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Serum vitamin D levels and hypogonadism in men

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SUMMARY

There is inconsistent evidence on a possible association of vitamin D and androgen levels in men. We therefore aim to investigate the association of 25-hydroxyvitamin D (25(OH)D) with androgen levels in a cohort of middle-aged men. This cross-sectional study included 225 men with a median (interquartile range) age of 35 (30–41) years. We measured 25(OH)D, total testosterone (TT) and SHBG concentrations. Hypogonadism was defined as TT <10.4 nmol/L. We found no significant correlation of 25(OH)D and androgen levels. Furthermore, androgen levels were not significantly different across 25(OH)D quintiles. The overall prevalence of hypogonadism was 21.5% and lowest in men within 25(OH)D quintile 4 (82–102 nmol/L). We found a significantly increased risk of hypogonadism in men within the highest 25(OH)D quintile (>102 nmol/L) compared to men in quintile 4 (reference) in crude (OR 5.10, 1.51–17.24, $p = 0.009$) as well as in multivariate adjusted analysis (OR 9.21, 2.27–37.35, $p = 0.002$). We found a trend towards increased risk of hypogonadism in men within the lowest 25(OH)D quintile (≤43.9 nmol/L). In conclusion, our data suggest that men with very high 25(OH)D levels (>102 nmol/L) might be at an increased risk of hypogonadism. Furthermore, we observed a trend towards increased risk of hypogonadism in men with very low vitamin D levels indicating a U-shaped association of vitamin D levels and hypogonadism. With respect to risk of male hypogonadism, our results suggest optimal serum 25(OH)D concentrations of 82–102 nmol/L.

INTRODUCTION

Vitamin D is a steroid hormone and the vitamin D status is mainly determined by ultraviolet-B-induced vitamin D production in the skin, while vitamin D intake by nutrition and supplements plays only a minor role (Holick, 2007). Vitamin D from either source is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), which is used to determine a patient's vitamin D status. 25(OH)D is further hydroxylated to its active form, 1,25-dihydroxyvitamin D [1,25(OH)2D] in the kidney as well as in other tissues including human testis (Blomberg Jensen, 2014). Biological actions of vitamin D are mediated through the vitamin D receptor (VDR), which regulates about 3% of the human genome (Bouillon *et al.*, 2008). The VDR is almost ubiquitously expressed in human cells, which underlines the clinical significance of the vitamin D endocrine system (Kinuta *et al.*, 2000; Holick, 2007; Pludowski *et al.*, 2013). VDR and enzymes that metabolize vitamin D are concomitantly expressed in Sertoli cells, germ cells, Leydig cells, spermatozoa and in the epithelial cells lining the male reproductive tract (Blomberg Jensen *et al.*, 2010; Blomberg Jensen, 2014).

Interestingly, there is accumulating evidence suggesting a complex interplay of vitamin D and androgen metabolism. It has been shown that androgens increase 1-alpha-hydroxylase, a key enzyme in vitamin D metabolism which converts 25(OH)D to 1,25(OH)2D (Somjen *et al.*, 2007). Furthermore, it has been demonstrated that the regulation of gene expression by vitamin D metabolites is modified according to androgen levels (Mordan-McCombs *et al.*, 2010). In 2009, some of us demonstrated for the first time that androgen levels are associated with 25(OH)D levels in 2299 older men from the LURIC study (Wehr *et al.*, 2010). Furthermore, previous data indicated that vitamin D therapy might increase testosterone levels in obese men undergoing weight reduction (Pilz *et al.*, 2011). Interestingly, men with a combined vitamin D and androgen deficiency are at high risk for all-cause and cardiovascular mortality suggesting that a parallel deficiency is a powerful marker of poor health (Lerchbaum &

Obermayer-Pietsch, 2012; Lerchbaum *et al.*, 2012). Despite these previous studies suggesting an association of vitamin D and androgen levels in men, several recent studies among young and healthy men failed to find an association of vitamin D and androgen levels in men (Chen *et al.*, 2008; Ceglia *et al.*, 2011; Ramlau-Hansen *et al.*, 2011; Hammoud *et al.*, 2012).

