Introduction: The biological actions of vitamin D are mediated through vitamin D receptor (VDR). Numerous single-nucleotide polymorphisms (SNPs) in the VDR gene have been identified, and some have been associated with cardiovascular disease (CVD) risk factors. This study aims to evaluate the association of five SNPs in the VDR gene with 25-hydroxyvitamin D (25\[OH\]D) levels in patients with at least one CVD risk factor.

Material and methods: Genomic DNA was sequenced using standard Sanger methods for five VDR SNPs (BsmI rs1544410; Apal rs7975232; Cdx2 rs11568820; TaqI rs731236; FokI rs2228570) in 50 Mediterranean subjects having hypovitaminosis D with at least one documented CVD risk factor, aged 18 years or more. The collected variables were serum levels of (25[OH]D), HbA1c, fasting plasma glucose, triglycerides, LDL cholesterol, and total cholesterol.

Results: BsmI, Apal, and TaqI were moderately to highly intercorrelated. Cdx2 was less frequent than expected. With respect to the number of mutations in FokI, levels of (25[OH]D) were 11.2 ±5.5 ng/ml in the absence of mutations, 12.6 ±4.7 ng/ml in the presence of one mutation, and 16.5 ± 5.5 ng/ml in the presence of two mutations.

Conclusions: FokI polymorphism is more frequent in subjects with cardiovascular risk factors than in the general Caucasian population.

Key words: vitamin D receptor, single-nucleotide polymorphism, FokI, cardiovascular disease, 25-hydroxyvitamin D.
receptor (RXXR). The RXR-VDR complex translocates from cytoplasm into the nucleus to bind specific DNA sequence elements and vitamin D response elements (VDRE), up- or down-regulating the expression of hundreds of genes. The biological effects of vitamin D are possible only after activation of the VDR-RXR-VDRE complex [3]. The VDR was identified in many cell types, including cardiomyocytes, vascular smooth muscle cells, and endothelium [4].

Association studies showed an elevated risk of cardiovascular disease (CVD) when vitamin D is deficient [5–7]; emerging evidence shows a correlation between vitamin D deficiency and the stage of coronary atherosclerosis [8–10]. In addition, a study by Faridi et al. showed an association of vitamin D deficiency with non-lipid biomarkers of cardiovascular risk [11]. Moreover, in a previous work we demonstrated an association between vitamin D deficiency and lipid and non-lipid markers of CVD in the Middle East region [12]. The gene encoding for VDR is located on the long arm of chromosome 12 (locus 12q11-q14) [13]. VDR single nucleotide polymorphisms (SNPs) have been identified, and their association with vitamin D status or risk of CVD, diabetes, obesity, hypertension, tuberculosis, and breast cancer has been reported in different ethnicities [14–19]. However, all these studies, especially in the Middle East region, analysed few VDR SNPs in relation to CVD risk factors [20–24]. Whether VDR affects vitamin D levels and whether it could be a risk factor for CVD is still not well elucidated. This study evaluates the association of five VDR SNPs (BsmI, Apal, Cdx2, TaqI, and FokI), with 25-hydroxyvitamin D (25(OH)D) levels, in a sample of subjects with at least one CVD risk factor.

**Material and methods**

**Study design and subjects**

Participants were Mediterranean subjects having hypovitaminosis D with at least one documented cardiovascular disease risk factor, aged 18 years or more. The study was approved by the Saint Joseph University Ethical Committee (reference number USJ 2015-34). Each participant gave written and informed consent before enrolment. Exclusion criteria were pregnancy, supplementation with vitamin D, any medical condition prone to altering parathyroid hormone (PTH) concentration (hyper or hypocalcaemia, renal insufficiency, certain medications: bisphosphonates, anticonvulsants medications, lithium), established cardiovascular disease, and cancer patients. The collected variables were serum levels of 25 OH vitamin D, glycated haemoglobin (HbA1c), fasting plasma glucose, triglycerides, low-density lipoprotein (LDL) cholesterol, total cholesterol, hypertensive status, and body mass index (BMI). Subjects who were consulting at three cardiology outpatient clinics in North Lebanon between January 1, 2017 and March 31, 2017, and who fulfilled the inclusion/exclusion criteria were enrolled in the study using convenience sampling, to reach a total of 50 participants.

