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Low serum free testosterone level is associated with carotid intima-media thickness in middle-aged Japanese men

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Abstract. In the present study, we measured carotid artery intima-media thickness (CIMT) and assessed several metabolic factors in middle-aged healthy Japanese men to clarify the relation between testosterone and atherosclerosis. The study comprised 176 male employees aged ≥40 years who visited Osaka University Health Care Center for their annual health examinations. Serum total testosterone (TT) concentration was measured using radioimmunoassay (RIA) and serum free testosterone concentration was measured using analog ligand RIA (aFT). A multivariate model adjusted for age, body mass index, mean arterial pressure and treatment for hypertension demonstrated a significant association between aFT and CIMT. Even after adjustment for other clinically relevant factors, the significant association between aFT and CIMT was not attenuated. After adjustment for all other clinically relevant factors, both univariate and multivariate models ascertained the stepwise association that a level of aFT of ≤10.0 pg/mL was significantly associated with CIMT. However, the association between TT and CIMT was not significant in either univariate or multivariate models. We conclude that our finding showing that low serum aFT level is an influencing and independent risk factor for CIMT is of value in the clinical setting because no other studies, to our knowledge, have conducted multivariate analyses using the various metabolic factors included in the present analyses.

Key words: Free testosterone, Carotid artery, Intima-media thickness, Atherosclerosis, Men

IT IS WELL KNOWN that androgen plays many physiological roles in various organs and tissues such as skin, muscle, liver, bone and bone marrow, brain and sexual organs. Late-onset hypogonadism (LOH) is defined as "a clinical and biochemical syndrome associated with advancing age and characterized by typical symptoms and a deficiency in serum testosterone levels" by the International Society of Andrology, the International Society for the Study of the Aging Male, and the European Association of Urology [1]. Reported symptoms of LOH include diminished sexual desire and erectile quality, particularly in nocturnal erections, changes in mood with concomitant decreases in intellectual activity and spatial orientation, fatigue, depression and anger, decrease in lean body mass with associated decreases in muscle volume and strength, decrease in body hair and skin alterations, and decreased bone mineral density resulting in osteoporosis [2-7]. Recently, testosterone has received increased attention not only from the standpoint of LOH but also that of cardiovascular disease (CVD) because it has been reported that low serum testosterone levels are associated with increased cardiovascular mortality in men [8-10]. Furthermore, an association of low testosterone level with decreased flow-mediated dilation, which is a measurable marker of endothelial dysfunction causing atherosclerosis, has been reported in Japanese men with high CVD risk factors [11]. It was also reported that serum testosterone concentration inversely and independently correlates with the presence of aortic calcified plaques and the progression of aortic atherosclerosis in a large population-based study [12].

In the present study, we measured carotid artery inti-

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ma-media thickness (CIMT), which is another widely accepted noninvasive measure of preclinical atherosclerosis, and assessed several metabolic factors, such as diabetes, hypertension and hyperlipidemia, in healthy middle-aged men to clarify the relation between testosterone and atherosclerosis after eliminating the effects of other factors.

Methods

Participants

Between April 2009 and March 2010, 1,382 male employees of Osaka University aged ≥40 years visited Osaka University Health Care Center for their annual health examinations. In Japan, an annual health examination was made mandatory for all employees by the Labor Standards Act [13]. Among 719 employees who underwent carotid ultrasonography to evaluate CIMT, serum testosterone level was measured in 177 employees. After excluding one employee with missing data, the present study assessed an association between serum testosterone level and CIMT in 176 employees. The study protocol was approved by the ethical committee of Osaka University Health Care Center.

Measurements

Demographic, physical, and laboratory data included age, sex, body mass index (BMI = body weight [kg] / height^2 [m^2]), and mean arterial pressure (MAP [mmHg] = diastolic blood pressure + (systolic blood pressure - diastolic blood pressure) / 3)), hemoglobin A1c, serum concentrations of creatinine, total cholesterol, and total and free testosterone, and urinary protein by dipstick test. Serum total testosterone (TT) concentration was measured using radioimmunoassay (RIA) and serum free testosterone (FT) concentration was measured using analog ligand RIA (aFT). Estimated glomerular filtration rate (eGFR) was calculated using the Japanese equation for eGFR ($194 \times \text{serum creatinine [mg/dL]}^{-1.094} \times \text{age [years]}^{-0.287} \times 0.739$ [if female]) [14]. Smoking status and current treatment for hypertension and dyslipidemia were determined from responses to a self-reported general questionnaire. All ultrasound examinations were performed by a single well-trained sonographer who regularly participate in quality control measurement sessions and is totally blinded to all clinical information, using LOGIQ 5 (GE Yokogawa Medical Systems Co., Tokyo, Japan) with an 8.8- MHz linear transducer. Three different longitudinal images (anterior oblique, lateral, and posterior oblique) of the left common carotid artery (CCA) of a 1.0-1.5 cm section at the distal end of the CCA proximal to the carotid bulb was obtained as described previously [15, 16]. Mean IMT was obtained using computer software (IntimaScope, SoftMedical Co., Ltd, Tokyo, Japan) that automatically traces the intima-media edge of the far wall [17].