Considering these inconsistent previous data, we aim to study the association of 25(OH)D levels with TT, free testosterone (FT) and SHBG levels in a cohort of middle-aged men. Moreover, we aim to examine the association of vitamin D status with hypogonadism.

METHODS

Subjects

The study cohort consisted of 225 healthy men, aged 20–58 years, who were routinely referred to the outpatient clinic of the Department of Gynecology and Obstetrics at the Medical University of Graz (n = 179, trial site 1) and the Kinderwunsch Institut Dobl (n = 46, trial site 2) between 2010 and 2012. These men were either part of an infertile couple or simply in desire of having children and therefore came for endocrine evaluation. None of the subjects had a history of hypogonadism or any known disease associated with hypogonadism (except obesity). None of the patients had diabetes and none of the men took medications known to affect endocrine parameters. Four men reported intake of vitamin D supplementation. Those men were included in the analyses as 25(OH)D levels were similar in men with and without vitamin D supplementation [78 (67.5–87.0) nmol/L vs. 74 (46.5–94.0) nmol/L, p = 0.889]. The study protocol was approved by the ethics committee of the Medical University of Graz. Written informed consent was obtained from each patient.

Data from a part of the cohort (men without azoospermia from trial site 1) have been published previously (Schwetz *et al.*, 2013).

Procedures

Standard anthropometric data [height, weight, waist circumference (WC) and hip circumference, blood pressure (BP)] were obtained from 200 subjects. WC was measured in a standing position midway between the lower costal margin and the iliac crest. Hip circumference was measured in a standing position at the maximum circumference over the buttocks. The BMI was calculated as the weight in kilograms divided by the square of height in metres. Overweight was defined as BMI 25.0–29.9 kg/m² and obesity was defined as BMI ≥ 30 kg/m².

Basal blood samples for endocrine parameters [25(OH)D, TT, free testosterone (FT), SHBG, LH, FSH, estradiol] were collected between 8.00 and 9.00 a.m. after an overnight fast in all 179 men at trial site 1. At trial site 2, blood samples were collected between 7.15 and 10.00 a.m. in 23 men, between 10.30 a.m. and 12.00 noon in 10 men and after 12 noon in 13 men. Routine laboratory parameters were immediately measured on a daily (SHBG, LH, FSH, estradiol) to weekly (FT) basis. Remaining blood samples were frozen and stored at −80 °C until further analysis. Serum levels of 25(OH)D and TT were measured by Isotope-Dilution Liquid Chromatography Tandem Mass Spectrometry (ID-LC-MS/MS) in 2013. FT values ($FT_{Vermeulen}$) were calculated from TT, SHBG and albumin according to Vermeulen (Vermeulen *et al.*, 1999). The free androgen index (FAI) was calculated as TT (nmol/L)/SHBG (nmol/L) × 100. Male hypogonadism was defined as TT levels <10.4 nmol/L measured by ID-LC-MS/MS according to widely used cut-offs (to convert serum TT levels to ng per litre, divide by 3.467) (Tajar *et al.*, 2010). Men with hypogonadism were further classified depending on their LH concentrations using a cut-off of 7.4 IU/L as suggested by the manufacturer and validated in our laboratory. A low TT level and LH ≤7.4 IU was defined as secondary hypogonadism and a low TT level and LH >7.4 IU/L was classified as secondary hypogonadism. According to widely used cut-offs for vitamin D status classification, subjects were divided into three groups: vitamin D sufficiency (25(OH)D ≥75.0 nmol/L), vitamin D insufficiency (25(OH)D 50.0–74.9 nmol/L) and vitamin D deficiency (<50.0 nmol/L) (to convert serum 25(OH)D levels to μg per litre, divide by 2.496). As the prevalence of subjects with sufficient vitamin D levels was high (48%), we further calculated quintiles of 25(OH)D concentrations. To study the influence of seasonal variation on hormones, we subdivided the year into 3-month measurement periods: January–March (season 1); April–June (season 2); July–September (season 3); October–December (season 4) to address the seasonal changes in availability of sunlight.

