Vitamin D and Biomarkers of Sex Steroid Hormones Are Non-Linearly and Inversely Related to All-Cause Mortality: Results from NHANES III

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Vitamin D and biomarkers of sex steroid hormones are non-linearly and inversely related to all-cause mortality: results from NHANES III

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Abstract

Background: In men, hypovitaminosis D as well as high and low testosterone levels have been linked to adverse events, including death. A biological interaction has been previously suggested between vitamin D and androgens. In a cohort study using Third National Health and Nutrition Examination Survey data, we simultaneously investigated circulating vitamin D and biomarkers of sex steroid hormones as predictors of all-cause mortality.

Methods: Age-adjusted and fully-adjusted Cox regression models were constructed to estimate hazard ratios (HR) and their 95% confidence intervals (CI). Whereas the vitamin D sufficient group (25(OH)D ≥ 30 ng/ml) was selected as a referent, biomarkers of sex steroid hormones (testosterone, estradiol, SHBG) were defined as Loge-transformed continuous variables.

Results: Of 1,472 men with a mean age of 42.1 years at baseline, 382 died over a median of 192 months of follow-up. Estradiol levels were significantly higher among vitamin D deficient compared to vitamin D sufficient men and sex hormone binding globulin level was significantly higher in vitamin D sufficient compared to vitamin D insufficient or deficient groups. An inverse non-linear relationship was observed between all-cause mortality rate and levels of testosterone, estradiol and vitamin D, in fully-adjusted models. There were no significant interaction effects between vitamin D and sex steroid hormones in relation to all-cause mortality rate.

Conclusions: Vitamin D and sex steroid hormones, but not sex hormone binding globulin, may be inversely and non-linearly related to all-cause mortality among adult men, after adjustment for baseline demographic, socioeconomic, lifestyle and clinical characteristics.

Keywords: All-cause mortality, androgens; cohort study, estradiol, sex hormone binding globulin, testosterone, vitamin D

Introduction

In men, hypovitaminosis D as well as both high or low testosterone levels have been linked to adverse events, including death. Vitamin D is a fat-soluble secosteroid hormone endowed with pleiotropic effects [1,2]. Besides the well-known function of vitamin D in promoting in vitro bone mineralization and prevention of osteoporosis and bone fracture through intestinal calcium absorption [3,4], mounting evidence has linked hypovitaminosis D to musculoskeletal diseases [5], cancer [6], autoimmune diseases, type 2 diabetes, cardiovascular disease [6,7] and all-cause mortality [5]. The main source of vitamin D is cholecalciferol (vitamin D3), synthesized in the skin by ultraviolet radiation [2]; pro-vitamin D3 (7-dehydrocholesterol) is converted to pre-vitamin D3, which isomerizes into cholecalciferol and is later converted to calcitriol (1,25(OH)2D3) through a two-step enzymatic pathway involving liver 25-hydroxylase (CYP2R1) and kidney 1-α-hydroxylase (CYP27B1) [2,8,9]. Vitamin D status is assessed by measuring 25(OH)D3 [8]. Currently, the
Institute of Medicine advises 25(OH)D $\geq$ 20 ng/ml but Endocrine Society advises 25(OH)D $\geq$ 30 ng/ml, partly due to lack of adequate assays for vitamin D quantification; thus, individuals are considered sufficient if 25(OH)D $\geq$ 30 ng/ml ($\geq$ 74 nmol/L), insufficient if 25(OH)D $\geq$ 20-29 ng/ml (50-74 nmol/L), and deficient if 25(OH)D $< 20$ ng/ml ($< 50$ nmol/L) [5,7]. A biological interaction has been previously suggested between vitamin D and androgens [5,10-12]. Specifically, androgens may increase 1-$\alpha$-hydroxylase, a key enzyme in vitamin D metabolism, and regulation of gene expression by vitamin D metabolites is modified according to androgen levels [5]. Numerous biological mechanisms have been proposed in an effort to explain the joint protective effects of vitamin D and sex steroids on bone fracture which is generally less among males compared to females [12], while other studies have suggested a contribution for testicular vitamin D metabolism [11].

