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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 ($25(OH)D_3 \geq 30$ 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 ($1,25(OH)_2D_3$) through a two-step enzymatic pathway involving liver 25-hydroxylase (CYP2R1) andkidney 1-α-hydroxylase (CYP27B1) [2,8,9]. Vitamin D status is assessed by measuring $25(OH)D_3$ [8]. Currently, the

Institute of Medicine advises 25(OH)D_3 ≥20 ng/ml but Endocrine Society advises 25(OH)D_3 ≥30 ng/ml, partly due to lack of adequate assays for vitamin D quantification; thus, individuals are considered sufficient if 25(OH)D_3≥30 ng/ml (≥74 nmol/L), insufficient if 25(OH)D_3 is 20-29 ng/ml (50-74 nmol/L), and deficient if 25(OH)D_3<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-α-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 deleteriousoutcomes [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 andhigher 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 a 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_3 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 $\log_e$-transformed or defined as a categorical variable (Deficient: <20 ng/ml; Insufficient: 20-<30 ng/ml; Sufficient: ≥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, $\log_e$-transformation and categorization in quintiles were performed for biomarkers of sex steroid hormones.

Testosterone

Elecsys Testosterone was used to measure circulating testos-

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terone concentration and was based on a competitive test principle using a monoclonal antibody directed against testosterone. 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/m^2), *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 bronchitis, 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$_e$-transformed, as needed. One-way ANOVA tests with post-hoc comparisons evaluated using Bonferronicorrections were used for comparing log$_e$-transformed testosterone, estradiol and sex hormone-binding globulin concentrations 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, smoking 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, lifestyle 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 healthand 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), estradiol ($r_{spearman}$=-0.07) and SHBG ($r_{spearman}$=0.09). When log$_e$-transformed testosterone, estradiol and SHBG, were compared among vitamin D sufficient, insufficient and deficient 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 of382 men died over a median of192 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 estimated all-cause mortality rate of 16.7% (95%CI:14.8%-18.8%). Kaplan-Meier curves were constructed to examineall-cause

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Table 1. Demographic, socioeconomic, lifestyle and clinical characteristics in adult men in the study sample (n=1472)–Third National Health and Nutrition Examination Survey.

Characteristic	Mean±SEM or %
N	1472
Age (years):	
Mean±SEM	42.1±0.6
<20	3.1
20-39	47.5
40-59	31.5
60+	17.9
Race / Ethnicity:	
Non-Hispanic White	77.4
Non-Hispanic Black	9.9
Mexican-American	5.1
Other	7.4
Marital status:	
Married / Cohabiting	69.6
Not Married	30.4
Education:	
Less than High School	23.6
High School	29.2
More than High School	47.2
Poverty-Income Ratio:	
Mean±SEM	3.1±0.1
<100%	9.8
100%-<200%	17.4
≥200%	65.6
Unknown	7.2
Urban-Rural Residence:	
Metropolitan	45.1
Other	54.9
Smoking status:	
Current smoker	33.2
Ex-smoker	31.4
Never smoker	35.4
	Mean±SEM or %
Alcohol use – past 12 months:	
Yes	70.8
No	21.7
Unknown	7.5
Body Mass Index (kg/m^2):	
Mean±SEM	26.7 ± 0.4
<25	44.9
25-<30	36.7
≥30	18.3
Waist-to-Hip Ratio:	
≤0.9	29.4
>0.9	70.6
Self-Rated Health:	
Excellent	25.5
Very Good	29.9
Good	30.5
Fair	11.3
Poor	2.7
History of Major Chronic Conditions *:	
Yes	28.4
No	71.6
Physical Activity (Metabolic Equivalents):	
Mean±SEM	139.3±8.2
0	8.4
>0-<100	41.5
100-<200	25.9
200-<300	11.3
≥300	12.8
Vitamin D (ng/ml)	**Mean±SEM or %**
Median [IQR]	26.7 [20.2-34.3]
Sufficient (≥ 30)	50.3
Insufficient (20-<30)	33.1
Deficient (<20)	16.5
Testosterone (ng/mL):	
Median [IQR]	5.4±0.15.15 [3.97-6.56]
Estradiol (pg/mL):	
Median [IQR]	37.2±0.735.4 [29.5-43.7]
Sex hormone-binding globulin (nmol/L):	
Median [IQR]	37.9±0.836.1 [25.9-49.7]

* 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

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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.