Biochemical analyses

All samples (trial sites 1 and 2) were measured in the same laboratory using the same assays. FT was determined using a radioimmunoassay (DSL, Webster, TX, USA; intra- and interassay CVs of <10%). SHBG was measured by luminescence immunoassay (Cobas; Roche, Basel, Switzerland) with an intra- and interassay CV of 1.3 and 2.1% respectively. LH and FSH were measured by enzyme immunoassay (DiaMetra S.r.l., Segrate (MI), Italy; intra- and interassay CVs of <10%) and estradiol was measured by chemiluminescent immunoassay (Immulite, SIEMENS Healthcare, UK; intra- and interassay CVs of 15 and 16% respectively).

MS

Total testosterone and 25(OH)D levels were measured by ID-LC-MS/MS in 225 men. 25(OH)D measurements by ID-XLC-MS/MS were performed at the Endocrine Laboratory of the VU University Medical Center (Amsterdam, the Netherlands) as described before (Heijboer *et al.*, 2012), with only minor adjustments. In short, deuterated internal standard (IS) [25(OH)D3-d6] (Synthetica, Oslo, Norway) was added to the samples and 25(OH)D was released from its binding proteins with acetonitrile. Samples were extracted and analysed by XLC-MS/MS [a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands)] coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The limit of quantitation (LOQ) was 4.0 nmol/L; intraassay CV was <6%, and interassay CV was <8% for concentrations between 25 and 180 nmol/L. 25(OH)D2 and 25(OH)D3 were measured separately.

Total testosterone measurements by ID-XLS-MS/MS were performed at the Endocrine Laboratory of the VU University Medical Center (Amsterdam, the Netherlands) as described before (Bui *et al.*, 2013). In short, a stable isotopically labelled internal standard (testosterone-2,2,4,6,6-D5;D5T; CDN Isotopes, Pointe-Claire, Canada) was added to every sample. Testosterone was extracted with hexane/ether (4/1, v/v), dried and derivatized with methoxylamine hydrochloride (T-mox), followed by another hexane/ether extraction. Separation was achieved on a

C8 analytical column (XBridge, 2.1 × 50 mm, 2.5 μm particle size: Waters Corp.) by gradient elution, using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. A Quattro Premier XE tandem mass spectrometer (Waters Corp.) with electrospray in positive mode was used for detection. The LOQ was 0.10 nmol/L (2.88 ng/dL); inter-assay variation at 0.21, 2.1 and 15.8 nmol/L was 9, 7 and 4% respectively. This method correlates well with a ID-GC-MS reference method for TT (Thienpont *et al.*, 2008; Bui *et al.*, 2013).

Semen analyses

Semen analysis was carried out using standard procedures as recommended by the World Health Organization (World Health Organization, 2010). Oligozoospermia was defined as a sperm concentration of <15 mio/mL, azoospermia was defined as a sperm count of 0/mL and oligo-astheno-teratozoospermia (OAT) was defined as sperm concentration <15 mio/mL, motility <40% and strict morphology <4% according to the WHO criteria 2010 (WHO, 2010).

Statistical analyses

Anthropometric and biochemical data were completely available in 200 men. Thus, all analyses (except correlation analyses between endocrine parameters) were performed among 200 men. Data are presented as median with interquartile range unless otherwise stated. The distribution of data was analysed by descriptive statistics and Kolmogorov–Smirnov test. All parameters except 25(OH)D and TT were found to be non-normally distributed. All non-normally distributed variables were log-transformed and checked for normal distribution before being entered in parametric tests. Pearson correlation analysis was used for correlation analysis of hormonal and anthropometric parameters. *T*-test, ANOVA and chi-squared test were used for comparisons between groups. We calculated binary logistic regression analyses using hypogonadism as dependent variables and 25(OH)D quintiles, age, BMI, trial site and ethnical background as independent variables. Because TT levels measured in morning samples are considered more accurate, we repeated all analyses after excluding 23 men in whom blood samples were drawn after 10 a.m. Furthermore, we repeated all analyses after excluding men with primary hypogonadism, azoospermic men and after adjusting for time of blood sampling. All statistical procedures were performed with SPSS version 20 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Baseline characteristics of men are presented in Table 1. Overweight and obesity were prevalent in 44.7% and 14.3% of men respectively. We found vitamin D deficiency, insufficiency and sufficiency in 53 (26.5%), 51 (23.5%) and 96 (48%) of 200 men respectively. Furthermore, 126 men (63.0%) had normozoospermia, 47 men (23.5%) had oligozoospermia, 27 men (13.5%) had azoospermia and 42 men (21.0%) presented with OAT.