The key androgen, in men, is testosterone, 50-60% of which is bound with high affinity to sex hormone binding globulin (SHBG), and the remaining 40-50% is bound loosely to albumin and other proteins, leaving 1-3% as ‘free’ testosterone [13]. The clinical significance of testosterone level in men remains controversial [5,14,15], with low and high levels of testosterone linked to deleterious outcomes [16]. Starting in the 3rd-4th decade of life, male aging is accompanied by 0.4-2.6% annual decline in total testosterone and 0.2% annual decline in ‘free’ testosterone resulting from gradual rise in SHBG, implying a clinical significance for measuring ‘free’ testosterone [5,17,18]. Age-related decline in endogenous testosterone, or male hypogonadism, has been associated with reduced libido and vitality, depression, sarcopenia, obesity, dyslipidemia, hypertension, metabolic syndrome, type 2 diabetes, stroke, atherosclerosis, subclinical inflammation, osteoporosis, trauma fracture and mortality risk [13,16,19-21]; an increasing level of SHBG, which predominantly carries testosterone and estradiol in the blood, has also been associated with cardiovascular risk factors and higher risk for premature death [22]. Similarly, when administered at high doses, exogenous testosterone has been correlated with sudden cardiac death and liver disease [16,23,24]. However, perceived health benefits of low dose exogenous testosterone may have contributed to its widespread use without approved change in indication [16,22,25-27].

The purpose of this population-based cohort study is to simultaneously investigate vitamin D and biomarkers of sex steroid hormones (testosterone, estradiol, SHBG) as predictors of all-cause mortality among adult men in the Third National Health and Nutrition Examination Survey (NHANES III).

Methods

Study population

The National Center for Health Statistics implemented NHANES III between 1988 and 1994 in two phases (Phase I: 1988-1991, Phase II: 1991-1994) using a complex multistage probability sample design [28]. This study utilized questionnaire, physical examination and laboratory data from Phase I of NHANES III. Public-use datasets, including adult (n=20,050), exam (n=31,311), examdr (n=30,818), lab (n=29,314), lab2 (n=29,314), nhanes3 (n=33,994) and sshormon (n=1,636) were merged to select male adults (18 years and older) with serum specimens analyzed for vitamin D, biomarkers of sex steroid hormones and all-cause mortality data. A total of 1,472 NHANES III participants were included in the study. NHANES III is compliant with ethical rules of human experimentation stated in the Declaration of Helsinki, including approval by institutional review board and informed consent.

Measures

All-cause mortality

Mortality linkage of NHANES III with the National Death Index provides the opportunity to investigate associations of wide range of characteristics at baseline with mortality rates at follow-up through December 31, 2006. Variables provided in this linked mortality file include sequence number, eligibility status, assigned vital status, mortality source, person-months of follow-up from interview date, person-months of follow-up from Mobile Examination Center (MEC) or home examination date and underlying multiple causes of death [29]. In this analysis, an event was defined as death from any cause during the follow-up period, starting at date of MEC examination and ending before December 31, 2006. Sensitivity analyses were performed to examine shorter durations (5 years and 10 years) of follow-up, yielding similar results to the total follow-up period.

Vitamin D

Serum 25(OH)D concentration was determined in ng/mL (range: 5-160.3 ng/mL; limit of detection is 3.5 ng/mL) and nmol/L (range: 12.5-400.1 nmol/L; limit of detection is 8.7 nmol/L) at the National Center for Environmental Health using DiaSorin radioimmunoassay kit (Stillwater, MN) [30,31]. NHANES documentation did not include inter- or intra-assay coefficients of variation for vitamin D. Because of skewed distribution, vitamin D concentration was log$_{10}$-transformed or defined as a categorical variable (Deficient: <20 ng/mL; Insufficient: 20-<30 ng/mL; Sufficient: $\geq$ 30 ng/mL), taking the sufficient group as a referent.

Biomarkers of sex steroids

Serum testosterone, estradiol and SHBG concentrations were quantitatively determined using immunoassay techniques. NHANES documentation did not include inter-assay and intra-assay coefficients of variation for sex steroid hormones. Because of skewed distributions, log$_{10}$-transformation and categorization in quintiles were performed for biomarkers of sex steroid hormones.

Testosterone

Elecys Testosterone was used to measure circulating testost-
terone concentration and was based on a competitive test principle using a monoclonal antibody directed against test-
tosterone. The measuring range for testosterone is 0.069-52.00
nmol/L or 0.020-15.00 ng/mL, with a lower detection limit of
0.069 nmol/L (0.02 ng/mL) and a functional sensitivity (level
at which between-run coefficient of variation is less than or
equal to 20%) of 0.42 nmol/L (0.12 ng/mL) [32].

