Interaction between bone, the neuroendocrine system and metabolism
Limonard, E.J.

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Osteocalcin and the pituitary-gonadal axis in older men: a population-based study

Eelkje J. Limonard, Natasja M. van Schoor, Renate T. de Jongh, Paul Lips, Eric Fliers, Peter H. Bisschop

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Abstract

Objective Osteocalcin is a well-known marker of bone formation. Recently, mice lacking osteocalcin or its receptor were reported to be subfertile with low testosterone and high luteinizing hormone concentrations. In parallel, in humans, a loss-of-function mutation of the osteocalcin receptor was associated with hypergonadotropic hypogonadism. This suggests that osteocalcin is necessary for normal pituitary-gonadal axis function. Our objective was to determine the association between physiological variations in osteocalcin and the pituitary-gonadal axis in older men.

Design and patients Data were used from the Longitudinal Aging Study Amsterdam (LASA), an ongoing cohort study in a representative sample of the older Dutch population (65-88 years).

Measurements Serum levels of total (T), free (FT) and bioavailable (bioT) testosterone, luteinizing hormone (LH) and osteocalcin were determined. Data were analyzed using linear regression analyses and adjusted for age, BMI, 25-hydroxyvitamin D, parathyroid hormone and vitamin K antagonist use.

Results A total of 614 men participated in the study. The median age was 75.4 (69.8-81.2) years, and the median osteocalcin level was 1.8 (1.3-2.4) nmol/l. Serum osteocalcin was inversely associated with FT (adjusted B = -0.22 ± 0.09 ng/dl, p = 0.012) and bioT (adjusted B = -0.26 ± 0.08 nmol/l, p < 0.01), but not with total T. Furthermore, osteocalcin was positively associated with LH (adjusted B = 0.09 ± 0.03 U/l, p < 0.01).

Conclusions Serum osteocalcin was negatively associated with free and bioavailable testosterone and positively with luteinizing hormone levels.

Introduction

Bone physiology is regulated by many factors, including hormones. Among these hormones, sex steroids are among the most powerful regulators of bone (re)modeling (1). Oestrogen and testosterone positively influence bone growth and maturation and are essential for maintaining bone mass integrity throughout adult life (1, 2). This is best exemplified by the fact that a decrease or absence of sex steroids, either with aging or in gonadal failure, triggers bone loss in both genders (3, 4).
Recent studies indicate that vice visa, bone is a regulator of gonadal function through bone-derived osteocalcin (OC). Osteocalcin is a bone-specific protein synthesized by osteoblasts (5). Osteocalcin accumulates in the bone matrix and binds to mineral, where it is assumed to regulate bone remodeling and mineralization. However, the precise function of osteocalcin as a bone matrix component remains subject to speculation (6). Low concentrations of osteocalcin are detectable in the circulation and are serving as a marker of bone formation.
Circulating osteocalcin exists in two forms, that is carboxylated on three glutamate residues or undercarboxylated (5). Recent findings have identified the testis as a target of osteocalcin. Male mice lacking osteocalcin or its receptor have reduced testosterone levels, smaller reproductive organs and poor reproductive activity (7). Osteocalcin administration to male osteocalcin-deficient mice dose-dependently increased circulating testosterone levels, while ex vivo osteocalcin increased testosterone production by Leydig cells (7). A remarkable feature of the osteocalcin-null mice is that the fertility phenotype developed in the face of an increase in circulating levels of luteinizing hormone, the main regulator of sex steroid synthesis by Leydig cells (8). The phenotype of osteocalcin knockout mice in humans is called hypergonadotropic hypogonadism (9-11). A systematic genomic analysis of a cohort of 59 with unexplained primary hypergonadotropic hypogonadism identified a loss-of-function mutation in the osteocalcin receptor in two subjects, establishing genetic evidence that osteocalcin also fulfills a hormonal function in humans (8). These results indicate that both luteinizing hormone (LH) and osteocalcin are necessary for adequate circulating testosterone levels. This raises the question if biological variations in osteocalcin concentrations are associated with testosterone and LH levels in the general population. To date, one large population-based study showed osteocalcin to be positively associated with total testosterone levels (12). Others did not find an association between osteocalcin and testosterone (13, 14) or an association only in selected populations, including young subjects and subjects with metabolic disorders (15-17). To elucidate the role of physiological variations in osteocalcin on the pituitary-gonadal axis, we determined the relationship of serum osteocalcin with testosterone, free testosterone and bioavailable testosterone, and with LH concentrations in an unselected cohort of older Dutch men.

