Full title: Vitamin D status is not associated with reproductive parameters in young Spanish men.

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Short title: Vitamin D status and male reproductive outcomes.

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ABSTRACT

Background: Relatively low sperm count was reported among young Spanish men in 2013. Several potential culprits have been suggested as explanations for reported trends in sperm counts in Western men, including lifestyles. Although controversial, some studies suggest that semen parameters, such as low sperm motility or abnormal morphology, may be associated with low serum vitamin D levels.

Objectives: To evaluate associations between semen parameters and reproductive hormones and serum 25-hydroxyvitamin D (25OHD) status in young Spanish men and to examine these associations in relation to dietary intake of vitamin D.

Materials and Methods: This cross-sectional study includes 198 university students recruited in 2010-2011 in southern Spain, who provided samples of blood and semen and food frequencies. Semen quality was evaluated by measuring volume, concentration, sperm counts, motility and morphology, according to the WHO guidelines. Serum samples were analyzed for total 25OHD and reproductive hormones, including FSH, LH, testosterone, inhibin B and estradiol. Dietary vitamin D intake was assessed using a validated food frequency questionnaire. Associations with semen quality and reproductive hormones were examined using linear regression, adjusting for potential confounders.

Results: Almost all men had adequate levels of serum vitamin D - only 3 men (1.5%) were vitamin D deficient (<30 nmol/L) and 17% were insufficient (<50 nmol/L). However, dietary vitamin D intakes were relatively low (below recommended 600 IU/day in 99% of men). Neither dietary intake nor serum vitamin D levels were associated with any sperm parameter or any reproductive hormone (all $p \geq 0.09$).

Discussion: We did not observe an association between vitamin D status and any reproductive parameter in our study population.

Conclusions: Our results suggest that serum vitamin D levels are sustained in Spanish men despite low dietary intake and therefore low vitamin D does not explain the poor semen quality previously observed in these young Spanish men.

INTRODUCTION

Relatively low sperm count was reported among young Spanish men in 2013 (Mendiola *et al*., 2013). While declines in sperm count and concentration have been described in Western men (Levine *et al*., 2017), sperm counts for students from the Murcia region of Spain (median concentration: 44 mill/ml) (Mendiola *et al*., 2013) are lower than those reported in most other studies of unselected western men (Blomberg Jensen *et al*., 2011; Hammoud *et al*., 2012; Levine *et al*., 2017; Zareba *et al*., 2013).

While several studies have examined possible causes of low sperm count, including personal or maternal exposures to environmental toxins and lifestyle factors, such as smoking, diet and physical activity, low sperm counts in Western men remains unexplained (Gaskins *et al*., 2015; Jensen *et al*., 2004; Lassen *et al*., 2014; Priskorn *et al*., 2016). Some studies reported that impaired semen parameters (sperm counts, motility or morphology) may be associated with low serum vitamin D levels (Abbasihormozi *et al*., 2017; Blomberg Jensen *et al*., 2011; Hammoud *et al*., 2012). The US Institute of Medicine recommended daily dietary allowance is 600 IU (15µg) (Ross *et al*., 2011), and the same recommendation is considered adequate for Spanish adults (Moreiras *et al*., 2015). However, as stated in the Scientific Report of the 2015 Dietary Guidelines Advisory Committee, this amount is almost impossible to reach from a normal diet, even with careful food choices, such as fortified products (McGuire *et al*., 2016). According to a recent report, low vitamin D is also relevant for the Spanish population, where only about 10% of participants showed vitamin D intake higher than 80% of recommended value (Partearroyo *et al*., 2018). Low consumption of products which are food sources of vitamin D, such as cod liver oil, oily fish or fortified dairy products, may lead to insufficiencies, especially during the seasons with low sun exposure (Olza *et al*., 2017).

Traditionally, vitamin D was known for its well-established role in calcium homeostasis, but it is now recognized as a versatile signaling hormone with multiple effects on human health (Holick, 2007; Lorenzen et al., 2017; Norman, 2008). In clinical practice, deficiency and insufficiency of serum vitamin D levels are defined as concentrations below 12 ng/mL (30 nmol/L) and 20 ng/mL (50 nmol/L), respectively (Institute of Medicine, 2011; Ross *et al*., 2011). Vitamin D receptors (VDRs) have been found in human liver, pancreas, lung, brain, skin, breast, muscle and adipose tissue (Prentice *et al*., 2008), as well in spermatozoa, epididymis, prostate, and seminal vesicles (Aquila *et al*., 2009; Blomberg Jensen *et al*., 2010; Blomberg Jensen, 2014; Corbett *et al*., 2006). Finding these receptors in the male reproductive system led to the hypothesis that vitamin D levels may have a positive influence on

spermatogenesis. Furthermore, the testis, which produce both sperm and reproductive hormones, are sensitive to nutrition, hormones or vitamins status (Mortimer *et al*., 2013).