**Estradiol**

Elecsys Estradiol II was used to measure circulating estradiol
concentration and employs a competitive test principle using a
polyclonal antibody specifically directed against 17β-estradiol.
The measuring range for estradiol is 18.4-15,781 pmol/L (5.00-
4,300 pg/mL), with a lower detection limit of 18.4 pmol/L (5.0
pg/mL) and a functional sensitivity (level at which between-
run coefficient of variation is less than or equal to 20%) of 44
pmol/L (12 pg/mL) [33].

**Sex hormone binding globulin**

Elecsys SHBG is used to measure SHBG concentration and
employs two monoclonal antibodies specifically directed
against it. The measuring range for SHBG is 0.350-200 nmol/L,
with a lower detection limit of 0.35 nmol/L [34].

**Demographic, socioeconomic, lifestyle and clinical
characteristics**

Age (continuous; ‘18-20’; ‘20-39’; ‘40-59’; ‘60+’ years), education
(‘Less than High School’; ‘High School’; ‘More than High School’),
race/ethnicity (‘Non-Hispanic White’; ‘Non-Hispanic Black’;
‘Hispanic’; ‘Other’), area of residence (‘Metropolitan’; ‘Other’),
poverty income ratio (continuous; ‘<100%’; ‘100%–<200%’;
‘≥200%’), marital status (‘Married/Co-habiting’; ‘Not married’),
smoking status (‘Current smoker’; ‘Ex-smoker’; ‘Never smoker’),
alcohol consumption (at least 12 glasses in the past 12 months)
(‘yes’; ‘no’), body mass index (BMI) based on directly measured
weight and height (continuous; ‘<25’; ‘25–<30’; ‘30+ kg/m2’),
waist-to-hip ratio defined as ratio of directly measured waist
and hip circumferences (continuous; ‘≤0.9’; ‘>0.9’), self-rated
health (‘excellent’; ‘very good’; ‘good’; ‘fair’; ‘poor’), history of
chronic conditions including arthritis, asthma, chronic bron-
chitis, emphysema, congestive heart failure, stroke, diabetes
and cancer (‘yes’; ‘no’), and physical activity (continuous; ‘0’;
‘>0–<100 METS’; ‘100–<200 METS’; ‘200–<300 METS’; ‘≥300 METS’).

**Statistical analysis**

Statistical analyses were performed using STATA version 12
(STATA Corporation, College Station, TX). Whereas frequencies
and percentages were computed for categorical variables, for
continuous variables mean, median, standard deviation (SD),
standard errors of the mean (SEM) and inter-quartile ranges
were computed, as appropriate. Shapiro-Wilk’s normality
test was applied and continuous exposure variables were
log-transformed, as needed. One-way ANOVA tests with
post-hoc comparisons evaluated using Bonferronicorrections
were used for comparing log-transformed testosterone,
estriadiol and sex hormone-binding globulin concentra-
tions across vitamin D status groups. Kaplan-Meier curves
were constructed and log-rank tests were used to examine
bivariate relationships between exposure variables (defined
in quintiles) and survival. For multivariable analyses, hazard
ratios (HR) and their 95% confidence intervals (CI) were
calculated using Cox regression models. Fully-adjusted models
were controlled for age (continuous), race/ethnicity, smok-
ing status, and physical activity (categorical). Furthermore,
we examined non-linearity by including quadratic terms for
exposure variables and evaluated the interaction between
vitamin D status and biomarkers of sex steroid hormones in
age-adjusted and fully-adjusted models. Several aspects of
NHANES III design were taken into account in data analyses,
including sampling weights and complex survey design
(primary sampling unit (PSU) and strata). Three full-sample
and four sub-sample weights are available in NHANES III. For
this analysis, we applied the MECweights with strata and PSU
based on Phases I of NHANES III (i.e., 3 years). Sampling weights
were used to correctly estimate population prevalence rates,
means and other statistics while accounting for differential
probabilities of selection, non-coverage, non-response and
over-sampling of sub-populations. Two-sided statistical tests
were performed at alpha of 0.05.