We found no significant correlation of 25(OH)D levels with other endocrine or metabolic parameters (data not shown). We observed significant positive correlations between TT and FT ($r = 0.379$, $p < 0.001$), $FT_{Vermeulen}$ ($r = 0.812$, $p < 0.001$), FAI ($r = 0.302$, $p < 0.001$), SHBG ($r = 0.574$, $p < 0.001$), FSH ($r = 0.170$, $p = 0.036$) and LH ($r = 0.342$, $p < 0.001$) levels and significant negative correlations with age ($r = -0.239$, $p < 0.001$), BMI ($r = -0.326$, $p < 0.001$), WC ($r = -0.415$, $p < 0.001$) and WHR ($r = -0.395$, $p < 0.001$).

Anthropometric characteristics of subjects according to 25(OH)D quintiles are shown in Table 2. We found no significant differences in anthropometric and biochemical parameters between men in different 25(OH)D quintiles. The prevalence of hypogonadism and ethnical background was significantly different across 25(OH)D quintiles.

Hypogonadism

We found hypogonadism in 43 of 200 men (21.5%); five men had primary hypogonadism and 38 men had secondary hypogonadism. Men with hypogonadism had significantly higher BMI, WC and WHR and lower FAI, FT, $FT_{Vermeulen}$ and SHBG levels, whereas no significant differences were found in age, 25(OH)D, estradiol, LH, FSH, ethnic background (Table 1), season or month of blood sampling and semen parameters (data not shown).

Table 1 Characteristics of all subjects and according to hypogonadism

	All men (n = 200)		Eugonadal men (n = 157)		Hypogonadal men (n = 43)		p-value
	Median	IQ range	Median	IQ range	Median	IQ range	
Age (years)	35	31–41	35	31–41	38	33–42	0.056
BMI (kg/m²)	25.7	23.6–28.5	25.3	23.5–27.7	28.6	24.9–31.4	<0.001
WC (cm)	91	85–99	90	84–96	98	90–106	<0.001
WHR	0.89	0.84–0.93	0.88	0.82–0.92	0.92	0.88–0.96	<0.001
Systolic BP	135	124–145	135	124–145	134	122–146	0.909
Diastolic BP	85	79–94	84	78–94	86	80–91	0.552
TT (nmol/L)	13.9	10.6–16.9	15.3	13.2–18.0	8.6	7.2–9.5	<0.001
$FT_{Vermeulen}$ (pmol/L)	281	229–350	312	267–364	191	170–220	<0.001
FT (pmol/L)	353	259–469	387	279–486	293	196–350	0.048
FAI	43.48	35.19–54.04	458	357–549	356	300–445	<0.001
SHBG (nmol/L)	32.4	23.81–41.65	34.4	26.3–44.4	23.6	18.0–29.8	<0.001
FSH (IU/L)	4.3	2.8–6.0	4.3	2.9–6.0	3.6	2.3–5.9	0.276
LH (IU/L)	3.2	2.2–4.5	3.5	2.5–4.7	2.3	1.5–3.7	0.527
Estradiol (pmol/L)	100	70–130	100	70–130	100	70–140	0.509
25(OH)D (nmol/L)	67.8	44.9–94.6	65.0	44.4–94.0	70.3	48.4–107.8	0.413
Caucasian white (%)	74.5		72.7		74.5		0.806

Comparisons between men with and without hypogonadism were performed using *t*-test. BMI, body mass index; WC, waist circumference; WHR, waist-to-hip ratio; TT, total testosterone; FT, free testosterone; FAI, free androgen index; 25(OH)D, 25-hydroxyvitamin D.

