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Keywords

Salivary testosterone

Late-onset hypogonadism (LOH)

Liquid chromatography/ mass spectrometry (LC-MS)

Enzyme-linked immunosorbent assay (ELISA)

# Diagnostic significance of salivary testosterone measurement revisited: using liquid chromatography/mass spectrometry and enzyme-linked immunosorbent assay

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## Abstract

**Background & Objectives:** The use of saliva as a material for screening biomarkers has several advantages in the study of large research populations. Since testosterone is not bound to protein in saliva, salivary testosterone determination provides an excellent approach for the evaluation of serum bioavailable or free testosterone. Liquid chromatography/mass spectrometry (LC-MS) has been considered to be a gold standard for estimating serum total testosterone levels in male serum. Our objective was to evaluate the reliability of salivary testosterone levels as measured by LC-MS. We also investigated the association between salivary testosterone measured by LC-MS and that measured by enzyme-linked immunosorbent assay (ELISA) in order to evaluate the clinical application for ELISA measures.

**Methods:** The study involved 51 healthy male volunteers (median age = 57 years old; range = 30–85 years) and 29 patients with late-onset hypogonadism (LOH) (median age = 65 years; range = 55–78 years) in order to include a wide range of testosterone levels (median age of all subjects = 65 years; range = 30–85 years). Serum total testosterone was measured using LC-MS, and sex hormone binding globulin (SHBG) by immunoradiometric assay. Serum free testosterone and bioavailable testosterone levels were calculated using an international formula. Salivary testosterone levels were measured using LC-MS and ELISA.

**Results:** Salivary testosterone levels measured by LC-MS were in accordance with calculated serum free testosterone levels ($r = 0.655$, $p < 0.001$, $y = 0.91x+27.04$; where $x$ is the salivary testosterone measured by LC-MS and $y$ is the calculated free testosterone). Salivary testosterone measured by LC-MS and ELISA showed a strong correlation ($r = 0.808$, $p < 0.001$).

**Conclusion:** Salivary testosterone measured by LC-MS and ELISA is a non-invasive, reliable substitute for serum calculated free or bioavailable testosterone. Considering its cost advantage and technical

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convenience, ELISA for salivary testosterone is now recommended for the purpose of screening the androgen bioavailability level especially in a large population-based study. 

## Introduction

Interest and debate regarding late-onset hypogonadism (LOH), or testosterone deficiency syndrome are increasing throughout the world [1–5]. The Endocrine Society and the European Association of Urology define LOH as a clinical and biochemical syndrome associated with advanced age and characterized by physiological, psychological and sexual symptoms and a deficiency in serum testosterone levels [6,7]. To screen for and diagnose LOH, and to evaluate the efficacy of treatment, an accurate and reliable biochemical index is needed. A robust assessment of the accuracy of testosterone assays remains challenging [8]. Total testosterone circulates mostly in the blood and is 98% bound to serum proteins, primarily sex hormone binding globulin (SHBG) and albumin; only 1–2% of serum testosterone is free of bound protein [9]. The combination of albumin-bound (weakly bound) testosterone and free testosterone is referred to as bioavailable testosterone, which is available to target tissues for androgenic action [10,11]. Serum free and bioavailable testosterone levels calculated from measured total testosterone and SHBG, using the International Society for the Study of the Aging Male (ISSAM) formula are widely accepted [12]. Liquid chromatography/mass spectrometry (LC-MS) has been validated as a gold standard method for estimating serum testosterone levels in male serum using protocols specified by the Federal Drug Administration. It includes the determination of the limits of detection, the limit of quantification (LOQ), the characteristics of the calibration curve, and the within- and between-day reproducibility [13]. The direct measurement of free testosterone levels in serum is currently possible only by using radioimmunoassay with equilibrium dialysis methods, which cannot be used for routine analysis [6]. In addition, this procedure is time-consuming and expensive because at least two low testosterone values must be obtained to confirm the diagnosis of hypogonadism, due to intra-individual variation [14]. In addition, since testosterone levels in healthy men follow a circadian rhythm [15], blood collection should be done early in the morning, which is often difficult in outpatient services.

Testosterone, like all steroid hormones, exists in the saliva in the free (bioavailable) form. Measurement of salivary testosterone levels has a great advantage, overcoming the problems associated with serum testosterone measurements. Saliva collection is simple, non-invasive, and repeatable. The subjects collect their saliva at home at a particular time specified by the researchers. Salivary testosterone is neither bound to proteins nor conjugated [16]. Thus, measuring salivary testosterone does not require dialysis (separation according to the molecule size) before assaying. Previous studies have shown significant correlations between serum free and bioavailable testosterone and salivary testosterone levels measured using radioactive isotopes [17]. The aim of this study was to re-evaluate the use of salivary testosterone levels measured using LC-MS and enzyme-linked immunosorbent assay (ELISA) as a substitute for serum bioavailable or free testosterone levels for clinical practice.

## Subjects and Methods

### Subjects

This study was approved by the Institutional Review Board at Teikyo University. In order to measure a wide range of testosterone levels, participants included 29 newly diagnosed LOH patients (median age = 65 years; range = 55–78 years) whose serum total testosterone levels were less than 300 ng/dl, which is the generally acceptable lower limit of the normal testosterone range in healthy young men [7], and 51 healthy male volunteers (median age = 57 years; range = 30–85 years: median age of all patients = 65 years; range = 30–85 years).

In order to diagnose LOH, the aging males' symptoms rating scale (AMS), which was developed by Heinemann et al. [18] was utilized as well as serum total testosterone levels. The severity of LOH symptoms according to the

AMS total score was classified as none/little (17–26), mild (27–36), moderate (37–49), and severe (50 or more) [19]. At the time of the first visit, the median AMS score of the 29 newly diagnosed LOH patients in this study was 44 (range = 29–52).

All participants provided written informed consent.

### Saliva collection

Subjects were provided with two bakelite test tubes in which to collect saliva twice between 9 am and 9:30 am on a single day. They were asked to avoid brushing their teeth and smoking for at least 1 hour before saliva sampling, as testosterone levels in saliva have been shown to increase post-micro-injury due to brushing teeth [20]. They rinsed their mouths with tap water three times and waited 5 mins, then expectorated at least 1 ml of saliva directly into a collection vial. The 5-min delay was added to prevent the rinse from diluting the salivary testosterone, as it is measured in concentration per volume units (e.g. pg/ml). Patients were asked to refrain from taking sugar-free chewing gum within 1 hour of sample collection, as this gum can change salivary testosterone results [18]. Salivary samples were stored at −20 °C for up to 1 month in laboratory freezers until analyzed.

### Serum collection

For the simultaneous collection of saliva and blood, blood was drawn immediately after saliva sampling to avoid any increase in testosterone levels due to taking a blood sample, which may be a stressor. Blood samples were centrifuged to isolate serum, and serum was stored at −70 °C until analysis.