**Results**

Table 1 describes baseline demographic, socioeconomic, life-
style and clinical characteristics of study participants. Of 1,472
men with (mean±SEM) age of (42.1±0.6) years at baseline, 77%
were non-Hispanic White, 70% were married or cohabiting,
47% had more than high school education and nearly 10%
were below the PIR. Whereas 35% were never smokers and
71% drank alcohol in the past 12 months, 18% were obese,
71% exhibiting central obesity, 13% reported fair or poor
self-rated health 28% reported having major chronic
conditions, and physical activity was, on average, 139.3±8.2
METs. Descriptive statistics for vitamin D and biomarkers of
sex steroid hormones are also presented in Table 1.

Vitamin D was weakly correlated with biomarkers of sex
steroid hormones including testosterone (r spearman =0.08),
estriadiol (r spearman =–0.07) and SHBG (r spearman =0.09). When
log-transformed testosterone, estradiol and SHBG, were
compared among vitamin D sufficient, insufficient and defi-
cient men, estradiol levels were significantly higher among
vitamin D deficient compared to vitamin D sufficient men and
sex hormone binding globulin level was significantly higher
in vitamin D sufficient compared to vitamin D insufficient or
deficient groups (Table 2).

A total of 382 men died over a median of 192 months with
an interquartile range of 183 to 202 months of follow-up
(<5 years: 100; 5–<10 years: 105; ≥10 years: 1,267), for an es-
estimated all-cause mortality rate of 16.7% (95%CI:14.8%–18.8%).
Kaplan-Meier curves were constructed to examine all-cause

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Beydoun et al. *Hormonal Studies* 2015,
http://www.hoajonline.com/journals/pdf/2052-8000-3-1.pdf
doi: 10.7243/2052-8000-3-1
Table 1. Demographic, socioeconomic, lifestyle and clinical characteristics in adult men in the study sample (n=1472)–Third National Health and Nutrition Examination Survey.

<table>
<thead>
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<th>Characteristic</th>
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<tr>
<td><strong>Age (years):</strong></td>
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<tr>
<td>Mean±SEM</td>
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<tr>
<td>&lt;20</td>
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<td>20-39</td>
<td>47.5</td>
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<td>40-59</td>
<td>31.5</td>
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<td>≥60+</td>
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<td>Non-Hispanic Black</td>
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<td>Mexican-American</td>
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<tr>
<td>Other</td>
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<tr>
<td>Not Married</td>
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<td><strong>Education:</strong></td>
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<td>23.6</td>
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<td>High School</td>
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<tr>
<td>More than High School</td>
<td>47.2</td>
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<td><strong>Poverty-Income Ratio:</strong></td>
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<tr>
<td>Mean±SEM</td>
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<tr>
<td>&lt;100%</td>
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<tr>
<td>100%-&lt;200%</td>
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<tr>
<td>≥200%</td>
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<tr>
<td>Other</td>
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<td><strong>Smoking status:</strong></td>
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<td>Never smoker</td>
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<td><strong>Alcohol use – past 12 months:</strong></td>
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<td>Yes</td>
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<td><strong>Body Mass Index (kg/m²):</strong></td>
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<tr>
<td>Mean±SEM</td>
<td>26.7 ± 0.4</td>
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<tr>
<td>&lt;25</td>
<td>44.9</td>
</tr>
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<td>25-&lt;30</td>
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<td>≥30</td>
<td>18.3</td>
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<td><strong>Waist-to-Hip Ratio:</strong></td>
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<td>≤0.9</td>
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<tr>
<td>&gt;0.9</td>
<td>70.6</td>
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<tr>
<td><strong>Self-Rated Health:</strong></td>
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<tr>
<td>Excellent</td>
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<td>Very Good</td>
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Continuation of Table 1.

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<thead>
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<th>Characteristic</th>
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<td><strong>History of Major Chronic Conditions:</strong></td>
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<td>No</td>
<td>71.6</td>
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<tr>
<td><strong>Physical Activity (Metabolic Equivalents):</strong></td>
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<tr>
<td>Mean±SEM</td>
<td>139.3±8.2</td>
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<td>0</td>
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<td>&gt;0-&lt;100</td>
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<td>100-&lt;200</td>
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<td>200-&lt;300</td>
<td>11.3</td>
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<tr>
<td>≥300</td>
<td>12.8</td>
</tr>
<tr>
<td><strong>Vitamin D (ng/ml):</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>26.7 [20.2-34.3]</td>
</tr>
<tr>
<td>Sufficient (≥ 30)</td>
<td>50.3</td>
</tr>
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<td>Insufficient (20-&lt;30)</td>
<td>33.1</td>
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<tr>
<td>Deficient (&lt;20)</td>
<td>16.5</td>
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<tr>
<td><strong>Testosterone (ng/mL):</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>5.4±0.15.15</td>
</tr>
<tr>
<td>[3.97-6.56]</td>
<td></td>
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<tr>
<td><strong>Estradiol (pg/mL):</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>37.2±0.735.4</td>
</tr>
<tr>
<td>[29.5-43.7]</td>
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<tr>
<td><strong>Sex hormone-binding globulin (nmol/L):</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>37.9±0.836.1</td>
</tr>
<tr>
<td>[25.9-49.7]</td>
<td></td>
</tr>
</tbody>
</table>