The economic and psychosocial burden of infertility is substantial. Poor semen quality is an important contributor to this problem, an estimated 20-50% of infertile couples have been found to have one or more abnormal semen parameter (Agarwal *et al*., 2015). Associations between vitamin D and semen quality in men were examined in several studies (Abbasihormozi *et al*., 2017; Akhavizadegan & Karbakhsh, 2017; Blomberg Jensen *et al*., 2011, 2016; Hammoud *et al*., 2012; Ramlau-Hansen *et al*., 2011; Tartagni *et al*., 2015). Some of them found a positive association between vitamin D concentration and sperm motility (Abbasihormozi *et al*., 2017; Blomberg Jensen *et al*., 2011, 2016) and sperm concentration in fertile and infertile men (Akhavizadegan & Karbakhsh, 2017). Yet, others reported that not only insufficient, but also excessive levels of vitamin D (over 50 ng/mL = 125 nmol/L) were associated with poorer semen quality -sperm concentrations, morphology or progressive motility- (Hammoud *et al*., 2012). Clinical trials have shown that vitamin D supplementation did not affect men's semen parameters or testosterone levels (Blomberg Jensen *et al*., 2018; Heijboer *et al*., 2015).

Possible associations between vitamin D and male reproductive parameters are still uncertain. To the best of our knowledge, there are no studies exploring associations between serum vitamin D levels and reproductive parameters in young Mediterranean men. Therefore, the aim of this study was to examine associations between vitamin D status (as measured by blood serum levels and dietary intake) and semen parameters and serum reproductive hormone levels in young Spanish men.

MATERIALS AND METHODS

Study population

The Murcia Young Men's Study (MYMS) is a cross-sectional study of university students 18-23 years old in the Murcia Region (Southern Spain). Study details are described elsewhere (Mendiola *et al*., 2013). Briefly, a total of 215 students agreed to participate and completed the study visit between October 2010-November 2011. To be included, men had to be university students, born in Spain after 31 December 1987, and able to contact their mother and ask her to complete a questionnaire. At the study visit men underwent an andrological examination, provided semen and blood samples and completed questionnaires on lifestyle and food frequency. Complete data on vitamin D serum levels and vitamin D intake were available for 204 men. Six men were excluded from the analysis because of their daily calorie intake exceeded 5000 kcal. A total of 198 young men were included in the final analysis. The Research

Ethics Committee of the University of Murcia approved this study, and written informed consent was obtained from all subjects.

Physical examination and semen analysis

Body weight and height were measured using a digital scale (Tanita SC 330-S, London, UK). Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. The presence of varicocele or other scrotal abnormalities was evaluated and recorded. Semen analyses methods are described in detail elsewhere (Mendiola *et al*., 2013). Briefly, men were asked to abstain from ejaculation for at least 48 hours before sample collection. Ejaculation abstinence time was recorded as the time between current and previous ejaculation as reported by the study subject. Ejaculate volumes were estimated by specimen weight, assuming a semen density of 1.0 g/ml. Sperm concentration was evaluated by hemocytometer (Improved Neubauer; Hauser Scientific, Inc., Horsham, PA, USA). The spermatozoa were classified as either motile or immotile to report the percentage of motile spermatozoa [progressive (PR) and non- progressive (NP)] (World Health Organization, 2010). Total sperm count (volume × sperm concentration) was also calculated. Smears for morphology were made, air-dried, fixed, Papanicolaou stained and assessed using strict criteria (Menkveld *et al*., 1990). A single trained semen analyst reviewed all samples. Throughout the study period an external quality control program was carried out in collaboration with the University of Copenhagen's Department of Growth and Reproduction.

Hormonal analyses

Hormone analysis methods have been described previously (Asklund *et al*., 2007; Cutillas-Tolín *et al*., 2015). Briefly, blood samples were drawn from participants' cubital veins on the same time of the day of semen sample collection and were stored and frozen. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone–binding globulin (SHBG) were determined using time-resolved immunofluorometric assays (DELFIA; PerkinElmer, Skovlund, Denmark). Intra- and interassay variations were <5% in each of the three assays. Serum testosterone (T) levels were determined using a time-resolved fluoroimmunoassay (DELFIA; PerkinElmer) with intra- and interassay variation of <8%. Estradiol (E2) was measured by radioimmunoassay (Pantex, Santa Monica, CA) with an intraassay variation of <8% and an interassay variation of <13%. Inhibin B levels were determined by a specific two-sided enzyme immunometric assay (Oxford Bio-Innovation Ltd, Bicester, UK) with intra- and interassay variation of 13% and 18%, respectively. Free

testosterone was calculated using the equation of Vermeulen and colleagues (1999) assuming a fixed albumin of 43.8 g/L.

Vitamin D Status

Dietary Assessment

We used a validated 101-food item semi-quantitative food frequency questionnaire (FFQ) to assess the usual diet (available at: http://bibliodieta.umh.es/files/2011/07/CFA101.pdf) (Vioque *et al*., 2007, 2013). Subjects were asked to report how often, on average, they had consumed each food item over the past year. The questionnaire offered nine options for frequency of consumption for each food, ranging from never or less than once a month to six or more times a day. Nutrient values for each food were obtained from the US Department of Agriculture and supplemented with Spanish sources (Palma *et al*., 2008; U.S. Department of Agriculture, 2010). The reproducibility and validity of this FFQ are comparable with other widely used FFQs (Willett, 2013). The average of correlation coefficients between nutrient intakes estimated using prospectively collected diet records and those estimated with the FFQ were 0.44 for reproducibility and 0.44 for validity (Vioque, 2006). The FFQ also showed satisfactory biochemical validity when compared with the plasma levels of carotenoids and vitamin C in further validation studies with other adult populations (Vioque *et al*., 2007, 2013). Nutrient intakes were adjusted for total energy intake by calculating the residuals from a linear regression with the natural logarithm of the nutrient modeled as the dependent variable and the natural logarithm of total energy intake as the independent variable (Willett *et al*., 1985).