Table 2 Characteristics of subjects according to 25(OH)D quintiles

	25(OH)D quintiles										*p*-value
	Quintile 1 (≤43.9 nmol/L)		Quintile 2 (44.0–67.9 nmol/L)		Quintile 3 (68.0–82.1 nmol/L)		Quintile 4 (82.2–101.8 nmol/L)		Quintile 5 (>101.8 nmol/L)		
	Median	IQ range	Median	IQ range	Median	IQ range	Median	IQ range	Median	IQ range	
Age (years)	35	30–38	34	30–39	35	32–44	36	33–41	37	34–41	0.300
BMI (kg/m²)	25.7	23.6–23.4	25.7	23.9–29.0	25.1	23.2–27.9	26.1	23.5–28.3	25.1	24.7–27.4	0.975
WC (cm)	89	86–101	91	85–97	92	85–99	91	84–97	90	84–98	0.926
WHR	0.89	0.84–0.94	0.87	0.84–0.91	0.90	0.83–0.93	0.88	0.83–0.93	0.88	0.82–0.92	0.743
TT (nmol/L)	14.7	10.3–16.8	14.2	10.9–18.2	12.8	10.9–15.9	15.1	11.1–19.0	13.9	9.1–17.3	0.310
$FT_{Vermeulen}$ (pmol/L)	279	222–352	312	260–374	277	198–347	291	246–354	258	220–342	0.321
FT (pmol/L)	333	293–422	437	294–500	372	301–437	388	319–426	344	269–477	0.730
FAI	44.98	32.52–53.26	47.27	37.07–58.1	41.13	33.92–53.95	41.37	36.68	41.35	34.57–51.7	0.362
SHBG (nmol/L)	30.4	24.06–38.5	32.6	23.2–38.5	30.5	22.25–37.2	35.9	29.4–47.5	32.5	23.6–46.5	0.327
FSH (IU/L)	4.6	3.6–5.9	4.7	3.6–7.0	3.5	2.6–4.5	4.5	2.3–6.8	3.7	2.8–6.1	0.125
LH (IU/L)	3.6	2.5–4.7	4.0	2.9–5.2	2.6	2.0–3.8	2.9	2.1–4.2	3.0	2.0–4.0	0.400
Estradiol (pmol/L)	94.5	51.6–114.5	117.3	73.4–143.5	101.7	73.4–122.6	103.7	73.4–127.2	110.0	74.1–136.2	0.129
25(OH)D (nmol/L)	32.0	24.0–41.0	57.0	48.0–62.0	74.0	72.0–78.0	91.0	87.0–94.0	118.5	107.0–133.0	<0.001
Hypogonadism (%)	26.2		11.1		21.1		10.5		37.5		0.021
Caucasian white (%)	38.1		68.4		89.5		92.3		75.1		<0.001

Comparisons between 25(OH)D quintiles were performed using ANOVA and chi-squared test. Data are available in 200 men. 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; WC, waist circumference; WHR, waist-to-hip ratio; TT, total testosterone; FT, free testosterone; FAI, free androgen index.

We performed binary logistic regression using hypogonadism as dependent variable and 25(OH)D quintiles, age, BMI, ethnic background and study site as explanatory variables. As the prevalence of hypogonadism was lowest in men within 25(OH)D quintile 4 (Table 2), we used quintile 4 as reference. We found a significantly increased risk of hypogonadism in men within the highest 25(OH)D quintile compared with men in quintile 4 in crude as well as in multivariate adjusted analysis (Table 3). Furthermore, we found a trend towards increased risk of hypogonadism in men within the lowest 25(OH)D quintile in all models. As the majority of men had secondary hypogonadism, we repeated our binary regression analyses after exclusion of men with primary hypogonadism, but our results remained materially unchanged (data not shown).

The prevalence of hypogonadism was higher at trial site 2 (16.7% vs. 37%, $p = 0.003$). To explore this difference, we compared all variables displayed in Table 2 between the trial sites. We found significantly lower TT and FAI levels ($p < 0.001$ for both) and significantly higher WHR levels ($p = 0.032$) in men from trial site 2, whereas all other parameters including 25(OH)D levels were similar (p-value >0.300 for all). After exclusion of 23 men with non-morning blood samples, the prevalence of hypogonadism still tended to be higher at trial site 2 ($p = 0.052$). To further address this difference, we excluded men from trial site 2 and repeated all analyses including only men from trial site 1. This did, however, not significantly change our results (data not shown).