* Major chronic conditions include self-reported physician diagnosis of arthritis, asthma, chronic bronchitis, congestive heart failure, diabetes, emphysema, stroke, skin cancer and other cancer.

mortality across quintiles of testosterone, estradiol and SHBG, and according to vitamin D status. Log-rank tests suggested statistically significant crude differences (P<0.0001) in survival according quintiles of testosterone and SHBG, but not according to estradiol level or vitamin D status (Figures 1A-1D).

Table 3 presents Cox regression models for circulating vitamin D (log_e-transformed and categorical) and biomarkers of sex steroid hormones (log_e-transformed) as predictors of all-cause mortality, while examining linearity and interaction effects. Vitamin D deficiency but not insufficiency was significantly related to all-cause mortality in age-adjusted but not in fully-adjusted models. By contrast, log_e-transformed vitamin D concentration and biomarkers of sex steroid hormones did not exhibit a linear relationship to all-cause mortality rate in either age-adjusted or fully-adjusted models. By including quadratic terms, we observed an inverse non-linear relationship between all-cause mortality rate and levels of testosterone, estradiol and vitamin D, in fully-adjusted models. Furthermore, there were no significant interaction effects between vitamin
D and sex steroid hormones in relation to all-cause mortality rate. We found no significant interaction effects between age group and the selected exposure variables; therefore, no stratified analyses were performed according to age group (data not shown).

Discussion
In a population-based cohort study, we examined vitamin D and biomarkers of sex steroid hormones—separately and simultaneously—as predictors of all-cause mortality among adult men, 18 years and older, who participated in NHANES III. Significant differences in estradiol and SHBG concentrations were found across levels of vitamin D status. In age-adjusted but not fully-adjusted models, vitamin D deficiency was positively linked to all-cause mortality. Log-transformed levels of vitamin D, testosterone and estradiol were non-linearly related to all-cause mortality, independently of baseline demographic, socioeconomic, lifestyle and clinical characteristics. There were no statistically significant interactions between vitamin D and biomarkers of sex steroid hormones in relation to all-cause mortality in age- or fully adjusted models.

Previously, Sempos and colleagues who analyzed the complete cohort of 15,099 NHANES III participants aged ≥20 years with 3,784 deaths reported over a 9-year follow-up period (1991-2000) found a reverse J-shaped association between serum vitamin D and all-cause mortality [35]. In another study of 1,114 NHANES III participants followed-up for a period of 18 years, differences in all-cause mortality were noticed between 90th and 10th percentiles of the sex hormone levels, including free and bioavailable testosterone [36]. Discrepancies between these studies and ours may be due to differences in exposure assessment and ability to detect

<p>| Table 1. Circulating Biomarkers of Sex Steroid Hormones Concentrations among Vitamin D Sufficient, Insufficient and Deficient Adult Men in the Study Sample (n=1472)–Third National Health and Nutrition Examination Survey. |
|-----------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Vitamin D status</th>
<th>Mean±SD</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>P-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/mL)</td>
<td>5.5±2.1</td>
<td>5.2±1.9</td>
<td>5.1±2.0</td>
<td>0.1*</td>
<td>0.03 0.4</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>36.8±13.3</td>
<td>36.9±11.0</td>
<td>38.9±13.4</td>
<td>0.6*</td>
<td>0.006 0.02</td>
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<tr>
<td>Sex hormone-binding globulin (nmol/L)</td>
<td>42.9±21.6</td>
<td>38.1±17.9</td>
<td>39.2±20.3</td>
<td>&lt;0.001*</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* P values are determined based on one-way ANOVA tests for comparing loge-transformed testosterone, estradiol, sex hormone-binding globulin concentrations across vitamin D status groups.