Serum 25OHD levels

The concentration of total Vitamin D in serum was determined by the Liaison® 25-OH-Vitamin D total kit of Diasorin®, which uses the chemiluminescent immunoassay technology (CLIA) for the quantitative determination of 25-hydroxy-vitamin D (25OHD) and other hydroxylated metabolites in serum or plasma. Studies have shown that CLIA is an effective replacement for high-performance liquid chromatography (HPLC) which is widely used for measuring 25-hydroxyvitamin D levels (Kushnir *et al*., 2010; Pal *et al*., 2013), and among automated immunoassays the Liaison® kit demonstrated the best performance (Farrell *et al*., 2012). Detection limit was 4.0 ng/ml with a measuring range between 4.0-150 ng/ml.

Statistical analyses

In addition to looking at continuous measures of vitamin D, study participants were categorized based on quartiles of dietary intake or levels of serum 25OHD levels: “high” >75 nmol/L (30 ng/mL), “sufficient” 50–75 nmol/L (20–30 ng/mL) and “insufficient and deficient” <50 nmol/L (20 ng/mL). These categories were set up in accordance with previous used cut-off points (Blomberg Jensen *et al*., 2011, 2016; Lee *et al*., 2012; Lips, 2004). The definition of “insufficient and deficient” (below 50 nmol/L) follows the classification of the Institute of Medicine, which recognizes it as inadequate for bone and overall health in healthy individuals (Institute of Medicine, 2011). Semen parameters were considered as continuous variables as well as dichotomized at the World Health Organization reference values (the lowest “normal” values as the fifth percentile of fertile men) (World Health Organization, 2010). Semen volume, sperm concentration, total sperm count (TSC), percentage of morphologically normal sperm, serum FSH and estradiol levels showed skewed distributions and were transformed using the natural log before analysis. To test for associations of baseline characteristics across recommended serum levels or quartiles of intake, Kruskal-Wallis and chi-squared tests were used for continuous and categorical variables, respectively.

Multiple linear regression was used to examine associations between serum levels or dietary intake of vitamin D and semen parameters or serum reproductive hormones. Tests for linear trend were performed using the median values of vitamin D categories in each subgroup or quartile as a continuous variable and reproductive outcomes as the response variable. We also used analysis of covariance (ANCOVA) to calculate multivariable adjusted semen quality and reproductive hormone levels for each subgroup of recommended serum levels or quartile of dietary intake by relevant covariates. Regression coefficients for outcomes that were log-transformed for analysis were exponentiated (“back-transformed”) to allow the presentation of adjusted means in the original scale.

The effects of several potential confounders were assessed [e.g., age, BMI, smoking, presence of varicocele, cryptorchidism, prolonged disease, medication use, physical activity (hours/week), season (winter vs. spring, summer or fall), total energy intake (kcal/day), etc.]. When inclusion of a potential confounder resulted in a change in the ß-coefficient of <10%, the variable was not retained in final models. The technical variables ejaculation abstinence time, time from semen collection to analysis (for sperm motility), and total kcal dietary intake (for vitamin D intake models) were included. All tests were two tailed, and the level of statistical significance was set at 0.05. Statistical analyses were performed with the IBM Statistical Package for the Social Sciences (SPSS) 19.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Study participants

Participant characteristics are summarized in Table 1. Study subjects were young (median age=20.4 years; IQR 5^{th}-95^{th}: 18.2-22.3) and most had a BMI within the normal range (median=23.6 kg/m^2; 19.4-29.8). Almost one-third of the subjects smoked (30.6%), and varicocele was detected in 15% of the participants. Only 16.2% of the semen samples were delivered during the winter season, and the others were collected in spring, summer or fall. Serum vitamin D levels were significantly higher in younger men (p=0.001), those with more intense physical activity (p=0.001) and lower alcohol consumption (p=0.03) (Table 1). Subjects' characteristics according to quartiles of vitamin D dietary intake are shown in Supplemental Table 1.

Vitamin D dietary intake and serum vitamin D levels

Nearly all participants (99%) had dietary intake of vitamin D below the recommended level of 600 IU/day (15 µg/day) (Ross *et al*., 2011). The average total daily vitamin D (vitamin D_2 and D_3) intake from food sources alone was 167 IU (4.2 µg), and the median was 137 IU (3.4 µg). None of the participants reported taking vitamin D supplements. Only 3 men (1.5%) were vitamin D deficient (<30 nmol/L), and 17% were insufficient (<50 nmol/L). The average vitamin D serum level was 69.8 nmol/L and was equal to the median. Figure 1 shows the relationship between these two measures of Vitamin D. Both, the linear and the quadratic terms were significant (*P*, value=0.03 and *P*, value=0.01, respectively).

