function, sexual desire, intercourse satisfaction and overall

**Table 1** Correlation coefficient between the age and hormonal profile

	Testosterone				Cortisol		
	Total	Free	Bio	Saliva	Total	Bio	Saliva
Age	0.01	-0.35***	-0.30**	-0.19*	0.07	0.10	0.17
<i>Testosterone</i>							
Total	—	0.52****	0.65****	0.47****	0.02	0.06	0.08
Free	—	—	0.77****	0.67****	0.18*	0.15	0.16
Bio	—	—	—	0.71****	0.05	0.14	0.08
Saliva	—	—	—	—	0.06	0.13	0.12
<i>Cortisol</i>							
Total	—	—	—	—	—	0.91****	0.80****
Bio	—	—	—	—	—	—	0.79****
Saliva	—	—	—	—	—	—	—

n = 103.  
\*P < 0.10, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001.  
Speaman's rank test.

satisfaction) and IIEF5 was evaluated simultaneously.<sup>11</sup> Men who did not complete the questionnaire were excluded from statistical analysis.

### Statistical analysis

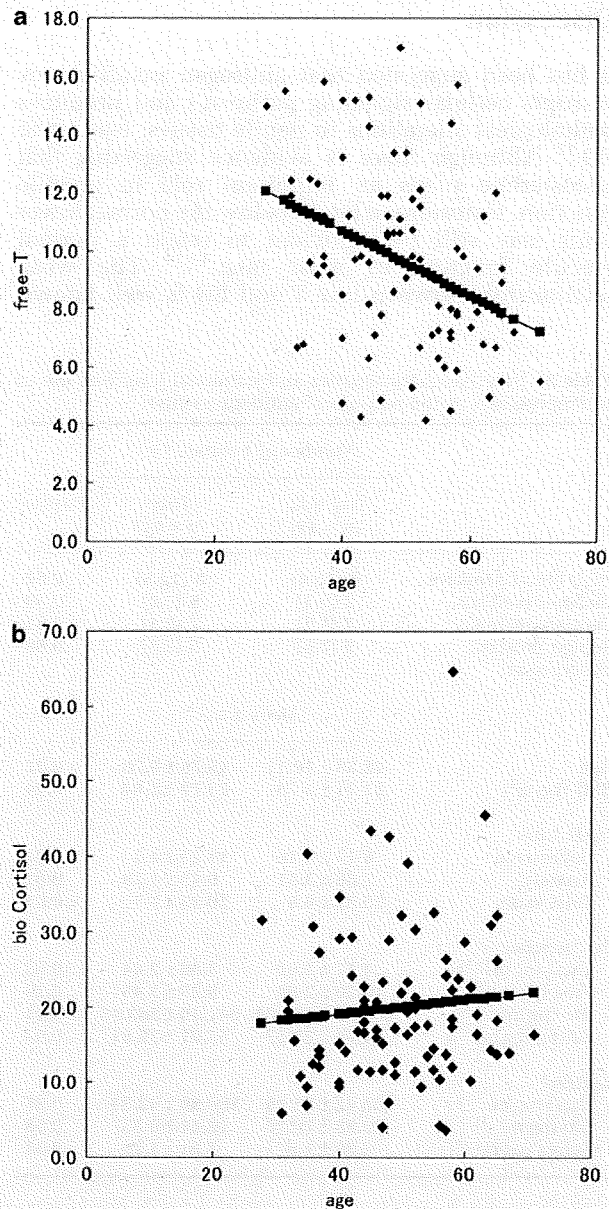
Data from completed questionnaires were input into a Microsoft Excel spreadsheet. All analyses were performed with a SAS package (version 8.1) and differences were considered significant at  $P < 0.05$ . The hormonal values were subjected to logarithmic transformation and controlled for age. Thereafter, we examined correlations between IIEF responses and hormonal levels. Spearman's rank test was used to test the correlations between age and hormonal profile, IIEF score and hormonal profile.

