ORIGINAL ARTICLE

Male Health

Serum vitamin D content is associated with semen parameters and serum testosterone levels in men

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The present study aimed to evaluate the influence of serum vitamin D levels on semen quality and testosterone levels. This is a cross-sectional study conducted at Androscience, Science and Innovation Center in Andrology and High-Complex Clinical and Andrology Laboratory in Sao Paulo, Brazil, with 508 male patients, aged 18–60 years, from 2007 to 2017. Seminal parameters and serum sexual hormones were correlated with serum vitamin D concentrations in 260 men selected by strict selection criteria. Patients were divided into normozoospermic group (NZG, $n = 124$) and a group with seminal abnormalities (SAG, $n = 136$). Evaluation included complete physical examination, past medical history, habits and lifestyle factors, two complete seminal analysis with sperm functional tests, serum levels of 25-hydroxy-vitamin D_3 (25(OH)VD$_3$), total and free testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormone-binding globulin (SHBG), total cholesterol, homeostatic model assessment of insulin resistance (HOMA-IR) index, and karyotype. The mean concentration of 25(OH)VD$_3$ was significantly lower in the SAG ($P < 0.001$) and positively correlated with all baseline seminal parameters and total testosterone levels. In addition, serum vitamin D_3 concentration was found to be positively correlated with sperm concentration ($\beta = 2.103$; $P < 0.001$), total number of spermatozoa with progressive motility ($\beta = 2.069$; $P = 0.003$), total number of motile spermatozoa ($\beta = 2.571$; $P = 0.015$), and strict morphology ($\beta = 0.056$; $P = 0.006$), regardless of other variables. This is the first comparative study to address the issue of serum vitamin D_3 content between normozoospermic patients and those with sperm abnormalities. It clearly demonstrates a direct and positive relationship between serum vitamin D level and overall semen quality, male reproductive potential, and testosterone levels.

INTRODUCTION

Classically, vitamin D (VD$_3$) is recognized as a key regulator of calcium, phosphorus, and bone health homeostasis.[1] However, in addition to bones, VD$_3$ targets a wider range of biological organs and processes, including fat metabolism,[2] thyroid function,[3] immune response,[4] cardiovascular function,[5] the central nervous system,[6] and the reproductive system.[7,8] Vitamin D deficiency (VDD) secondary to modern lifestyles, food habits (such as a strict vegan diet or poor nutrition), milk allergies, and, more recently, the excessive use of sunscreens for the adequate prevention of skin cancer and melanoma in particular, is considered a public health problem in both developed and developing countries.[9] According to the Dietary Reference Intakes for Calcium and Vitamin D (Institute of Medicine, 2011),[10] the prevalence of VDD (estimated by a serum concentration of 25-hydroxy-vitamin D$_3$ [25(OH)VD$_3$] ≤20 ng ml⁻¹) has been estimated between 30% and 93%, even in tropical countries where sun exposure is not a limitation.[9] In a recent meta-analysis, the prevalence

of VDD in Brazil reached 28.2%, while VD$_3$ insufficiency affected up to 45.3% of the general population, with an average serum level of 25(OH)VD$_3$ at about 27 ng ml⁻¹.[11]

Adequate testicular function requires a cascade of complex events, the detailed investigation of which has been the objective of many studies.[12,13] It is generally accepted that male infertility is largely underestimated using only basic sperm analysis as recommended by the World Health Organization (WHO). When the etiology of male infertility is unclear and the cause for sperm dysfunction is unknown, idiophatic cases are often directly guided for the *in vitro* fertilization (IVF) lab, not allowing more relevant, better cost-effective and less risky andrological treatments.[14,15] An ongoing debate on global VDD in the general population, and the recognition that VD$_3$ is a pleiotropic signaling molecule, has encouraged studies of its action on nonconventional target organs and tissues, *i.e.*, other than bone health and metabolism.[1,16,17] The multiple localization of VD$_3$ receptor (VDR) in human spermatozoa (including the sperm head, the postacrosomal

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region, the neck, and sperm midpiece) supports VD_3 potential actions on sperm functions.[18–21] In addition, the fact that the VDR has been detected in spermatogonia, spermatids, spermatozoa, Leydig, Sertoli, and maturing germ cells supports the idea that VD_3 could be a potent actor in male reproductive performance.[19–21] Finally, the fact that all the VD_3 metabolic enzymes (including 25-hydroxylase, 1α-hydroxylase, and 24-hydroxylase) are found broadly expressed in the male reproductive tract and are present in spermatozoa[18,22] also supports the idea that VD_3 is likely to modulate male reproductive functions. In rodent VDD models, sperm motility, concentration, and normal morphology were reported to decrease and these effects were mainly attributed to VDD-induced hypocalcemia as they were increased by calcium and phosphorus supplementation.[23] In human VDD, similar sperm phenotypical and functional impairments have been reported together with impacts on female reproductive performances and offspring; however, the link with hypocalcemia remains obscure and controversial.[24,25] In addition, the link between VDD and serum androgen levels is still poorly understood.[26] Several studies suggesting that VDD may influence male reproductive function have been reported in both fertile and infertile men.[24,25,27–30] However, the cohort size limits the conclusions and all the associations disappeared after adjustment for confounders using multivariate analysis.[27] This prompted us to evaluate serum VD_3 and testosterone concentrations in relation to semen parameters in normozoospermic patients compared with patients showing defective semen parameters.