Semen parameters and reproductive hormone levels

Median ejaculation abstinence time was 71.5 hours (IQR 5^{th}-95^{th}: 39.0-138 hours), median sperm concentration 42.9 mill/mL (8.4-133 mill/mL), total sperm count 119 mill (17-407 mill), morphologically total sperm 9.0% (2.0-23.0%), 48.3% (31.6-64.6%) for motile sperm (PR+NP) and 3.0 mL (1.0-6.6 mL) for semen volume. In general, all hormones showed serum levels within normal ranges. Although the majority (70%) of men had all semen parameters in the normal range, 7-16% of each parameter was below the lower limit (Table 2). There was a small but not significant difference in serum vitamin D levels between participants below or above the lower limit of semen parameters. Slightly lower median 25OHD was observed for men with values below the lower limit for semen volume, sperm concentration, total sperm count, and sperm motility (PR+NP). Semen quality parameters did not differ significantly by serum

25OHD concentration, and there was no evidence that men with 25OHD insufficiency had poorer mean semen quality (Table 3). Serum hormone measures did not differ significantly across categories of serum 25OHD concentration, and only in unadjusted analyses inhibin B levels were significantly different across serum vitamin D categories (*P*, value=0.02), but not in the final adjusted models (Table 3).

Further, we reran these analyses using different models: a) serum vitamin D values as a linear term; b) serum vitamin D values as tertiles o quartiles (non-linear associations); c) excluding all men below 30 nmol/L; and d) assessing effect modification by BMI (above/below 25, modeling 25(OH)D as a linear term). All of them produced similar findings to those already presented, not changing the results. We also did not observe any association between vitamin D dietary intake and reproductive outcomes (Supplemental Table 2).

DISCUSSION

In this study of young Spanish men, we investigated the association between serum levels and intake of vitamin D and semen parameters and reproductive hormone concentrations, which might have contributed to the low sperm count among young Spanish men unselected for fertility status, with primarily normal reproductive parameters. However, we found no association between vitamin D status and male reproductive outcomes in this population.

Almost all of participants had very low vitamin D intake with daily diet, but only 3 (1.5%) of them were considered vitamin D deficient, according to the US Institute of Medicine cut-off of 30 nmol/L (Institute of Medicine, 2011). This finding may be explained by sufficient sun exposure, as reported previously in other Mediterranean regions (Carnevale *et al*., 2001). Although the prevalence of vitamin D deficiency has been reported to be approximately 30% among adults in Spain (González-Molero *et al*., 2011), young men living near subtropical regions are at low risk for deficiency (Carnevale *et al*., 2001). Interestingly, in our study population, even small amounts of vitamin D from food sources were related to vitamin D serum levels (Figure 1).

Though some epidemiologic studies support an association between vitamin D and semen quality (Abbasihormozi *et al*., 2017; Akhavizadegan & Karbakhsh, 2017; Hammoud e*t al*., 2012; Blomberg Jensen *et al*., 2011), we observed no association in our study population. It has been known for many years that vitamin D is essential to maintain reproductive functions in male rats (Fu *et al*., 2017; Kwiecinski *et al*., 1989). However, the mechanism through which vitamin D affects male reproductive functions remains unclear (Ding *et al*., 2016; Fu *et al*., 2017). It has been suggested that vitamin D affects function and survival of mature spermatozoa

(Blomberg Jensen *et al*., 2011, 2016). In 2009 researchers reported that 1,25(OH)2D3 was locally produced in sperm (Aquila *et al*., 2009). Sperm responds to 1,25(OH)2D3 by a VDR-independent mechanism, and VDRs are expressed in human mature sperm, mainly in the head and nucleus but also in the neck of spermatozoa (Aquila *et al*., 2008; Blomberg Jensen, 2014). It has also been observed that in pathologic semen samples that had severe oligoastenozoospermia, a lower amount of VDRs was expressed (Aquila *et al*., 2009).

Results from studies investigating these associations in healthy young males from the general population are inconsistent (Blomberg Jensen *et al*., 2011; Ramlau-Hansen *et al*., 2011). In one of these previous studies, as in ours, low vitamin D levels were not associated with poor semen quality (Ramlau-Hansen *et al*., 2011). These authors reported a borderline significant association with sperm motility in crude analyses, which was no longer significant after covariate adjustment. Like our study, in both of these populations, few men had deficient or insufficient levels of vitamin D (less than 6% and 24%, respectively). No significant associations were found in a study from Iran (subfertile men), where over 50% of participants had insufficient levels of vitamin D (<50 nmol/L) (Abbasihormozi *et al*., 2017). Likewise, in a pilot study on Italian couples, neither dietary intake nor serum vitamin D were associated with semen parameters or reproductive hormone levels (Tartagni *et al*., 2015). On the other hand, a Danish group found that men with vitamin D <25 nmol/l had lower sperm motility, but did not detect any significant difference between the groups 25-50, 50-75 and >75 nmol/L (Blomberg Jensen *et al*., 2011). Moreover, the same group reported a positive effect of vitamin D serum levels on sperm motility in an *in vitro* study, consistent with previous *in vitro* findings (Blomberg Jensen *et al*., 2010, 2011, 2016). Interestingly, in a very recent study, a group from China reported similar findings in an *in vitro* study but did not observed effect on sperm motility *in vivo* neither with vitamin D blood serum levels nor seminal plasma vitamin D levels. However, seminal plasma vitamin D levels were positively related to sperm velocity (Jueraitetibaike *et al*., 2019). On balance, the role of Vitamin D in semen quality remains inconsistent.