## Results

The relationships of testosterone and cortisol to age are shown in Table 1. Free-T and Bio-T showed significant inverse correlations with age ( $P < 0.01$ ). Sa-T showed a tendency toward a negative correlation, but the relation was not significant. Cortisol did not have a meaningful correlation with age (Figure 1). In addition, the levels of each type of testosterone (Total-T, Free-T, Bio-T and Sa-T) showed very strong correlations to the other forms of testosterone and there were also correlations among cortisol levels (Total-F, Bio-F and Sa-F).

Table 2 shows the baseline characteristics and the levels of each hormone in men who did and did not take psychotropic drugs. Sixty-four of the 103 patients included in the investigation (62%) took psychotropic drugs. The HAD scale was significantly high in the group taking psychotropic drugs. It was suggested that populations of men who took psychotropic drugs suffered from anxiety and depression. With regard to different types of psychotropic drugs, 15 patients took only selective serotonin reuptake inhibitors (SSRI), 14 took serotonin and norepinephrine reuptake inhibitors (SNRI), 9 took tricyclic antidepressants, 10 took minor tranquilizers and 16 patients took antidepressants and minor tranquilizers. Men who did not take psychotropic drugs showed significantly lower Total-T levels. There were no significant differences in levels of hormones other than those of Total-T.

When we examined the correlation between hormonal level and IIEF response, the hormonal levels were controlled for age, as it is well known that testosterone shows a significant inverse correlation with age. Table 3 shows the correlation coefficients for each domain of IIEF (erectile function, orgasmic function, sexual desire, intercourse satisfaction and overall satisfaction), IIEF5 and total score of IIEF, with various hormones in men who did not take psychotropic drugs. Bio-F showed negative correlations with erectile function, sexual



**Figure 1** (a) Free testosterone showed significant inverse correlations with age ( $n = 103$ ,  $R = -0.35$ ,  $P < 0.001$ ). (b) On the other hand, bioavailable cortisol did not have a meaningful correlation with age ( $n = 103$ ,  $R = 0.10$ ,  $P = 0.10$ ).

desire, IIEF5 and total IIEF score ( $P < 0.05$ ). In addition, Sa-F showed negative correlations with intercourse satisfaction and total IIEF score ( $P < 0.05$ ). However, testosterone did not show a meaningful correlation with any of the IIEF domains (Figure 2).

There were no significant correlations between the various hormones and IIEF in this group who took psychotropic drugs.

## Discussion

It has been suggested that androgen insufficiency disrupts cellular signaling pathways and produces pathological alterations in penile tissues, leading to ED.<sup>12</sup> Although there is evidence suggesting that testosterone plays an important role in erectile function, testosterone levels below the normal lower limit may still be sufficient to retain a normal erectile function in most men.<sup>13,14</sup> The weak relationship between low T and ED is well known