**Subjects and Methods**

**Data collection**

Data for this study were collected in the context of the Longitudinal Aging Study Amsterdam (LASA). LASA is an ongoing multidisciplinary longitudinal cohort study initiated by the Dutch Ministry of Health to determine predictors and consequences of changes in physical, cognitive, emotional, and social functioning in the ageing population in the Netherlands. Detailed information on data collection and sampling have been described in more detail elsewhere (18). Briefly, a representative sample of the older Dutch population was drawn from the population registers of 11 municipalities in three regions in the Netherlands. The sample consisted of men and women aged 55-85 years, predominantly Caucasian (> 99%), stratified by age, sex, urbanization grade, and expected five-year mortality rate. Subjects were examined at baseline (1992-1993) and every three years thereafter.

**Study population**

Data for the present study were collected during the second cycle (1995-1996) from respondents who were born in 1930 and before (aged 65 years or older as of 1 January, 1996) and were living in Amsterdam, Zwolle or Oss and surroundings. Of the 1720 eligible respondents, 1509 (response rate 87.8%) took part in both the main interview and medical interview. The
interviews were conducted by specially trained interviewers (main interview) and nurses (medical interview). All interviews were tape-recorded in order to monitor quality.

Blood samples were available for 1318 participants (87.3% of 1509), of whom 656 were men. Participants were invited to the VU University Medical Center (VUMC) (respondents living in Amsterdam and surroundings) or a health care centre (respondents living in Zwolle and Oss and surroundings). Urine and blood samples were obtained in the morning after a light breakfast (no dairy products). Blood samples were put on ice, centrifuged and analyzed immediately or stored at -20 °C or -80 °C until measurement. Serum levels of testosterone, luteinizing hormone and osteocalcin were determined in 628 men (95.7% of 656). Subjects using anti-androgens (n = 3), gonadotropin-releasing hormone analogues (n = 4) and/or testosterone-5-alpha reductase inhibitors (n = 8) were excluded from all analyses, leaving 614 respondents. Informed consent was obtained from all participants. The study was approved by the Medical Ethics Committee of the VUMC.

Assessment of biochemical markers

Testosterone (T) concentrations were measured by radioimmunoassay (Radio immunoassay Coat-A-Count, DPC, Los Angeles USA), with an intra-assay coefficient of variation (CV) of 7% at 5 nmol/L and 6% at 30 nmol/L. The interassay CV was 7% at 11.5 nmol/L and 6% at 30 nmol/L. Sex hormone-binding globulin (SHBG) concentrations were determined by an immunoradiometric assay (Orion Diagnostica, Espoo, Finland), with an intra- and interassay CV of 6% at 10 nmol/L and 5% at 18 nmol/L. Luteinizing hormone (LH) levels were measured by an immunometric assay (Delfia Wallac, Turku, Finland), with an intra- and interassay CV of 3% and 7% at 3 U/L. The detection limits of testosterone, SHBG and LH were 1 nmol/L, 6 nmol/L and 0.3 U/L, respectively. The lower reference limit was 8 nmol/L for testosterone and the reference range was 1.0-8.4 U/L for LH. Levels of free testosterone (FT) and bioavailable testosterone (bioT) were calculated according to the method described by Vermeulen et al. (19). Serum 25-hydroxyvitamin D (25(OH)D) levels were measured by a competitive protein binding assay (Nichols Diagnostics Capistrano, CA, USA) and parathyroid hormone (PTH) by an immunoradiometric assay (Incstar Corp., Stillwater, MN, USA). The interassay coefficients of variation were 10% and 12%, respectively. The detection limit was 10 nmol/L for 25(OH)D and 0.7 pmol/L for PTH. Osteocalcin (OC) was measured using an immunoradiometric assay (Biosource Diagnostics, Fleurus, Belgium) in 1997/1998. The intra- and interassay coefficients of variation were 3% at 1.7 nmol/L and 8% at 3.5 nmol/L, respectively. The detection limit was 0.1 nmol/L.