PATIENTS AND METHODS

Study design and patients

This cross-sectional study collected data from the records of 508 male patients, aged 18–60 years, who attended Androscience, Science and Innovation Center in Andrology and High-Complex Clinical and Andrology Laboratory in Sao Paulo, Brazil, a reference center for male infertility, hypogonadism, and male health, from 2007 to 2017. The main reasons for consulting these patients were infertility, erectile dysfunction, loss of libido, premature ejaculation and other sexual problems, prevasectomy visit, or as part of a general health checkup. The data collected included sex hormone evaluation, semen analysis, and functional testing of semen (WHO standards).[31] Serum $25(OH)VD_3$ (in ng ml^{-1}) concentrations were included and each subject was classified according to the Endocrine Society guidelines.[32] When $25(OH)VD_3$ serum concentration falls below 20 ng ml^{-1}, the patient is in a state of VDD, from 20 ng ml^{-1} to 30 ng ml^{-1}, and is qualified as VD_3 insufficiency; when $25(OH)VD_3$ serum concentration is over 30 ng ml^{-1}, the patient is considered normal. Data also included information on medications, physical exercise, tobacco, alcohol, and other drugs. The presence of clinical varicocele according to the criteria modified by Dubin and Amelar[33] was verified as well as other testicular abnormalities. Overweight and obesity status of each patient were also assessed by defining the body mass index (BMI) with the following reference values (BMI: eutrophic <25 kg m^{-2}, overweight ≥25 to <30 kg m^{-2}, and obesity ≥30 kg m^{-2}).[34]

Rigorous exclusion criteria included any previous history of medical, lifestyle, or serious unhealthy habits that could affect sperm quality or impair testicular function: genetics or infectious and inflammatory diseases, use of synthetic or illicit drugs, and cancer and related therapies, as described in **Figure 1**. In addition, data collection was strictly limited to a 6-month interval between the initial assessment and blood and semen testing. As a consequence, out of the 508 patients, final sample size was reduced to 260 subjects (**Figure 1**). These were subsequently divided into two groups according to the World Health Organization Laboratory Manual for
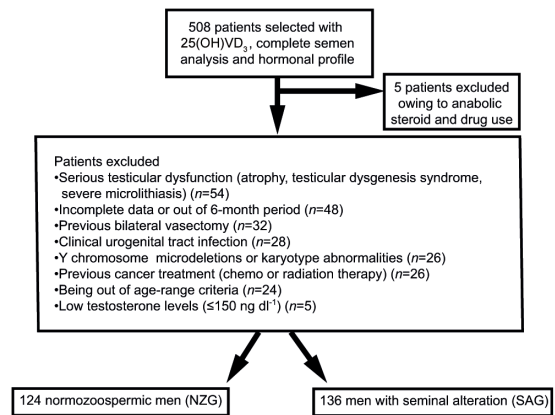

Figure 1: Selection diagram representing study design.

the Examination and Processing of Human Semen (WHO, 2010).[31] The seminal abnormalities group (SAG; $n = 136$) included patients with one or more seminal abnormalities, based on the 5th centile of recommendations: concentration <15 × 10^6 ml^{-1}, progressive motility <32%, and semen morphology <4%. The normozoospermic group (NZG, $n = 124$) consisted of individuals with no identifiable differences from any of the semen's basic parameter reference values. This study was approved by the Research Ethics Committee of the School of Medicine of the University of Sao Paulo, Sao Paulo, Brazil (trial registration number: CAAE 48393715.9.0000.0065). Informed consent form was exempt by Ethics Committee because all data were extracted from medical records and there was no clinical intervention.

Semen and biochemical analyses

Blood tests come from a specific database and were carried out in high-quality certified laboratories. Serum concentrations of $25(OH)VD_3$ (in ng ml^{-1}) were obtained by competitive chemiluminescent immunoassay (ECLIA binding assay, Diasorin, Stillwater, MN, USA) or by high-performance liquid chromatography (HPLC) with mass spectrometry. Serum luteinizing hormone (LH, in μU l^{-1}), follicle-stimulating hormone (FSH, in μU l^{-1}), sex hormone-binding globulin (SHBG, in nmol l^{-1}), and free and total testosterone (in ng ml^{-1}) were measured by an electrochemiluminescent immunoassay kit (ECLIA kit; Roche Diagnostics, Mannheim, Germany). Free testosterone levels were calculated from the Vermeulen formula.[35] The homeostatic model assessment of insulin resistance (HOMA-IR) index and total cholesterol (in mg dl^{-1}) were adopted as potential confounding variables. The HOMA-IR index was calculated from the formula: HOMA-IR = fasting glucose (in mg dl^{-1}) × fasting insulin (in μUI ml^{-1})/22.5 as described by Bergman *et al*.[36] in 2003. Fasting glucose was evaluated by calorimetric enzyme, whereas fasting insulin was monitored by ECLIA kit (Roche Diagnostics). All semen analyses were performed at Androscience, High Complexity Clinical and Research Andrology Laboratory. Samples were collected by masturbation after a period of 2–7 days of abstinence. Semen analysis was performed using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and included the evaluation of macroscopic and microscopic semen parameters: volume (in ml), pH, concentration (×10^6 ml^{-1}), progressive motility (%PR) and total motility (%TM), total number of spermatozoa (×10^6), total number of motile spermatozoa (×10^6), and total number of spermatozoa with progressive motility (×10^6) according to the WHO guidelines.[31] The morphological analysis was carried out taking into account WHO[31] and strict criteria by Kruger *et al*.[37] The presence of

antisperm antibodies was assessed using the Marscreen® commercial kit (Bioscreen, New York, NY, USA).