### Hormone determination

Serum total testosterone and salivary testosterone levels were measured by LC-MS, as described elsewhere [21]. Serum samples (0.1 ml) and saliva samples (1 ml) were analyzed using an API4000 electrospray-ionization mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada). An Agilent 1100 device (Agilent Technologies Inc, Santa Clara, California, USA) was used for high-performance liquid chromatography. An HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a Cadenza CD-C18 column (3 μm, 3 × 150 mm; Imtakt Corporation, Kyoto, Japan) were used for the separation of steroids.

For the mobile phase, we used 0.1% formic acid solution (Solution A) and a mixture of acetonitrile and methanol (1:1, Solution B). Initially, a mixture of Solutions A and B at a ratio of 35:65 was used, and, subsequently, gradient setting was performed so that the volume of Solution B reached 100% within 1.5 mins. This state was maintained for 2.5 mins. After 0.01 mins, the solution was set so that the Solution A:Solution B ratio returned to 35:65 (first system), and this state was maintained for 3 mins. The flow rate was 0.35 ml/min, and the duration of the analysis was 8.5 mins. Measurements were performed in the positive ion mode, and the infusion volume was 10 μl. Measurements were performed under the following conditions: for the quantification of cortisol, we used a product ion m/z 327.1 (IS: m/z 331.3) produced from a precursor ion m/z 363.3 (IS: m/z 367.3). For the quantification of testosterone, we used a product ion m/z 97.3 (IS: m/z 97.3) produced from a precursor ion m/z 289.2 (IS: m/z 292.2). Ion spray voltage and ion source temperature were established as 5000 V and 500 °C, respectively. Serum at 100 μl was diluted with purified water to prepare a capacity of 1 ml. T-d3 (D/N/P, Toronto, Canada) was used as the internal standard methanol solution and its purity was 98.5%. Samples, 1 ml, of saliva and the diluted serum samples were added to 1 ng/100 μl of Td-3, and then stirred. Following the previous process, the samples were mixed with 4 ml of ethyl acetate, shaken for 10 mins, and centrifuged at 3,000 rpm for 10 mins. After the aqueous layer was frozen, ethyl acetate was isolated, and evaporated using a centrifugal evaporator. The extract was dissolved in 100 μl of 70% acetonitrile solution, and 10 μl of this solution was infused into an LC-MS device. SHBG was measured by immunoradiometric assay. Both serum free testosterone and bioavailable testosterone were calculated by measuring total testosterone, SHBG, and albumin concentrations and using the international formula [12]. Saliva testosterone levels were also measured by ELISA (DE-SLV3013, Demeditec Diagnostics, Kiel, Germany) [22]. Briefly, 0.5 ml of saliva sample was applied to the microtiter wells that were

**Table 1** Features of healthy volunteers and LOH patients

| | Healthy volunteers $N = 51$ | LOH patients $N = 29$ |
|---|---|---|
| Age (mean ± SD) (years) | 56.75 ± 10.35 | 65.52 ± 7.7* |
| Serum albumin (mean ± SD) (g/dl) | 4.21 ± 0.28 | 4.4 ± 0.16 |
| Sex hormone binding globulin (mean ± SD) (nmol/l) | 52.94 ± 27.14 | 48.28 ± 19.52 |
| Serum total testosterone (mean ± SD) (ng/dl) | 466.96 ± 147.70 | 238.71 ± 41.03*** |
| Calculated free testosterone (mean ± SD) (pg/ml) | 74.45 ± 21.02 | 40.27 ± 20.61*** |
| Calculated bioavailable testosterone (mean ± SD) (ng/dl) | 171.46 ± 51.26 | 96.93 ± 50.46*** |
| Salivary testosterone (LC-MS) (mean ± SD) (pg/ml) | 40.22 ± 16.90 | 33.00 ± 7.07* |
| Salivary testosterone (ELISA) (mean ± SD) (pg/ml) | 42.85 ± 10.16 | 31.78 ± 10.65* |

LOH, late onset hypogonadism; SD, standard deviation; LC-MS, liquid chromatography/mass spectrometry; ELISA, enzyme-linked immunosorbent assay. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

coated with a mouse monoclonal antibody directed toward an antigenic site on the testosterone molecule. Endogenous testosterone from a salivary sample competed with a testosterone–horseradish peroxidase conjugate for binding to the coated antibody.

## Statistical analysis

SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis.

Statistical differences in each indicator between the healthy volunteers and patients with LOH were examined using a *t*-test. Pearson's correlation coefficient was used to examine the association between two valuables. A *p*-value of <0.05 was considered statistically significant.

## Results

The mean age, serum testosterone, albumin, SHBG, calculated free and bioavailable testosterone and salivary testosterone, as measured by both LC-MS and ELISA in healthy volunteers and LOH patients, are shown in Table 1. There were statistically significant differences in age ($p = 0.012$), serum total testosterone ($p < 0.001$), calculated free ($p < 0.001$) and bioavailable tes-

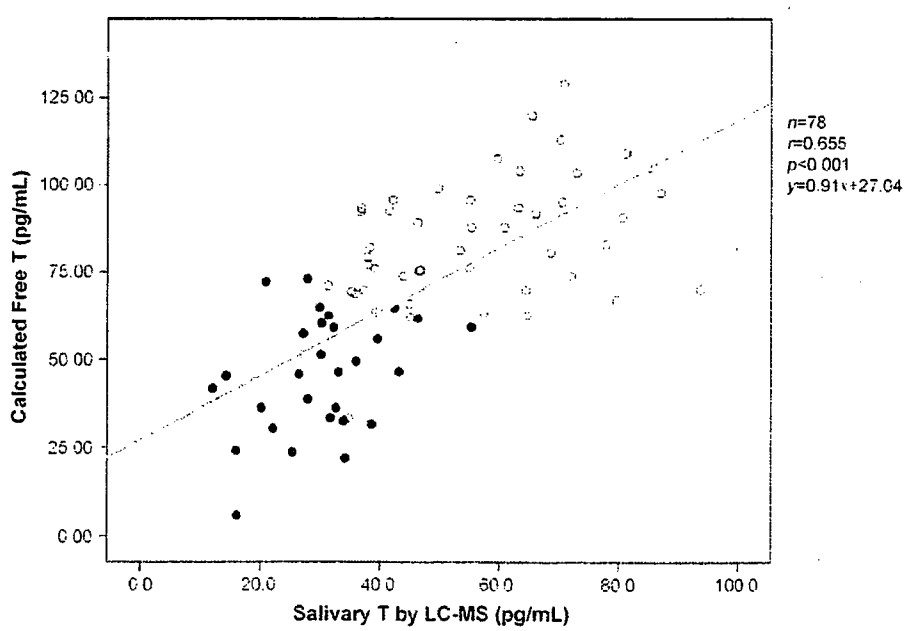


**Figure 1** Significant correlation between salivary testosterone (T) as measured by liquid chromatography/mass spectrometry (LC-MS) and calculated serum free testosterone. Comparison of healthy volunteers (○) with LOH patients (●).