Confounders

Age, body mass index (BMI), serum 25-hydroxyvitamin D, parathyroid hormone, alcohol use, smoking, number of self-reported chronic diseases and use of medication were considered as potential confounders. Data on age was derived from the population registries at baseline. Height was measured using a stadiometer and body weight was measured using a calibrated balance scale (without clothing and shoes). Waist circumference was determined as the average of two measurements measured midway between the lower rib margin and the iliac crest after normal expiration. BMI was calculated by dividing body weight (kg) by the height squared (m²). Self-reported alcohol use, smoking status, chronic diseases and use of medication were
registered during the main interview. Alcohol consumption was classified according to the Garretsen index into three categories: non-drinkers, moderate drinkers and (very) excessive drinkers based on the number of days drinking alcohol per month and the number of alcohol consumptions each time (20). Smoking was divided into currently smoking, used to smoke, and never smoked. Chronic disease included chronic nonspecific lung disease (including asthma and chronic obstructive pulmonary disease [COPD]), cardiac disease, diabetes mellitus, cerebrovascular accident (CVA) or stroke, rheumatoid arthritis or osteoarthritis, cancer and hypertension. Use of vitamin K antagonists, systemic corticosteroids and β-blockers was included in the analysis.

**Statistical analysis**

All analyses were performed using SPSS for Windows (version 20.0; SPSS, Inc., Chicago, IL, USA). The mean and standard deviation (SD) or the median and interquartile range (IQR) are reported depending on the distribution. To study the association between serum osteocalcin and (free, bioavailable) testosterone and the association between serum osteocalcin and luteinizing hormone linear regression analysis was performed. A log-transformed luteinizing hormone variable was used, because of the skewed distribution. All analyses were also performed with osteocalcin divided in quartiles, with the lowest quartile as reference group. The analysis was repeated after exclusion of subjects with testosterone levels below the lower reference limit and LH levels within the reference range (n = 23), and after exclusion of subjects with testosterone levels below the lower reference limit and LH levels above the upper reference limit (n = 12). The study was powered (80%) to detect a small effect ($R^2 = 0.025$) with seven covariates with a two-sided significance level of 0.05. All analyses were tested at the 0.05 level of significance. B-values were reported with standard errors (SE). Unadjusted analysis was performed first. The potential confounders were separately added to the first model. The confounder that showed the largest change in the regression coefficient was than included in the model, after which the remaining confounders were again separately included in the second model. This was repeated until no confounder showed a change of more than 10% in the regression coefficient.