	Vitamin D status			
Mean±SD	**Group 1 Sufficient**	**Group 2 Insufficient**	**Group 3 Deficient**	**P-value * *2 vs. 1* *3 vs. 1* *2 vs. 3***
Testosterone (ng/mL)	5.5±2.1	5.2±1.9	5.1±2.0	0.1^{ns} 0.03^{ns} 0.4^{ns}
Estradiol (pg/mL)	36.8±13.3	36.9±11.0	38.9±13.4	0.6^{ns} 0.006^{sign} 0.02^{ns}
Sex hormone-binding globulin (nmol/L)	42.9±21.6	38.1±17.9	39.2±20.3	$<0.0001^{sign}$ 0.003^{sign}

* 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_e-transformed levels of vitamin D, testosterone and estradiol were non-linearly 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

Table 3. Cox proportional hazards models for Circulating Vitamin D and Loge-transformed Biomarkers of Sex Steroid Hormone Concentrations as predictors of Age-Adjusted and Fully-Adjusted All-Cause Mortality Rate among Adult Men in the Study Sample (n=1472)–Third National Health and Nutrition Examination Survey.

	Age-Adjusted		Fully-Adjusted	
Overall	**HR**	**95%CI**	**HR**	**95%CI**
Testosterone	1.08	0.82-1.43	0.92	0.70-1.22
Estradiol	1.53	0.87-2.70	1.06	0.61-1.81
Sex hormone binding globulin:	1.42	0.79-2.54	1.23	0.70-2.17
Vitamin D-continuous	0.84	0.61-1.17	0.99	0.66-1.48
Vitamin D–categorical:				
Sufficient Vitamin D	Ref.	--	Ref.	--
Insufficient Vitamin D	0.97	0.69-1.35	0.92	0.65-1.29
Deficient Vitamin D	1.47	1.04-2.08	1.33	0.88-2.01
Linearity	**HR**	**95%CI**	**HR**	**95%CI**
Testosterone	0.89	0.76-1.04	0.79	0.66-0.96
Testosterone^2	1.22	1.08-1.40	1.19	1.03-1.38
Estradiol	0.08	0.01-0.54	0.05	0.004-0.76
Estradiol^2	1.53	1.13-2.05	1.11	1.09-1.12
Sex hormone binding globulin	0.07	0.003-1.63	0.24	0.041-3.89
Sex hormone binding globulin^2	1.50	0.98-2.31	1.25	0.84-1.86
Vitamin D	0.003	0.0002-0.07	0.01	0.0004-0.29
Vitamin D^2	2.33	1.46-3.72	2.00	1.98-3.36
Interactions	**HR**	**95%CI**	**HR**	**95%CI**
Testosterone	1.13	0.75-1.71	1.00	0.63-1.61
Insufficient Vitamin D	1.04	0.43-2.54	1.08	0.47-2.52
Deficient Vitamin D	1.67	0.49-2.54	1.79	0.58-5.54
Testosterone*Insufficient Vitamin D	0.96	0.51-1.79	0.90	0.48-1.68
Testosterone*Deficient Vitamin D	0.91	0.39-2.14	0.80	0.37-1.73
Estradiol	1.94	0.71-5.31	1.53	0.52-4.49
Insufficient Vitamin D	4.09	0.030-660.10	10.59	0.059-1876.57
Deficient Vitamin D	14.80	0.07-3342.88	14.12	0.04-4989.95
Estradiol*Insufficient Vitamin D	0.66	0.16-2.70	0.50	0.12-2.09
Estradiol*Deficient Vitamin D	0.52	0.12-2.33	0.51	0.10-2.56
SHBG	1.41	0.67-2.94	1.26	0.62-2.54
Insufficient Vitamin D	1.21	0.02-75.17	1.20	0.03-43.7
Deficient Vitamin D	0.62	0.017-23.04	0.99	0.05-20.9
SHBG*Insufficient Vitamin D	0.95	0.32-2.88	0.94	0.35-2.49
SHBG*Deficient Vitamin D	1.26	0.49-3.29	1.09	0.48-2.44

Fully-Adjusted Cox proportional hazards models included age, race/ethnicity, smoking status and physical activity.