D and sex steroid hormones in relation to all-cause mortality rate. We found no significant interaction effects between age group and the selected exposure variables; therefore, no stratified analyses were performed according to age group (data not shown).

Discussion
In a population-based cohort study, we examined vitamin D and biomarkers of sex steroid hormones—separately and simultaneously—as predictors of all-cause mortality among adult men, 18 years and older, who participated in NHANES III. Significant differences in estradiol and SHBG concentrations were found across levels of vitamin D status. In age-adjusted but not fully-adjusted models, vitamin D deficiency was positively linked to all-cause mortality. Log-transformed levels of vitamin D, testosterone and estradiol were non-linearly related but inversely related to all-cause mortality, independently of baseline demographic, socioeconomic, lifestyle and clinical characteristics. There were no statistically significant interactions between vitamin D and biomarkers of sex steroid hormones in relation to all-cause mortality in age- or fully adjusted models.

Previously, Sempos and colleagues who analyzed the complete cohort of 15,099 NHANES III participants aged ≥20 years with 3,784 deaths reported over a 9-year follow-up period (1991-2000) found a reverse J-shaped association between serum vitamin D and all-cause mortality [35]. In another study of 1,114 NHANES III participants followed-up for a period of 18 years, differences in all-cause mortality were noticed between 90th and 10th percentiles of the sex hormone levels, including free and bioavailable testosterone [36]. Discrepancies between these studies and ours may be due to differences in exposure assessment and ability to detect

<p>| Table 2. Circulating Biomarkers of Sex Steroid Hormones Concentrations among Vitamin D Sufficient, Insufficient and Deficient Adult Men in the Study Sample (n=1472)–Third National Health and Nutrition Examination Survey. |
|-----------|------------------|------------------|------------------|------------------|</p>
<table>
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<tr>
<th>Vitamin D status</th>
<th>Mean±SD</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>Testosterone (ng/mL)</td>
<td>5.5±2.1</td>
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<td>Estradiol (pg/mL)</td>
<td>36.8±13.3</td>
<td>36.9±11.0</td>
<td>38.9±13.4</td>
<td>0.6*</td>
<td>0.006 0.02</td>
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<td>Sex hormone-binding globulin (nmol/L)</td>
<td>42.9±21.6</td>
<td>38.1±17.9</td>
<td>39.2±20.3</td>
<td>&lt;0.001*</td>
<td>0.003</td>
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* P values are determined based on one-way ANOVA tests for comparing loge-transformed testosterone, estradiol, sex hormone-binding globulin concentrations across vitamin D status groups.

D and sex steroid hormones in relation to all-cause mortality rate. We found no significant interaction effects between age group and the selected exposure variables; therefore, no stratified analyses were performed according to age group (data not shown).

Discussion
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small risk changes among groups. For instance, we defined testosterone as log$_e$-transformed variables or in quintiles (taking the 3$^{rd}$ quintile as a referent) and our sample consisted of NHANES III participants with data available on vitamin D and biomarkers of sex steroid hormones [35,36].

To our knowledge, this epidemiological study is one of few to have simultaneously examined vitamin D, biomarkers of sex steroid hormones and all-cause mortality and the first to assess these associations using a nationally representative sample. Our study findings pertaining to vitamin D and biomarkers of sex steroid hormones as predictors of all-cause mortality are somewhat consistent with two previous studies [36,37]. In a large cohort of coronary angiography patients, Lerchbaum and colleagues observed that vitamin D and free T deficient men experienced the highest risk for all-cause mortality even after multivariable adjustment. Moreover, in men with vitamin D deficiency, low free T levels were associated with fatal events, whereas no association of free T with fatal events was found in men with vitamin D insufficiency or sufficiency [5]. In another study of 782 French men $\geq$50 years, Szulc and colleagues identified the lowest quartile of vitamin D as predictor of mortality (HR=1.44, 95% CI: 1.03-2.03); estradiol level predicted mortality after the third year (HR=1.21 per 1 SD increase, 95% CI: 1.09-1.35), where by risk of death increased per quartiles of estradiol level and was higher in third and fourth quartiles compared with the lowest quartile (HR=1.80, 95% CI: 1.09-2.98 and HR=2.83, 95% CI: 1.71-4.67); mortality risk was not significantly associated with free testosterone level [37]. Unlike the present study, no formal testing for interaction between vitamin D and biomarkers of sex steroid hormones was performed in these studies [36,37].