**Results**

Baseline characteristics of the study sample are shown in Table 1. The median age was 75.4 (69.1-81.2) years. The median osteocalcin level was 1.8 (interquartile range 1.3-2.4) nmol/L. Osteocalcin was divided in quartiles with a range of Q1, 0.3-1.3 nmol/L, Q2, 1.3-1.8 nmol/L, Q3, 1.8-2.4 nmol/L and Q4, 2.4-9.4 nmol/L. Baseline data and serum concentration of sex hormones are presented for the whole cohort and according to the osteocalcin quartiles (Table 1). Age, BMI, waist circumference, serum levels of LH and FSH, serum levels of free and bioavailable testosterone, smoking status, use of vitamin K antagonists and β-blockers use were significantly different between the osteocalcin quartiles. Total testosterone levels, alcohol use, presence of self-reported chronic diseases, use of systemic corticosteroids and bisphosphonate use did not differ significantly with respect to the osteocalcin quartiles.
The results of the linear regression analyses are presented in Tables 2-4. Neither serum osteocalcin levels nor serum osteocalcin in quartiles were associated with total testosterone levels (data not shown). There was however a significant inverse association between serum osteocalcin levels and free testosterone (unadjusted $B = -0.41 \pm 0.09$ ng/dL, $p < 0.001$) (Table 2) and between serum osteocalcin and bioavailable testosterone (unadjusted $B = -0.43 \pm 0.07$ nmol/L, $p < 0.001$) (Table 3). Men in Q4 of osteocalcin had significantly lower free testosterone (12.5%, $p < 0.001$) and bioavailable testosterone levels than those in Q1 (17.2%, $p < 0.001$).

After adjustment for relevant confounders (age, BMI, LH, PTH and 25(OH)D levels and vitamin K antagonist use), these relationships were still present. Furthermore, serum osteocalcin levels were positively associated with LH levels (unadjusted $B = 0.13 \pm 0.03$ U/L, $p < 0.001$) (Table 4). Men in the lowest quartile of osteocalcin had lower levels of LH than those in Q4 (28.6%, $p < 0.001$). This association remained significant after adjustment for confounders (age, BMI, bioavailable or free testosterone and PTH levels). After exclusion of men with low testosterone levels and normal LH levels ($n = 23$) and men with low testosterone levels and elevated LH levels ($n = 12$), linear regression analyses revealed similar results (data not shown).
### Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Osteocalcin (OC) 1.8 (1.3-2.4) nmol/L</th>
<th>OC, Q1 0.4-1.3 nmol/L</th>
<th>OC, Q2 1.3-1.8 nmol/L</th>
<th>OC, Q3 1.8-2.4 nmol/L</th>
<th>OC, Q4 2.4-9.5 nmol/L</th>
<th>p-value &lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>614</td>
<td>153</td>
<td>156</td>
<td>152</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.4 (69.8-81.2)</td>
<td>73.5 (68.9-81.0)</td>
<td>74.9 (68.8-79.8)</td>
<td>74.4 (69.9-81.7)</td>
<td>78.4 (72.1-82.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 ± 3.4</td>
<td>27.0 ± 3.2</td>
<td>26.6 ± 3.3</td>
<td>26.0 ± 3.0</td>
<td>24.6 ± 3.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>99.2 ± 10.3</td>
<td>101.3 ± 9.5</td>
