

Article

Vitamin D Receptor and Its Influence on Multiple Sclerosis Risk and Severity: From Gene Polymorphisms to Protein Expression

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Abstract: Multiple sclerosis (MS) is a multifactorial neurodegenerative disease. Low levels of vitamin D are a risk factor for MS and alterations in the vitamin D receptor (VDR) might be a risk factor as well. This study aimed to evaluate whether the VDR rs731236 (Taq-I) and rs4334089 (HpyCH4V) gene polymorphisms and VDR protein expression are associated with MS risk and severity. Vitamin D plasma levels were analyzed in a group of patients. Additional analyses of VDR protein expression and vitamin D levels of patients with different forms of MS (MSSS < 3 and MSSS ≥ 3) were performed. The analysis of the genotypic and allelic frequencies revealed that the rs731236 (Taq-I) gene polymorphism is significantly associated with MS presence. Although the total, cytosolic and nuclear VDR protein contents do not change between MS patients and healthy controls and between patients with different MS severity, vitamin D levels decrease in parallel with an increase in MSSS.

Keywords: multiple sclerosis; vitamin D; VDR; polymorphism

1. Introduction

Multiple Sclerosis (MS) is a neurodegenerative disease with an inflammatory and autoimmune pathogenesis that affects the central nervous system (CNS). Characterized by an early onset, it usually appears between 20 and 40 years of age and it is the most common cause of neurologic disability in young adults [1,2]. The incidence varies according to geographic areas: Canada, United States and northern Europe are the areas where the disease occurs with a greater frequency [3]. Currently, 109,000 people are estimated to be affected by MS in Italy, with an average prevalence rate of 176/100,000 in mainland Italy and Sicily and 299/100,000 in Sardinia [4].

MS has a multifactorial origin and develops in genetically predisposed individuals: it is characterized by a high level of familiarity so that siblings of an affected individual have a 10- to 30-fold greater risk of developing MS than the general population; second- and third-degree relatives also present a modest increased risk [5,6]. Moreover, environmental factors, which contribute to the development of the disease, are extremely important. Among them, latitude affects the frequency of MS occurrence, and both incidence and prevalence increase with increasing latitude [7].

A potential explanation for the association between the incidence of MS and geographic location is the effect of sun exposure on the synthesis of vitamin D. Indeed, low

vitamin D levels are associated with a higher risk of developing MS [8] and vitamin D supplementation reduces the risk [9]. Furthermore, vitamin D replacement therapy contributed to a decreased annual relapse rate in treated MS patients in several clinical trials [10]. Epidemiological evidence about the effect of month of birth on MS further supports the concept that maternal deficiency of vitamin D could increase MS risk. In line with these data, an increase in MS risk has been reported among subjects who were born in April and a reduction in individuals who were born in October and November [11].

The precise mechanism of action of vitamin D in modulating MS onset and course remains to be determined. Vitamin D is essential for correct regulation of the immune system, being a potent immunomodulator that controls both innate and adaptive immune responses. The biologically active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), inhibits dendritic cells differentiation and maturation [12] and reduces the responsiveness of monocytes to pathogen-associated molecular patterns [13]. Moreover, it prevents the proliferation and enhances apoptosis of activated B cells [14], inhibits the expression of Th1 cytokines [15] and promotes the development of a Th2 phenotype [16].

Vitamin D acts via the vitamin D receptor (*VDR*), an intracellular receptor that is expressed in almost all the cell types of the body, including immune cells [17], and is a ligand-activated transcription factor that mediates the genomic effects of 1,25(OH)₂D₃. The gene encoding the *VDR* is located on chromosome 12 (12q13.11) and contains nine exons. Several common allelic variants have been identified [18], and some variants may have consequences on *VDR* function and activity, thus potentially influencing the effect of vitamin D on immune system function [19].

The aim of our research was to study the role of *VDR* polymorphisms in MS. Specifically, allelic and genotypic frequencies of *VDR* rs731236 (TaqI T/C) and *VDR* rs433408 (HpyCH4V G/A) polymorphisms were analyzed in Italian MS patients and healthy controls. The first polymorphism is located in exon 9 of the *VDR* gene and causes a substitution from T to C, which results in a silent codon change. The second polymorphism, *VDR* rs4334089, is located in the 5'UTR. Furthermore, total, cytoplasmic and nuclear *VDR* protein levels were evaluated to understand whether an altered expression and/or distribution of *VDR* could be associated with MS risk. In addition, plasma vitamin 25(OH)D₃ levels were determined in MS patients to assess a possible association with *VDR* protein levels.