In a recent review, most of the studies analyzed supported a positive association between 25(OH)D concentrations and androgens (Karras *et al*., 2016; Wehr *et al*., 2010). In a study from Germany among a large cohort of older men, subjects with adequate levels of 25(OH)D had significantly higher levels of testosterone than patients with insufficient levels (Wehr *et al*., 2010). Later studies among healthy European men confirmed this tendency by showing that lower levels of vitamin D were significantly correlated with lower total and free testosterone amounts (Lee et al., 2012). However, after supplementing subjects with vitamin D, the increase

of amount of testosterone was not statistically significant (Heijboer *et al*., 2015). On the other hand, a study of men in China did not find correlations between 25(OH)D and testosterone levels (Yang *et al*., 2012). A potential explanation for the inconsistent results on reproductive hormones could be effect modification by age on vitamin D functioning, supported by studies comparing effects in adolescent and adult men (Spiro & Buttriss, 2014). In line with our results, levels of testosterone and LH were similar in vitamin D insufficient and replete groups.

There is also no consistency about vitamin D levels cut-offs among participants in the studies we cited. Regarding the pattern recommended by the US Institute of Medicine deficiency is below 30 nmol/L (Institute of Medicine, 2011). These concentrations were developed in the context of parathyroid hormone response and skeletal health, and recent research suggests alternate cut-off values for a wider range of health outcomes: above 30 ng/mL (~75 nmol/L) (Holik, 2010) or even higher, 32 ng/mL (~80 nmol/L) which is dictated by factors other than calcium homeostasis, insulin resistance or beta-cell function (Hollis & Wagner, 2005). Previous studies of vitamin D and semen parameters have used cut points of <25 nmol/L; 25-50 nmol/L; 50-75 nmol/L; >75 nmol/L (Blomberg Jensen *et al*., 2011, 2016), or other sample-based quantiles (Ramlau-Hansen *et al*., 2011). Next difference between the studies is methodology used to measure vitamin D serum levels. The most widely used assay is liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Blomberg Jensen *et al*., 2011, 2016; Heijboer *et al*., 2015; Ramlau-Hansen *et al*., 2011; Wehr *et al*., 2010). Another popular method is chemiluminescent immunoassay (CLIA) (Hammoud *et al*., 2012; Tartagni *et al*., 2015), which was also used in our study.

Recent research has suggested that circulating concentrations of the active form of vitamin D, 1,25(OH)2D3, may be a more useful measure than total 25OHD. For example, a study of Chinese men found no correlation between 25(OH)D3 and reproductive parameters, but did observe a positive association between 1,25(OH)2D3 and semen parameters (progressive motility and total sperm count) (Zhu *et al*., 2016). Extrarenal (e.g., in the testes) expression of CYP27B1, the enzyme activating vitamin D, may affect semen quality independently of circulating 25OHD concentrations. However, this hypothesis is unlikely to be testable in human observational studies.

There are several limitations of this study. Both, serum vitamin D and reproductive outcomes were based on samples at a single time point. However, it has been shown that one sample is enough to assess semen quality in epidemiological studies (Chiu *et al*., 2017) and that a single sample can be used to classify men's reproductive hormones (Vermeulen &Verdonck, 1992). Exposure measurement error or misclassification in terms of dietary intake cannot be

ruled out and, if non-differential, may be expected to bias effect estimates toward the null. Moreover, our study may be subject to a healthy volunteer bias. That is, men with poor health may be underrepresented in the sample. Nonetheless, because of their young age and no reproductive experience, participants had no previous knowledge of their reproductive health status such as semen quality parameters or reproductive hormone levels, which means they were blinded to the outcome measures. That could have eliminated reverse causality. Finally, our sample size was relatively small, although similar to the usual size of published semen quality studies.

CONCLUSIONS

We found no associations between vitamin D status and reproductive outcomes in a population of young Spanish men unselected for fertility status. Therefore, low vitamin D is not a potential explanation for the poor semen quality previously observed in these young men. Future studies that include men with vitamin D deficiency and low semen and reproductive hormone values may be better suited to evaluate whether vitamin D plays a role in poor male reproductive outcomes. Lack of consistency between the studies on vitamin D and reproductive parameters suggest that we cannot expect major effects of vitamin D -or other general lifestyle factors- on semen quality or reproductive hormone levels. Notwithstanding, our findings may not generalize to fertile, subfertile or vitamin D deficient men.

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AUTHORS' CONTRIBUTION SECTION

AMTC, JM, NJ, JEC and SHS were involved in study conception and design. JANV, JV, FCH and JM were involved in study execution and acquisition of data. AR, EA, CMN, JM, NJ, JEC, SHS and AMTC contributed to data analysis and interpretation. AR, EA, CMN, JM and AMTC drafted the manuscript. All authors provided substantial intellectual contributions and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

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Table 1. Characteristics of participants in the Murcia Young Men's Study according to category of serum 25OHD (n=198).