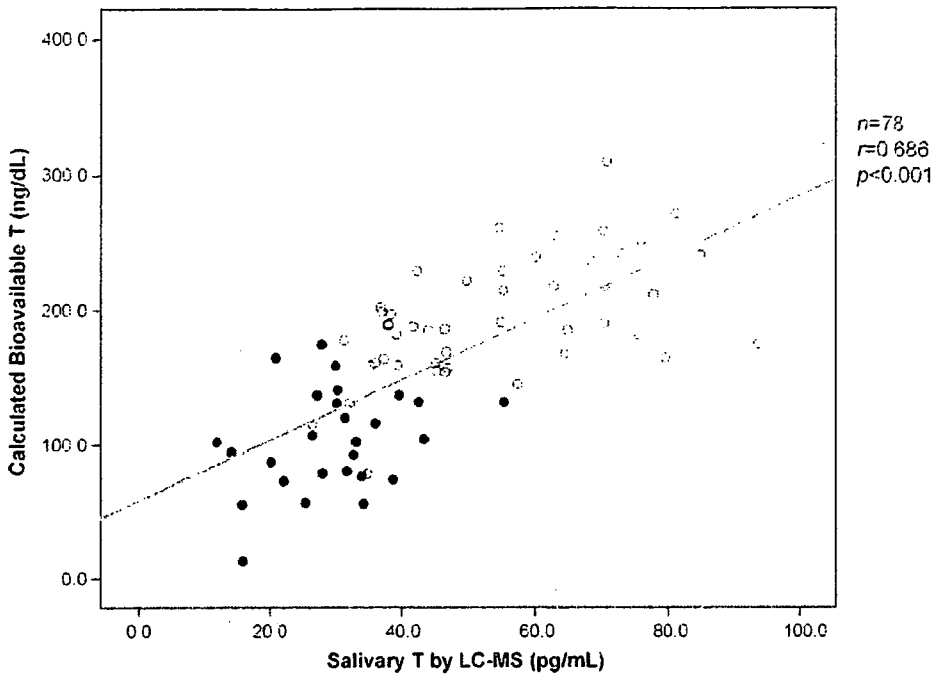


Figure 2: Significant correlation between salivary testosterone (T) as measured by liquid chromatography/mass spectrometry (LC-MS) and calculated bioavailable testosterone. Comparison of healthy volunteers (○) with LOH patients (●).

tosterone ($p < 0.001$) and in salivary testosterone as measured both by LC-MS ($p = 0.045$) and ELISA ($p = 0.048$) between the two groups. We confirmed that we could include subjects whose testosterone levels ranged from low to normal.

First, we examined the correlation between age and testosterone level and SHBG level. The

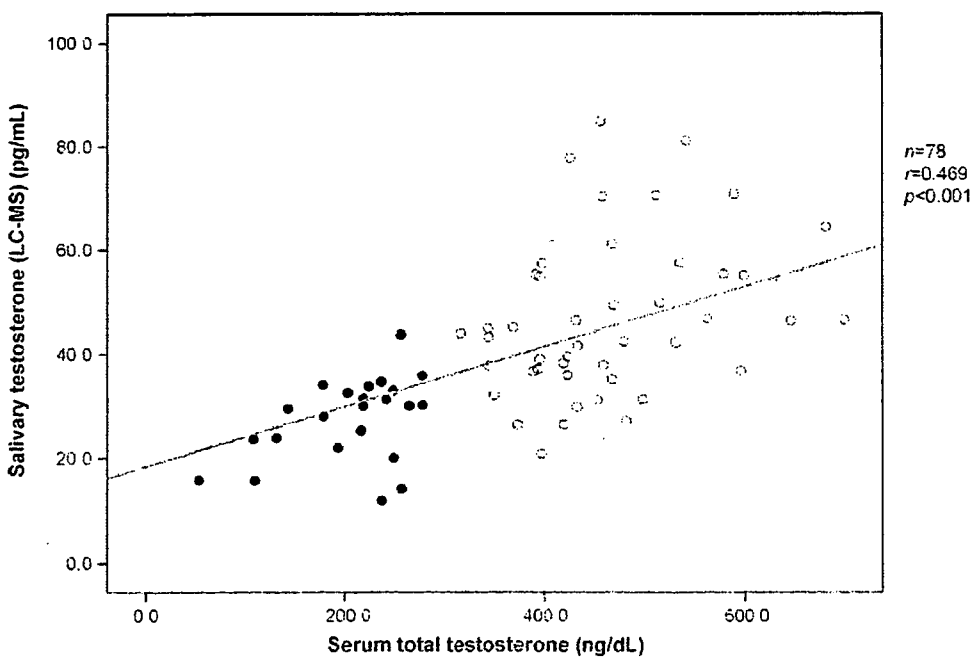


Figure 3: Correlations between salivary testosterone (T) and serum total testosterone. There was a parallel trend at high salivary testosterone levels, while a significant correlation was shown at lower testosterone levels. Comparison of healthy volunteers (○) with LOH patients (●).

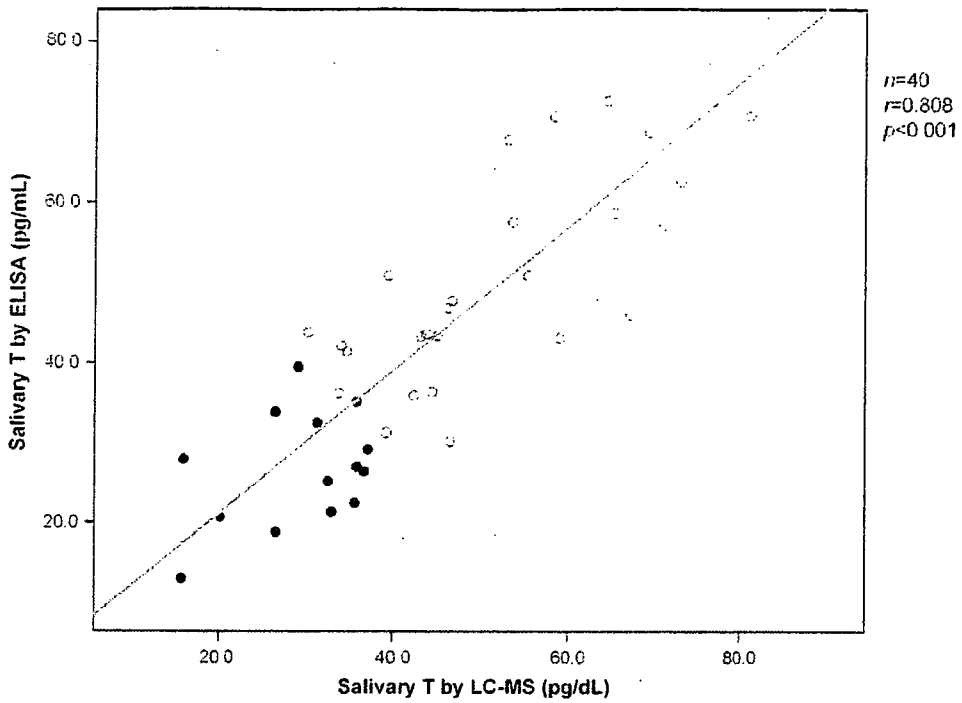


Figure 4 Strong correlation between salivary testosterone (T) as measured by liquid chromatography/mass spectrometry (LC-MS) and salivary testosterone as measured by enzyme-linked immunosorbent assay (ELISA). Comparison of healthy volunteers (○) with LOH patients (●).

serum total testosterone level measured by LC-MS was not significantly correlated with age ($p = 0.336$), whereas SHBG level significantly increased with age ($r = 0.338$, $p = 0.002$). Calculated serum free testosterone ($r = -0.387$, $p < 0.001$) and salivary testosterone as measured by LC-MS significantly, but slowly, decreased with age ($r = -0.381$, $p < 0.001$.)