2. Materials and Methods

2.1. Subjects and Ethics Statement

Allele and genotype frequencies of TaqI T/C and HpyCH4V A/G polymorphisms were first investigated in 105 subjects from the MS center of the IRCCS National Neurological Institute C. Mondino, Pavia, Italy, who were diagnosed with MS according to 2010 McDonald criteria (Polman et al., 2011). The patients suffered from RRM and were under multiple therapies. In this study, we also tested 282 healthy controls provided by the Immunogenetics Laboratory, Immunohematology and Transfusion Centre, Fondazione IRCCS, Policlinico San Matteo, Pavia, Italy. At the time of blood sampling all the controls had been assessed to be free from any kind of disorder, whether physical or mental. MS patients' and healthy controls' demographical and clinical characteristics were recorded at the time of blood collection (Table 1), and the subjects with no missing information were included in the analyses.

The study was approved by the local ethics committees and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All patients and controls signed informed consent.

Table 1. Demographic and clinical characteristics of the examined MS patients and healthy controls. Data are expressed as number of subjects or median (95% CI). The two-sample Wilcoxon rank-sum (Mann–Whitney) test and the χ^2 test were used to compare age and sex in MS cases and healthy controls, respectively. $p < 0.05$ was considered statistically significant. Abbreviations: EDSS = expanded disability status scale; F/M = female to male ratio. MS = multiple sclerosis; MSSS = multiple sclerosis severity score.

	MS Patients	Healthy Controls	<i>p</i> -Value
	<i>n</i> = 105	<i>n</i> = 282	
Age	45.0 (38.0–53.0)	34.5 (29.0–41.0)	$p < 0.0001$
Sex (F/M ratio)	1.69	1.19	$p = 0.129$
MS duration (years)	12.0 (8.0–19.0)		
EDSS	2.0 (1.5–4.5)		
MSSS	2.3 (1.0–5.0)		

2.2. Gene Polymorphism Analyses

Whole blood was collected by venipuncture in Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Blood samples from MS patients were collected during the stable period and not during relapses. Human genomic DNA was obtained from 200 μ L of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's instructions. The concentration and purity of DNA were determined by spectrophotometric analysis. In order to establish alleles and genotypes for the *VDR* rs731236 polymorphism polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed. PCR-RFLP amplifications were carried out in a total volume of 25 μ L containing 1 μ L of genomic DNA (final concentration 20 ng/ μ L), 0.1 μ L of Taq polymerase (Eurobio, Les Ulis, France) (final concentration 0.02 U/ μ L), 1 μ L of each primer (final concentration 4 ng/ μ L), 2.5 μ L PCR Buffer 10X (Eurobio), 0.75 μ L MgCl₂ (Eurobio, Les Ulis, France) (final concentration 1.5 mM) and 2.5 μ L dNTPs (final concentration 2 mM each), using the I-Cycler (BioRad, Hercules, CA, USA). Amplification was performed under the following conditions: initial incubation at 95 °C for 5 min, followed by 30 cycles each of 95 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s; final extension was carried out at 72 °C for 7 min. The PCR products were visualized by electrophoresis in 2% agarose gel (Agarose Standard, Eurobio, Les Ulis, France). Genotypes were determined by digestion with the restriction enzyme TaqI, following the protocol: 15 μ L PCR product, 3.5 μ L NE Buffer 10 \times , 0.5 μ L TaqI (final concentration: 0.2 U/ μ L) and 6.0 μ L H₂O. The restriction patterns were obtained by gel electrophoresis in 3% agarose gel (Eurobio, Les Ulis, France). Table 2 shows the sequence of primers and restriction fragments obtained for the *VDR* rs731236 polymorphism.

Table 2. Sequence of primers and restriction fragments. Primers and restriction enzymes used for the investigation for the *VDR* rs731236 gene polymorphisms.

Polymorphism	Primers	Size	Enzyme	Fragments
<i>VDR</i> rs731236	F: 5'-GTCAGTGGAGGGCTTTGG-3' R: 5'-GCTGCACTCAGGCTGGAA-3'	381 bp	TaqI	381 (T) 203 (C) 178

VDR rs4334089 was analyzed by Real-Time PCR using the specific assay C__2880798_10 (Applied Biosystem, Foster City, CA, USA). Briefly, 1 μ L of DNA was aliquoted into each well of a 384-well plate (final concentration: 10 ng/ μ L); the reaction was carried out in a final volume of 5 μ L, adding 2.5 μ L of 2X Master mix Probe (Biorad, Hercules, CA, USA), 0.0625 μ L of assay and 1.4375 μ L of sterile H₂O. The single-nucleotide polymorphism (SNP) detection was carried out in the LightCycler 480 (Roche, Roche, Basel, Switzerland), following the thermal cycling conditions: one denaturation cycle of 10 min at 96 °C,

followed by 45 cycles each of 92 °C for 15 s and 60 °C for 90 s. Fluorescence was measured by dedicated software (RealPlex 2.0) and the genotype was determined.