Characteristics	Serum 25OHD (nmol/L)				
	Insufficient and deficient	Adequate	High	All men (n=198)	*P*, value
	<50 (n=34)	50-75 (n=92)	>75 (n=72)		
Serum 25OHD (nmol/L)	44.6 (24.2-49.7)	61.8 (50.9-74.4)	88.6 (76.0-116)	69.8 (41.4-102)	<0.001
Age (years)	21.3 (18.2-22.6)	20.6 (18.5-23.0)	19.7 (18.1-22.8)	20.4 (18.2-22.9)	0.001
BMI (kg/m^2)	24.8 (20.0-31.8)	23.7 (19.6-30.2)	23.0 (19.0-28.5)	23.6 (19.4-29.8)	0.09
Ejaculation abstinence time[a] (hours)	65.5 (37.3-105)	75.0 (38.0-146)	72.5 (38.7-159)	71.5 (39.0-138)	0.02
Current smoker, n (%)	13 (38.2)	33 (36.3)	14 (19.7)	60 (30.6)	0.08
Prolonged disease[b], n (%)	2 (5.9)	11 (12.0)	2 (2.8)	15 (7.6)	0.17
Take any medication[c], n (%)	5 (14.7)	23 (25.0)	15 (20.8)	43 (21.7)	0.36
Physical activity, hours/week	4.0 (0.0-13.3)	4.3 (0.0-14.0)	7.0 (0.0-20.0)	5.0 (0.0-15.2)	<0.001
Dietary intake					
Calories (kcal/day)	2106 (1090-4177)	2374 (1336-3854)	2266 (1511-3895)	2273 (1304-3862)	0.41
Alcohol (g/day)	13.1 (0.0-38.6)	6.2 (0.0-23.4)	5.5 (0.0-22.7)	6.7 (0.0-24.5)	0.03
Coffee intake (g/day)	51.9 (14.7-413)	71.7 (9.5-392)	82.7 (5.8-395)	76.7 (8.3-397)	0.73
Vitamin D (µg/day)	2.9 (0.66-8.9)	3.5 (0.99-8.9)	3.8 (1.5-12.2)	3.4 (1.1-11.4)	0.13
Calcium (mg/day)	10.0 (9.6-10.4)	9.9 (9.4-10.4)	10.0 (9.6-10.5)	10.0 (9.5-10.4)	0.15
Andrological exam					
Mean testis size (ml)	21.0 (14.9-27.1)	21.3 (14.5-26.0)	21.0 (16.0-26.0)	21.0 (15.0-26.0)	0.86
Varicocele, n (%)	4 (11.8)	15 (16.3)	11 (15.3)	30 (15.0)	0.72

Values presented are median (5th-95th) or number (n) and percentage (%).
P-value is for Kruskal-Wallis test for continuous variables and $\chi 2$ test for categorical variables. [a]Ejaculation abstinence time: period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation; [b]Long-lasting disease (including diabetes/thyroid disease), sexually transmitted diseases (diagnosed with epididymitis, chlamydia or gonorrhea).[c]Taken any medication during 3 months prior to participation in study (mostly antibiotics or medication against allergy).

Table 2. Proportion of men with poor semen parameters and differences in serum 25OHD concentrations (n=198).

Semen parameter	WHO lower limit[49]	Below lower limit, n (%)	Serum 25OHD, median (5^{th}-95^{th} percentile)		*P*, Wilcoxon rank-sum test
			Below lower limit of each semen parameter	At or above lower limit of each semen parameter	
Seminal volume (mL)	1.5	20 (10)	60.4 (44.5-117)	69.9 (41.2-103)	0.66
Sperm concentration (mill/mL)	15	31 (16)	62.2 (33.4-99.1)	69.9 (42.4-103)	0.46
Total sperm count (mill)	39	28 (14)	60.7 (29.2-112)	69.9 (43.8-102)	0.19
Motile sperm (PR+NP) (%)	40	13 (7)	69.9 (24.5-89.5)	68.9 (42.0-104)	0.83
Normal morphology (%)	4	21 (11)	73.4 (40.2-92.3)	67.9 (41.4-104)	0.82

Table 3. Adjusted mean (95% CI) semen parameters and reproductive hormone concentrations by serum 25OHD concentration category (and range) in the Murcia Young Men's Study of vitamin D (n=198).

Variables	Serum 25OHD (nmol/L)			
	Insufficient and deficient <50 (n=34)	Adequate 50-75 (n=92)	High >75 (n=72)	*P,* trend
Semen parameters[a]				
Seminal volume (mL)	3.3 (2.6-4.3)	2.6 (2.3-3.0)	2.6 (2.2-3.1)	0.33
Sperm concentration (mill/mL)	36.8 (25.1-54.2)	37.0 (30.0-45.6)	39.3 (30.5-50.6)	0.73
Total sperm count (mill)	124 (81.9-188)	96.2 (76.8-121)	103 (78.3-136)	0.77
Motile sperm (PR+NP) (%)	60.3 (56.2-64.3)	56.3 (54.1-58.5)	56.6 (53.9-59.3)	0.29
Normal morphology (%)	8.4 (6.6-10.7)	8.6 (7.6-9.8)	8.5 (7.2-9.9)	0.95
Serum reproductive hormones[b]				
FSH (IU/L)	2.3 (1.8-2.8)	2.3 (2.0-2.5)	2.5 (2.1-2.8)	0.42
LH (IU/L)	4.0 (3.4-4.7)	4.4 (4.0-4.7)	4.2 (3.7-4.6)	0.94
Inhibin B (pg/mL)	188 (160-214)	199 (185-214)	208 (190-227)	0.24
Total testosterone (T, nmol/L)	24.1 (21.6-26.7)	21.4 (20.2-22.8)	21.0 (19.3-22.7)	0.16
SHBG (nmol/L)	32.5 (28.5-36.5)	31.6 (29.4-33.8)	30.0 (27.3-32.7)	0.31
Calculated free T (cFT, pmol/L)	565 (499-631)	497 (461-533)	493 (448-537)	0.23
Estradiol (E2, pmol/L)	81.8 (72.9-91.7)	75.0 (70.5-79.8)	74.1 (68.6-80.1)	0.32

[a]Adjusted for age, BMI, current smoker, physical activity, season, ejaculation abstinence time, and time to start of semen analysis (for sperm motility only).