Next, we examined the correlation between salivary testosterone as measured by LC-MS and serum testosterone. Salivary testosterone measured by LC-MS had a significant correlation with calculated serum free testosterone and both values were similar ($r = 0.655$, $p < 0.001$, $y = 0.91x+27.04$: where $x$ is salivary testosterone as measured by LC-MS, and $y$ is the calculated free testosterone; Fig. 1). Salivary testosterone as measured by LC-MS was significantly associated with calculated bioavailable testosterone ($r = 0.686$, $p < 0.001$; Fig. 2).

A significant correlation was shown between salivary testosterone level as measured by LC-MS and serum total testosterone ($r = 0.469$, $p < 0.001$; Fig. 3). The correlation between salivary testosterone levels as measured both by LC-MS and by ELISA was examined for the possibility of the clinical application of ELISA methods. A strong correlation was shown between salivary testosterone level measured by LC-MS and by ELISA ($r = 0.808$, $p < 0.001$; Fig. 4).

## Discussion

Serum total testosterone levels are most commonly used to diagnose hypogonadism [6,7]. However, it should be noted that these testosterone values may not reflect the level of metabolically active testosterone. The most appropriate parameter for determining hypogonadism is probably the measurement of bioavailable testosterone [23]. In community studies, age-related declines in testosterone levels have been seen more frequently in bioavailable or free testosterone than in total testosterone [24,25] due to an increase in SHBG [23,25,26]. In our study, calculated serum free testosterone and salivary testosterone, as measured by LC-MS, significantly but slowly decreased with age. These observations were in accordance with previous studies [1,6,21]. It has been recommended that serum free testosterone level should be accurately measured using equilibrium dialysis or calculated from total testosterone, SHBG, and albu-

min concentrations using the international formula. Bioavailable testosterone levels can be measured using the ammonium sulfate precipitation method, the concanvalin A aggregation method, or it can be calculated in the same way as serum free testosterone using the international formula. However, these methods are time-consuming and costly for clinical practice. For example, measuring one SHBG level costs $150 in Japan, and this method is not covered by national health insurance.

In previous studies in the field of anthropology, salivary testosterone has been frequently adopted as an index for the assessment of examined subjects' androgen levels for several reasons [27–30]. Firstly, subjects feel more comfortable providing saliva rather than allowing blood to be drawn. Secondly, saliva can be collected without medical assistance. Thirdly, salivary testosterone is stable at room temperature for a few days before measurement. Fourthly, as testosterone is not bound to protein in saliva, measuring salivary testosterone does not require dialysis. However, in other studies, direct radioimmunoassay (RIA) was mainly used for the measurements of salivary testosterone [31]. In most of those previous studies, RIA had a good correlation for salivary testosterone with serum bioavailable and free testosterone [32]. Johnson et al. showed that $R = 0.83$ ($p < 0.001$) and Sannikka et al. showed $R = 0.75$ ($p < 0.001$) [33,34]. Vittek et al. also used direct RAI of salivary testosterone and found a strong correlation between serum free and salivary testosterone ($r = 0.97$, $p < 0.05$) [16]. However, Rey et al. performed a critical evaluation of free testosterone levels in plasma and saliva as measured by direct RIA. They failed to reveal a detectable testosterone concentration in saliva using the direct RIA method and concluded that salivary testosterone determined by this method did not reflect the true free testosterone values and that the significance of salivary testosterone remained to be established [35]. Although they agreed with the idea that salivary testosterone determination provided an excellent approach for the evaluation of androgen activity, they emphasized the discrepancy between salivary testosterone and serum free testosterone levels. They suggested that it might be due to the local conversion of androstenedinone to testosterone in the salivary glands or the possibility of the extraction of steroid-binding protein (such as SHBG) or albumin, which could bind free testosterone.

In addition to those contradictions, RIA is not a procedure that can be easily carried out in the laboratory nor is it an environmentally-friendly test. The advantages of the LC-MS for the measurement of salivary testosterone include easy and simple preparation (non-derivatized steroids can be directly analyzed), high recovery with improved signal-to-noise ratio, enhanced specificity, and low interference due to MS technology [36]. Moreover, it has been validated using protocols specified by the Federal Drug Administration and is considered to be a gold standard for determining testosterone levels. The superiority of the present study lies in the measurement of both serum total and salivary testosterone in order to investigate the association between salivary testosterone and serum testosterone bioavailability.

Notwithstanding, LC-MS is not an easy assay to perform, requiring numerous procedures. In measuring testosterone, ELISA is more cost effective ($5 per sample) and easier to perform than RIA and LC-MS (more than $40 per sample).

Travison et al. conducted a prospective cohort study with three data collection waves (1987–89, 1995–97, 2002–2004) and they reported that the past 20 years had seen a substantial age-independent population-level decrease in serum total and bioavailable testosterone. They concluded that the prevalence of low testosterone will exhibit an increase in excess of that expected given the projected aging of the population [37]. To screen biomarkers for evaluating health-related quality of life in community-based studies, sampling of salivary testosterone can be done anywhere in the community without medical help (e.g. at home for bedridden men) and make the examination of diurnal rhythms easy [38]. Based on our results, the values of salivary testosterone could be used as an alternative index to those of calculated serum testosterone without any need for complex calculations.

In conclusion, salivary testosterone is a non-invasive and reliable substitute for serum testosterone availability. Salivary testosterone level, as measured by ELISA, is recommended for use in large, population-based studies because of its clinical reliability, convenience and cost-effectiveness.

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# Low testosterone level of middle-aged Japanese men – the association between low testosterone levels and quality-of-life

**Keywords**

Salivary testosterone

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Quality of life

Late-onset hypogonadism

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**Abstract**

**Background:** Late-onset hypogonadism (LOH) is due to age-related steep declines in free testosterone levels in middle age. LOH can induce a variety of signs and symptoms that deteriorate the quality-of-life (QOL) of middle-aged men. This study examined the circadian rhythm of salivary testosterone levels in three age cohorts: 20s–30s, 40s–50s, and 60s+ to investigate the association between QOL and testosterone levels in adult Japanese men.

**Subjects and Methods:** Eighty-one healthy male Japanese volunteers and 20 LOH patients in their 40s–50s were recruited. Their salivary testosterone levels were measured as were independent variables including Body Mass Index (BMI) and smoking rates. The SF-36 v2 was used as the health-related questionnaire to evaluate QOL. Saliva samples were collected at 2-hour intervals between 9:00 am and 9:00 pm. Salivary testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA: Demeditec Diagnostics, Germany).