2.3. Peripheral Blood Mononuclear Cells Isolation from Whole Blood

Ten mL of blood, collected by venipuncture in Vacutainer tubes containing EDTA, from MS patients and healthy controls, were diluted 1:1 with Ficoll (Histopaque-1077, Sigma-Aldrich, Munich, Germany) and centrifuged at 450× g for 30 min to isolate peripheral blood mononuclear cells (PBMCs). The cells were collected from the ring above Ficoll and washed with phosphate buffered saline 1× (PBS) by centrifuging them at 300× g for 10 min, and the dried pellet was stored at −80 °C.

2.4. Cell Fractionation

Nuclear and cytoplasmic fractions of PBMCs from MS patients and healthy controls were prepared using the Nuclear Extract Kit (Active Motif, Active Motif, La Hulpe, Belgium) following the manufacturer's instructions, and stored at −80 °C for subsequent Western blot analysis of VDR protein.

2.5. Protein Expression Analysis

To evaluate the total VDR protein expression, PBMCs isolated from peripheral blood of MS patients and healthy controls were used. The dried pellets were homogenized in a buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM 2-mercaptoethanol and 0.32 mM sucrose, with the addition of a protease inhibitor cocktail at the dilution suggested by the manufacturer (Roche, Basel, Switzerland) by using a Teflon/glass homogenizer and sonicating twice for 10 s.

The protein content of each sample of nuclear, cytoplasmic and whole-cell fractions was measured via Bradford's method using bovine serum albumin (BSA; Sigma Aldrich, Munich, Germany) as standard.

Proteins were diluted in a protein gel loading solution 2× containing 0.125 M Tris HCl (pH 6.8), 20% glycerol, 4% SDS, 0.05% bromophenol blue, 10% mM 2-mercaptoethanol and boiled for 5 min at 95 °C to denature the proteins and subsequently separated by 12% SDS-polyacrylamide gel electrophoresis and processed as previously described [20].

The mouse monoclonal anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:750 and the mouse monoclonal antibody anti- α -tubulin (Sigma-Aldrich, Munich, Germany; T9026) was diluted at 1:1000 in TBST buffer [10 mM Tris-HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5] containing 6% (v/v) milk. The nitrocellulose membrane signals were detected by chemiluminescence; α -tubulin was used to normalize the data. The analyses were performed on the densitometric values obtained using ImageJ, an NIH software package, after image acquisition.

2.6. Evaluation of Vitamin D Plasma Levels

The plasma was collected when PBMCs were separated with Ficoll and centrifuged at 2500× g for 10 min before storing it at −20 °C. Vitamin D plasma levels were evaluated by using the Elecsys® Vitamin D total assay on the cobas e601 immunoanalyzer (Roche, Basel, Switzerland), following manufacturers' instructions.

2.7. Statistical Analysis

Genotypic and allele frequencies of the VDR rs731236 (TaqI T/C) and VDR rs4334089 (HpyCH4V G/A) polymorphisms were calculated in MS patients and healthy controls. Allelic frequencies in controls were examined to detect any significant deviation from Hardy-Weinberg Equilibrium using a goodness of fit χ^2 test.

An unconditional logistic regression analysis, adjusted by sex and age, was performed to assess the association between the analyzed polymorphisms and MS; adjusted odds ratios (OR) with 95% confidence intervals (95% CI) were derived and used as measures of effect. For the VDR rs731236 polymorphism, a genotypic model was fitted to estimate

the heterozygous TC versus wild-type TT risk and the homozygous CC versus wild-type TT genotype risk. For the *VDR* rs4334089 polymorphism, a genotypic model was fitted to estimate the heterozygous GA versus wild-type GG risk and the homozygous AA versus wild-type GG genotype risk. For both polymorphisms, dominant and recessive genetic models were tested and selected based of statistical significance. A dominant model was applied considering at least one minor allele: C allele for *VDR* rs731236 and A allele for *VDR* rs4334089.