[b]Adjusted for age, BMI, current smoker, physical activity, season and time to blood sampling.

Supplemental Table 1. Characteristics of participants in the Murcia Young Men's Study according to quartiles of Vitamin D dietary intake (n=198)

Characteristics	Vitamin D intake (quartile and range)					
	Q1 (0.21-2.3) (n=49)	Q2 (2.4-3.4) (n=50)	Q3 (3.5-4.9) (n=50)	Q4 (5.0-20.1) (n=49)	All men (n=198)	*P*, value
Age (years)	20.6 (17.9-22.3)	20.6 (18.3-23.2)	20.4 (18.2-23.2)	20.4 (18.3-23.1)	20.4 (18.2-22.9)	0.91
BMI (kg/m^2)	24.5 (19.5-31.2)	23.1 (19.1-32.1)	23.6 (19.4-29.6)	23.4 (18.9-27.7)	23.6 (19.4-29.8)	0.65
Ejaculation abstinence time[a] (hours)	75.0 (41.0-118)	73.5 (33.7-126)	70.5 (40.2-144)	68.0 (35.5-166)	71.5 (39.0-138)	0.78
Current smoker, n (%)	16 (32.7)	18 (36.0)	10 (20.4)	16 (33.3)	60 (30.6)	0.34
Prolonged disease[b], n (%)	2 (4.1)	3 (6.0)	7 (14.0)	3 (6.1)	15 (7.6)	0.25
Take any medication[c], n (%)	13 (26.5)	6 (12.0)	10 (20.0)	14 (28.6)	43 (21.7)	0.18
Physical activity, hours/week	3.5 (0.0-12.0)	5.0 (0.0-12.5)	5.0 (0.0-18.4)	6.0 (0.0-20.0)	5.0 (0.0-15.2)	0.01
Dietary intake						
Calories (kcal/day)	2090 (1162-3134)	2122 (1135-3390)	2262 (1642-3863)	3012 (1945-4430)	2273 (1304-3862)	<0.001
Alcohol (g/day)	11.9 (0.61-34.7)	5.9 (0.0-20.4)	5.4 (0.0-26.6)	4.9 (0.63-22.5)	6.7 (0.0-24.5)	0.002
Coffee intake (g/day)	40.1 (7.7-410)	85.4 (9.8-374)	39.8 (5.2-380)	84.9 (12.6-424)	76.7 (8.3-397)	0.07
Vitamin D (µg/day)	1.9 (0.53-2.3)	2.8 (2.4-3.4)	4.1 (3.5-4.9)	6.8 (5.0-14.5)	3.4 (1.1-11.4)	
Calcium (mg/day)	9.9 (9.4-10.5)	9.9 (9.6-10.3)	10.0 (9.3-10.5)	10.0 (9.5-10.4)	10 (9.5-10.4)	0.51
Andrological exam						
Mean testis size (ml)	21.0 (15.3-25.3)	21.0 (15.1-25.0)	22.0 (13.4-26.7)	22.0 (15.0-26.8)	21.0 (15.0-26.0)	0.28
Varicocele, n (%)	9 (18.4)	3 (6.0)	9 (18.0)	9 (18.4)	30 (15.0)	0.23

Values presented are median (5th-95th) or number (n) and percentage (%). P-value is for Kruskal-Wallis test for continuous variables and $\chi 2$ test for categorical variables. [a]Ejaculation abstinence time: period calculated as difference between time of current ejaculation and self-reported time of previous ejaculation; [b]Long-lasting disease (including diabetes/thyroid disease), sexually transmitted diseases (diagnosed with epididymitis, chlamydia or gonorrhea).[c]Taken any medication during 3 months prior to participation in study (mostly antibiotics or medication against allergy).

Supplemental Table 2. Adjusted mean (95% CI) semen parameters and reproductive hormone concentrations by vitamin D intake quartile (and range) in the Murcia Young Men's Study of vitamin D (n=198).

Variables	Vitamin D intake (µg/day) (quartile and range)				*P*, trend
	Q1 (0.21-2.3) (n=49)	Q2 (2.4-3.4) (n=50)	Q3 (3.5-4.9) (n=50)	Q4 (5.0-20.1) (n=49)	
Semen parameters[a]					
Semen volume (mL)	2.3 (1.9-3.0)	3.0 (2.5-3.6)	3.0 (2.5-3.6)	2.8 (2.3-3.4)	0.52
Sperm concentration (mill/mL)	36.8 (27.4-49.0)	42.9 (32.2-55.9)	34.8 (26.2-44.9)	35.9 (26.1-47.9)	0.63
Total sperm count (mill)	85.4 (62.4-117)	128 (95.3-173)	102 (75.6-136)	97.4 (70.4-135)	0.94
Motile sperm (PR+NP) (%)	55.6 (52.4-58.8)	58.2 (55.2-61.2)	57.3 (54.4-60.3)	56.3 (52.9-59.6)	0.91
Normal morphology (%)	8.2 (6.8-10.0)	8.3 (6.9-10.0)	8.1 (6.7-9.6)	9.5 (7.8-11.6)	0.32
Serum reproductive hormones[b]					
FSH (IU/L)	2.1 (1.8-2.4)	2.6 (2.3-3.1)	2.5 (2.1-2.9)	2.2 (1.8-2.6)	0.83
LH (IU/L)	4.1 (3.6-4.6)	4.5 (4.0-4.9)	4.5 (4.1-5.0)	3.9 (3.4-4.4)	0.25
Inhibin B (pg/mL)	204 (181-227)	184 (163-206)	203 (182-224)	220 (196-243)	0.15
Total testosterone (T, nmol/L)	21.9 (19.9-23.9)	23.6 (21.7-25.5)	20.6 (18.7-22.5)	21.0 (18.9-23.1)	0.26
SHBG (nmol/L)	30.7 (27.2-34.3)	33.8 (30.4-37.2)	31.4 (28.1-34.7)	30.1 (26.4-33.8)	0.50
Calculated free T (cFT, pmol/L)	520 (469-570)	534 (486-582)	475 (428-522)	496 (443-549)	0.37
Estradiol (E2, pmol/L)	75.9 (69.7-82.8)	78.7 (72.5-85.4)	74.8 (69.1-81.0)	73.2 (66.9-80.0)	0.39