Statistical analyses

Patients were characterized by age and vitamin D_3 deficiency as assessed according to the Endocrine Society guidelines.[32] The presence of varicocele, smoking, alcohol consumption, physical activity level and other lifestyle habits, BMI, HOMA-IR index, basal serum hormone concentrations, and seminal parameters were recorded. Continuously changing data were described by mean and standard deviation (s.d.), whereas categorical data were described by absolute number, frequency, and proportion. Correlations between serum $25(OH)VD_3$ concentrations, semen parameters, sex hormones, HOMA-IR index, total cholesterol, BMI, and age were analyzed by Spearman's correlation coefficient. The Mann–Whitney U test was used to verify the association between $25(OH)VD_3$ and other clinical or epidemiological characteristics. This test was also used to identify an association between semen parameter values and sex hormones and all the variables described above. A multiple linear regression was performed to predict which variables (VD_3, age, presence of varicocele, smoking, alcohol consumption, BMI, HOMA-IR index, and total testosterone concentration) could have a positive or negative influence on seminal parameters. The normality of semen parameters was confirmed and evaluated by partial regression graphs and residue

tables in the predicted values. These data were also evaluated for residue independence by the Durbin–Watson test and homoscedasticity (variance homogeneity) by visual inspection of a residue graph against nonstandard predicted values. It was observed that both the collective linearity and the dependent variable with all independent variables were linear. The significance level was set at $P < 0.05$. All analyses were performed with SPSS 23.0 (IBM Corp, Armonk, NY, USA).

RESULTS

Sample characteristics

Table 1 presents the clinical and laboratory characteristics of the patients' seminal samples. The mean age was similar in the SAG and NZG groups. The mean serum $25(OH)VD_3$ concentration was significantly lower in the SAG group than the NZG group (**Figure 2**). The prevalence of VDD was significantly higher in patients with abnormal seminal parameters than in the normozoospermic group (33.1% in the SAG group and 25.0% in the NZG group, $P < 0.001$). The $25(OH)VD_3$ concentration was considered sufficient in 25.7% of SAG men and 41.9% of NZG men. The prevalence of overweight and obesity was significantly higher in patients with abnormal seminal characteristics than normozoospermic patients (77.1% for the SAG group and 54.6% for the NZG group). The same was true for serum insulin levels ($P = 0.054$). The incidence of alcohol consumption and smoking was similar in both groups (54.4% in the SAG group *vs*

Table 1: Baseline clinical and laboratory features of the samples according to seminal analysis diagnosis group

Clinical/laboratory characteristics	SAG (n=136)	NZG (n=124)	P
Age (year), mean±s.d. (range)	38.73±8.45 (19.00–59.00)	37.96±8.77 (18.00–60.00)	0.615
$25(OH)VD_3$ (ng dl^{-1}), mean±s.d. (range)	25.18±8.89 (7.00–68.00)	30.06±12.10 (9.00–75.00)	<0.001*
Sufficient (>30.1 ng ml^{-1}), n (%)[a]	35 (25.7)	52 (41.9)	0.01*
Insufficient (20.1–30 ng ml^{-1}), n (%)[a]	56 (41.2)	41 (33.1)	0.01*
Deficient (≤20 ng ml^{-1}), n (%)[a]	45 (33.1)	31 (25.0)	0.01*
BMI (kg m^{-2}), mean±s.d. (range)[b,c]	27.72±4.16 (17.03–42.80)	26.63±3.62 (20.86–36.90)	0.013*
Underweight, n (%)	1 (0.9)	-	
Normal BMI, n (%)	23 (21.9)	44 (45.4)	0.01*
Overweight, n (%)	54 (51.4)	39 (40.2)	0.01*
Obese, n (%)	27 (25.8)	14 (14.4)	0.01*
Presence of varicocele (yes), n/total (%)[c]	56/102 (54.9)	56/102 (54.9)	1.000
Currently smoker (yes), n/total (%)[c]	18/102 (17.6)	16/97 (16.5)	0.829
Alcohol use (yes), n/total (%)[c]	55/101 (54.4)	60/98 (61.2)	0.334
Insulin (µU ml^{-1}), mean±s.d. (range)	10.12±6.42 (2.20–40.00)	8.58±5.79 (2.00–29.00)	0.054
HOMA-IR, mean±s.d. (range)	2.25±1.42 (0.46–8.39)	1.98±1.50 (0.20–8.19)	0.071
Total cholesterol (mg dl^{-1}), mean±s.d. (range)	199.04±39.21 (103.00–317.00)	197.33±40.74 (90.00–304.00)	0.734
Seminal parameters, mean (range)[d]			
Semen volume (ml)	3.00 (0.25–10.00)	2.50 (0.50–8.50)	0.244
Total number of motile spermatozoa (10^6)	23.25 (0–559.00)	147.00 (11.0–1152.00)	<0.001*
Total number of spermatozoa (10^6)	43.60 (0–866.70)	205.75 (1.50–1440.00)	<0.001*
Sperm concentration (10^6 ml^{-1})	16.00 (0–577.80)	82.00 (15.00–800.00)	<0.001*
Total number of spermatozoa with progressive motility (10^6)	5.41 (00–426.00)	79.60 (6.93–748.00)	<0.001*
Total progressive motility (%)	16.00 (0–65.00)	50.00 (32.00–75.00)	<0.001*
Total motile sperm (%)	45.00 (0–80.00)	70.00 (15.00–90.00)	<0.001*
Hormonal parameters, median (range)			
LH (µU l^{-1})	4.50 (0.08–19.20)	3.88 (0.06–10.10)	0.203
FSH (µU l^{-1})	6.17 (0.22–41.50)	4.31 (0.29–23.90)	0.018*
Total testosterone (ng dl^{-1})	463.84 (164.00–1123.00)	522.99 (175.00–1231.00)	0.019*
Free testosterone (ng dl^{-1})	9.88 (3.56–32.00)	14.22 (3.70–374.00)	0.018*
SHBG (nmol l^{-1})	37.57 (6.70–413.0)	36.27 (8.00–415.00)	0.641*