For analyzing the total, cytoplasmic and nucleic *VDR* protein expression, the protein levels in both the group of MS patients and in that of healthy controls were checked for normality by using the Shapiro–Wilk test. Since the values do not always meet these assumptions, the Mann–Whitney U test was used to check whether the total, cytoplasmic and nucleic *VDR* protein expression differed between MS patients and healthy controls. The Mann–Whitney test was also used to check for differences in the total *VDR* protein levels between patients with a mild form of MS (MSSS < 3) and subjects with a moderate to severe form (MSSS \geq 3). The Multiple Sclerosis Severity Score corrects the Expanded Disability Status Scale (EDSS) for the disease duration by using an arithmetically simple method to compare an individual’s disability with the distribution of scores in cases having equivalent disease duration [21].

In order to check for a possible effect of the polymorphisms on the total *VDR* protein expression in MS patients, the total *VDR* protein samples were subdivided into three groups according to their *VDR* rs731236 genotype and the protein levels were analyzed applying the Kruskal–Wallis test.

The vitamin D levels were evaluated in a subgroup of MS patients and the levels between patients with a MSSS < 3 and subjects with MSSS \geq 3 were compared by using the Mann–Whitney test. Furthermore, a linear regression model was used to test whether plasma vitamin D levels affect total *VDR* protein expression; sex, age and MS severity were included as covariates.

Statistical analyses were performed using Plink 1.07 (Purcell et al., 2007) and Stata 17 statistical software (Stata Corporation, College Station, TX, USA).

3. Results

3.1. *VDR* rs731236 (TaqI T/C) and *VDR* rs433408 (HpyCH4V G/A) Polymorphisms

A total of 105 MS patients and 282 controls were analyzed. MS patients were older compared to healthy controls ($p < 0.0001$), whereas the MS female-to-male ratio was not significantly different from that of healthy controls ($p = 0.129$) (Table 1).

In order to assess the possible association between the two polymorphisms and MS, the statistical analyses were adjusted by age and gender as possible MS risk confounders. The *VDR* rs731236 gene polymorphism significantly associated with MS risk. Based on a dominant model (the most statistically significant genetic model tested), the presence of at least a C allele raised the risk of MS of almost twofold (OR = 1.93, $p < 0.017$).

On the other hand, the genetic models for the *VDR* rs433408 polymorphism do not show any statistically significant difference between MS patients and healthy controls ($p > 0.05$) (Table 3).

Table 3. *VDR* rs731236 (TaqI T/C) and *VDR* rs433408 (HpyCH4V G/A) genotypic and dominant association with MS using logistic models corrected by age and gender.

Genotype/Allele	MS Patients <i>n</i> = 105	Healthy Controls <i>n</i> = 282	Adjusted OR (95% CI)	<i>p</i> -Value
<i>VDR</i> rs731236 (TaqI T/C)				
TT	32 (30.5%)	121 (42%)	1	
TC	53 (50.5%)	130 (46%)	1.75 (0.99–3.08)	0.053
CC	20 (19.0%)	31 (11%)	2.61 (1.20–5.64)	0.015

Table 3. Cont.

Genotype/Alele	MS Patients <i>n</i> = 105	Healthy Controls <i>n</i> = 282	Adjusted OR (95% CI)	<i>p</i> -Value
TT	32 (30%)	121 (43%)	1	
CC or TC	73 (70%)	161 (57%)	1.93 (1.13–3.29)	0.017
VDR rs433408 (HpyCH4V G/A)				
GG	50 (48%)	158 (56%)	1	
AG	41 (39%)	98 (35%)	1.24 (0.72–2.12)	0.446
AA	14 (13%)	26 (9%)	1.82 (0.80–4.16)	0.156
GG	50 (48%)	158 (56%)	1	
AA or AG	55 (52%)	124 (44%)	1.34 (0.81–2.22)	0.247

3.2. Vitamin D Receptor Protein Expression

3.2.1. Total VDR Protein Expression

To evaluate the total VDR protein expression, protein quantification was performed using Western blot on PMBCs from 100 MS patients and 54 healthy controls, randomly selected (Figure 1A,B).

