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S1PR1 genetic variants may modulate disability outcomes and therapy response in relapsing-remitting multiple sclerosis

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Abstract: Sphingosine-1-phosphate receptor 1 (S1PR1) modulators are an effective therapy for patients with relapsing-remitting multiple sclerosis (RRMS), although considerable inter-individual variability in treatment response remains. This study aimed to investigate genetic variability within the coding regions of S1PR1 and to assess its potential association with treatment response and clinical disability measures, including the Expanded Disability Status Scale (EDSS) and Multiple Sclerosis Severity Score (MSSS), in patients with RRMS. Thirty-one RRMS patients from Serbia underwent sequencing of the 5′ regulatory region and coding exons of S1PR1 using an Applied Biosystems 3130 Genetic Analyzer. Three polymorphic variants were identified. None of the analyzed variants demonstrated a significant association with treatment response. Despite the small sample size, rs41287280 showed a significant association with disability measures; in a dominant inheritance model, carriers of the G allele had lower EDSS and MSSS values (P=0.01 and P=0.006, respectively) compared with the CC genotype. In multivariable analyses, the rs41287280 G allele was associated with lower disability, whereas the rs3737577 T allele was associated with higher disability compared to the wild-type GG genotype after adjustment. These results provide preliminary evidence suggesting that S1PR1 genetic variation may be related to disease pathogenesis in RRMS, warranting further confirmation in larger, independent cohorts and, potentially, functional investigation.

Keywords: *S1PR1*, modulating therapy, multiple sclerosis, therapy response

Abbreviations: MS – multiple sclerosis; RRMS – relapsing-remitting multiple sclerosis; S1P – sphingosine-1-phosphate; S1PR1 – sphingosine-1-phosphate receptor 1; EDSS – expanded disability status scale; MSSS – multiple sclerosis severity score; DMT – disease-modifying therapy; SNV – single nucleotide variant; CNS – central nervous system; ENCODE – Encyclopedia of DNA Elements; HWE – Hardy-Weinberg equilibrium; CAD – coronary artery disease; GWAS – genome-wide association study; MAF – minor allele frequency; MAPK – mitogen-activated protein kinase; eQTL – expression quantitative trait locus

INTRODUCTION

Multiple sclerosis (MS) is a complex progressive chronic inflammatory neurological disorder of the central nervous system (CNS) in which the adaptive immune system activity causes axonal demyelination, lesions in the CNS tissue, and, consequently, neurological disability [1]. MS is the leading cause of nontraumatic neurological disability in young adults and affects approximately 2.8 million individuals worldwide [2].

The *sphingosine-1-phosphate receptor 1* (*S1PR1*) gene encodes a G protein-coupled receptor that binds the signaling lipid sphingosine-1-phosphate (S1P). S1P modulators are an effective disease-modifying therapy (DMT) for relapsing-remitting MS (RRMS) [3,4]. The primary mechanism of action of S1PR modulators in MS involves binding to S1PR1 receptors on lymphocytes. Signaling via S1PR1 is essential for the ability of lymphocytes to migrate from lymphoid organs to

peripheral tissues [5,6], including the CNS [7,8]. S1P modulators bind to and negatively modulate S1PR1 by causing intracellular receptor sequestration and receptor degradation [9,10], leading to a downstream suppression of S1PR1-induced signaling [10,11]. Despite the advancements in the discovery of novel therapies, the effectiveness of DMTs is still limited [12-14] and heterogeneous [12,14,15]. Second-generation S1P receptor modulators were developed to enhance functional selectivity toward S1PR1 while minimizing activity at other S1P receptor subtypes, thereby reducing off-target effects unrelated to immune cell trafficking [16]. Approved therapeutics target some of the other S1PR subtypes (2-4), but the clinically relevant functions of these receptor subtypes are less well established. According to a recent computational study, numerous *S1PR1* genetic variants may affect the binding kinetics between therapeutic agents and S1PR1 [17,18]. In addition, genetic variants also have the potential to alter receptor expression or internalization kinetics, ultimately influencing treatment efficacy [17].

There are currently no validated genetic markers to guide and optimize DMT selection in MS, limiting the implementation of more personalized and effective therapeutic strategies in clinical practice [19,20]. Given the important role of S1PR1 modulation in MS treatment, particularly with second-generation therapeutics, understanding patient-specific genetic profiles may offer insights into differential treatment outcomes. The 5′ region of the gene, encompassing the first exon and part of the second exon, is of particular importance because it encodes the receptor N-terminal helix, which is involved in ligand recognition and binding [21]. The ENCODE Project Consortium identified a substantial number of candidate cis-regulatory elements located at the 5′ end of the *S1PR1* gene, highlighting the regulatory importance of this region [22].

In this study, we aimed to perform targeted sequencing of the *S1PR1* gene to investigate genetic variability that may influence response to therapy in a follow-up group of RRMS patients from Serbia. Furthermore, we investigated the association of the described *S1PR1* genetic variants with clinical parameters of MS progression.

MATERIALS AND METHODS

Ethics statement

All procedures were performed in compliance with approvals of the Ethics Committee of the Military Medical Academy of 25/02/2010 and 04/08/2020 (ref. No. 6/2020), and consent of the Ethics Committee of "Vinča" Institute of Nuclear Sciences of 05/10/2020 (ref. No. 116-28- 5/2020-000). All procedures were in accordance with the Declaration of Helsinki Ethical Guidelines. Privacy rights of the study participants were observed, and informed consent was obtained before recruitment.