**Table 2** Baseline characteristics and hormonal level (the use of antidepressants vs the non-use of antidepressants)

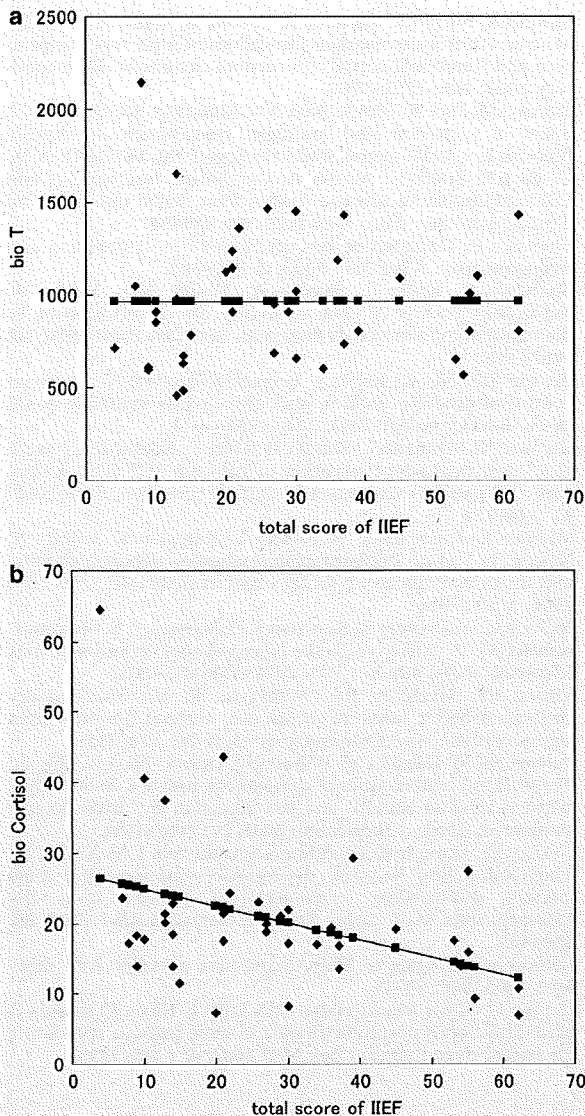
	Number of patients (%)		P-value
	Not take (n = 39)	Take (n = 64)	
High blood pressure	6 (15.4)	8 (12.5)	0.67
Diabetes mellitus	2 (5.1)	5 (7.8)	0.59
Cardiac treatment	2 (5.1)	2 (3.1)	0.61
Benign prostatic hyperplasia	3 (7.6)	5 (7.8)	0.98
	Mean ± s.d.		
Age	48.38 ± 10.11	49.76 ± 9.78	0.52
BMI (kg/m <sup>2</sup> )	22.41 ± 2.78	23.77 ± 2.64	0.44
<i>HAD scale</i>			
Depression	8.13 ± 4.16	11.23 ± 4.2	<0.01
Anxiety	7.68 ± 3.61	9.45 ± 4.29	0.02
Total score	15.81 ± 6.8	20.55 ± 7.5	<0.01
<i>Testosterone</i>			
Total (ng ml <sup>-1</sup> )	4.77 ± 1.60	3.86 ± 2.44	0.03
Free (pg ml <sup>-1</sup> )	10.20 ± 2.85	9.27 ± 3.43	0.16
Bio (pg ml <sup>-1</sup> )	965.73 ± 359.81	841.45 ± 383.46	0.11
Saliva (pg ml <sup>-1</sup> )	55.70 ± 23.57	55.91 ± 29.81	0.97
<i>Cortisol</i>			
Total (ng ml <sup>-1</sup> )	108.42 ± 35.05	100.89 ± 42.34	0.35
Bio (ng ml <sup>-1</sup> )	20.58 ± 10.78	19.41 ± 9.95	0.58
Saliva (ng ml <sup>-1</sup> )	2.36 ± 1.57	1.91 ± 1.26	0.13

and supported by the results of both human and animal studies.<sup>15</sup> A lack of association between T and IIEF-5 has been documented in a large consecutive series of almost 1000 elderly individuals with or without ED.<sup>16</sup> In ED patients, hypogonadism is often associated with reduced sexual desire and nocturnal penile erections, whereas association with sex-induced erection is less evident. This is because T regulates not only cyclic guanosine monophosphate formation through nitric oxide synthase stimulation but also its catabolism through PDE5 activity. Androgens positively regulate PDE5, thus providing a possible explanation for the highest levels of this enzyme in the male genital tract.<sup>17</sup> The androgen-dependent PDE5 expression could explain the reduced effectiveness of PDE5 inhibitors in the treatment of ED in hypogonadal patients. As T positively regulates both the initiation (nitric oxide synthase) and the end (PDE5) of the erectile process, its net effect on erection is modest. Hence, erections are still possible under hypogonadal conditions in which decreased cyclic guanosine monophosphate formation because of impaired NO production is most likely counterbalanced by regulated PDE5 activity and cyclic guanosine monophosphate hydrolysis. The main physiological action of T is therefore to ensure the timely adjustment of the erectile process as a function of sexual desire, therefore finalizing erection associated with sex. A trophic effect of T on the penile architecture has also been shown in different animal species.<sup>18</sup> For all of the above reasons, treating hypogonadism restores impaired penile erections in experimental animal models, as well as in a clinical setting. Conversely, T administration to otherwise eugonadal individuals is rather ineffective. There have been many reports that sexual function is improved by androgen replacement therapy.<sup>19-21</sup> All the above considerations explain the well-known weak correlation between ED and T. In this study, there was no correlation between testosterone and IIEF responses. That is, testosterone is not an appropriate biomarker for an evaluation of sexual function.