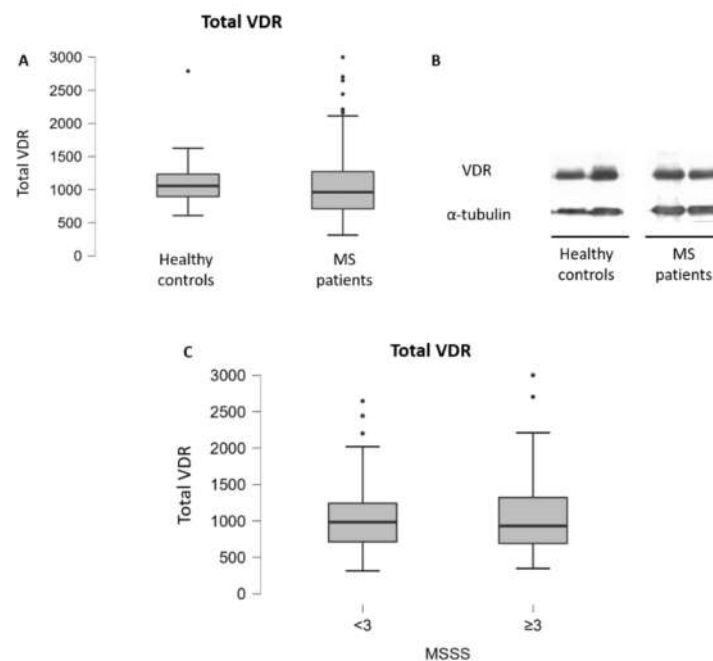


Figure 1. (A) Total VDR protein expression. MS patients and healthy controls; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significant difference between MS patients and healthy controls was observed (Mann–Whitney test, $p > 0.05$). (B) Representative Western blot images of total VDR and α -tubulin protein content in PMBCs from MS patients and controls. α -Tubulin was used to normalize the data. (C) Total VDR protein expression in patients with a mild form (MSSS score < 3) and patients with moderate to severe forms (MSSS ≥ 3) of MS; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significant differences were observed in total protein levels (Mann–Whitney test, $p > 0.05$).

Total VDR protein expression did not significantly vary between MS patients and healthy controls; MS patients had a median VDR protein expression of 963 (711–1272) arbitrary units (A.U.), and healthy controls showed a median VDR protein expression of 1057 (895–1234) A.U. ($p > 0.05$) (Figure 1A).

The total *VDR* protein expression was also analyzed in the group of MS patients in order to evaluate the possible expression difference between patients with a mild form (MSSS score < 3; 58% of patients) and patients with moderate to severe forms (MSSS \geq 3; 42% of patients) of MS and no differences were found ($p > 0.05$) (Figure 1C).

In addition, the total *VDR* protein expression was analyzed according to the *VDR* polymorphisms. However, no difference in the total *VDR* expression was observed, neither according to the *VDR* rs731236 genotype nor the *VDR* rs433408 genotype ($p > 0.05$) (Figure 2).

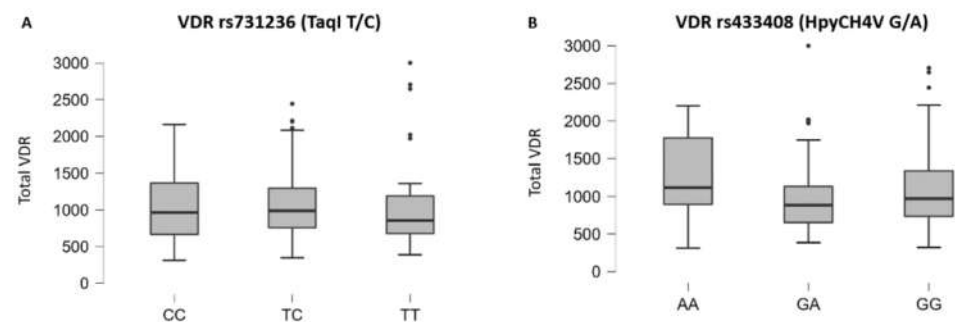


Figure 2. Total *VDR* protein expression according to (A) *VDR* rs731236 (TaqI T/C) and (B) *VDR* rs433408 (HpyCH4V G/A) polymorphisms; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significant differences are observed (Kruskal–Wallis test, $p > 0.05$).

3.2.2. Cytoplasmic and Nuclear *VDR* Expression

To test the activation of *VDR* and its nuclear translocation [22], PBMCs from a subgroup of 63 MS patients and 33 healthy controls randomly selected were used; the cytoplasmic and nuclear fractions were separated, and Western blot was performed to assess *VDR* protein expression levels in the cytoplasm and in the nucleus (Figure 3C).

No statistically significant differences between MS patients and healthy controls were observed both for the *VDR* expression in the cytoplasm and in the nucleus. PBMCs from MS patients showed a median cytoplasmic protein expression of 947 (702–1213) A.U., whereas PBMCs from healthy donors had a median cytoplasmic protein expression of 930 (754–1275) A.U. ($p > 0.05$) (Figure 3A). Regarding the *VDR* protein levels in the nucleus, PBMCs from MS patients had a median protein expression of 1124 (851–1441) A.U. and those from healthy subjects showed a median protein expression of 1046 (930–1421) A.U. ($p > 0.05$) (Figure 3B).

