

# Vitamin D Levels in Serum, Vitamin D Receptor Polymorphisms and Semen Quality Correlations in Lebanon: A Pilot Cross-Sectional Study

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**Abstract** Background: The role of the steroid hormone Vitamin D (VD) and its nuclear receptor (VDR) in skeletal metabolism is well known. Furthermore, research suggests that VD plays a role in female and male reproduction. However, semen quality status is not clear in infertile men with different VD serum concentrations. The aim of this study is to measure serum VD levels in infertile Lebanese men, to investigate semen quality parameters and their correlation to serum VD levels and polymorphic variations in the VDR. Materials and methods: From March to April 2013, 40 men presenting to an IVF center located in Mount-Lebanon to undergo IUI or ICSI procedure were recruited to our study. VD in serum was evaluated using ELISA method. The polymorphic regions were amplified using PCR followed by digestion with restriction enzymes FokI (rs10735810), ApaI G/T(rs11168271), TaqI T/C(rs731236). Results: No correlation was found between the ApaI and FokI polymorphisms and both VD levels in serum ( $P=0.367$ ,  $P=0.75$  respectively) and sperm count ( $P=0.919$ ). Positive correlation was found between ApaI polymorphisms and the number of non progressive and immotile spermatozoa ( $P=0.012$ ,  $P=0.033$  respectively). Also, positive correlation was found between TaqI polymorphisms and VD serum levels ( $P=0.038$ ). Conclusions: More studies on VD could be relevant of a potential VD supplementation that might improve semen quality of involuntary infertile men and would be beneficial both for the infertile couples and the society in general. VD supplementation can opens for a safe treatment of some cases of "idiopathic" impaired semen quality.

**Keywords** Serum VD, Semen Parameters, Male Infertility, VDR Polymorphism

## 1. Introduction

During the past decade, important advances in the study of vitamin D (VD) have been made. In addition to its important role in skeletal development and maintenance, evidence is mounting that VD produces beneficial effects on both female and male reproduction. Despite ample sunshine, the Middle East ( $15^{\circ}$ - $36^{\circ}$ N) registers the highest rates of rickets worldwide. This is in large part explained by limited sun exposure due to cultural practices and prolonged breast-feeding without vitamin D supplementation in the Middle East [1]. However, this region also has a high prevalence for hypovitaminosis D. The reported proportions were 32% in Lebanese girls and between 9% and 12% in Lebanese adolescent boys [2, 3].

Infertility is the inability of a non-contracepting couple to achieve pregnancy within one year of regular sexual intercourse [4, 5]. About 25% of couples do not achieve pregnancy within 1 year, 15% of these couples seek medical treatment for infertility and less than 5% remain unwillingly childless. Infertility can be due to male or female factors. However, in many couples, both male and female are implicated [4].

Male reproduction has been the spotlight of the studies because it has significantly decreased, resulting in a serious problem in modern society [6]. Reduced male fertility can be the result of congenital and acquired urogenital abnormalities, infections of the genital tract, varicocele, endocrine disturbances, genetic abnormalities and immunological factors. No causal factor is found in 60-70% of cases; it is though considered an idiopathic male infertility. In these cases, men have no previous history of fertility problems or abnormal findings on physical or endocrine laboratory testing [7].

VD is metabolized locally in the male reproductive tract and VD receptor expression has been shown in testis from rodents, chickens, roosters, humans, and in ejaculated human spermatozoa [8, 9]. The importance of testicular VDR expression is highlighted by impaired fertility due to

decreased sperm counts, reduced sperm motility, and histological testicular abnormalities in one of the VDR knock-out mice strains [10].

Studies have proven that men with higher dietary and supplement intake of certain micronutrients may produce sperm with less DNA damage. Therefore, higher intakes of antioxidants and micronutrients, such as selenium, zinc, vitamin E, vitamin C, L-carnitine and folic acid, might protect somatic as well as germ cells against age-associated genomic damage [8, 9]. Furthermore, relatively current studies have also shown that VD is correlated with semen quality as well as androgen levels and that VD treatment could increase testosterone levels, thus having a beneficial effect on human reproduction [4].