**Table 3** Correlation coefficient between the IIEF score and hormonal profile (The group who did not take psychotropic drugs)

	Testosterone				Cortisol		
	Total	Free	Bio	Saliva	Total	Bio	Saliva
Erectile function	-0.26	-0.07	-0.01	-0.13	-0.28	-0.39**	-0.30
Orgasmic function	-0.10	-0.06	0.05	0.04	-0.20	-0.27	-0.19
Sexual desire	-0.08	-0.05	-0.02	-0.20	-0.30	-0.44**	-0.35*
Intercourse satisfaction	-0.23	-0.12	0.00	-0.11	-0.26	-0.33*	-0.37**
Overall satisfaction	-0.07	-0.33*	-0.16	-0.17	-0.19	-0.29	-0.32*
Total score of IIEF	-0.24	-0.12	-0.01	-0.13	-0.31*	-0.43**	-0.37**
IIEF5	-0.25	-0.08	0.00	-0.15	-0.29	-0.38**	-0.30*

\*P < 0.10, \*\*P < 0.05.  
Spearman's rank test.  
n = 32.



**Figure 2** (a) Bioavailable testosterone did not show a meaningful correlation with the total score of IIEF ( $n=32$ ,  $R=-0.01$ ,  $P=0.98$ ). (b) Bioavailable cortisol showed negative correlations with the total score of IIEF score ( $n=32$ ,  $R=-0.43$ ,  $P<0.05$ ).

Bio-F and Sa-F showed significant negative correlations with some domains of IIEF in this study. In a rat model of adrenal insufficiency, it was shown earlier that the rat adrenal gland contributes to the maintenance of the erectile mechanism and may affect the neuronal nitric oxide synthase content in the penis.<sup>22</sup> In a human study, on the other hand, there were no differences in cortisol levels between individuals with and without ED.<sup>23</sup> These studies did not include detailed differential counts of cortisol. However, in our study, Bio-F and Sa-F (not Total-F) showed negative correlations with the IIEF score. This suggested that Bio-F and Sa-F, which are active forms of cortisol, likely decrease

sexual function. One reason why ED occurred in patients with high levels of cortisol is because cortisol is increased by stress. It is known that the blood cortisol level increases at the same time as the blood norepinephrine level when sympathetic nervous activity is dominant. Sympathetic nervous activity has a restraining effect on erection, and sexual function is thought to be reduced under stress.<sup>24,25</sup> Our results indicate that increases in plasma and salivary cortisol may play causative roles in ED induced by social stress.

In studies of stress, cortisol and norepinephrine have been overused as standard stress markers. Cortisol level is an index of the endocrine response to stress and shows comparatively high normal values in blood ( $100-150\text{ ng ml}^{-1}$ ). We can analyze cortisol from saliva if we use a high sensitivity analytical procedure such as ELISA or LC-MS/MS.<sup>26</sup> Measuring cortisol in saliva may become a useful index for evaluating the sexual function non-invasively. In addition, saliva-based measurement methods have attracted attention for measuring amylase in saliva as a marker of the sympathetic nervous system response to stress.<sup>27</sup> Further studies in this field are needed.

In the men who took antidepressants in this study, there were no significant correlations between hormone values and IIEF. Neither testosterone nor cortisol values played an important role in erectile function in the group taking antidepressants. This was thought to be because ED and ejaculation disorder are induced by the actions of antidepressant medication. Furthermore, psychiatric disorder in itself may be a cause of ED.<sup>28</sup> Patients with depression may have elevated cortisol levels.<sup>29</sup> In addition, this type of drug could result in an increase in the prolactin level determining the reduction of T.<sup>30</sup> Therefore, we could not obtain meaningful data from the patients taking psychotropic drugs.

## Conclusions

The active forms of cortisol (Bio-F and Sa-F) showed negative correlations with sexual function in men who did not take psychotropic drugs, although there was no such correlation for testosterone. ED is thought to occur in patients with high levels of cortisol because of the relations between cortisol and stress. Cortisol may thus become a useful index for the evaluation of sexual function.

## Conflict of interest

The authors declare no conflict of interest.

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