Additionally, in this case, both the cytoplasmic and the nucleic *VDR* protein expression were analyzed in the group of MS patients by comparing patients with a mild form (MSSS score < 3; 62% of patients) and patients with moderate to severe forms (MSSS \geq 3; 38% of patients) of MS, but no differences were found ($p > 0.05$) (Figure 3D,E).

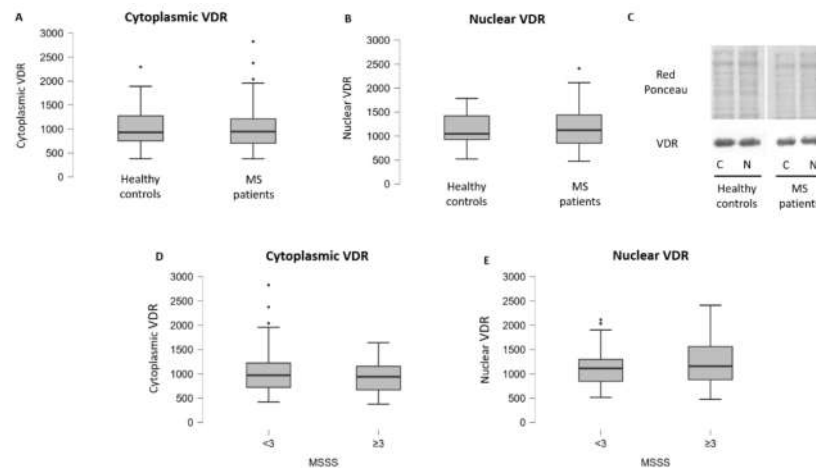


Figure 3. (A) Cytoplasmic and (B) nuclear VDR protein expression in MS patients and healthy controls; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significantly difference between MS patients and healthy controls was observed (Mann–Whitney test, $p > 0.05$). (C) Representative Western blot images of VDR protein content in PBMCs from MS patients and controls subdivided in cytoplasmic (C) and nuclear (N) fractions. Red ponceau was used to normalize the data. (D) Cytoplasmic and (E) nuclear VDR protein expression in patients with a mild form (MSSS score < 3) and with moderate to severe forms (MSSS ≥ 3) of MS; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significant differences were observed in total, cytoplasmic and nuclear protein levels (Mann–Whitney test, $p > 0.05$).

3.3. Vitamin D Levels

Given the importance of vitamin D levels in the susceptibility to MS, the plasma levels of 25(OH)D₃ in a subgroup of 73 MS patients, randomly selected, were determined. The mean age of this subgroup was 46.0 (95% CI: 36.0–53.0), and the MSSS was 2.8 (95% CI: 1.1–5.4). MS patients showed mean 25(OH)D₃ levels of 22.51 ± 13.71 ng/ mL, and subjects with moderate to severe forms of the disease (MSSS ≥ 3 ; 44% of patients) had significantly lower 25(OH)D₃ levels compared to patients with a mild form of MS (MSSS score < 3 ; 57% of patients) ($p = 0.048$) (Figure 4).

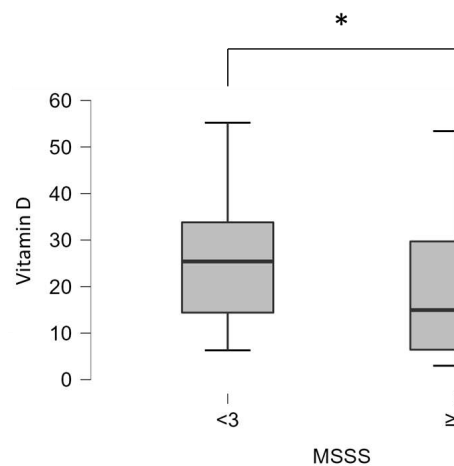


Figure 4. 25(OH)D₃ levels in patients with a mild form (MSSS score < 3 ; 41 subjects) and patients with moderate to severe forms (MSSS ≥ 3 ; 32 subjects) of MS; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). The two groups have significantly different 25(OH)D₃ levels (Mann–Whitney test, * $p = 0.048$).