Thus, we studied the correlation between VD levels in serum and sperm parameters including motility and count on 40 infertile Lebanese men presented to IVF Center in Mount Lebanon region, Lebanon.

Moreover, genetic studies provide excellent opportunities to link molecular insight with epidemiological data. We, therefore, observed the polymorphism of three adjacent restriction fragment length polymorphisms for *FokI*, *ApaI* and *TaqI* of the VDR gene in the 40 Lebanese patients and its correlation to VD serum level and spermatoc parameters.

## 2. Materials and Methods

### 2.1. Study Subjects

From March to April 2013, 40 men presenting to an IVF center located in Mount-Lebanon region, Lebanon, to undergo IUI or ICSI procedure, as a treatment to their couple infertility, were recruited to our study. Oral informed consent was obtained from all participants. All men completed a general health and reproductive health questionnaire and donated 2 tubes of blood, one EDTA tube and one chemistry tube, and semen samples for serum VD analysis, semen parameters analysis and molecular analysis for VDR gene polymorphism. All men have respected a period of abstinence between 3 and 5 days. Subjects were aged between 25 and 50 years old. All subjects had normal secondary sexual characteristics and normal ejaculation. Men suffering from azoospermia either obstructive or non-obstructive, cryopreserved sperm or patients undergoing percutaneous epididymal or testicular sperm aspiration (PESA or TESA) were not included in our study. The study questionnaires included detailed questions regarding age, tobacco use, VD intake through diet, profession, hours of daily sun exposure, medical and surgical history, familial history of infertility and use of long term medications. The ethics committee of the Lebanese University approved the study protocol.

### 2.2. Semen Analysis

Semen samples were obtained by masturbation after 3–5

days of abstinence and were analyzed within 1 hour of ejaculation. Sperm parameters analysis included: sperm concentration (million/ml) and sperm motility (%). Spermatozoa were classified according to WHO classification progressive motile (PGR class A+B), non-progressive motile (NPRG class C) or immotile (class D) [11]. The latter parameters were assessed after adding 10  $\mu$ l of liquefied, well mixed semen on a glass slide and counting the average of few fields at a magnification of 200. The mean of spermatozoa per field was counted as millions/ml.

### 2.3. Assessment of Serum VD

Serum 25(OH)D concentrations serve as a biomarker for vitamin D stores. They were measured using the ELISA Kit (Euroimmun, Germany). For the classification of 25(OH)D status, the following ranges were used: Deficiency if VD < 10 ng/ml; insufficiency if VD is between 10 and 30 ng/ml; sufficiency if VD is between 30 and 100 ng/ml and toxicity if VD > 100 ng/ml.

### 2.4. DNA Extraction and Genotyping

DNA was extracted from whole blood samples collected in EDTA tubes using Sigma GenElute™ Blood Genomic DNA Kit. The *FokI*, *ApaI* and *TaqI* polymorphic sites were considered. Polymerase chain reaction (PCR) amplification was performed using 2 sets of primers - VDR2 (for *FokI*), and VDR3 (for both *ApaI* and *TaqI*) using Applied Biosystems 9700 thermocycler. PCR mixture consists on 50ng/ $\mu$ l of DNA, 200 mM of dNTP, 10  $\mu$ M of each primer (forward and reverse), 1.25 U of Taq polymerase in a total volume of 25  $\mu$ l per tube, with 10X PCR buffer and 2.5 mM magnesium chloride concentrations of 1X each. After initial denaturation for 5 min, at 94<sup>o</sup> C, samples were subjected to 35 cycles of amplification, 30 s at 94<sup>o</sup> C, 30 s at the relevant primer pair annealing temperature and 30 s at 72<sup>o</sup> C. The final step was a 5 min hold at 72<sup>o</sup> C. PCR products were then migrated on 1.5% electrophoresis gel (agarose gel). Following amplification, PCR products were digested overnight with *ApaI* and *FokI* at 37<sup>o</sup> C, and for 4 hours with *TaqI* at 65<sup>o</sup> C. 8  $\mu$ L of the PCR products were mixed with 1X buffer and 10U of the restriction enzyme in a total volume of 15  $\mu$ L. The digested products were analyzed for the presence or absence of recognition sites after ethidium bromide staining of fragments, separated through a 2% agarose gel. The alleles were designated *F* (196 bp and 69 bp fragments) and *f* (265 bp) for *FokI*, *A* (280 bp and 210 bp) and *a* (490 bp) for *ApaI* and *T* (290bp and 200 bp fragments) and *t* (490bp fragment) for *TaqI*.