[a]According to the Endocrine Society guidelines (2011);[32] [b]according to the WHO 2000;[34] [c]there were some missing data in the medical records; [d]according to the WHO 2010.[31] *$P≤0.05$ is considered statistically significant difference. SAG: seminal abnormalities group; NZG: normozoospermic group; $25(OH)VD_3$: 25-hydroxy-vitamin D_3; BMI: body mass index; HOMA-IR: homeostatic model assessment insulin resistance; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone-binding globulin; s.d.: standard deviation

61.2% in the NZG group, and 17.6% in the SAG group *vs* 16.5% in the NZG group, respectively). The frequency of varicocele was exactly the same in both groups (54.9%). No differences were observed in total cholesterol and HOMA-IR between the two groups ($P = 0.734$ and $P = 0.071$, respectively). Regarding hormonal profile, the SAG group had lower total and free testosterone levels ($P = 0.019$ and $P = 0.018$, respectively) and higher FSH ($P = 0.018$) than the NZG group.

Vitamin D and semen quality

We identified a significant correlation between serum 25(OH)VD$_3$ concentrations and all basic seminal parameter values, mainly with percentage of total sperm motility ($P = 0.001$; **Supplementary Figure 1**), even using multivariate analysis (**Table 2**). The best-correlated sperm parameters were associated with a 25(OH)VD$_3$ concentration of about 30 to 40 ng ml^{-1} (**Figure 2**). Total serum testosterone concentration was positively correlated with values of three semen parameters: total sperm motility ($\beta = 0.025$; $P < 0.004$), total progressive motility ($\beta = 0.020$; $P < 0.009$), and sperm morphology according to the WHO criteria ($\beta = 0.007$; $P = 0.021$). In contrast, varicocele had a statistically significant impact only in the total number of spermatozoa ($\beta = 62.930$; $P = 0.039$). In particular, the 25(OH)VD$_3$ levels positively and independently influenced four sperm quality parameters, including sperm concentration ($\beta = 2.103$; $P < 0.001$), total number of motile spermatozoa ($\beta = 2.571$; $P = 0.015$), total number of spermatozoa with progressive motility ($\beta = 2.069$; $P = 0.003$), and Kruger's strict criteria morphology ($\beta = 0.056$; $P = 0.006$).

Vitamin D and clinical and laboratorial parameters

We found a negative correlation between serum 25(OH)VD$_3$ concentrations and HOMA-IR concentrations ($r = -0.21$; $P = 0.009$). No correlation with BMI ($P = 0.330$), total serum cholesterol levels ($P = 0.850$), smoking ($P = 0.500$), or alcohol intake ($P = 0.104$) was established.

Supplementary Figure 2 shows the positive Spearman's correlation between 25(OH)VD$_3$ concentrations and total testosterone levels ($r = 0.161$; $P = 0.014$). In addition, 25(OH)VD$_3$ concentration and BMI concentration were predictors of total testosterone concentration ($\beta = 2.751$, $P = 0.019$, and $\beta = -9.621$, $P = 0.002$, respectively; **Table 3**). Smoking, alcohol consumption, and the presence of varicocele had no influence on sex hormone outcomes in either group and were not independent predictors of any controlled variables.

DISCUSSION

An ongoing debate regarding global VDD deficiency in the general population has encouraged intensive investigation of its action upon nonclassical target organs and tissues, other than bone health and metabolism.[1,16,17] Vitamin D has been recognized as an all-around signaling molecule and male reproductive organs are among its target tissues.[38]

In this cross-sectional study, with strict inclusion criteria, we found that 66.5% of patients (from a cohort of 260 men in a tropical country where sun exposure is not a limitation) consulting for sexual and reproductive health problems were in a situation of vitamin D insufficiency. Considering their functional and structural sperm parameters, we clearly demonstrated here that serum 25(OH)VD$_3$ levels were positively correlated with values of all seminal parameters that are part of the standardized WHO-recommended semen analysis. The strongest correlation was with sperm motility, as it has already been reported elsewhere.[25] Nevertheless, these results further indicate that regardless of a wide number of confounding variables, serum VD$_3$ concentration had a positive influence on the total number of spermatozoa with progressive motility. An easily understandable and useful equation formulated from our findings demonstrates that every "one unit" (ng ml^{-1}) increase in 25(OH)VD$_3$ serum concentrations correlates with a 2.1% increase in progressive motile spermatozoa in the ejaculate. Other factors that may affect sperm quality and reduce fertility status, such as exposure to tobacco and alcohol, had no influence and were not significant as confounders for seminal
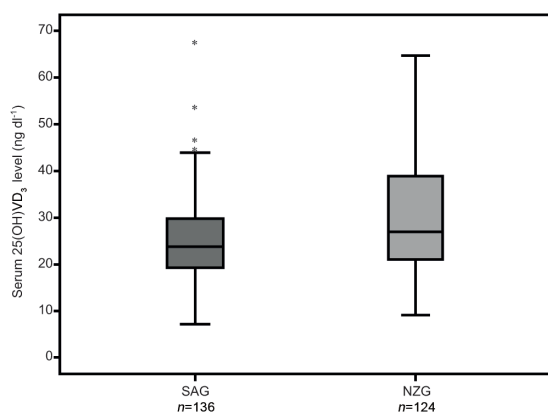

Figure 2: Box-plot graph of 25-hydroxy-vitamin D$_3$ serum levels distribution in both groups. The box displays the first quartile, median, and third quartile value distribution, whereas the horizontal line within it represents the median. Error bars represents the maximum and minimum VD$_3$ levels and the black dots represents few outliers. *SAG: seminal abnormalities group; NZG: normozoospermic group; 25(OH)VD$_3$: 25-hydroxy-vitamin D$_3$; VD$_3$: vitamin D.