<td>100.3 ± 9.2</td>
<td>99.6 ± 9.6</td>
<td>95.4 ± 11.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LH (U/L)</td>
<td>5.65 (3.82-7.92)</td>
<td>5.25 (3.25-7.62)</td>
<td>5.29 (3.62-7.07)</td>
<td>5.47 (4.13-7.70)</td>
<td>6.75 (4.32-11.61)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FSH (U/L)</td>
<td>7.37 (4.75-12.59)</td>
<td>6.75 (4.51-11.49)</td>
<td>6.63 (4.58-12.18)</td>
<td>7.28 (4.90-10.55)</td>
<td>9.19 (5.31-21.63)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>15.52 ± 4.81</td>
<td>15.61 ± 4.82</td>
<td>15.49 ± 4.52</td>
<td>15.52 ± 4.83</td>
<td>15.45 ± 5.11</td>
<td>0.022</td>
</tr>
<tr>
<td>Bioavailable testosterone* (nmol/L)</td>
<td>6.40 ± 1.94</td>
<td>6.93 ± 1.83</td>
<td>6.58 ± 1.85</td>
<td>6.33 ± 1.92</td>
<td>5.74 ± 2.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Free testosterone† (ng/dL)</td>
<td>8.05 ± 2.33</td>
<td>8.53 ± 2.17</td>
<td>8.16 ± 2.11</td>
<td>8.04 ± 2.32</td>
<td>7.46 ± 2.58</td>
<td>0.001</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>41.1 (30.5-52.3)</td>
<td>36.7 (28.2-47.0)</td>
<td>40.0 (28.5-50.2)</td>
<td>40.5 (30.5-52.7)</td>
<td>46.7 (35.7-59.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>57.8 ± 24.6</td>
<td>56.3 ± 22.3</td>
<td>61.2 ± 24.2</td>
<td>60.9 ± 25.5</td>
<td>52.7 ± 25.5</td>
<td>0.006</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>3.18 (2.41-3.36)</td>
<td>2.85 (2.23-3.76)</td>
<td>2.90 (2.27-4.03)</td>
<td>3.08 (2.44-4.12)</td>
<td>4.02 (2.87-5.49)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Alcohol intake‡ (%)</td>
<td>14.3</td>
<td>16.3</td>
<td>10.9</td>
<td>15.1</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Non-drinkers</td>
<td>75.1</td>
<td>69.9</td>
<td>78.2</td>
<td>73.7</td>
<td>78.4</td>
<td></td>
</tr>
<tr>
<td>Moderate drinkers</td>
<td>10.6</td>
<td>13.7</td>
<td>10.9</td>
<td>11.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>(very) excessive drinkers</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.036</td>
</tr>
<tr>
<td>Never</td>
<td>10.1</td>
<td>11.8</td>
<td>9.0</td>
<td>9.9</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>24.8</td>
<td>17.0</td>
<td>21.8</td>
<td>26.3</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>65.1</td>
<td>71.2</td>
<td>69.2</td>
<td>63.8</td>
<td>56.2</td>
<td></td>
</tr>
<tr>
<td>Chronic disease‡ (%)</td>
<td>74.3</td>
<td>80.4</td>
<td>70.5</td>
<td>72.4</td>
<td>73.8</td>
<td>0.216</td>
</tr>
<tr>
<td>Medication use (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic corticosteroid</td>
<td>1.6</td>
<td>1.3</td>
<td>1.3</td>
<td>3.3</td>
<td>0.7</td>
<td>0.291</td>
</tr>
<tr>
<td>Bisphosphonate</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>0.389</td>
</tr>
<tr>
<td>Vitamin K antagonist</td>
<td>8.5</td>
<td>18.3</td>
<td>3.8</td>
<td>8.6</td>
<td>3.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>β-blocker</td>
<td>16.0</td>
<td>24.2</td>
<td>15.4</td>
<td>12.5</td>
<td>11.8</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Values are represented as mean ± standard deviation or median (interquartile range).