### 2.5. Statistical Analysis

For descriptive statistics, mean  $\pm$  S.D. and median (fifth and ninety-fifth percentiles) were reported when appropriate. SPSS version 21.0 was used for the statistical analysis. The

comparisons between groups were performed using One-way Anova. Multiple linear models were built to assess the association between serum VD levels, semen VD levels and semen parameters. Pearson correlation analysis was used to analyse the correlations of variables. Chi-squared tests were applied to detect the difference in the rates among different groups. Finally, P-values of less than 0.05 were considered significant.

### 3. Results

#### 3.1. Sample Description and Serum VD Level

In this study, the mean age was  $38 \pm 6.7$  (mean $\pm$ SD) with a range between 28 and 47 years old. 25(OH)D levels were measured in 37 sera using EUROIMMUN 25(OH)D ELISA method. The mean level of VD was  $14.93 \pm 7.41$  ng/ml with a range between 7.29 and 40.49 ng/ml. The median level of vitamin D was 12.07 ng/ml.

Overall, 21.6 % (n=8) of the subjects were deficient in VD (25 (OH)D < 10 ng/mL), 75.7% (n= 28) were insufficient in VD (10 ng/mL < 25(OH)D < 30 ng/mL), and only 2.7 % (n=1) were sufficient in VD (25(OH)D = 40.5 ng/mL  $\geq$  30 ng/mL).

#### 3.2. Sperm Parameters

The mean sperm concentrations in our population was 36.11 million/ml with a range between 0.1 and 110 million/ml and the median value was 30 million/ml. Sperm concentration values were classified into two categories: oligozoospermia (<20 million/ml) and normozoospermia (concentration  $\geq$ 20 million/ml). 29.7% of our study population belong to the oligozoospermia group whereas, 70.3% fall in the normozoospermia category.

The mean of total progressive motility was 27% with a range between 0% and 60%. The median of total progressive motility was 30%. We categorized the progressive motility into 2 groups: Asthenozoospermia < 35% and normozoospermia  $\geq$  35%. We found that most of our subjects (65%) were asthenozoospermic (Table 1).

Our results did not show any significant correlation between sperm concentration and both its progressive motility and different serum VD levels.

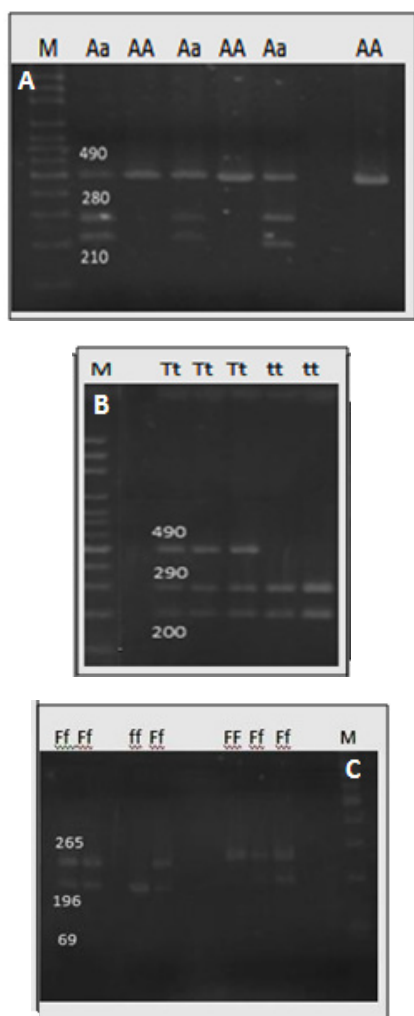
#### 3.3. VDR Polymorphism, VD Serum Level and Semen Parameters