Table 2: Contribution of each variable to seminal quality parameters by β coefficient (significance value, *P*) from multivariate regression analysis

Constant	Total number of spermatozoa	Sperm concentration	Total number of motile spermatozoa	Total number of spermatozoa with progressive motility	Total progressive motility	Total sperm motility	Normal morphology (WHO 2010)	Morphology (strict)
25(OH)VD$_3$	3.231 (0.033*)	2.103 (<0.001*)	2.571 (0.015*)	2.069 (0.003*)	0.366 (0.015*)	0.426 (0.013*)	0.117 (0.053*)	0.056 (0.006*)
Age	−1.439 (0.480)	0.394 (0.615)	−0.967 (0.499)	−0.536 (0.565)	−0.120 (0.554)	−0.331 (0.152)	0.007 (0.930)	−0.011 (0.681)
BMI	−0.974 (0.522)	0.195 (0.739)	−0.591 (0.580)	−0.519 (0.455)	−0.014 (0.925)	−0.124 (0.470)	0.035 (0.569)	0.015 (0.468)
Total testosterone	0.081 (0.294)	0.033 (0.262)	0.069 (0.200)	0.038 (0.281)	0.020 (0.009*)	0.025 (0.004*)	0.007 (0.021*)	0.002 (0.058)
Total cholesterol	0.248 (0.439)	0.139 (0.261)	0.148 (0.510)	0.034 (0.817)	−0.025 (0.436)	−0.008 (0.829)	0.003 (0.840)	−0.002 (0.582)
HOMA-IR	−6.558 (0.509)	3.177 (0.405)	−5.536 (0.427)	−2.170 (0.632)	0.094 (0.924)	1.070 (0.341)	0.363 (0.363)	−0.057 (0.582)
Alcohol	−51.649 (0.069)	−20.007 (0.066)	−33.197 (0.095)	−13.701 (0.289)	−1.234 (0.661)	−5.481 (0.087)	0.685 (0.547)	−0.052 (0.891)
Smoking	−0.886 (0.973)	−5.282 (0.605)	−2.210 (0.906)	−5.855 (0.630)	−1.332 (0.615)	−0.700 (0.816)	−0.698 (0.506)	0.164 (0.638)
Varicocele	62.930 (0.039*)	16.758 (0.152)	31.890 (0.135)	20.889 (0.132)	−2.291 (0.448)	−4.348 (0.206)	−0.593 (0.626)	0.332 (0.411)

Predictors (constant): 25(OH)VD$_3$, varicocele, HOMA-IR, age, alcohol, total testosterone, BMI, smoking. Dependent variable: total number of motile spermatozoa, total number of spermatozoa, total number of spermatozoa with progressive motility, total progressive motile, total motile, morphology by WHO, morphology by Kruger. *$P \leq 0.05$ is considered statistically significant difference. 25(OH)VD$_3$: 25-hydroxy-vitamin D$_3$; BMI: body mass index; HOMA-IR: homeostatic model assessment insulin resistance; WHO: World Health Organization

Table 3: Contribution of each individual variable to sexual hormone parameters by standardized β coefficient from multivariate regression analysis

Constant	Total testosterone	FSH	SHBG
25(OH)VD$_3$	2.751 (0.019*)	3.306 (0.112)	0.133 (0.177)
Age	−1.927 (0.180)	0.039 (0.111)	0.401 (0.001*)
BMI	−9.621 (0.002)	0.069 (0.175)	−0.935 (<0.001*)
Total cholesterol	0.017 (0.956)	−0.012 (0.022*)	−0.008 (0.754)

Data are expressed as β coefficient (*P value*). Predictors (constant): 25(OH)VD$_3$, age, total cholesterol, BMI, smoking, varicocele. Dependent variable: total testosterone, free testosterone, LH, FSH, SHBG. *$P \leq 0.05$ is considered statistically significant difference. 25(OH)VD$_3$: 25-hydroxy-vitamin D$_3$; BMI: body mass index; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SHBG: sex hormone-binding globulin

parameters in our multivariate analysis. Progressive motility is one of the most important indicators of sperm quality provided by basic semen analysis and has a positive predictive value for natural conception and even intrauterine insemination (IUI), as demonstrated in a large retrospective review of 1728 IUI cycles, demonstrated that among 38 clinical, hormonal, metabolic and semen variables analyzed, only 3 of them were associated with successful IUI outcome: female age <37.7 years at the time of treatment, absence of pelvic surgery, and the strongest correlation with postwash sperm motility higher than 40.0% ($P = 0.006$).[39] Therefore, theoretically, VD$_3$ supplementation could, in selected cases, decrease the invasiveness of proposed assisted reproductive technologies and turn previous IVF or intracytoplasmic sperm injection (ICSI) cases into a promising less invasive with minor risks and more cost-effective scenario of simple IUIs.