**Results:** There were no significant differences in BMI and smoking rates among the three healthy groups. However, scores from the SF-36 related to body pain were significantly lower in the 40s–50s cohort than in the 20s–30s group. The mean salivary testosterone levels in the 40s–50s group were the lowest at any point of time, except for 9:00 am among all healthy cohorts, and were similar to those of LOH patients. A circadian rhythm was seen in salivary testosterone levels in the 20s–30s and 40s–50s groups, whereas it was lost in the 60s+ group and in LOH patients.

**Conclusion:** Middle-aged Japanese men had the lowest levels of salivary testosterone and the worst QOL scores in relation to body pain, which may affect their overall QOL. 

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## Introduction

Promoting health and preventing disease requires a thorough understanding of the complex of social and behavioral factors that affect the health of individuals and the condition of communities.

Saliva has the advantage of allowing the measurement of biomarkers in a non-invasive and repeatable manner and this has created opportunities for behavioral scientists to test biosocial models of individual differences and intra-individual changes in mood, cognition, behavior and psychopathology [1].

The concept of age-related androgen deficiency in men, also termed late-onset hypogonadism (LOH), has opened up public awareness to the significance of men's health. Low testos-

terone levels affect physical, mental, and sexual activities, manifesting as a loss of muscle mass and bone strength, increased body fat, decreased energy, less interest in sex, erectile dysfunction, irritability and depression [2].

In contrast to women, men do not experience a sudden cessation of gonadal function comparable to the menopause, although there is a progressive reduction in hypothalamic-pituitary-gonadal (HPG) function in aging men, hence serum testosterone levels decline through both central (pituitary) and peripheral (testicular) mechanisms [3]. Serum total testosterone gradually declines with advancing age, particularly after the age of 50 [4]. Although serum testosterone levels are generally measured in the morning when they are at a peak, this circadian rhythm may be abolished or blunted in men with advancing age [3,4]. In healthy men, only 1–3% of biologically active steroids circulate freely, with the balance being tightly bound to sex hormone binding globulin (SHBG) or loosely bound to albumin. Free testosterone and the fraction bound loosely to albumin are readily available for entry into tissues. Unlike serum total testosterone, the concentration of SHBG significantly increases with age [4]. Consequently, serum free testosterone level decreases steeply, and this is considered to be more closely associated with the incidence of LOH than is the decline in total serum testosterone [5,6]. Moreover, physical and psycho-social stresses challenge homeostasis, increasing glucocorticoid secretion and decreasing testosterone levels [7].

Most patients who visit LOH clinics in Japan are in their 40s to 50s. They have more responsibilities than other generations, both at work and at home, placing them in a stressful environment. In Teikyo Hospital, many of the patients who came to LOH outpatient services were white-collar workers in their 40s to 50s, and half of them suffered from depression [8]. Japan is facing a rapidly increasing suicide rate in middle-aged men, mostly due to depression that may be associated with LOH. Previous studies of age-related testosterone decline have not focused on the testosterone levels in middle-aged men. We hypothesized that, aside from aging, environmental stressors such as overwork escalate the steep decline of free testosterone levels, leading to the onset of LOH. To investigate the complexity of declining testosterone levels in later life we examined the circadian rhythm of salivary testosterone levels in three age cohorts: 20s–30s, 40s–50s and 60s+. We used salivary testosterone as an alternative measure for evaluating serum free testosterone levels. Salivary testosterone level is a useful, non-invasive and repeatable method of assessing levels of free testosterone because testosterone is not bound to any protein in the saliva [9].

## Subjects and methods

### Subjects

This study was approved by our institutional review board. Sixty-four healthy, salaried men were included in this study upon written consent: 22 in a 20s–30s cohort (mean age = 30.32; range = 22–39), 32 in a 40s–50s cohort (mean age = 53.53; range = 40–59) and 10 in a 60s+ cohort. They were all white-collar workers in Tokyo. We also included 17 healthy retired men in their 60s–70s. The healthy 60s+ cohort thus consisted of 27 men in total (mean age = 66.25; range = 60–74). We compared the healthy 40s–50s cohort with 20 new LOH patients in their 40s–50s (mean age = 50.42; range = 41–57) whose calculated free testosterone, using the formula devised by the International Society for the Study of the Aging Male (ISSAM), was lower than 72 pg/ml, the generally acceptable lower limit considered to be normal for testosterone substitution [10]. We included information on both body mass index (BMI) and smoking habits, which could affect testosterone levels. The subjects were asked about their history of smoking. If a subject had quit smoking for more than 2 years, he was counted as a non-smoker, as being smoke-free for 2 years can eliminate many adverse effects on health [11].

### Health-related quality-of-life

The 36-item Short-Form Health Survey (version 2: SF-36 v2) was used to evaluate each subject's health-related quality-of-life (QOL) [12]. The SF-36 produces scores for eight dimensions of health status, namely, Physical health (PH), Role-physical (RF), Body pain (BP), General health (GH), Vitality (VT), Social function (SF), Role-emotional (RE), and Mental health (MH). The scores for each dimension were assigned a mean (±SD) score of 50 (±10) on the basis of an

assessment of a general Japanese population without chronic conditions; individual scores could then be compared with the normalized scores for the general population.

### Saliva collection

Subjects were provided with plastic sterile screw sputum processors to collect samples at 2-hourly intervals between 9 am and 9 pm. Subjects were asked to finish eating and brushing their teeth at least 1 hour before saliva sampling in order to avoid both food and blood contamination. Subjects rinsed their mouths with water three times and waited for a few minutes before expectorating at least 1 ml of saliva directly into a collection vial. Testosterone levels in saliva increase post-microinjury due to the brushing of teeth [1]. It is noteworthy that, in this study, the testosterone levels remained elevated over the baseline well after microinjury and even in samples that did not appear, visually, to be contaminated with blood. Importantly, the effect of microinjury was specific for testosterone. That is, after brushing, neither salivary cortisol nor dehydroepiandrosterone levels were different from the baseline levels [13]. Subjects were asked not to use sugar-free chewing gum, which can change salivary testosterone results [1].

### Sample storage

Salivary free testosterone levels were to be determined using an enzyme-linked immunosorbent assay (ELISA; Demeditec Diagnostics, Germany). The instructions from Demeditec Diagnostics for the use of free testosterone in saliva ELISAs stated that saliva samples, in general, were stable at ambient temperature for several days. Therefore, mailing of such samples by ordinary mail without cooling should not create a problem.

Specifically, Demeditec Diagnosics had done stability studies of salivary testosterone with the following results:

- Stability at ambient temperature: up to 1 week
- Stability at 4 °C: up to 1 month
- Stability at −20 °C: no limitation was detected

Notwithstanding these results, Demeditec Diagnosics recommended deep freezing samples whenever a freezer was available to avoid any potential risk.