Statistical methods

Continuous variables were expressed as mean ± SD or median (interquartile range), as appropriate, and categorical variables were expressed as number (proportion). Stepwise associations between the free testosterone concentration and other clinical characteristics were compared using the Cochran-Armitage test for trend and the Jonckheere-Terpstra test for trend, as appropriate. To identify the contributors to CIMT, the associations between CIMT and clinical parameters, including serum TT and aFT level, were assessed in univariate and multivariate linear regression models. CIMT was logarithmically transformed because of its skewed distribution in the linear regression models. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using Stata version 11.2 (Stata Corp., College Station, TX, USA) and R version 2.13.1 (The R Foundation for Statistical Computing, http://www.r-project.org/).

Results

Clinical characteristics of the 176 employees across the five groups stratified by serum testosterone concentration are described in Table 1. The numbers of employees with a serum aFT level of 3.8-8.0, 8.1-10.0, 10.1-12.0, 12.1-14.0, and 14.1-21.2 pg/mL were 18 (10.2%), 43 (24.4%), 58 (33.0%), 29 (16.5%), and 28 (15.9%), respectively. Employees with a lower serum level of aFT were statistically older ($P_{trend} = 0.003$). Mean CIMT was 0.84 (0.66-0.94), 0.81 (0.67-1.07), 0.69 (0.59-0.90), 0.63 (0.57-0.73), and 0.66 (0.57-0.98) mm in the employees with serum aFT levels of 3.8-8.0, 8.1-10.0, 10.1-12.0, 12.1-14.0, and 14.1-21.2 pg/mL, respectively, indicating that a lower aFT level was associated with a higher level of CIMT ($P_{trend} = 0.004$; Fig. 1).

Contributors to CIMT were identified in univariate and multivariate linear regression models (Table 2). In univariate linear regression models, higher CIMT was associated with older age, higher BMI, higher MAP,

Table 1 Clinical characteristics of 176 male employees

	Free testosterone by analog ligand radioimmunoassay (pg/mL)					P_{trend}
	3.8 - 8.0	8.1 - 10.0	10.1 - 12.0	12.1 - 14.0	14.1 - 21.2	
Number	18	43	58	29	28	
Age (year)	53 (46 - 56)	51 (45 - 57)	50 (41 - 60)	46 (41 - 50)	42 (40 - 55)	0.003
40 - 49 year, n (%)	7 (38.9)	18 (41.9)	26 (44.8)	18 62.1)	17 (60.7)	0.033[a]
50 - 59 year, n (%)	9 (50.0)	16 (37.2)	17 (29.3)	9 (31.0)	6 (21.4)	
60 - 62 year, n (%)	2 (11.1)	9 (20.9)	15 (25.9)	2 (6.9)	5 (17.9)	
Body mass index, kg/m^2	24.9 ± 2.2	24.5 ± 3.4	23.8 ± 3.2	23.5 ± 2.5	23.6 ± 3.3	0.091
Mean arterial pressure, mmHg	98 ± 9	98 ± 16	95 ± 14	95 ± 12	94 ± 11	0.085
Non-smokers, n (%)	11 (61.1)	30 (69.8)	36 (62.1)	23 (79.3)	15 (53.6)	0.701[b]
Past smokers, n (%)	4 (22.2)	9 (20.9)	12 (20.7)	3 (10.3)	2 (7.1)	
Current smokers, n (%)	3 (16.7)	4 (9.3)	10 (17.2)	3 (10.3)	11 (39.3)	
Hemoglobin A1c, %	4.8 ± 0.2	5.1 ± 0.4	5.1 ± 0.5	4.9 ± 0.3	5.0 ± 0.3	0.683
Total cholesterol, mg/dL	214 ± 50	200 ± 35	203 ± 34	212 ± 32	220 ± 36	0.453
eGFR, mL/min/1.73m^2	73 ± 12	77 ± 12	77 ± 11	78 ± 14	79 ± 13	0.147
Urinary protein ≥1+, n (%)	0 (0.0)	3 (7.0)	7 (12.1)	1 (3.5)	1 (3.6)	0.919
Treatment for hypertension, n (%)	1 (5.6)	6 (14.0)	7 (12.1)	2 (6.9)	2 (7.1)	0.59
Treatment for dyslipidemia, n (%)	1 (5.6)	2 (4.7)	4 (6.9)	2 (6.9)	1 (3.6)	0.927