The results of observational studies concerning serum 25(OH)D$_3$ concentrations and seminal parameters are divergent. Some studies have reported a positive correlation with sperm motility and morphology,[24,25,40,41] while others have shown a negative[27] or even no effect on seminal parameters.[28] Our data concur with Hammoud *et al*.[24] who also suggested that low serum VD$_3$ concentrations may be associated with poor seminal parameter values. These authors reported an inverted U-shaped relationship between serum VD$_3$ concentration and five seminal parameters, including sperm concentration, percentage of progressively motile spermatozoa, total number of spermatozoa with progressive motility, total number of spermatozoa, and percentage of normal sperm heads.[24] In a cross-sectional study of 300 Danish men, serum VD$_3$ concentrations were also found to be correlated with total and progressive sperm motility as well as with normal sperm morphology.[30] In addition, in another Danish cross-sectional study involving 307 men, a high level of VD$_3$ (37.0–90.0 ng ml^{-1}) was associated with a lower median total number of spermatozoa and a lower percentage of sperm of normal morphology.[27] This unexpected finding in this latest study probably reflects a low number of men in the low-VD$_3$ group insufficient to detect an effect on sperm quality, rather than indicating that low VD$_3$ levels are not associated with poor seminal parameters. *In vitro*, serum 25(OH)VD$_3$ concentrations are correlated with increased intracellular calcium concentration in human spermatozoa.[40] This hypothesis was recently strengthened by the observation that in a randomized and placebo-controlled trial, higher live-birth rates were seen in oligozoospermic men with low serum VD$_3$ basal levels after they received a combined VD$_3$ and calcium supplementation.[41] The same group reported that serum calcium levels, VD$_3$ levels, and progressive sperm motility are correlated and that 25(OH)VD$_3$ induces a rapid increase in intracellular calcium concentration through a VDR-mediated action.[40] In addition, progressive sperm motility also has a positive

correlation with a higher probability of obtaining hyperactivated sperm motility in the female environment.[42] With these elements at hand, we propose a potential mechanism of action in which sperm motility is modulated by VDR-regulated calcium flows in the male genital tract (**Figure 3**). This model could explain the improvement in sperm motility and the stimulation of the acrosomal response in men with higher serum VD$_3$ levels. It could also explain the broad role of VD$_3$ that will act at both the mature spermatozoon and testicular levels, since VDR has been detected in mature spermatozoa and also in Leydig, Sertoli, and differentiating germ cells from spermatogonia and spermatids to spermatozoa.[19–22] The multiple locations of VDR in human spermatozoa (including the sperm head, the post-acrosomal region, the neck, and sperm midpiece) also support its various actions on functional sperm characteristics.[18–21]

In our cohort, we found a negative correlation between serum VD$_3$ concentration and HOMA-IR, suggesting the possible influence of serum VD$_3$ on glucose metabolism and sperm function. Recently, Ding *et al*.[43] looked at the impact of VD$_3$ supplementation on spermatogenesis in diabetic rats. They concluded that high blood sugar levels could be harmful to germ cells and affect the quantity and quality of spermatozoa. However, when animals received supplements with high doses of VD$_3$, they had significantly fewer abnormalities (mainly in sperm concentration and motility) than the control group. The authors concluded that VD$_3$ could play a protective role in overall testicular function, including increased reproductive capacity to withstand harmful effects of diabetes. It has been suggested that this effect was caused by the attenuation of inflammation, inactivating the caspase-dependent cascades that regulate apoptosis. In addition, the authors suggested that VD$_3$ could be seen as a promising form of treatment for testicular dysfunction in diabetic patients. Furthermore, serum VD$_3$ and BMI were determined to be positive and negative predictors of total testosterone levels, respectively. BMI also has a negative influence on SHBG, which leads to a better understanding of the lack of correlation between VD$_3$ and free testosterone levels. Nevertheless, the overall influence of VD$_3$ action on the increase in testosterone levels observed in our study is probably less important than the negative effects represented by the increase in BMI (**Table 3**). These variables appear to be an important factor influencing seminal quality.[12] However, the multicollinearity analysis excluded this influence in our cohort.