Furthermore, when we restricted our analyses to Caucasian white men, or to men with morning blood samples or to men without azoospermia, and after adjusting our results for time of blood sampling, our results did not materially change (data not shown).

Seasonal variation

Vitamin D levels were highest in season 3 [91.0 (73.0–115.0) nmol/L] and lowest in season 1 [52.5 (40.0–74.0) nmol/L], season 2 [69.0 (46.5–86.5) nmol/L] and season 4 [85.0 (51.0–101.0) nmol/L] respectively ($p < 0.001$).

We found no significant differences in TT, FT, $FT_{Vermeulen}$, FAI, SHBG, FSH, LH and estradiol levels or prevalence of hypogonadism regarding season or month of blood sampling ($p > 0.200$ for all, data not shown).

DISCUSSION

We present evidence that 25(OH)D levels >102 nmol/L are associated with increased risk of hypogonadism in otherwise healthy men with a median age of 35 (31–41) years. 25(OH)D levels between 82 and 102 nmol/L appear most favourable

Table 3 Odds ratio with 95% CI for hypogonadism according to 25(OH) quintiles

	Model 1		Model 2		Model 3	
	OR	*p*-value	OR	*p*-value	OR	*p*-value
Quintile 1 (≤43.9 nmol/L)	3.02 (0.87–10.46)	0.082	3.47 (0.95–12.75)	0.060	3.67 (0.84–16.11)	0.085
Quintile 2 (44.0–67.9 nmol/L)	1.06 (0.25–4.61)	0.935	0.97 (0.20–4.67)	0.968	1.05 (0.21–5.37)	0.949
Quintile 3 (68.0–82.1 nmol/L)	2.27 (0.62–8.29)	0.216	2.32 (0.59–9.16)	0.231	2.68 (0.64–11.25)	0.178
Quintile 4 (82.2–101.8 nmol/L)	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Quintile 5 (>101.8 nmol/L)	5.10 (1.51–17.24)	0.009	6.39 (1.76–23.22)	0.005	9.21 (2.27–37.35)	0.002

Binary logistic regression analysis was performed using hypogonadism as dependent variable and 25(OH)D quintiles, age, BMI, ethnic background and study site as explanatory variables. Data available in 200 men. Model 1: crude. Model 2: adjusted for age and BMI. Model 3: adjusted for age, BMI, ethnic background, study site. 25(OH)D, 25-hydroxyvitamin D; OR, odds ratio; BMI, body mass index.

regarding male hypogonadism. We further observed a trend towards increased risk of hypogonadism in men with low 25(OH)D levels (<44 nmol/L) suggesting a U-shaped association of vitamin D status and hypogonadism in middle-aged men. Of note, this is the first study evaluating the association of vitamin and androgen levels in men using a state-of-the-art ID-LC-MS/MS for measuring both 25(OH)D and TT levels which is considered the gold standard. Furthermore, the inclusion of a relatively large proportion of men with 25(OH)D levels ≥75 nmol/L allows a balanced evaluation of high 25(OH)D levels with the possibility to evaluate non-linear associations.