* Calculated according to Vermeulen et al. (19)
† Non-drinkers: 0 glasses/week; moderate drinkers 1-21 glasses/week; (very) excessive drinkers: > 21 glasses/week.
‡ Self-reported (chronic nonspecific lung disease, cardiac disease, diabetes mellitus, cerebrovascular accident/stroke, rheumatoid arthritis, osteoarthritis, cancer and hypertension).
§ Differences in mean values between quartiles (Q) were examined with one-way ANOVA (mean ± SD), Kruskal Wallis test (median) or chi-square test for categorical variables.
Testosterone: 1 ng/dL = 0.0347 nmol/L, osteocalcin: 1 μg/L = 0.171 nmol/L,
25-hydroxyvitamin D: 1 ng/mL = 2.496 nmol/L.
Table 2 Association between serum osteocalcin and free testosterone

<table>
<thead>
<tr>
<th>Osteocalcin (n = 614)</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Q2</td>
<td>-0.38 ± 0.26 (0.148)</td>
<td>-0.32 ± 0.25 (0.205)</td>
<td>-0.40 ± 0.25 (0.106)</td>
<td>-0.42 ± 0.25 (0.089)</td>
</tr>
<tr>
<td>Q3</td>
<td>-0.50 ± 0.26 (0.061)</td>
<td>-0.42 ± 0.25 (0.102)</td>
<td>-0.40 ± 0.25 (0.108)</td>
<td>-0.43 ± 0.25 (0.081)</td>
</tr>
<tr>
<td>Q4</td>
<td>-1.08 ± 0.26 (0.000)</td>
<td>-0.61 ± 0.26 (0.019)</td>
<td>-0.49 ± 0.26 (0.054)</td>
<td>-0.57 ± 0.26 (0.032)</td>
</tr>
<tr>
<td>Continuous</td>
<td>-0.41 ± 0.09 (0.000)</td>
<td>-0.28 ± 0.08 (0.001)</td>
<td>-0.19 ± 0.08 (0.023)</td>
<td>-0.22 ± 0.09 (0.012)</td>
</tr>
</tbody>
</table>

Data are presented with osteocalcin divided in quartiles (Q): Q1 = (0.4-1.3), Q2 = (1.3-1.8), Q3 = (1.8-2.4), Q4 = (2.4-9.5).

The lowest quartile of osteocalcin is being used as reference group.

Results are presented as B-value ± SE ng/dL (p-value).

Model 1: univariate.
Model 2: as model 1 and adjusted for luteinizing hormone.
Model 3: as model 2 and adjusted for age.
Model 4: as model 3 and adjusted for vitamin K antagonist use and BMI.

Table 3 Association between serum osteocalcin and bioavailable testosterone

<table>
<thead>
<tr>
<th>Osteocalcin (n = 614)</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Q2</td>
<td>-0.36 ± 0.22 (0.101)</td>
<td>-0.31 ± 0.21 (0.142)</td>
<td>-0.33 ± 0.20 (0.132)</td>
<td>-0.43 ± 0.20 (0.034)</td>
</tr>
<tr>
<td>Q3</td>
<td>-0.60 ± 0.22 (0.006)</td>
<td>-0.54 ± 0.21 (0.010)</td>
<td>-0.50 ± 0.20 (0.013)</td>
<td>-0.58 ± 0.20 (0.005)</td>
</tr>
<tr>
<td>Q4</td>
<td>-1.18 ± 0.22 (0.000)</td>
<td>-0.80 ± 0.22 (0.000)</td>
<td>-0.67 ± 0.21 (0.002)</td>
<td>-0.70 ± 0.22 (0.002)</td>
</tr>
<tr>
<td>Continuous</td>
<td>-0.43 ± 0.07 (0.000)</td>
<td>-0.31 ± 0.07 (0.000)</td>
<td>-0.24 ± 0.07 (0.001)</td>
<td>-0.26 ± 0.08 (0.002)</td>
</tr>
</tbody>
</table>

Data are presented with osteocalcin divided in quartiles (Q): Q1 = (0.4-1.3), Q2 = (1.3-1.8), Q3 = (1.8-2.4), Q4 = (2.4-9.5).

The lowest quartile of osteocalcin is being used as reference group.

Results are presented as B-value ± SE nmol/L (p-value).

Model 1: univariate.
Model 2: as model 1 and adjusted for luteinizing hormone.
Model 3: as model 2 and adjusted for age and BMI.
Model 4: as model 3 and adjusted for vitamin K antagonist use, parathyroid hormone and 25-hydroxyvitamin D.
### Table 4 Association between serum osteocalcin and the natural logarithm of luteinizing hormone