Mean ± SD, median (interquartile range); eGFR, estimated glomerular filtration rate
[a]40-49 *vs*. 50-59 and 60-62 years, [b]non-smokers *vs*. past and current smokers

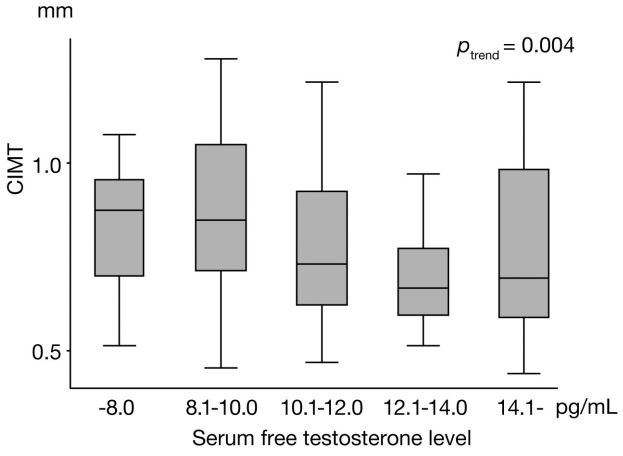

Fig. 1 Graph shows the stepwise association between serum free testosterone level measured by analog ligand radioimmunoassay and carotid intima-media thickness (CIMT).
Lower aFT level is associated with a higher level of CIMT (P_{trend} = 0.004 by the Jonckheere-Terpstra test).

higher hemoglobin A1c, proteinuria, current treatment for hypertension, and higher aFT concentration. A multivariate model adjusted for age, BMI, MAP, and treatment for hypertension, showed a significant association between aFT and CIMT (per 5 pg/mL, -0.08 [-0.16 to 0.00], P = 0.043) (multivariate model 1 in Table 2). Even after adjustment for other clinically relevant factors, the significant association between aFT and CIMT was not attenuated (per 5 pg/mL, -0.09 [-0.17 to 0.00], P = 0.043), identifying aFT as a significant contributor, along with older age (40-49 years as a reference; 50-59 years, 0.19 [0.09 to 0.30], P <0.001; 60-62 years, 0.33 [0.20 to 0.46], P <0.001) and current treatment for hypertension (0.18 [0.03 to 0.33], P = 0.019) (multivariate model 2 in Table 2).

To clarify the stepwise association between aFT and CIMT, each β [95% confidence interval] of the five categories of aFT (3.8-8.0, 8.1-10.0, 10.1-12.0, 12.1-14.0, and 14.1-21.2 pg/mL) was calculated in univariate and multivariate linear regression models (Table 3). β was significantly elevated for an aFT value of ≤10.0 pg/mL in a univariate model (*vs*. 12.1-14.0 pg/mL; 3.8-8.0 pg/mL, 0.22 [0.03 to 0.41], P = 0.021; 8.1-10.0 pg/mL, 0.24 [0.09 to 0.39], P = 0.002). A multivariate model also ascertained that an aFT value of ≤10.0 pg/mL was significantly associated with CIMT (*vs*. 12.1-14.0 pg/mL; 3.8-8.0 pg/mL, 0.19 [0.02 to 0.36], P = 0.028; 8.1-10.0 pg/mL, 0.14 [0.00 to 0.28], P = 0.048).

Although an association between TT and CIMT was also assessed, their association was not significant in either the univariate or multivariate models (per 5 ng/mL, 0.02 [-0.12 to 0.17], P = 0.752 in the univariate model; 0.03 [-0.12 to 0.17], P = 0.713 in the multivariate model adjusted for age, BMI, MAP, smoking status, hemoglobin A1c, total cholesterol, eGFR, urinary protein, and treatment for hypertension and dyslipidemia). These results identified a lower concentration of aFT, especially that of ≤10.0 pg/mL, and not TT, as a sig-