The RFLP-PCR method was used in order to determine the genotype of each individual included in this study. PCR was done using *ApaI* primers in order to amplify the *ApaI* VDR region and yielded a product of 490 bp. Digestion of the 490 bp PCR product with *ApaI* enzyme results in two fragments of 280 bp and 210 bp. When an individual is homozygous for the presence of the *ApaI* restriction enzyme site (genotype AA), only these two fragments would be expected. For the individuals who are homozygous for the absence of the *ApaI* restriction site (genotype aa), the PCR product would not be digested and therefore, the only fragment expected would be 490 bp. For those who are heterozygous (genotype Aa), the fragments expected were 490 bp, 280 bp and 210 bp (Figure 1A).

The same PCR product obtained with *ApaI* primers was digested using the *TaqI* enzyme. Digestion of the 490 bp PCR product with *TaqI* results in two fragments of 290 bp and 200 bp for homozygote genotype (TT) where restriction enzyme site is present; whereas homozygote genotype (tt) is shown when there is one fragment of 490 bp due to the absence of *TaqI* restriction site. Heterozygosis (genotype Tt) is indicated when *TaqI* restriction results in 3 products of 490, 290 and 200 bp (Figure 1B). PCR was done using *FokI* primers, in order to amplify the *FokI* VDR region, and yielded a product of 265 bp. Digestion of the 265 bp PCR product with *FokI* results in two fragments of 196 bp and 69 bp for homozygote genotype (FF) where restriction enzyme site is present; whereas homozygote genotype (ff) is shown when there is one fragment of 265 bp due to the absence of *TaqI* restriction site. Heterozygosis genotype (Ff) is indicated when *TaqI* restriction results in 3 products of 265, 196 and 69 bp (Figure 1C).

**Table 1.** Analysis of sperm parameters and Vitamin D serum levels

Sperm parameters		Sufficient VD levels $\geq 30$ g/ml	Insufficient VD levels <30 mg/ml	P-value	Total N=37
COUNT	Oligozoospermia	1(4.2%)	23(95.8%)	0.075	24
	Normozoospermia	0(0%)	13(100%)		13
PRG	Asthenozoospermia	1(9.1%)	10(90.9%)	0.813	11
	Normozoospermia	0(0%)	26(100%)		26
IM	Asthenozoospermia	1(5.6%)	17(94.4%)	0.515	18
	Normozoospermia	0(0%)	19(100%)		19



**Figure 1.** Gel electrophoresis separation pattern (2%, 1X TBE) of different samples for *ApaI*, *TaqI* and *FokI* digestion. A. Gel electrophoresis separation pattern after *ApaI* digestion of 490 bp PCR product. Lane M- 100 bp ladder; AA and Aa (*ApaI* genotypes) B. Gel electrophoresis separation pattern (2%, 1X TBE) after *TaqI* digestion of 490 bp PCR product. Lane M-100 bp ladder; TT, Tt, tt (*TaqI* genotypes) C. Gel electrophoresis separation pattern (2%, 1X TBE) after *FokI* digestion of 265 bp PCR product. Lane M-100 bp ladder; FF, Ff, ff (*FokI* genotypes).

Table 2 shows genotypes of 38 individuals. This study shows that AA genotype (51%) for *ApaI* polymorphism and the least common genotype in this study was aa (0%). For *TaqI* polymorphism, TT genotype (50%) is the most common and the least common genotype is tt (13%). For *FokI* polymorphism, the most common genotype was Ff (50%) and the least common genotype was ff (5%).