Since it is known that 25(OH)D₃ can induce *VDR* expression [23], the total *VDR* protein expression levels and the 25(OH)D₃ plasma levels were evaluated to analyze a possible relation. However, the linear regression model, also including the clinical parameters, did not show any significant correlation between 25(OH)D₃ plasma levels and the total *VDR* protein expression ($p > 0.05$) (Table 4).

Table 4. Total, cytoplasmic and nucleic *VDR* protein expression levels related to 25(OH)D₃ plasma levels in MS patients. β -Coefficient and standard error of analyzed factors are derived from a linear regression model. $p < 0.05$ is considered statistically significant.

	β Coefficient	Standard Error	p -Value
Total <i>VDR</i>			
25(OH)D ₃	−9.24	5.31	0.09
Sex (male vs. female)	−14.20	144.38	0.92
Age	2.97	6.98	0.67
MSSS	−3.77	32.95	0.91
Cytoplasmic <i>VDR</i>			
25(OH)D ₃	6.52	5.46	0.24
Sex (male vs. female)	−185.83	146.59	0.21
Age	−7.60	8.52	0.38
MSSS	−5.96	36.92	0.87
Nuclear <i>VDR</i>			
25(OH)D ₃	5.49	5.19	0.30
Sex (male vs. female)	189.44	144.69	0.197
Age	4.46	8.25	0.59
MSSS	31.20	36.04	0.39

4. Discussion

Low levels of vitamin D constitute an important environmental risk factor for developing MS. Indeed, vitamin D is essential for correct regulation of the immune system, since it controls both innate and adaptive immune responses [24]. The biological actions of vitamin D are mediated by *VDR*. The interaction between 1,25(OH)₂D₃ and the receptor induces the heterodimerization with RXR and the translocation of the complex into the nucleus [25]. Here, *VDR* binds to specific DNA sequences, called VDRE, regulating the transcription of target genes [26]. Polymorphisms in the *VDR* gene might influence MS susceptibility, thus altering the action of vitamin D on immune cells. Namely, not only inadequate vitamin D intake but also impaired vitamin D signaling may contribute to the onset and progression of the disease [27]. Given the importance of the interaction between environmental and genetic factors, our attention was focused on *VDR*, which mediates vitamin D effects. Among MS risk-associated gene variants, some include alleles codifying molecules involved in the vitamin D pathway. The *VDR* gene is polymorphic, and several studies have analyzed the role of different *VDR* gene SNPs in MS, although the results are contradictory [28–30].

In our analysis, two SNPs of the *VDR* gene were considered: rs731236 (TaqI T/C) and rs433408 (HpyCH4V G/A). The *VDR* rs731236 is significantly associated with MS risk. On the other hand, MS patients and healthy controls did not show any significant difference either in allelic or genotypic frequencies for the *VDR* rs433408 polymorphism.

The association between the *VDR* rs731236 polymorphism and MS is supported by some other studies [31,32], while others did not find any association between it and MS [33,34]. Additionally, the conflicting results found by different meta-analyses highlight the difficulty in determining a conclusive indication of the involvement of the *VDR* rs731236

gene polymorphism in MS risk [35,36]. Of note, a possible reason for these contrasting results could be related to the population considered, with genetic differences between the studied groups.

To investigate whether altered expression of *VDR* could be associated with MS risk, the total *VDR* protein expression was analyzed. *VDR* protein levels do not change between MS patients and healthy controls, in our sample. The bioactive $1,25(\text{OH})_2\text{D}_3$ exerts its effects thanks to the association with *VDR*; in the absence of $1,25(\text{OH})_2\text{D}_3$, *VDR* shuttles between the nucleus and the cytoplasm. The binding with $1,25(\text{OH})_2\text{D}_3$ stabilizes the *VDR/RXR* heterodimer, which moves to the nucleus, where it binds to VDREs [37]. Considering such a central role for the bioactive molecule, we wondered whether a variation in the levels of *VDR* protein in the nucleus or in the cytoplasm could affect its availability for the ligand $1,25(\text{OH})_2\text{D}_3$, thus influencing indirectly the immunomodulatory effect of vitamin D. For this reason, we also analyzed the expression of *VDR* in the cytoplasm and in the nucleus. No statistically significant differences were obtained between MS patients and healthy controls, either in the cytoplasm or in the nucleus. In addition, the total, cytoplasmic and nucleic *VDR* levels do not appear to be associated with MS severity.