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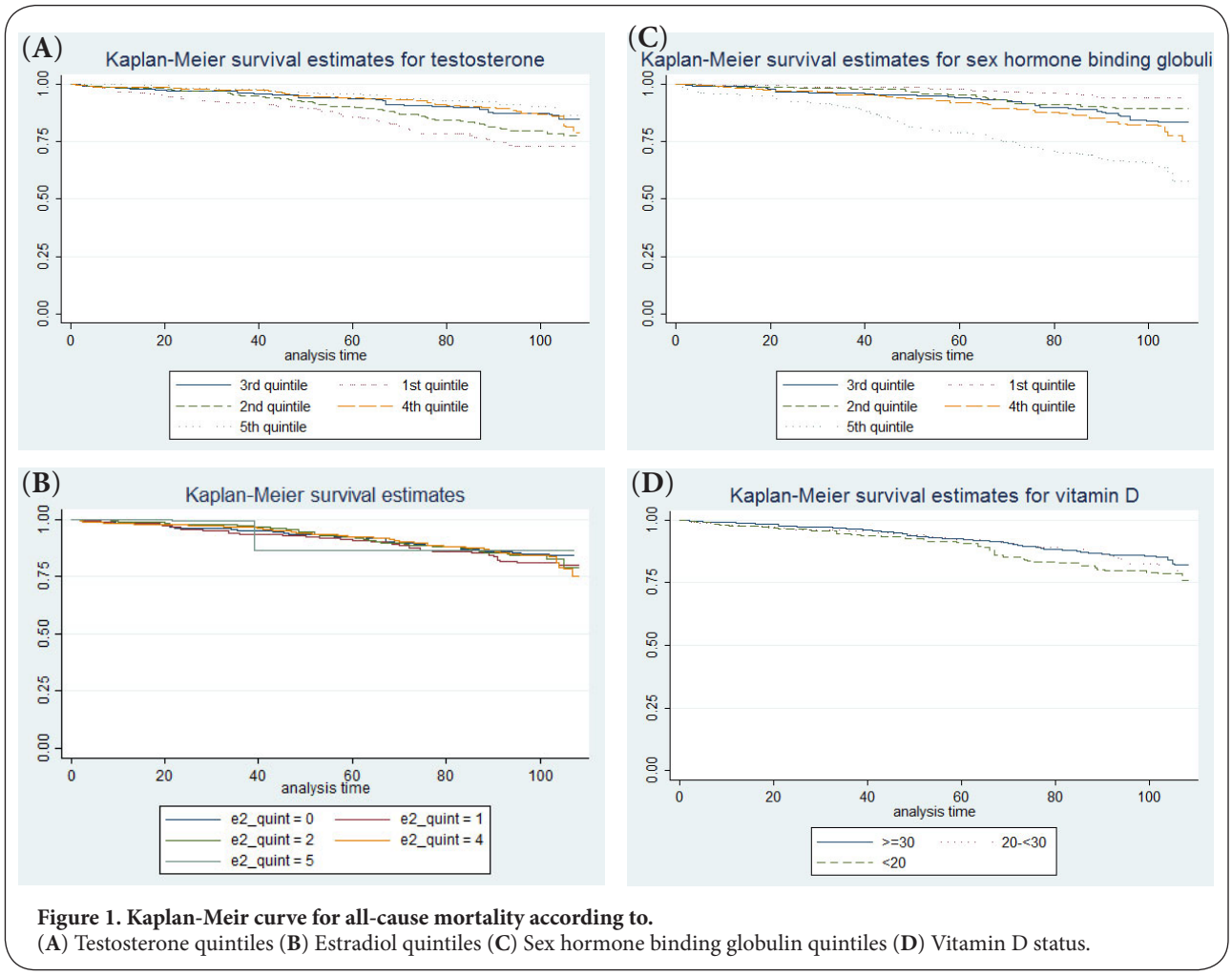

Figure 1. Kaplan-Meir curve for all-cause mortality according to.
(**A**) Testosterone quintiles (**B**) Estradiol quintiles (**C**) Sex hormone binding globulin quintiles (**D**) Vitamin D status.

small risk changes among groups. For instance, we defined testosterone as $\log_e$-transformed variables or in quintiles (taking the 3rd 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 ≥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 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 andlimited evidence suggests that SHBG may not be related [22] to mortality.

The finding that testosterone levelswere negatively and

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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 outcomesamong 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

Authors' contributions	HAB	SME	HAJ	ABZ	MAB
Research concept and design	✓	✓	✓	✓	✓
Collection and/or assembly of data	✓	--	--	--	--
Data analysis and interpretation	✓	✓	✓	✓	✓
Writing the article	✓	✓	✓	✓	✓
Critical revision of the article	✓	✓	✓	✓	✓
Final approval of article	✓	✓	✓	✓	✓
Statistical analysis	✓	--	--	--	✓

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