**Genotyping**

The five VDR SNPs investigated were: BsmI rs15444410, Apal rs7975232, both in intron 8; Cdx2 rs11568820 in the upper stream of exon 1e; TaqI rs731236 in exon 9; and FokI rs2228570 in exon 2. BsmI and Apal polymorphisms resulted from the substitutions in intron 8, whereas TaqI resulted from a substitution of cytosine (C) with thymine (T) in exon 9. All of these three SNPs are located near the 3′-3′-Untranslated Region (3′-UTR) and are thought to alter the stability of the VDR mRNA [25]. The G allele of the Cdx2 (rs11568820) decreases VDR transcriptional activity relative to the A allele [26]. The FokI restriction enzyme identifies a polymorphic site in exon 2, more specifically a T to C variation is found in the start codon of the VDR gene that implicate a change of ATG (FokI T) to ACG (FokI C) [27].

**DNA extraction**

Blood was collected after a 12-h fasting period on EDTA tubes and across all seasons. For DNA sampling, white cells were isolated using a lysis solution blood lymphocyte buffer containing NH4Cl, KHCO3, and EDTA, and then frozen at –80°C. Genomic DNA was extracted from 50 μl buffy coat samples by standard salt-precipitation methods.

**RFLP-PCR**

Polymerase chain reaction (PCR) was performed in a final volume of 50 μl containing approximately 100 ng of DNA, 0.25 mM dNTPs, 1.5 mM MgCl2, 100 ng of each primer, 1× PCR buffer, and 0.02 US Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The amplification conditions for each PCR were 95°C for 5 min, followed by 35 cycles of: 95°C for 60 s, 55°C for 60 s, and 72°C for 60s, with a post-cycling final extension of 10 min at 72°C.

Primers used for the amplification of Apal, BsmI, and Cdx2 SNPs were designed using Primer 3 and checked for specificity using Basic Local Alignment Search Tool (BLAST). DNA sequences were obtained from UCSC or Genbank databases. The primers used for the study, which were Apal, Cdx2, BsmI, TaqI, and FokI SNPs, were the same as those used respectively by Hajj et al. [28]. The PCR products were verified using 1% agarose gel con-
Table I. Primer sequences used in PCR

| Variable | Studied polymorphisms | Forward primer | Reverse primer | Product size [bp] |
|----------|------------------------|----------------|----------------|------------------|
| Intron 8 | BsmI (rs1544410)       | 5'-ctccactgcctatagctcg-3' | 5'-tgctcataaatcaggg-3' | 247              |
| Exon 1c  | Cdx2 (rs11568820)      | 5'-gagccccaaaaaggaagaa-3' | 5'-gtttcgatgtagaaatgatga-3' | 396              |
| Intron 8 | Apal (rs7975232)       | 5'-ggatcccaaaaggaagaa-3' | 5'-acgttcagtaggtatggagc-3' | 265              |
| Exon 9   | TaqI (rs731236)        | 5'-cagagctgcagacagggaa-3' | 5'-ccatccagcacaagggagc-3' | 501              |
| Exon 2   | FokI (rs2228570)       | 5'-agctgccctgcaacctgct-3' | 5'-atggaaacaccttctctcgc-3' | 267              |

DNA sequencing

DNA sequencing was performed for all the subjects according to Standard Sanger methods. Positive heterozygous and homozygous controls (defined by direct sequencing) and negative controls (water) were systematically included in experiments. In addition, genotypes were confirmed by Sanger sequencing. PCR products were purified using the Agilent Technologies® StrataPrep Purification Kit according to the manufacturer’s instructions. Strands of the obtained products were sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) under standard conditions. The labelled products were subjected to electrophoresis on an ABI 3130 Genetic Analyzer sequencing system (Applied Biosystems). Electropherograms were analysed using Sequence Analysis Software version 5.2 (Applied Biosystems) and compared with reference sequences using ChromasPro version 1.7.6.1 (Technology, Queensland, Australia).