Since we found an association between the *VDR* rs731236 polymorphism and MS risk, the total *VDR* protein levels were analyzed in accordance to its genotype. It is important to take into account that the *VDR* rs731236 polymorphism, located on exon 9, triggers T to C transition that is a synonymous change; the amino acid does not change, and the protein structure is therefore not altered [38]. However, this polymorphism is known to be in linkage disequilibrium (LD) with other *VDR* polymorphisms and the LD extends into the 3'UTR regulatory region, thus probably being involved in the regulation of *VDR* expression [39]. However, our analysis does not show any significant difference in the total *VDR* protein levels in MS patients with a different genotype of the *VDR* rs731236 polymorphism. Consistent with our results, a 2011 study reported no changes in *VDR* protein expression in PBMCs from MS patients according to the *VDR* rs731236 genotype [40]. The authors described an alteration in *VDR* protein expression in PBMCs only when stimulated with the myelin basic protein: the cells carrying the TT or CT genotypes have significantly higher *VDR* protein levels compared to CC genotype cells. More studies, in an enlarged group of patients, are needed to better understand the impact of the *VDR* rs731236 polymorphism in MS patients with different severity levels of the disease and to obtain insight into the putative association between this polymorphism and a specific form of the disease.

Regarding the *VDR* rs4334089 polymorphism, no studies correlate this polymorphism to MS so far. The *VDR* rs4334089 polymorphism is located in the 5' UTR and it does not affect the transcript. To our knowledge, our study is indeed the first one that analyzes the *VDR* rs4334089 polymorphism in MS pathogenesis. The *VDR* rs4334089 polymorphism has been studied in Parkinson disease (PD) [41,42]. Moreover, our analysis does not suggest an impact of this polymorphism on the total *VDR* protein levels. For now, our data, together with the other one present in the literature, suggest that the *VDR* rs4334089 polymorphism has no direct association with the development of neurodegenerative diseases, such as MS and PD, though further and larger investigations are required to confirm this concept.

Finally, given the important role of low levels of vitamin D for MS risk and the potential of vitamin D supplementation in MS therapy, the vitamin D levels of a subgroup of MS patients were analyzed.

Our patients have a mean in $25(\text{OH})\text{D}_3$ plasma levels of 22.51 ± 13.71 ng/mL. This is consistent with the fact that low $25(\text{OH})\text{D}_3$ levels, around 20 ng/mL, are usually observed in MS patients already at the beginning of the disease, such as in clinically isolated syndrome or during the first relapses in relapsing-remitting MS patients [43,44]. In addition, our results suggest that patients with moderate to severe forms of MS have lower vitamin D levels compared to patients with a mild form of disease, in agreement with other studies relating higher disability (EDSS) and lower vitamin D levels [45,46]. In our study the MSSS were considered instead of the more commonly used EDSS, since MSSS is a more

powerful parameter for detecting different rates of disease progression. In fact, it should be considered that the same level of neurological disability can be seen both in aggressive forms of MS, in which disability develops within months of clinical onset, and in relatively mild forms, in which disability develops after decades of the disease. By correcting the EDSS for disease duration, the MSSS allows to compare disability in patients with comparable disease durations [21].

It is known that, in some cell types, *VDR* expression is modulated by the presence of its own ligand $1,25(\text{OH})_2\text{D}_3$. The typical response to $1,25(\text{OH})_2\text{D}_3$ is the upregulation of *VDR* expression. This can be due to the ability of $1,25(\text{OH})_2\text{D}_3$ to induce *VDR* gene transcription, thanks to the presence of VDREs within the *VDR* gene, or to the fact that *VDR* can stabilize itself [23,47,48]. However, our analysis did not find any correlation between vitamin D levels and *VDR* protein levels. It is important to consider that a reduction in *VDR* mRNA levels has also been described in MS patients treated with vitamin D supplementation for 2 months [49]. *VDR* regulation is complicated and *VDR* expression is regulated, at the transcriptional level, in a tissue-specific manner by several other molecules, including calcium, hormones (such as parathyroid hormone) and all-trans retinoic acid [50].

Based on our results, the *VDR* rs731236 (TaqI T/C) polymorphism is related to MS risk in the Italian population, but it does not seem to impact the total *VDR* protein levels. *VDR* protein levels and its location between cytoplasm and nucleus seem not to be associated either with MS risk or with MS severity. However, it is essential to understand the impact of this gene polymorphism on *VDR* expression both in basal and in stimulated conditions to better understand its role within the complexity of MS. In addition, *VDR* protein levels do not seem to correlate with vitamin D levels, which are lower in patients with a more severe form of the disease. Further studies are necessary to better disclose the involvement of *VDR* in MS pathogenesis given the important role of low vitamin D levels in MS risk and the potential of vitamin D supplementation in MS therapy [51].

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