Table 2 Contributors to mean carotid intima-media thickness

	Univariate model		Multivariate model 1		Multivariate model 2	
	β [95%CI]	*P*	β [95%CI]	*P*	β [95%CI]	*P*
Age 40 - 49 year	Reference		Reference		Reference	
50 - 59 year	0.22 [0.11 to 0.31]	<0.001	0.17 [0.07 to 0.27]	0.001	0.19 [0.09 to 0.30]	<0.001
60 - 62 year	0.37 [0.26 to 0.49]	<0.001	0.33 [0.21 to 0.45]	0	0.33 [0.20 to 0.46]	<0.001
Body mass index (per 1 kg/m^2)	0.02 [0.00 to 0.03]	0.022	0.01 [-0.01 to 0.03]	0.187	0.01 [-0.01 to 0.02]	0.296
Mean arterial pressure (per 10 mHg)	0.06 [0.03 to 0.10]	0.001	0.01 [-0.02 to 0.05]	0.447	0.02 [-0.02 to 0.05]	0.442
Non-smokers	Reference				Reference	
Past smokers	0.06 [-0.07 to 0.20]	0.347			-0.03 [-0.15 to 0.09]	0.62
Current smokers	0.03 [-0.10 to 0.16]	0.638			-0.01 [-0.14 to 0.11]	0.846
Hemoglobin A1c (per 1 %)	0.16 [0.04 to 0.28]	0.007			0.05 [-0.06 to 0.16]	0.389
Total cholesterol (per 10 mg/dL)	0.00 [-0.01 to 0.02]	0.606			0.08 [-0.11 to 0.26]	0.41
eGFR (per 30 mL/min/1.73m^2)	-0.09 [-0.21 to 0.03]	0.13			0.07 [-0.04 to 0.18]	0.199
Urinary protein ≥1+	0.23 [0.04 to 0.42]	0.017			-0.01 [-0.02 to 0.01]	0.334
Treatment for hypertension	0.32 [0.17 to 0.48]	<0.001	0.21 [0.07 to 0.35]	0.005	0.18 [0.03 to 0.33]	0.019
Treatment for dyslipidemia	0.17 [-0.04 to 0.38]	0.116			0.11 [-0.08 to 0.30]	0.254
aFT (per 5 pg/mL)	-0.12 [-0.21 to -0.03]	0.009	-0.08 [-0.16 to 0.00]	0.043	-0.09 [-0.17 to 0.00]	0.043

95%CI, 95% confidence interval; eGFR, estimated glomerular filtration rate; aFT, free testosterone measured by analog ligand radioimmunoassay

Table 3 Association between free testosterone levels and mean carotid intima-media thickness

aFT	Univariate model		Multivariate model[a]	
	β [95%CI]	*P*	β [95%CI]	*P*
3.8 - 8.0 pg/mL	0.22 [0.03 to 0.41]	0.021	0.19 [0.02 to 0.36]	0.028
8.1 - 10.0 pg/mL	0.24 [0.09 to 0.39]	0.002	0.14 [0.00 to 0.28]	0.048
10.1 - 12.0 pg/mL	0.12 [-0.03 to 0.26]	0.112	0.02 [-0.11 to 0.16]	0.733
12.1 - 14.0 pg/mL	Reference		Reference	
14.1 - 21.2 pg/mL	0.08 [-0.09 to 0.25]	0.353	0.06 [-0.09 to 0.21]	0.443

95%CI, 95% confidence interval; aFT, free testosterone measured by analog ligand radioimmunoassay; [a]Adjusting for age, body mass index, mean arterial pressure, smoking status, hemoglobin A1c, total cholesterol, eGFR, urinary protein, use of antihypertensive agent, and use of lipid-lowering agent

nificant contributor to CIMT, although serum aFT correlated with serum TT significantly (Spearman correlation coefficient, 0.5124; P <0.001).

Discussion

Recently, a follow-up study of middle-aged Japanese men with coronary risk factors showed the startling finding that low serum testosterone level is associated with cardiovascular events [18]. Although the mechanism for this association has not been sufficiently elucidated, the individual association of low testoster one level with abdominal obesity [19], hypertension [20], diabetes [21] and metabolic syndrome [22] may be related. Furthermore, inverse association of CIMT, which is an independent predictor of future adverse events for CVD [23-25], with serum testosterone level has been reported in a study of very old men aged 74 to 92 years [26], men with type 2 diabetes [27] and obese men with glucose intolerance [28]. A large population-based study of 1482 men aged 25-84 years also showed serum total testosterone level to be inversely associated with age-adjusted CIMT [29]. In that study, a logistic regression model adjusted for the confounding effect of CVD risk factors showed that men with testosterone levels in the lowest quintile had an independent odds ratio (1.51) of being in the highest CIMT quintile [29]. Another recent study of middle-aged men with