To be on the safe side, salivary samples were stored for up to 3 days at 4 °C in regular household refrigerators, to avoid bacterial growth that could interfere with antibody binding, and they were then shipped cooled and stored at -20 °C for up to 1 month in laboratory freezers until analyzed.

### Hormone determinations

A previous study had shown that salivary concentration measured using a refined immunoassay was a reliable biomarker of serum free testosterone concentration [14,15]. Saliva testosterone levels were, therefore, measured using an ELISA (DE-SLV3013: Demeditec Diagnostics, Germany). The efficacy of using an ELISA was examined by the simultaneous measurement of free testosterone using liquid chromatography-mass spectrometry (LC-MS), which has high sensitivity and specificity.

Salivary testosterone, as measured by LC-MS and by ELISA, showed a strong correlation ($r = 0.84$), confirming the comparative validity with established kits of LC-MS for measuring salivary testosterone.

## Statistics

SPSS (version 15.0) was used for statistical analysis. Since the data were normally distributed, One-Way Analysis of Variance tests (ANOVA; post-hoc $t$ test) were used to compare the means of BMI, smoking rates and each dimension of QOL on the SF-36 between the three healthy cohorts.

We performed post-hoc $t$ tests on the results from each of the 2-hour saliva samples to determine if there were any differences in hormone levels at a particular time of the day for each age group. For outliers whose testosterone levels were more than 1.5 interquartile ranges below Q1 or above Q3 on the Box-and-Whiskers plots, data were visually inspected for each subject. If there were explanatory events at unexpected peaks of testosterone levels, those values were excluded; if there were no explanatory events, data were included as likely to represent part of the normal fluctuation in the HPG axis in response to day-to-day events.

In order to examine circadian rhythm, we analyzed the data using repeated measures

**Table 1** Baseline characteristic of the healthy subjects

| | Age ± SD(years) | BMI ± SD | *P* value | Smoking rates (%) | *P* value |
|---|---|---|---|---|---|
| 20s-30s (*n*=22) | 30.32 ± 4.45 | 22.73 ± 2.46 | 0.47 | 0.53 ± 0.51 | 0.99 |
| 40s-50s (*n*=32) | 53.53 ± 5.47 | 23.62 ± 2.37 | 0.83 | 0.52 ± 0.51 | 0.19 |
| 60+ (*n*=27) | 66.25 ± 4.88 | 23.20 ± 1.98 | 0.83 | 0.21 ± 0.43 | 0.16 |

Values given are Means ± SD.

ANOVA for each of the 2-hour samples, in accordance with previous work that used a similar design [16–18]. In addition, we used *t*-tests to compare the 40s–50s cohort with the LOH patients.

## Results

First, we examined the circadian rhythm of testosterone levels in three healthy cohorts. Then we compared age-matched LOH patients with the healthy 40s–50s cohort to see whether there were any similarities.

## Healthy cohorts

### Subjects' characteristics

For each age group, the average age, BMI, smoking rate, health-related QOL (as measured by SF-36 v2), and the *P* values for the post hoc *t* tests are shown in Tables 1 and 2.

There were no statistically significant differences in either BMI or smoking rates among the three groups.

On the SF-36 v2, there was a statistically worse QOL in relation to body pain in the 40s–50s age group than in the 20s–30s cohort.

### Salivary testosterone levels

A comparison of values for each age group from each 2-hour sample is shown in Table 3. Post-hoc analysis showed that there were significantly lower testosterone levels in the 40s–50s cohort than in the 20s–30s cohort at all time points, except for 7:00 pm ($P = 0.03$ for 9:00 am; $P < 0.001$ for 11:00 am; $P = 0.004$ for 1:00 pm; $P < 0.001$ for 3:00 pm; $P = 0.044$ for 5:00 pm; $P = 0.070$ for 7:00 pm; $P = 0.046$ for 9:00 pm). There were only two time block samples in which the testosterone levels were lower in the 60s+ cohort than in the 20s–30s cohort ($P = 0.007$ for 9:00 am; $P < 0.001$ for 3:00 pm). There were no significant differences in mean testosterone levels at any time blocks between the two older cohorts (40s–50s and 60s+).

## Analysis of the circadian rhythm

The repeated-measures ANOVA showed significant main effects of time in the 20s–30s cohort

**Table 2** Health-related quality-of-life using SF-36 v2 in healthy subjects

| | Physical function | Role-physical | Body-pain | *P* value | General health | Vitality | Social function | Role-emotional | Mental health |
|---|---|---|---|---|---|---|---|---|---|
| 20s-30s | 57.38 ± 2.37 | 55.32 ± 2.21 | 58.45 ± 4.31* | *0.025 | 52.48 ± 8.21 | 51.36 ± 9.05 | 54.11 ± 5.39 | 52.31 ± 6.01 | 52.0 ± 6.67 |
| 40s-50s | 54.84 ± 2.79 | 55.11 ± 3.03 | 51.13 ± 7.39* | | 52.54 ± 10.07 | 55.88 ± 6.40 | 51.62 ± 8.33 | 54.97 ± 2.19 | 53.32 ± 7.56 |
| 60+ | 54.88 ± 2.92 | 55.03 ± 3.69 | 54.98 ± 6.75 | | 52.21 ± 6.54 | 56.18 ± 7.18 | 52.46 ± 6.54 | 53.53 ± 4.23 | 53.10 ± 7.71 |

Values given are Means ± SD. * $P = 0.025$.

**Table 3** Comparison of testosterone levels from each 2-hour sample for each age group

| | 9 am | *P* value | 11 am | *P* value | 1 pm | *P* value | 3 pm | *P* value |
|---|---|---|---|---|---|---|---|---|
| 20s-30s | 66.82 ± 32.49 | * 0.03 | 63.17 ± 23.59 | *** <0.001 | 55.25 ± 10.60 | ** 0.004 | 61.47 ± 24.77 | *** <0.001 |
| 40s-50s | 47.33 ± 20.35 | ** 0.007 | 36.89 ± 7.30 | 0.135 | 37.60 ± 15.40 | 0.126 | 29.16 ± 13.22 | *** <0.001 |
| 60+ | 41.17 ± 26.18 | 0.704 | 50.77 ± 28.11 | 0.075 | 44.54 ± 21.98 | 0.348 | 35.54 ± 13.47 | 0.414 |

| | 5 pm | *P* value | 7 pm | *P* value | 9 pm | *P* value |
|---|---|---|---|---|---|---|
| 20s-30s | 51.58 ± 25.89 | * 0.044 | 45.51 ± 19.06 | 0.070 | 45.72 ± 18.87 | * 0.046 |
| 40s-50s | 36.64 ± 12.87 | 0.232 | 32.81 ± 13.38 | 0.490 | 32.50 ± 13.57 | 0.230 |
| 60+ | 41.04 ± 24.33 | 0.741 | 38.54 ± 20.21 | 0.524 | 36.72 ± 20.82 | 0.696 |

Values given are Means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Hotteling's Trace = 2.750, $F = 3.667$, $P = 0.047$) and the 40s–50s cohort (Hotteling's Trace = 2.539, $F = 4.231$, $p = 0.022$), confirming a circadian pattern. However, there was no main effect of time in the 60s+ cohort (Hotteling's Trace = 0.516, $F = 1.117$, $P = 0.404$), indicating the lack of circadian rhythm.