[a]Adjusted for total caloric intake, alcohol intake, coffee intake, physical activity, ejaculation abstinence time, and time to start analysis (for sperm motility only).

[b]Adjusted for total caloric intake, alcohol intake, coffee intake, physical activity and time to blood sampling.

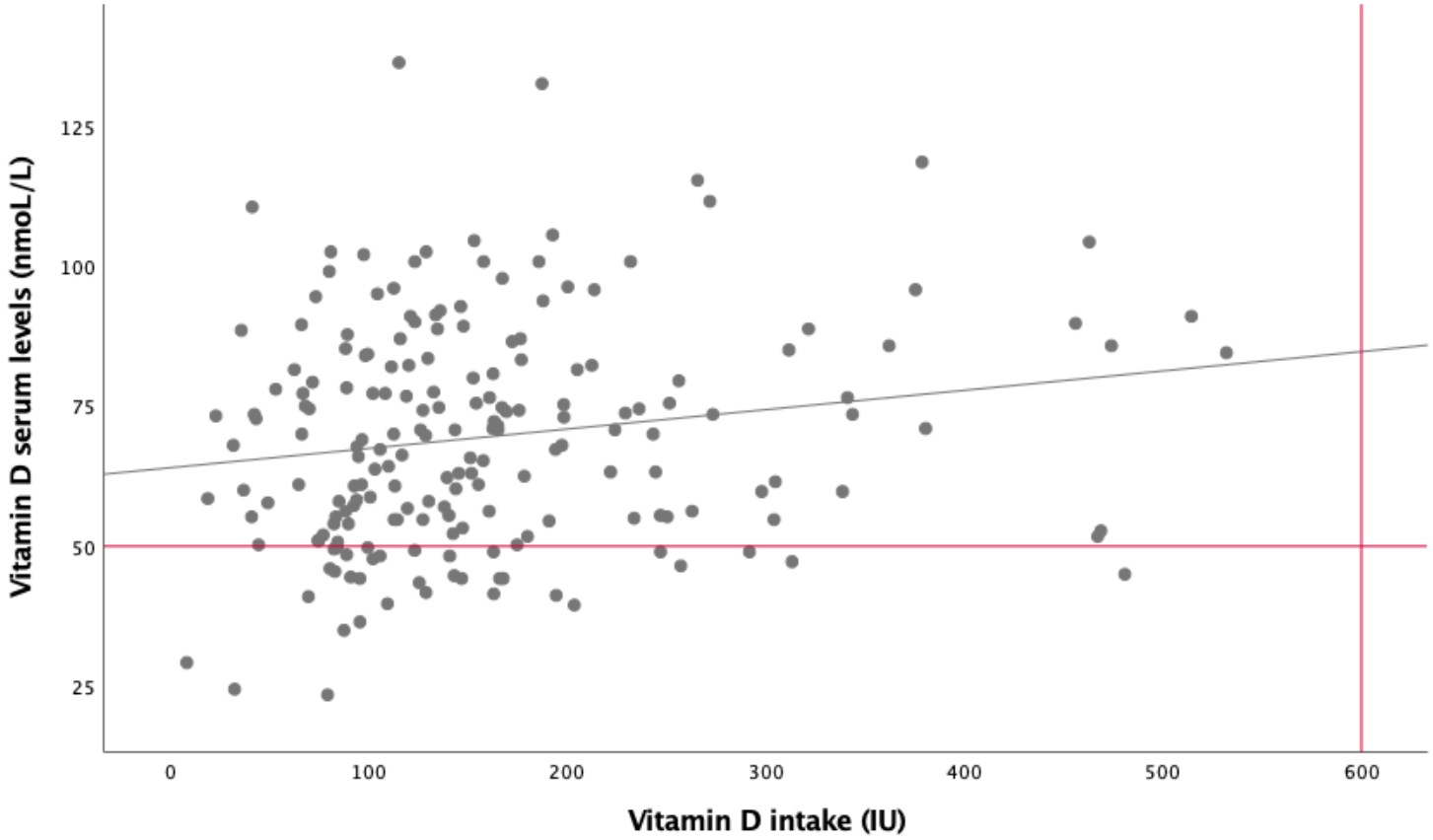

Figure 1. Vitamin D intake and serum vitamin D levels in the Murcia Young Men's Study of vitamin D (n=196).

Two outliers were dropped for the purposes of the graphing.

Red lines represent cut-off points for vitamin D recommended daily allowance (vertical) and serum vitamin D sufficient level (horizontal).