LOH symptoms together with testosterone deficiency reported that CIMT correlated inversely with serum testosterone level in multivariate models adjusted for age, total cholesterol, body mass index, blood pressure and smoking [12]. A prospective study of men aged 73-91 years found a higher rate of CIMT progression over 4 years with serum testosterone levels in the lowest tertile [30]. In the present study, we also found that lower aFT level was associated with a higher level of CIMT in middle-aged Japanese men. Furthermore, our multivariate model adjusted for age, BMI, MAP, hemoglobin A1c, total cholesterol and other clinically relevant factors demonstrated a significant association between aFT and CIMT (multivariate model 2 in Tables 2 and 3). Thus, on the basis of our data and that of others, we suggest that low serum testosterone level is an independent risk factor for atherosclerosis in middle-aged men.

A study in animals with a normal physiological level of serum testosterone has shown that androgens reduce atherosclerosis caused by diet and injury [31]. Although the mechanism of androgen in anti-atherosclerosis is still obscure, on the basis of findings in animal models, it may be speculated to be related to an anti-inflammatory effect, regulation of apoptosis and promotion of smooth muscle cell stability. In humans, a cross-sectional study of men aged 50-70 years found that serum testosterone level correlated inversely with the level of vascular cell adhesion molecule-1 (VCAM-1), in addition to CIMT [32]. Endothelial cells produce VCAM-1 by stimulation of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1 and so, the expression of VCAM-1 increases during inflammation. In addition, it is well known that VCAM-1 plays an important role in the development of atherosclerosis. On the basis of these findings, the inverse association of VCAM-1 with serum testosterone level also might support the possibility that progression of atherosclerosis is inhibited by the anti-inflammatory effects of testosterone.

Regarding the effect of exogenous testosterone on atherosclerosis, male animal studies with castrated cholesterol-fed rabbits [33] and low-density lipoprotein (LDL) receptor-deficient mice [34] have shown that testosterone administration has a protective effect against atherosclerosis. Furthermore, two randomized, double-blind, placebo-controlled studies have already reported the effect of testosterone replacement therapy with a long-acting injectable preparation on CIMT in hypogonadal men [35,36]. In the first study of elderly men (mean age 64.8 years) with stable, chronic angina pectoris, CIMT decreased more in the treatment group than in the placebo group at 12 months of treatment [36]. However, this difference did not reach statistical significance, perhaps because of the small sample size (testosterone group, 7; placebo group, 6). Interestingly, in another study of 50 older men (mean age, 57 years) with metabolic syndrome and/or type 2 diabetes mellitus, testosterone replacement therapy significantly improved CIMT as well as the homeostasis model assessment index of insulin resistance and high-sensitivity C-reactive protein at 12 months of treatment [35]. In the same study, an inverse relation between the variation of testosterone from baseline and CIMT reduction was also reported. This may suggest that the changes in CIMT were closely related to the percentage of increase in serum testosterone level from baseline. Thus, testosterone is considered to be one key factor affecting CIMT.

In the present study, we found that a low serum aFT level was associated with CIMT in Japanese men by multivariate analysis with age, BMI and other clinically relevant factors. However, we did not find any association of TT with CIMT in our subjects. Basically, testosterone circulates nonspecifically bound to albumin and specifically bound to sex hormone binding globulin, with a small percentage circulating as unbound or FT. The active testosterone in the blood is considered to be FT. Serum aFT level decreases with aging, whereas aging has little influence on serum TT level in a study with healthy Japanese men [37]. However, aFT has been criticized as unreliable because the values of aFT are substantially lower than the values obtained by equilibrium dialysis [38-40], which has been considered the gold standard technique for determining FT concentrations. Thus, the Endocrine Society and a group of international andrology societies have discouraged the use of aFT. FT and testosterone nonspecifically bound to albumin have been together called bioavailable testosterone (BT). BT reflects the physiological activity of testosterone and is accepted as the most reliable indicator of hypogonadism. Calculated FT (cFT) derived from TT and sex hormone binding globulin has also been used as a reliable marker in place of BT because BT is difficult to measure in the clinical setting [41]. Under these situations with regard to the unreliability of aFT, a strong correlation between aFT and cFT was reported in a study with ambulatory men in the United States [42] and in our study of men with

sexual dysfunction [7]. Therefore, we believe that the present finding that low serum aFT level is an influencing and independent risk factor for CIMT is of value in the clinical setting because no other studies, to our knowledge, have conducted multivariate analyses that have included the various metabolic factors analyzed in the present study to evaluate the relation between serum testosterone level and CIMT.

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