The repeated-measures ANOVA for the 20s–30s and 40s–50s cohorts showed significant group-by-time interactions (Hotteling's Trace = 1.068, $F = 4.096$, $P = 0.002$), suggesting that the two groups had a different circadian rhythm pattern.

## Comparison of the healthy 40s–50s cohort with LOH patients

LOH patients had significantly lower testosterone levels than the healthy 40s–50s cohort at 11am ($P = 0.016$), 7 pm ($P = 0.024$) and 9 pm ($P = 0.029$). In LOH patients, there was no circadian rhythm (Hotteling's Trace = 6.131, $F = 3.061$, $P = 0.19$). Despite the similarity of testosterone levels, the scores for each domain of SF-36 v2 were significantly lower in LOH patients than in the 40s–50s cohort (Table 4).

In summary, the mean testosterone levels in the 40s–50s cohort were the lowest at almost every time-point among the healthy cohorts and similar to those in LOH patients. A circadian rhythm of salivary testosterone levels was maintained in the 20s–30s and 40s–50s cohorts. However, their circadian patterns differed. The circadian rhythms were lost in the 60s+ and LOH patients.

## Discussion

Gender-focused health care has been gaining ground through the understanding of the biological, behavioral, and socio-cultural factors that account for differences in the health of

**Table 4** Scores from the SF-36 v2 for the 40s-50s cohort and the LOH patients

| | Physical function | Role-physical | Body-pain | General health | Vitality | Social function | Role-emotional | Mental health |
|---|---|---|---|---|---|---|---|---|
| 40′–50′ | 54.84 ± 2.79 | 55.11 ± 3.03 | 51.13 ± 7.39 | 52.54 ± 10.07 | 55.88 ± 6.40 | 51.62 ± 8.33 | 54.97 ± 2.19 | 53.32 ± 7.56 |
| LOH | 45.25* ± 7.56 | 37.77** ± 10.63 | 40.55** ± 7.24 | 35.42** ± 4.38 | 35.12** ± 7.92 | 32.43** ± 11.94 | 32.83** ± 16.26 | 39.37** ± 6.83 |

Values given are Means ± SD. LOH, late-onset hypogonadism. * $P < 0.01$, ** $P < 0.001$.

men and women. The health care community has paid much less attention to men's health than to woman's so that the health care of men has tended to be piecemeal and somewhat uncoordinated [19].

Striking differences in life expectancy and susceptibility to certain diseases between men and women point to opportunities for understanding and improving the health of men. In Japan, the average life expectancy was 78.6 years for men and 85.6 years for women in 2005 [20]. Although the life expectancy gap between men and women has gradually narrowed over several decades, it remains approximately 7 years and merits better understanding.

Previous studies have shown that a steep decline in serum free testosterone contributes to the onset of LOH [21,22]. Our preliminary study revealed two findings: (1) testosterone levels in healthy middle-aged Japanese men were low, even though they maintained a circadian rhythm; (2) the circadian rhythm was lost in the 60s+ cohort and in LOH patients.

There is a progressive reduction in HPG function in aging men [3]. Testosterone secretion declines through both central (pituitary) and peripheral (testicular) mechanisms and its circadian rhythm becomes blunt or diminished [23]. Our study was consistent with previous studies. In addition, our study showed that a progressive reduction in HPG function may occur in LOH patients as well as in aging men, which induces the loss of circadian rythmicity in saliva testosterone levels.

The decline of serum free testosterone is associated with aging [22] as well as with stressors that challenge the homeostasis of the endocrine environment. Stress and other conditions that elevate circulating adrenocorticotropine hormone (ACTH) and cortisol levels lead to depressed testosterone levels in animals and in men [24,25]. Excessive exposure to cortisol initiates apoptosis in rat Leidig cells, potentially contributing to the suppression of testosterone levels [26].

From a cross-cultural perspective, Japanese workers have reported higher levels of job stress, anxiety, depression, and psychosomatic tendencies [27,28].

In our study, the middle-aged cohort showed the worst QOL in relation to body pain on the SF-36 v2. This merits profound consideration. Cultural factors have an impact on men's attitude to seek health care. In Japan, like many parts of the world, masculinity is given priority in traditional men's values. Men are forced to be 'macho' and to show stoicism, independence, self-reliance, and strength [29]. This gender perspective prevents them from admitting to having any problems, especially with regard to showing mental problems such as fear or sadness [30]. Job strain has been reported to be associated with bodily pain [31]. Thus, if middle-aged men are placed in a stressful environment, socio-environmental factors may affect their mental health and thus influence physical health problems.

In Western countries, LOH is frequently reported as the result of an age-related decline in free and bio-available testosterone. Cross-cultural studies have shown that the pattern of age-related decline in male free testosterone, as represented by salivary levels, is not a uniform characteristic of all populations [32]. The etiology of LOH is a complex matter involving cultural and socio-environmental issues as well as individual biological changes.

Low testosterone in middle-aged men might lead to their future frailty as well as to the onset of LOH. Our study suggests that middle-aged Japanese men are susceptible to LOH and that there may be many LOH patients who are under-diagnosed and not treated.

In an extension of this research, we will include a paper interview in addition to the SF-36 in order to evaluate stress levels.

So far, there have been few interventions to raise the public awareness of LOH in Japan. In addition to education for health professionals to help them understand the complexity of LOH symptoms, the distribution of information and screening tests in workplaces and public facilities will enable middle-aged men to become aware that they should be screened for LOH. It worth screening the testosterone levels in middle-aged men and providing appropriate awareness of the risk of having a low testosterone level. Holistic approaches, including proper prescriptions for exercise, consultations for dietary habits, alternative medicine such as acupuncture and Chinese herbal medicine, hormone replacement therapy, and counseling to ease excessive stress will be needed to prevent the symptoms of LOH.

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# The prevalence of depressive symptoms and other variables among frail aging men in New York City's Personal Care Services program

**Keywords**

Depressive symptoms

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Hearing impairment

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**Abstract**

**Background:** New York City's Personal Care Service Program provides service-rich assistance to the frail elderly who would not be able to live at home without such support. However, gender-specific health care delivery has not been introduced. Depressive symptoms are common among elderly people. We conducted a cross-sectional study in order to investigate the prevalence of depressive symptoms and other variables among frail elderly men receiving personal care services.

**Methods:** Data were collected from administrative data available in the Human Resources Administration's computer system. Two hundred men aged 65 or older were randomly selected. We defined depressive symptoms by tracking the recording of depressed mood in the data system. We examined statistical differences in a variety of indicators between elderly men with and without depressive symptoms. Multiple logistic regression analysis was performed to determine which independent variables were associated with depressive symptoms.

**Results:** Of all cases, 10.5% had depressive symptoms. In multiple logistic regression models, the duration of services provision and hearing impairment were independently associated with depressive symptoms in frail elderly men.