Statistical analysis

Proportions of the different VDR polymorphisms were based on their allelic frequencies and were reported with their 95% binomial confidence intervals (Jeffrey’s formula). They were compared with the expected proportions derived from a Caucasian population because the allelic frequencies in our population are similar to those described in Caucasian populations (European HapMap control population, n = 113) [29]. The number of mutations of each VDR polymorphism (0 to 2) was correlated with 25 OH D levels, the number of risk factors, and age using Spearman correlation with bootstrapping for confidence limits [30, 31]. A generalised regression model was fitted for the number of mutations in FokI I (0, 1, or 2 mutations) as dependent variable, and 25 OH D, number of risk factors, age, gender, and BMI as independent predictors, using a Poisson regression model with identity link. The analyses were run using SPSS software (IBM Corp. Released 2013, SPSS Statistics for Windows Version 22.0, Armonk, NY).

Results

Among the 50 subjects who participated in the study, 32% were diabetic, 26% hypertensive, 48% had hypertriglyceridaemia, and 52% had hypercholesterolaemia. Their mean age was 51.9 ±12.7 years, their mean BMI was 27.7 ±4.8 kg/m², and 22% were obese. The proportions of the different VDR polymorphisms were depicted in Table II. While the observed proportions of BsmI, Apal, and TaqI were comparable to the expected ones, Cdx2 polymorphism was less frequent than expected, and FokI polymorphisms were more frequent than that of the Caucasian population. Polymorphisms of BsmI, Apal, and TaqI were moderately to highly intercorrelated. Neither age nor individual and number of risk factors was correlated with VDR polymorphisms. In respect to the number of mutations in FokI, the levels of 25 OH D were 11.2 ±5.5 ng/ml in the absence of mutations, 12.6 ±4.7 ng/ml in the presence of one mutation, and 16.5 ±5.5 ng/ml in the presence of two mutations (Spearman’s correlation coefficient rho 0.391 (95% CI: 0.153–0.613)), as shown in Figure 1. BMI was not correlated with VDR polymorphisms or with 25 OH D levels.

Using a Poisson regression model, the number of mutations in FokI I (0, 1, or 2 mutations) tended to be linked to 25 OH D levels but did not reach statistical significance (p = 0.082) and was not related to the number of risk factors, age, gender or BMI, as shown in Table III.

Discussion

To the best of our knowledge, this is the first study in the Middle East region that evaluates the association of five VDR SNPs (BsmI, Apal, Cdx2, TaqI, and FokI) with 25OH D levels. Herein we showed, in a sample of 50 Mediterranean subjects...
at risk of CVD, that FokI polymorphism is more frequent in subjects with cardiovascular risk factors than in the general Caucasian population.

VDR is classified among the nuclear receptors; the latter are known to control transcriptional regulation of hormone responsive genes [32]. Because VDR is expressed in cardiac cells [33] and several types of cells, it is not surprising that the ligand-activated VDR modulates the expression of multiple genes, and regulates the function of many organs in physiological and pathophysiological conditions [34]. In this context, many studies have shown an association between vitamin D deficiency and risk factors of CVD, metabolic syndrome, and various type of diseases [35]. In addition, numerous SNPs in the VDR have been identified and some were associated with CVD [36].

Three hypotheses can be drawn from the findings of the current report dealing with Caucasian subjects with cardiovascular risk factors.

First, polymorphisms of BsmI, ApaI, and TaqI co-occur more than pure chance would allow, albeit with no consequences to plasma 25OH vitamin D levels. BsmI, ApaI, and Cdx2 polymorphisms, being intronic, cannot directly modify the amino acid sequence of VDR protein [13]. G allele of Cdx2 decreases VDR gene transcription, whereas AA homozygosity increases the VDR gene transcription.