We also observed a positive correlation between total and free serum testosterone levels and serum 25(OH)VD$_3$ concentrations, in the NZG cohort. This is in agreement with the observation that in VDR-null mice, the testosterone/LH ratio is low, suggesting that testosterone levels are somehow regulated by VD$_3$.[22] However, a recent contradictory report demonstrated that a 6-month VD$_3$ + calcium supplementation did not have any significant effect on serum testosterone levels in young men.[44] It is thus clear that more investigations will be needed to elucidate the relationship existing between serum VD$_3$ and serum testosterone concentrations; however, owing to the high prevalence of VDD worldwide, this is an issue that cannot be ignored any longer. Our findings in testosterone levels are not limited to the reproductive age group, but could be extrapolated to benefit the growing population of older men, who universally demand for better health standards, including improved quality of overall sexual satisfaction, in order that these results could stimulate new therapeutic developments.[45] The elegant data reported here, in combination with nouvelle insights into the mechanisms of action of VD$_3$ and its receptor (**Figure 3**), serve like an orientational compass pointing to the direction of maintaining serum VD$_3$ concentration higher than 30 ng ml^{-1} for a better male reproductive performance. Whether higher VD$_3$ levels could result in
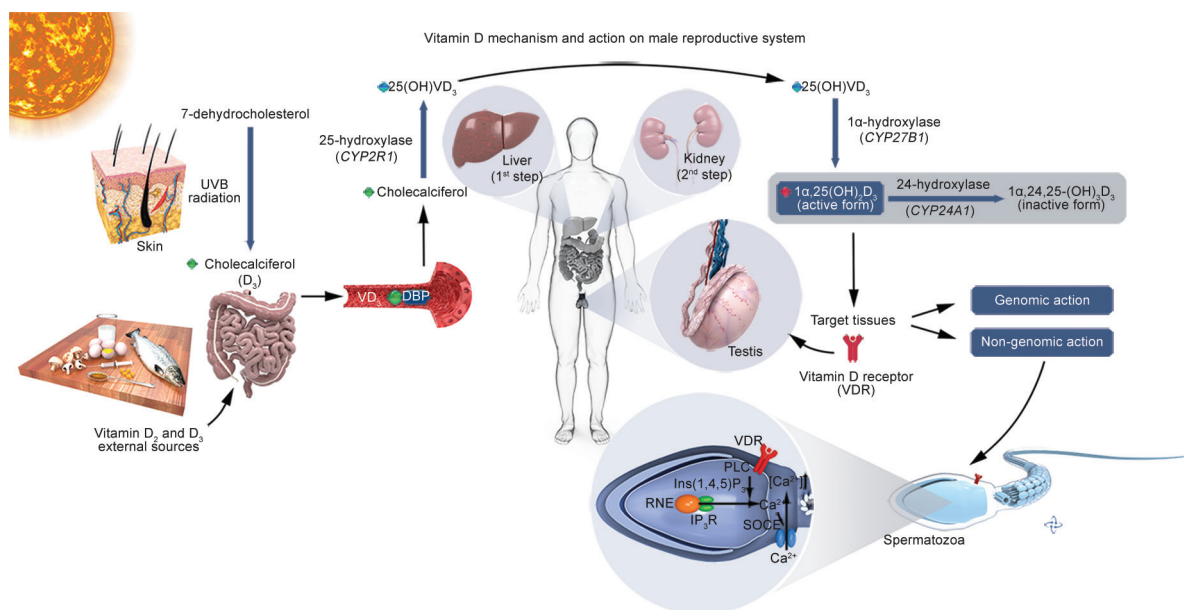
Figure 3: Vitamin D (VD_3) mechanism and action on male reproductive system. After sun exposure, the first step of VD_3 synthesis occurs in the skin, where ultraviolet B radiation converts 7-dehydrocholesterol into cholecalciferol (vitamin D_3). Vitamin D_2 and D_3 coming from food sources or external administration, are absorbed in the gut and transported to the bloodstream, where cholecalciferol couples with VD_3 binding protein (DBP) and is carried to the liver. Cholecalciferol (vitamin D_3) is biologically inactive, and must undergo two hydroxylation steps to form the active $1\alpha,25$-dihydroxy-vitamin D_3 ($1\alpha,25(OH)_2D_3$). The first step is a reaction mediated by the hepatic *CYP2R1* gene that codifies the 25-hydroxylase enzyme. The second step is conducted by renal *CYP27B1* that codifies the enzyme 1α-hydroxylase, leading to the formation of the biological active form, $1\alpha,25(OH)_2D_3$, responsible for a multitude of genomic and nongenomic actions through binding and activating VD_3 receptor (VDR) in many target tissues, including Leydig and Sertoli cells in testis, their precursor cells and spermatozoa. $1\alpha,25(OH)_2D_3$ can be inactivated by the 24-hydroxylase enzyme (codified by *CYP24A1* gene), transforming into the inactive form $1\alpha,24,25$-trihydroxy-vitamin D_3 ($1\alpha,24,25$-$(OH)_3D_3$). The nongenomic effect of VDR in human spermatozoa occurs when the active form, $1\alpha,25(OH)_2D_3$ activates VDR in the neck region inducing PLC activation leading to IP_3 production which subsequently opens IP_3R-gated calcium channels in the RNE increasing intracellular Ca^{2+} concentration. Subsequently, the initial Ca^{2+} release from RNE might be supported by SOCE.[38] VD_3: vitamin D; CYP: cytochrome P450 gene superfamily; *CYP2R1* gene: cytochrome P450 family 2 subfamily R member 1; *CYP27B1* gene: cytochrome P450 family 27 subfamily B member 1; *CYP24A1* gene: cytochrome P450 family 24 subfamily A member 1; VDR: vitamin D receptor; PLC: phospholipase C; IP_3: Inositol 1,4,5-trisphosphate; IP_3R: inositol 3-phosphate receptor; RNE: redundant nuclear envelope; SOCE: store-operated calcium entry. Illustration was developed by Androscience®.

further improvement in sperm quality and/or testosterone levels, as well as, mitigate the harmful effects of oxidative stress, lipid peroxidation and DNA damage, remains a subject for future research.[15,45]

Together, the known VDR-mediated positive endocrine action of serum VD_3 on the testis, as well as on spermatozoa and our present data showing that serum $25(OH)VD_3$ concentrations are positively correlated with sperm motility, nuclear integrity, and total testosterone levels, suggests that VD_3 supplementation could be part of a therapeutic strategy designed to improve male fertility.[46,47] Toward this goal, further investigation, including randomized, controlled, and double-blinded clinical trials as well as *in vitro* and *in vivo* studies, is needed to explore fully the therapeutic potential of VD_3 supplementation in cases of male infertility. Given growing concerns over the widespread and uncontrolled use of assisted reproductive technologies, in particular, ICSI beyond medical and ethical boundaries, any simple and cheap clinical treatment measure to improve testicular function and sperm quality *in vivo*, such as VD_3 supplementation, should be encouraged owing to its simplicity, low cost, and strong biological actions.[47]

AUTHOR CONTRIBUTIONS
JH, EMFC, PG, and RJA conceived the idea. IMC, JH, TAT, and JRP designed the study. IMC and JRP collected data. Data analysis was carried out by IMC, JRP, TAT, and PG. The first draft was written by IMC and EMFC and revised by TAT, JRP, EMFC, JRD, and JH. All authors read and approved the final version of the manuscript.