**Table 2.** Genotype and allele frequencies of 38 individuals

Polymorphism	Genotypes frequencies			Allele frequencies	
<i>ApaI</i>	AA	Aa	Aa	A	a
	19(51%)	18(49%)	0 (0%)	75.5%	24.5%
<i>TaqI</i>	TT	Tt	Tt	T	t
	19(50%)	14(37%)	5(13%)	68.5%	31.5%
<i>FokI</i>	FF	Ff	Ff	F	f
	16(45%)	18(50%)	2(5%)	69%	31%

Both Pearson and Anova tests were considered using the SPSS program in order to study significant correlations between the 3 SNPs on one hand and semen parameters (count and motility), as well as VD levels in serum on the other hand. Correlations were considered significant when  $P < 0.05$ . In the case of *ApaI*, a positive correlation was found between *ApaI* SNPs and the number of non progressive and immotile spermatozoa ( $P=0.012$ ,  $P=0.033$  respectively). No correlation was found between *ApaI* polymorphism and the VD in serum ( $P= 0.367$ ) nor with sperm count ( $P= 0.919$ ) (Table 3).

As for *TaqI* enzyme, statistical analysis using Pearson test revealed a positive correlation between *TaqI* SNPs and VD levels in serum obtained by ELISA method (Table 3). According to our study, no significant correlation was found either by using Anova or Pearson test between *FokI* genotypes and both semen parameters or VD serum levels (Table 3).

**Table 3.** Statistical analysis of Vitamin D polymorphisms and both Vitamin D serum levels and sperm parameters

Parameters	<i>ApaI</i>		P value	<i>TaqI</i>		P value	<i>FokI</i>		P value
	AA	Aa/aa		TT	Tt/tt		FF	Ff/ff	
VD Serum ng/ml	11.09	12.25	0.367	16.4	13.31	0.038*	15.56	14.51	0.75
Sperm Count millions	33	35.94	0.919	42.75	30.81	0.336	33.01	38.95	0.357
PGR %	23	32	0.226	28	27	0.578	27	29	0.889
NPGR %	14	21	0.012*	20	15	0.304	19	15	0.211
IM %	63	47	0.033*	52	58	0.417	54	56	0.764

## 4. Discussion

VD levels have been linked to various health outcomes including reproductive disorders in males as well as in females. Our study emphasizes particularly the role of VD in male reproductive function through exploring the association between serum VD and semen parameters. The purpose of this study is to search for possible correlations between serum VD levels (25OHD), VDR genotypes and semen parameters (sperm concentration and sperm motility). This cross-sectional pilot study included 40 men presenting to an IVF center to undergo IUI or ICSI procedure as a treatment to their couple infertility. Men completed general and reproductive health questionnaires, and donated blood and semen samples.

The mean age of the study population was  $38 \pm 6.7$  years old. The mean 25OHD level in serum was  $14.93 \pm 7.41$  ng/ml and all the subjects were deficient and insufficient in serum VD. This vitamin D deficiency was also demonstrated in most of the Lebanese studies reporting on vitamin D status in the Lebanese population [12,13].

29.7% of our subjects were oligozoospermic and 70.3% were asthenozoospermic. However, we didn't find any statistical correlation between serum VD levels and sperm parameters (sperm count and sperm progressive motility). The latter results were discordant with some of the studies conducted showing a significant association between serum VD levels and sperm parameters. This is probably due to the small size of our population. Evidence for an association of vitamin D with spermatogenesis comes from a recent study, which demonstrated that men with severe hypospermatogenesis or idiopathic Sertoli cell-only syndrome (SCOS) displayed lower plasma 25(OH)D concentrations compared with healthy controls, despite normal levels of total testosterone and oestradiol in both groups [14]. Another small study, a phase II randomised, placebo-controlled trial in patients with prostatitis, has attempted to provide evidence for a favorable effect of vitamin D supplementation on semen quality and fertility outcomes. In this phase IIa trial in patients with prostatitis, elocalcitol significantly reduced levels of IL-8 in semen, suggesting improved quality and forward motility of sperm [15].