<table>
<thead>
<tr>
<th>Osteocalcin (n = 614)</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>0.03 ± 0.08 (0.709)</td>
<td>0.02 ± 0.08 (0.752)</td>
<td>0.01 ± 0.08 (0.851)</td>
<td>0.01 ± 0.08 (0.863)</td>
<td>0.01 ± 0.08 (0.855)</td>
</tr>
<tr>
<td>Q2</td>
<td>0.10 ± 0.08 (0.197)</td>
<td>0.08 ± 0.08 (0.300)</td>
<td>0.07 ± 0.08 (0.387)</td>
<td>0.07 ± 0.08 (0.400)</td>
<td>0.07 ± 0.08 (0.389)</td>
</tr>
<tr>
<td>Q3</td>
<td>0.39 ± 0.08 (0.000)</td>
<td>0.32 ± 0.08 (0.000)</td>
<td>0.28 ± 0.08 (0.000)</td>
<td>0.28 ± 0.08 (0.001)</td>
<td>0.28 ± 0.08 (0.001)</td>
</tr>
<tr>
<td>Q4</td>
<td>0.13 ± 0.03 (0.000)</td>
<td>0.10 ± 0.03 (0.000)</td>
<td>0.08 ± 0.03 (0.002)</td>
<td>0.09 ± 0.03 (0.003)</td>
<td>0.09 ± 0.03 (0.003)</td>
</tr>
</tbody>
</table>

Data are presented with osteocalcin divided in quartiles (Q):
- Q1 = (0.4-1.3), Q2 = (1.3-1.8), Q3 = (1.8-2.4), Q4 = (2.4-9.5).
- The lowest quartile of osteocalcin is being used as reference group.
- Results are presented as B-value ± SE U/L (p-value).
- Model 1: univariate.
- Model 2: as model 1 and adjusted for age.
- Model 3: as model 2 and adjusted for BMI.
- Model 4: as model 3 and adjusted for parathyroid hormone and bioavailable testosterone.
- Model 5: as model 3 and adjusted for parathyroid hormone and free testosterone.
Discussion

In this population-based study of older subjects, serum osteocalcin levels were negatively associated with free and bioavailable testosterone levels and positively with LH levels, independently of confounders. No association with total testosterone levels was observed.

Circulating osteocalcin exists in two forms, carboxylated on three glutamate residues or undercarboxylated (5). Undercarboxylated osteocalcin is considered the biologically active form of osteocalcin (21, 22). In humans, an association between (undercarboxylated) osteocalcin and testosterone levels has been demonstrated in some, but not all studies. In the study of Kirmani et al., a correlation between total osteocalcin and total testosterone was found in 18 boys aged 11-14 years, but not in the other age groups (4-10 and 15-20 years) (15). This association was, however, no longer significant following adjustment for bone age. In a group of 2400 Chinese men aged 20-69 years, no association was found between total osteocalcin and total, free or bioavailable testosterone. In a subgroup of 357 men with any two factors of the metabolic syndrome, but not in men with one, three or more factors of the metabolic syndrome, osteocalcin was positively associated with total, free and bioavailable testosterone (17). In another, relatively small population of 69 patients with type 2 diabetes undercarboxylated osteocalcin and the undercarboxylated/total osteocalcin ratio was positively associated with free testosterone and negatively with LH (16).

To date only the study by Hannemann et al. has shown a positive association in healthy, adult subjects (12). In 1338, German men osteocalcin was positively associated with total, but not with free testosterone after adjustment for age and BMI. The major difference between this study and ours was the age of the study population. Subjects in our population were older, with a median age of 75.4 (IQR 69.8-81.2) years, compared to 53.5 (IQR 41.0-65.0) years. In the study by Hannemann et al., the strength of the association between osteocalcin and total testosterone was attenuated, and the association between osteocalcin and free testosterone disappeared after adjustment for age, suggesting that age is an important modifier of the relationship between osteocalcin and testosterone.

A randomized, placebo controlled trial by Bolland et al. (13) did not find a change in testosterone levels after a substantial reduction in total osteocalcin levels following zoledronate treatment in 28 HIV-infected men. In a study with 159 men from infertile couples, neither carboxylated, total nor percentage undercarboxylated osteocalcin was a predictor of testosterone in adjusted analysis (14). Only the association between percentage undercarboxylated osteocalcin and LH levels remained after adjustment.

The strength of the present study lies in the large sample of older persons representative of the older Dutch population, combined with the availability of a large number of outcome measures and potential confounders.