**Conclusion:** The results of this study indicate the low prevalence of depressive symptoms among frail elderly men compared with previous studies. The duration of services provision was a protective factor for depressive symptoms, i.e. personal care services provided high quality Activities of Daily Living (ADL) support, which keeps frail elderly men living at home for as long as possible. The significance of hearing impairment, which can induce social isolation, also needs to be stressed as an indicator of depressive symptoms, even though there was no discrete measurement of social isolation included in the data.



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## Introduction

Personal care services provide help to individuals in need of assistance with Activities of Daily Living (ADLs: e.g. bathing, dressing, personal grooming, getting out of bed or getting up from a seated position, eating, using the toilet etc) as well as household support, which are prerequisites for health and safety in their own homes. While personal care services – the Medicaid Optional Program – have been adopted by 26 states, 15 states have set limits

on the hours that services are made available and 10 states have set cost caps [1,2]. New York is the only state that has not placed limits on service benefits and provides generous and service-rich assistance to the frail elderly in the community. The program currently serves approximately 53,000 clients daily through 969 vendors. The high intensity of services, including 24-hour personal assistance care, allows individuals who are medically eligible for nursing home placement to live in their own homes. Elderly clients are more likely to be disabled than older community residents who do not participate in personal care services [3]. Depressive symptoms affect 8–20% of elderly individuals in the community [4]. Older adults with medical illness, somatic impairment and social isolation are more likely to be depressed [5]. We hypothesized that medical illness, physical impairment, and social isolation would be associated with administrative reports of depressive symptoms in elderly men. We sought to investigate the prevalence of depressive symptoms among those men aged 65 or older who had received personal care services from New York City for more than 1 year, and to examine the relationships between depressive symptoms and a variety of indicators, such as age, medical and functional indicators, use of personal care services, and caregiver support, in order to determine the variables associated with depressive symptoms. To do this, we used administrative data, such as Medicaid administrative claims, medical requests (M11Q) and social assessments (M11S).

## Methods

### Data collection

To receive personal care services, people need to submit a medical request form (M11Q) filled out by their physicians, a social assessment (M11S) performed by case workers, and a nurse's assessment (M27r) to New York City's Human Resources Administration (HRA). These data are collected at nine Community Alternative System Agency borough offices (CASA offices), which serve the clients in their geographic areas, and are stored in the centralized computer database.

### Subjects

Subjects were randomly drawn from the centralized computer database. In addition, we limited the subjects to those who were men aged 65 or older who had been receiving services for at least 1 year. Finally, we excluded clients who received only 'level 1' service (housekeeper services), since they were not receiving personal assistance care. Information on 200 elderly men was included in the study.

### Definition of depressive symptoms

According to DSM-IV [6], major depression is defined as the presence of five or more out of nine symptoms (depressed mood, loss of interest or pleasure, eating disturbance, sleep disturbance, psychomotor agitation, fatigue, a feeling of worthlessness or guilt, poor concentration and suicidal ideation) during the same 2 week period. At least one of the symptoms must be either depressed mood or loss of interest/pleasure. This level of either specificity or sensitivity was unavailable in the administrative data. In the absence of the full set of nine DSM-IV criteria, any mention of depressive mood in the medical report was used as an indicator of depressive symptoms, since depressive mood and anhedonia are two gateway symptoms, only one of which is needed to constitute a depressive disorder.

### Sociodemographic characteristics

Sociodemographic variables included age, living situation (living alone, living with caregivers or with non-caregivers), and caregiving support.

### Administrative claims

The number of service hours that each client was receiving in July 2005 was assessed as the number of billed service hours. For duration of home care services, we retrieved information on all periods of home care services from the initial authorization to July 2005.

### Medical status

Overall medical co-morbidity was indicated by the total number of ICD-9 (International Classification of Diseases) diagnoses.

### Cognitive impairment

Three mental status indicators in the M11Q, namely disorientation to place/time, short-term memory impairment, and impaired judgment, were used to identify cognitive impairment. If the clients had at least one of these three impairments, they were categorized as having 'dementia'. Clients with none of the three impairments were categorized as 'no impairment'.

### ADL status

ADL status was based on the number of needs in six ADLs: bathing, dressing, personal grooming, getting out of bed or getting up from a seated position, eating, and using the toilet.

### Functional status

Sensory impairment (speech, visual, or hearing impairments), muscular impairment (dominant hand, upper extremities, lower extremities), and bladder incontinence were assessed to examine whether these impairments were associated with depressive symptoms.

### Caregiver support

Data related to caregiver support were obtained from a social assessment (M11S). We investigated whether the clients lived with or without informal caregivers.

## Statistical analysis

The data were analyzed using an SPSS 13.0 statistics package. Statistical differences in each variable between those clients with and without depressed mood were examined using a t-test or a chi-square test. The level of significance was set at $P < 0.05$ (two-sided). In order to evaluate the role of depressive symptoms as indicators, multiple logistic regression analysis was performed considering depressive symptoms as a dependent variable.

## Results

The characteristics of groups designated as being with and without depressive symptoms are shown in Table 1. In all, 10.5% of the subjects were defined as having depressive symptoms, considered as the presence of depressed mood. Differences did not achieve a statistical significance with age, intensity of services (hours of services provided per week), number of ADL disabilities, number of comorbid conditions, and cognitive impairment. Regarding functional status, elderly men with depressive symptoms were significantly more likely to be visually impaired (66.7% vs. 44.9%, $P = 0.49$, $n = 200$, df = 1), to have a hearing impairment (76.2% vs. 43.3%, $P = 0.004$, $n = 200$, df = 1), an upper extremity impairment (70.0 vs. 43.8%, $P = 0.026$, $n = 200$, df = 1) and bladder incontinence (65.0% vs. 38.8%, $P = 0.024$, $n = 200$, df = 1). The two groups did not differ in either living situation or caregiver support. Although the difference did not achieve statistical significance, it should be noted that elderly men without depressive symptoms were more likely to live alone (59.3% vs. 44.4%, $P = 0.687$, $n = 200$, df = 2).

The associations between the independent variables and depressive symptoms, based on logistic regression analysis, are shown in Table 2. The probability of having depressive symptoms decreased with duration of services (odds ratio (OR) = 0.86, 95% confidence interval (95% CI) = 0.75–0.97), and increased with hearing impairment (OR = 3.67, 95% CI = 1.18–11.84).

## Discussion

Late-life depression is common. Estimates of the presence of depressive symptoms in community-dwelling elderly range from 8% to 20% [4]. Older adults with medical illness, somatic impairment, and social isolation are more likely to be depressed [5]. About 30–50% of nursing home residents and about 26–44% of housebound elderly adults have depressive symptoms [7,8]. Sex differences in depression are of clinical and scientific importance. These differences in depression prevalence rates have been reported previously with respect to phenomenology, level of distress and suffering, functional impairment, coping style, and treatment response [9]. Previous studies have shown that sex differences in brain neuroanatomy may be important in the pathophysiology of late onset depression. Men may be more susceptible than women to atrophy in the frontal subregions, which are related to geriatric