These two studies were followed by several cross-sectional studies searching for an association between serum vitamin D levels and sperm parameters. For example, Blomberg who conducted in 2011 a cross-sectional study of 300 men from the general Danish population found a positive correlation of 25(OH)D concentrations with sperm motility and progressive motility. Men with vitamin D deficiency [ $25(\text{OH})\text{D} < 25 \text{ nmol/l}$ ] displayed a lower percentage of motile, progressive motile and morphologically normal sperm compared with vitamin D-sufficient subjects [16].

Hammoud *et al* who conducted a cross-sectional study that included 170 healthy men in 2012 showed that sperm concentration, sperm progressive motility, sperm

morphology, and total progressively motile sperm count were lower in men with ' $25\text{OHD} > 50 \text{ ng/ml}$ ' when compared to men with ' $20 \text{ ng/ml} < 25\text{OHD} < 50 \text{ ng/ml}$ '. Total sperm count and total progressive motile sperm count were lower in men with ' $25\text{OHD} < 20 \text{ ng/ml}$ ' when compared to men with ' $20 \text{ ng/ml} < 25\text{OHD} < 50 \text{ ng/ml}$ '. Hammoud *et al* concluded that serum vitamin D levels at high and low levels can be negatively associated with semen parameters [5].

After investigating VDR genotypes, we found a positive correlation between *Apal* polymorphism and the number of non progressive and immotile spermatozoa and *TaqI* polymorphism and VD levels in serum. There was no mention in the literature of any previous correlations between either *TaqI* or *Apal* polymorphisms and semen parameters or VD levels in serum and in semen. However, no correlation was found between *FokI* polymorphism and both semen parameters and VD serum levels.

This study shows that AA genotype (51%) for *Apal* polymorphism is the most common which was also found by *Mitra et al.* (37%), but not by *Bid et al.* (36%) [17, 18]. The least common genotype in this study was aa (0%) which was also the least common in the other studies. For *TaqI* polymorphism, TT genotype (50%) is the most common genotype. Similar results were obtained in the findings of *Bid et al.* (49%) and *Mitra et al.* (35.8%) but not *Vupputuri et al.* (8.3%) [19]. The least common genotype tt (13%) was also the least common in all other studies; *Bid et al.* (11%) *Mitra et al.* (29.2%) and *Vupputuri et al.* (6.3%). For *FokI* polymorphism, the most common genotype was Ff (50%) which was similar to the findings of *Bid et al.* (49.1 %) but not *Vupputuri et al.* (37.5 %) and *Mitra et al.* (34.2 %). The least common genotype ff (5%) was also the least common in all the other studies; *Bid et al.* (6.9 %) *Mitra et al.* (26.4%) and *Vupputuri et al.* (4.2%). *FokI* polymorphism was identified in the literature upon comparison of the original Baker sequence of the VDR cDNA [20], where two potential translation initiation start sites (ATG) were observed and subsequent sequence comparisons have shown that a T to C polymorphism exists (ATG to ACG) at the first potential start site [21-23]. This polymorphism, also referred to as the start codon polymorphism or SCP, was later on defined using the *FokI* restriction enzyme in an RFLP test [24]. Thus, two protein variants can exist corresponding to the two available start sites: a long version of the VDR protein (the T-allele or the "F" allele; and also referred to as the M1 form, i.e., the methionine at first position) and a protein shortened by three amino acids (the C-allele detected as the "F" allele; also referred to as the M4 form, i.e., the methionine at fourth position). Therefore, this is the only "known" protein polymorphism in the VDR gene. However, the absence of correlation between semen parameters, VD levels and *FokI* polymorphism needs further investigations (e.g. by enlarging sample size) in order to be confirmed.

## 5. Conclusions

This pilot study in the Lebanese population is a milestone

to undergo further studies on VD concentrations in serum and male infertility that could be relevant for a potential VD supplementation for infertile men and for sperm preparation prior to assisted reproductive techniques. Additional studies are necessary to confirm our results, and public health measures could be taken to avoid male infertility through a potential VD supplementation.

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## Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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