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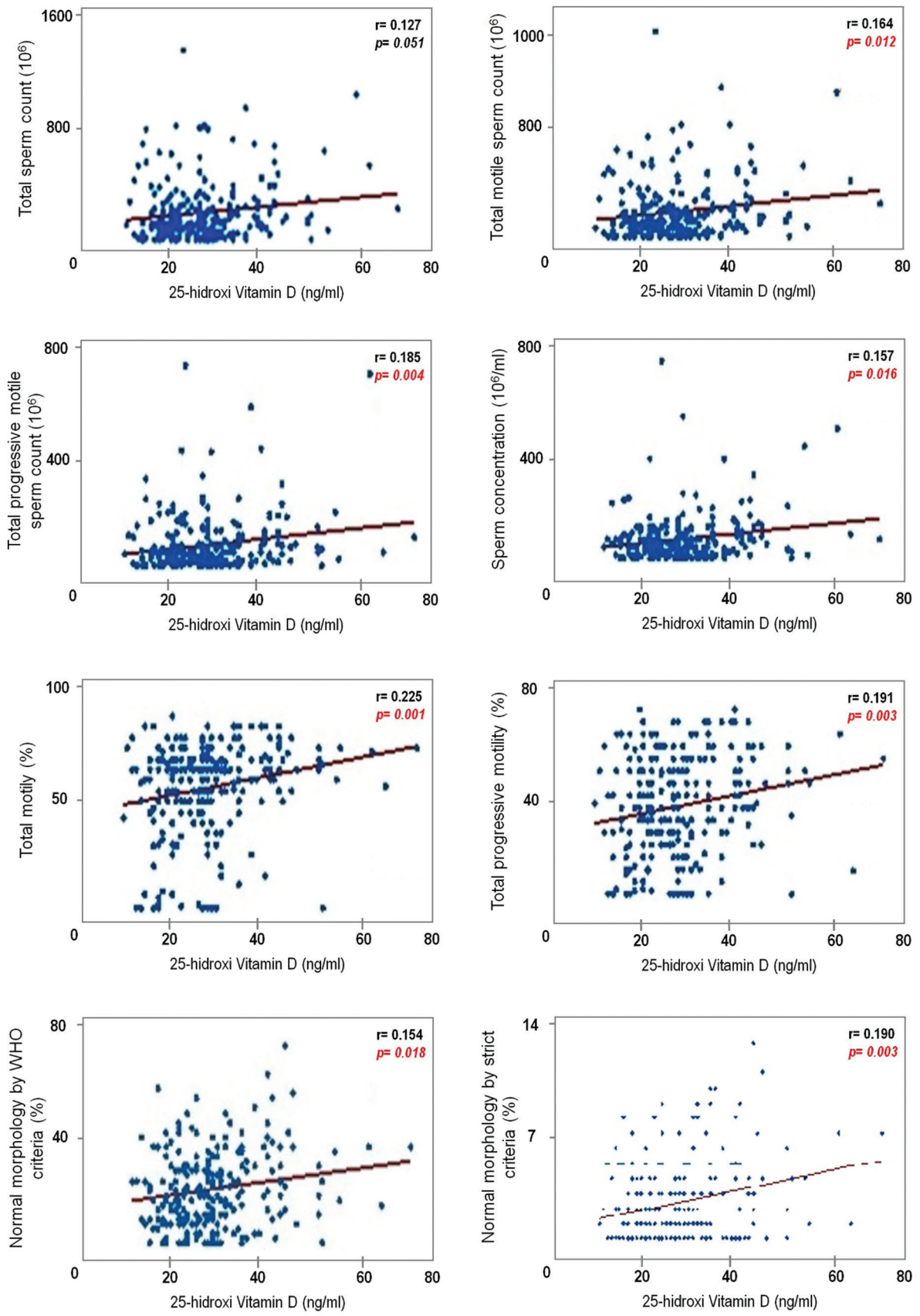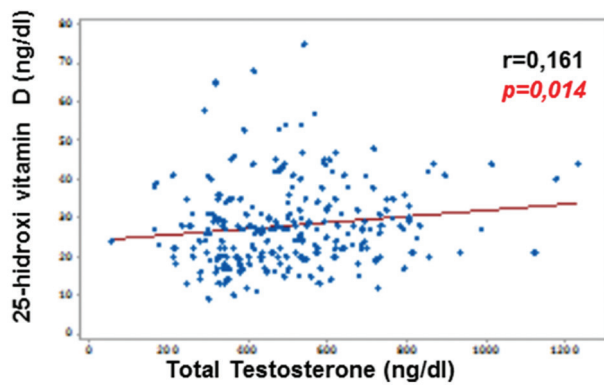
Supplementary Figure 1: Correlation between 25(OH)D$_3$ and seminal parameters in 260 male subjects. Each blue point represents 25(OH)D$_3$ concentration of one individual (each unity of measurement). Red line represents the correlation direct. 25(OH)D$_3$: 25-hydroxy-vitamin D$_3$; VD$_3$ vitamin D.

Supplementary Figure 2: Correlation between total testosterone levels and 25(OH)D$_3$ in 260 male subjects. Each blue point represents 25(OH)D$_3$ concentration for testosterone level in each patient. Red line represents the correlation direction tendency. r = Spearman's correlation coefficient. 25(OH)D$_3$: 25-hydroxy-vitamin D$_3$