Table II. Proportions of the different VDR polymorphisms with their 95% confidence limits and the expected population proportions

| Polymorphism | Proportion (95% CI) | Expected proportion ++ | Correlation between BsmI, ApaI, TaqI£ |
|--------------|---------------------|------------------------|---------------------------------------|
| BsmI         | 0.330 (0.244–0.426) | 0.296                  | 0.496 (0.250–0.688) 0.816 (0.641–0.938) |
| Cdx2         | 0.270 (0.190–0.363) | 0.457                  |                                       |
| ApaI         | 0.590 (0.492–0.683) | 0.529                  | 0.556 (0.367–0.714) |
| TaqI         | 0.310 (0.226–0.405) | 0.330                  |                                       |
| FokI         | 0.670 (0.574–0.756) | 0.570                  |                                       |

(+) : exact binomial confidence interval; (++) : Caucasian population; £ Spearman’s correlation. 95% confidence intervals derived by bootstrapping based on 1000 samples.

To our knowledge, BsmI, ApaI, and Cdx2 co-polymorphism have not been investigated previously, and its significance needs to be elucidated.

Second, FokI polymorphism is more frequent in subjects with cardiovascular risk factors than in the general Caucasian population. FokI SNP extends the length of the receptor protein from 424 to 427 amino acid residues [37]. The current study, by its exploratory nature, cannot provide definitive evidence, and further epidemiological studies are needed to assess the association between FokI polymorphism and cardiovascular risk.

Table III. Poisson regression model using the number of mutations in FokI (0, 1, or 2 mutations) as dependent count variable, and 25 OH D levels, number of risk factors, age, gender, and BMI as dependent predictors

| Parameter                  | Coefficient ± std. error | 95% Wald confidence interval | Wald $\chi^2$ df | P-value |
|----------------------------|--------------------------|------------------------------|------------------|---------|
| (Intercept)                | 0.333 ±1.280             | −2.175 – 2.841               | 0.068 1          | 0.795   |
| 25 OH D                    | 0.055 ±0.0314            | −0.007 – 0.116               | 3.025 1          | 0.082   |
| Number of risk factors     | −0.160 ±0.1827           | −0.518 – 0.198               | 0.771 1          | 0.380   |
| Age                        | 0.011 ±0.0156            | −0.019 – 0.042               | 0.529 1          | 0.467   |
| Male gender                | −0.424 ±0.3973           | −1.203 – 0.354               | 1.141 1          | 0.285   |
| BMI                        | 4.513E–05 ±0.0370        | −0.073 – 0.073               | 0.000 1          | 0.999   |

Df – degrees of freedom. Model’s deviance/df = 0.570. Model’s AIC = 136.4.
Third, the number of mutated alleles in FokI polymorphism correlates with plasma levels of 25(OH)D. Interestingly, this observation has been frequently reported in recent studies dealing with Hispanic, Afro-American [38, 39], Chinese [40], and Brazilian [41] subjects. What is more interesting is that the association between 25 OH vitamin D levels and FokI polymorphism was observed in cases as well as healthy controls, and was independent of the disease [39–43]. The mechanism underlying this observation could be related to a negative control of [1,25(OH)2D] over its own levels and the levels of its precursor 25 (OH)D through the VDR [44–46], knowing that the CC allele found in FokI encodes a shorter protein with higher transcriptional activity, thus enhancing VDR activity [47].

The current study is limited by its sample size, which could underpower the statistical tests, as well as its cross-sectional design, which would not allow us to follow-up subjects in time to document potential cardiovascular events. In conclusion, the study sheds light on FokI polymorphism and its potential association with cardiovascular risk, a finding to be corroborated by future epidemiological research.

Acknowledgments

The authors acknowledge the Lebanese University Doctoral School and the National Council for Scientific Research – Lebanon (CNRS-L) for providing financial support to this work through the Grant Research Program (GRP).

Conflict of interest

The authors declare no conflict of interest.

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