Whereas the link between vitamin D deficiency and all-cause mortality is well-established [38-41], the current epidemiological evidence linking endogenous total and free testosterone concentrations to mortality risk among men remains inconclusive. While total testosterone was mostly negatively related to mortality [13,14,16,20,42] with one study reporting a non-significant relationship [22], free testosterone was positively [22] or negatively [5] related to mortality and limited evidence suggests that SHBG may not be related [22] to mortality.

The finding that testosterone levels were negatively and
non-linearly related to all-cause mortality in fully-adjusted models is in line with the idea that as men age they experience a decline in testosterone [17,43], and is in line with another study [43]. In a case-cohort study of 495 elderly men (146 with ischemic arterial disease (IAD) and 349 controls), a J-shaped association was observed between total testosterone and IAD risk, whereby HR in the lowest and the highest quintiles relative to the second quintile were 2.23 (95% CI: 1.02; 4.88) and 3.61 (95% CI: 1.55; 8.45), respectively [43]. A similar J-shaped relationship was observed between IAD risk and free T, whereas SHBG was not significantly related to IAD risk [43].

The absence of a significant relationship between SHBG and all-cause mortality may be explained by the complex relationship among factors that determine SHBG level. Whereas the process of aging is accompanied by a rise in SHBG level, it is also accompanied by a rise in cardiovascular risk factors, such as obesity, insulin resistance, type 2 diabetes, hypertension, dyslipidemia and inflammation, which, in turn, are negatively correlated with SHBG level [17].

Our results should be interpreted with caution and in light of several limitations. First, the study design is observational and although cohort studies are considered as the gold standard in epidemiology, cause-and-effect relationships cannot be clearly established. Second, sex steroid hormones are known to vary on a diurnal basis, and the use of a single measurement for each of these biomarkers could potentially lead to non-differential misclassification bias. In addition, sex steroid hormones were measured by immunoassays and vitamin D was measured using DiaSorin (rather than mass spectrometry) which are now widely understood to be suboptimal for clinical research involving women, children or men with low serum testosterone [44,45] and especially for serum estradiol in men [46,47]. However, previous studies have indicated an association between testosterone levels and adverse events, irrespective of whether immunoassays or mass-spectrometry measurements were applied [5,14,15]. Third, the present study examined testosterone and SHBG levels but did not examine measures of free or bioavailable testosterone. Further analyses should be performed whereby free testosterone is calculated using validated equations [48,49]. Fourth, sample size limitations may have precluded our ability to identify significant interactions between vitamin D and biomarkers of sex steroid hormones. Also, complete-subject analyses based on availability of data on key variables of interest may have resulted in selection bias. Fifth, the role of chance cannot be ruled out given the large number of statistical tests being conducted. Sixth, although numerous covariates were included in the multivariate models, residual confounding cannot be ruled out as an explanation for the observed associations. For instance, total caloric intake was measured using 24-hour recall which may be inadequate as an assessment of usual food consumption. Finally, our findings can only be generalized to adult men, and further research is needed to evaluate the role of sex steroid hormones in women as well as individuals at different stages of life.

Conclusion
Vitamin D, testosterone and estradiol, but not sex hormone binding globulin, may be inversely and non-linearly related to all-cause mortality among adult men, after adjustment for baseline characteristics. Future studies should attempt to elucidate the complex relationships of circulating vitamin D with sex steroid hormones and their impact on health-related outcomes among men. In particular, studies should assess the potentially mediating role of vitamin D in the relationship between circulating sex steroid hormones and age-adjusted mortality as well as the potentially mediating role of cardiometabolic risk factors on the relationship between vitamin D and all-cause mortality.

List of abbreviations
Ci: Confidence Interval
CYP2R1: 25-hydroxylase
CYP27B1: 1-α-hydroxylase
IAD: Ischemic arterial disease
HR: Hazard Ratio
NHANES: National Health and Nutrition Examination Survey
PSU: Primary sampling unit
SEM: Standard errors of the mean
SHBG: Sex Hormone Binding Globulin

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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Acknowledgement and funding
This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Institute on Aging (NIA/NIH/IRP).

Publication history
EIC: Masayoshi Yamaguchi, Emory University School of Medicine, USA.
Received: 02-Nov-2015 Final Revised: 13-Dec-2015
Accepted: 19-Dec-2015 Published: 28-Dec-2015

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