The lack of a significant correlation between 25(OH)D and androgen levels can be explained by the non-linear relationship of the parameters. We further observed no significant differences in androgen levels across 25(OH)D quintiles which is in line with previous studies showing comparable testosterone levels in healthy young and middle-aged men (Chen *et al.*, 2008; Ceglia *et al.*, 2011; Ramlau-Hansen *et al.*, 2011; Hammoud *et al.*, 2012). Interestingly, one study among 307 healthy young men aged 18–21 years reported an inverse association of 25(OH)D levels with FAI in adjusted analyses (Ramlau-Hansen *et al.*, 2011). The significantly lower FAI levels were observed in men with 25(OH)D levels between 94 and 227 nmol/L. Moreover, results from the Health Professionals Follow-up Study including 1362 healthy men found a positive association between 25(OH)D and TT and FT (Nimptsch *et al.*, 2012). This association was linear at lower 25(OH)D levels (<75–85 nmol/L for TT and 75 nmol/L for FT) reaching a plateau at higher levels. The authors also found a significantly decreased risk of hypogonadism in men within the highest 25(OH)D quintile compared to men within the lowest quintile. The observed inconsistencies to our results indicating a U-shaped association might be explained by age (mean age 66 years vs. 35 years in our study), as striking differences between young and older men have been suggested and attributed to indirect effects vitamin D in older men (Blomberg Jensen, 2014). This notion is supported by the fact that the positive association of testosterone with 25(OH)D was mainly observed in older men (Nimptsch *et al.*, 2012) (Lee *et al.*, 2012) (Wehr *et al.*, 2010). Indirect vitamin D effects on testosterone levels in older men might be related to calcium and phosphate homeostasis, SHBG or osteocalcin production (Blomberg Jensen, 2014). This assumption is corroborated by the fact that Jorde *et al.* (2013) found a weak ($r = 0.08$) but significant correlation of vitamin D and TT levels in 893 healthy men aged 61 years. Consistently, data from the European Male ageing study, including 3369 community-dwelling men aged 40–79 years, suggest a positive association of 25(OH)D levels with TT and FT, which lost, however, significance after adjusting for age and lifestyle factors (Lee *et al.*, 2012). Nevertheless, an independent association was found between vitamin D deficiency and compensated as well as secondary hypogonadism. Furthermore, the prevalence of vitamin D deficiency was higher in diabetic men with hypogonadism compared to diabetic men without hypogonadism and healthy controls (Bellastella *et al.*, 2014). Results from the LURIC study, including 2299 men aged 67 years at high cardiovascular risk, demonstrated an independent linear association of high 25(OH)D levels with higher TT and FAI levels and men with vitamin D deficiency had an increased risk of hypogonadism compared to men with sufficient vitamin D levels (Wehr *et al.*, 2010). The studies (Wehr *et al.*, 2010; Lee *et al.*, 2012; Bellastella *et al.*, 2014) used men with 25(OH)D levels >75 nmol/L as reference groups and no further evaluation of men with "sufficient" levels was performed.

As most previous studies investigated the association between vitamin D and androgens or hypogonadism using men with 25(OH)D levels >75 nmol/L as reference group, it was not possible to detect a U-or J-shaped association. Furthermore, most previous studies include only a small proportion of men with vitamin D sufficiency and it was therefore not possible to separately analyse men with higher vitamin D levels (>100 nmol/L). Nevertheless, vitamin D concentrations of 75–100 nmol/L have been previously suggested as optimal and the need for studies using optimal vitamin D dosage regimes (e.g. with the aim to reach the 25(OH)D target levels of ~75–100 nmol/L) has been underlined (Pilz *et al.*, 2012). Nevertheless, the increased risk of hypogonadism in men with very high vitamin D concentration is somewhat unexpected and we can only speculate on the underlying mechanisms. In this context, it should be considered that high vitamin D concentrations may affect vitamin D metabolism within the target tissue, leading to increased 24-hydroxylation (Miller *et al.*, 1995). Thus, in the setting of high circulating 25(OH)D levels, the concentration of the biologically active 1,25(OH)2D might be reduced in target tissues such as testis and the pituitary gland. Hence, both high as well as low vitamin D concentrations might cause harm. This hypothesis along with our findings on a U-shaped association between 25(OH)D and hypogonadism fit well to previous reports on similar association curves between vitamin D status and adverse outcomes such as mortality. However, as we found no significant differences in androgen or LH levels across vitamin D quintiles, our results remain difficult to interpret.