In our study we did not measure undercarboxylated osteocalcin levels, which has been identified as the hormonally active form of osteocalcin (7, 21). Most studies that showed a
significant association with testosterone levels in humans did assess only total osteocalcin levels (12, 13, 17) or did not find an association with undercarboxylated osteocalcin concentrations (14, 15). The measurement of undercarboxylated osteocalcin remains a challenge due to its close relationship with total osteocalcin levels (23, 24). This makes interpretation of the relationship between undercarboxylated osteocalcin and testosterone levels difficult and prone to bias. Post-translational carboxylation of osteocalcin is vitamin K dependent (5, 25). In our study population 8.5% of the subjects used a vitamin K antagonist, which might have resulted in a higher concentration of undercarboxylated osteocalcin (26). Inclusion of vitamin K antagonists as a confounder in the regression model did not change the results, and results were also not different after exclusion of subjects using a vitamin K antagonist, a β-blocker or systemic corticosteroids (data not shown). Several studies have indicated that undercarboxylated osteocalcin acts via the G protein-coupled receptor family C, group 6, member A (GPRC6A) receptor present in the Leydig cells of the testes to stimulated testosterone production (7, 27). However, a recent study could not confirm that undercarboxylated osteocalcin activates GPRC6A in transfected cells (28).

There are several challenges when investigating the effects of osteocalcin on testosterone concentrations. First of all, low levels of testosterone are associated with high bone turnover, reflected in increased osteocalcin levels (29-32). Therefore, we cannot rule out that the detected negative association of osteocalcin with testosterone is caused by an effect of testosterone on bone metabolism, rather than the other way around. The main endocrine pathway regulating male fertility is the hypothalamic-pituitary axis, in which LH favours testosterone synthesis. The positive association seen between osteocalcin and LH in our study seems merely the result of feedback loop between testosterone and LH, rather than the existence of a direct relationship between LH and osteocalcin. Second, none of the subjects participating in our study were osteocalcin deficient. It could well be that only a minimal amount of osteocalcin is needed to exhibit positive effects on testosterone concentrations, with a low threshold above which osteocalcin has no additional stimulatory effects.

We observed a negative association between osteocalcin and free testosterone, and between osteocalcin and bioavailable testosterone, but not between osteocalcin and total testosterone. The majority of plasma testosterone is protein bound, mostly to SHBG with a high affinity, with the remainder nonspecifically bound to albumin. Only 1-2% of testosterone, referred to as the free fraction, circulates in an unbound form. The sum of free and albumin-bound testosterone is referred to as the bioavailable fraction. Total testosterone levels can be measured reliably by radioimmunoassays or tandem mass spectrometry. Several methods are available to estimate free and bioavailable testosterone. The gold standard for free testosterone measurement is equilibrium dialysis (19, 33). Immunoassays involving an analogic ligand are also used, but correspond poorly with the gold standard, and should therefore not be used (19, 34). Bioavailable testosterone can be obtained from serum total T concentrations and determination of the non-SHBG-T by the ammonium sulphate precipitation technique (35). Bioavailable and free testosterone can also be calculated by measuring total testosterone, SHBG and albumin concentrations and using the equilibrium-binding constants of testosterone.

Osteocalcin and the pituitary-gonadal axis in elderly men
to SHBG and albumin (19). This is regarded as a reliable index of free testosterone and bioavailable testosterone (19). Most studies, including ours, used an immunoassay to determine total testosterone levels (12-14, 17) and calculated bioavailable and free testosterone levels (12, 17). One study used tandem mass spectrometry for total testosterone levels (15). And two studies measured free testosterone levels, one with a radioimmunoassay (16), the other did not state the method used (14). These small methodological differences can however not account for the differences in associations with osteocalcin found.

In conclusion, serum osteocalcin levels were negatively associated with free and bioavailable testosterone levels and positively with luteinizing hormone levels in older men.