Our findings are, however, supported by previous inconsistent results with possible U-shaped or non-linear associations that have been suggested for vitamin D and cancer (Pilz *et al.*, 2013), cardiovascular disease (Wang *et al.*, 2008) and mortality (Zittermann *et al.*, 2012; Sempos *et al.*, 2013). Indeed, the literature reports a non-linear association of 25(OH)D with outcomes such as breast cancer (Abbas *et al.*, 2009), incident cardiovascular disease (Wang *et al.*, 2008) and all-cause mortality (Zittermann *et al.*, 2012; Sempos *et al.*, 2013). Interestingly, recent data from the NHANES III cohort suggest a reverse J-shaped association between serum 25(OH)D and all-cause mortality (Sempos *et al.*, 2013). A meta-analysis including 14 prospective studies involving 5562 deaths supports this reverse J-shaped association (Zittermann *et al.*, 2012). The suggested optimal 25(OH)D concentrations were 70–90 nmol/L (Sempos *et al.*, 2013) and 75–87.5 nmol/L (Zittermann *et al.*, 2012) respectively. Furthermore, an increased risk at low and high levels has been suggested for prostate cancer (Tuohimaa *et al.*, 2004), overall and cancer mortality (Michaëlsson *et al.*, 2010) and incident cardiovascular disease (Wang *et al.*, 2008). Of note, previous studies on vitamin D and female fertility demonstrated promising results regarding in vitro fertilization outcome as well as other aspects (Lerchbaum & Obermayer-Pietsch, 2012; Lerchbaum *et al.*, 2012; Lerchbaum & Rabe, 2014). In light of the results of this study, future studies should also focus on optimal vitamin D concentrations and critically examine the endocrine effects of very high 25(OH)D levels in women of reproductive age. In addition, the U-shaped association between 25(OH)D and androgens as reported in our study may also explain inconsistent results from

previous RCTs on the effect of vitamin D supplementation on testosterone levels (Pilz *et al.*, 2011; Jorde *et al.*, 2013).

Our study has several limitations that should be noted. First, because of the cross-sectional design of our study no conclusions with respect to causality or directionality of the vitamin D-hypogonadism association can be drawn. Furthermore, in some men, blood samples were taken in the afternoon which probably influences TT levels. To address this limitation, we repeated all analyses excluding men with non-morning blood samples and adjusted our analyses for time of blood sampling which did not materially change our results. Furthermore, as the majority of men had secondary hypogonadism, we cannot make a statement on vitamin D and primary hypogonadism. Moreover, diagnosis of hypogonadism is defined biochemically using TT and LH without the inclusion of clinical signs or symptoms.

The strengths of our study include the measurement of TT as well as 25(OH)D levels using a state-of-the-art LC-MSMS which is considered the gold standard. Of note, this is the first study evaluating the association of vitamin and androgen levels in men using ID-LC-MS/MS for measuring both 25(OH)D and TT levels. The inclusion of a relatively large proportion of men with 25(OH)D levels ≥75 nmol/L allowed a more balanced evaluation of high 25(OH)D levels compared to previous studies that included mainly subjects with 25(OH)D levels <75 nmol/L. As we observed a significantly higher risk of hypogonadism in men with 25(OH)D levels >102 nmol/L, future studies on vitamin D and testosterone might also focus on men with 25(OH)D levels in higher ranges. Overall, our study contributes to literature suggesting that there might be an optimal 25(OH)D concentration in humans that is between 75 and 100 nmol/L. Future RCTs might therefore include only subjects with insufficient or deficient vitamin D levels and should titrate vitamin D supplementation to reach and maintain 25(OH)D concentrations between 75 and 100 nmol/L.

In summary, we found a significantly increased risk of hypogonadism in men with 25(OH)D levels >102 nmol/L, whereas hypogonadism risk was lowest in men with 25(OH)D levels between 82 and 102 nmol/L. We further observed a trend towards increased risk of hypogonadism in men with very low vitamin D levels suggesting a U-shaped association of vitamin D status and hypogonadism in middle-aged men. Our study therefore contributes to the accumulating evidence suggesting that 25(OH)D concentrations between 75 and 100 nmol/L are optimal and that not only low but also very high 25(OH)D levels should be avoided.

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

E.L. contributed to research design, analysis of data and drafting of the manuscript; S.P. contributed to research design and revised the manuscript critically; C.T. contributed to acquisition of data and drafting of the manuscript; T.R. contributed to interpretation of data and revised the manuscript critically; M.S. contributed to data acquisition and revised the manuscript critically; A.H. contributed to acquisition and interpretation of data and drafting of the manuscript; B.O. contributed to research design and revised the manuscript critically. All authors approved the final version of the manuscript.

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