Study group

MS was diagnosed according to the revised McDonald criteria [23,24]. The clinical course of the disease was defined by trained clinicians [25]. All participants were recruited from the Clinic for Neurology at the Military Medical Academy (MMA), where they were followed for 5 years. All participants were unrelated, of Serbian origin, and diagnosed with the RRMS form of the disease. In this study, we used clinical data, including disease duration, Expanded Disability Status Scale (EDSS) score, and Multiple Sclerosis Severity Score (MSSS), measured at the time of sampling. EDSS [26] and MSSS [27] were used for disease severity estimation. Samples were collected during the remission phase of RRMS. All patients received clinically approved S1PR-modulating therapies for at least 12 months, one of which, ponesimod, targeted S1PR1 (19 patients), while the other, ozanimod, targeted both S1PR1 and S1PR5 (12 patients). None of the patients received fingolimod therapy. Responders were classified based on evidence of slowed disability progression, with minimal deterioration from baseline EDSS scores. Non-responders were defined as patients who experienced a clinically confirmed relapse associated with an increase in EDSS, sustained 24 weeks after relapse onset, as well as patients with disability progression, defined by an increase in MSSS accompanied by an EDSS increase of $\geq$1 point confirmed after 24 weeks. Patients with autoimmune disorders, chronic inflammatory diseases, severe comorbidities (e.g., malignancies, severe metabolic diseases, chronic systemic diseases), and patients on steroid medications were not included in the study.

DNA extraction

Peripheral blood samples (3 ml) were obtained from all participants using commercial EDTA-containing tubes. Genomic DNA was extracted using the Zymo Research Quick-DNA™ Miniprep Plus Kit, following the manufacturer's instructions (Zymo Research, Irvine, California, USA). DNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

Sequencing analysis

Targeted sequencing of the *S1PR1* gene was performed by PCR reaction and Sanger sequencing. The study focused on a portion of the promoter region, exon 1, the first intron, and part of exon 2 of the S1PR1 gene located on chromosome 1 (sequence range 101,238,814-101,239,888; NC_000001.11, GRCh38.p14, GCF_000001405.40 assembly). Two targeted sequencing reactions were performed. The first amplicon covered exon 1, the adjacent intron, and part of exon 2, spanning 489 nucleotides including primers (sequence range 101,238,814-101,239,303). A 36-nucleotide region between the two amplicons was not analyzed.

The primers (Supplementary Table S1) were designed to amplify selected regions of the gene based on the human genome reference sequence (GRCh38.p14), using Primer-BLAST (Primer design tool – NCBI; www.ncbi.nlm.nih.gov/tools/primer-blast). PCR amplification was carried out in a total volume of 25 μL containing 100 ng DNA template, 0.5 U of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA), 10 pmol of each primer, 0.2 mM of each dNTP, and 2.0 mM $MgCl_2$. The PCR conditions were as follows: denaturation at 95°C for 7 min, followed by 33 cycles of denaturation at 95°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min. The amplified product was electrophoresed using a 2% agarose gel stained with ethidium bromide and visualized via UV transillumination.

Purification of PCR products was performed using the QIAquick PCR Purification Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Purified PCR products were sequenced by Macrogen Europe Inc., using the forward primer (Amsterdam, Netherlands). Sequencing chromatograms and corresponding nucleotide sequences were provided by the company. Electropherograms were visually inspected using Sequence Analysis version 5.2 (Applied Biosystems, Foster City, California, USA). Obtained sequences were aligned against the reference sequence, GenBank accession no. NG_016181.1, using the BLAST tool. Raw electropherogram data for both targeted sequences are presented in the publicly available data set as Raw data S2 (exon 1, the adjacent intron, and part of exon 2, 489 bp) and Raw data S3 (subsequent portion of exon 2, 548bp) available at: https://hdl.handle.net/21.15107/rcub_vinar_16340.

Statistical analysis

Statistical analyses were conducted using Statistica v14.2.0 software (Spotfire Statistica Desktop, StatSoft GmbH, Hamburg, Germany). Genotype and allele frequencies were calculated for each genetic variant. Categorical variables were compared across groups using Pearson's chi-square (χ^2) test, which also served to assess departures from Hardy-Weinberg equilibrium (HWE). For continuous variables, normality of distribution was evaluated using the Shapiro-Wilk test. Based on these results, parametric tests (Student's t-test or ANOVA) were used when assumptions were met; otherwise, non-parametric alternatives (Mann-Whitney U-test or Kruskal-Wallis test) were applied. Associations between S1PR1 genetic variants and clinical outcomes (EDSS and MSSS) were initially assessed using univariate analyses. Subsequently, multivariable linear regression models were applied to evaluate the independent effects of treatment response, genetic variants, and disease duration on disability measures, including EDSS and MSSS. Disease duration was included as a covariate in EDSS models but not in MSSS analyses, as MSSS is inherently adjusted for disease duration. Regardless of distributional characteristics, continuous variables were reported as the mean±standard deviation (SD). Statistical significance was defined as $P<0.05$.

RESULTS

Study groups

A total of 31 patients diagnosed with RRMS were enrolled in the study. Participants were stratified according to their clinical response to DMT, with 58.1%

Table 1. Study group characteristics

Variable	RRMS patients n=31	Responders* n=18 (58.1%)	Non-responders* n=13 (41.9%)	P
Age (years)	42.8±9.2	41.6±10.7	44.5±6.7	0.489[b]
Sex				
Male, n (%)	16 (51.6)	9 (50)	7 (53,8)	0.83[a]
Female, n (%)	15 (48.4)	9 (50)	6 (46,2)	
Smoking				
Yes, n (%)	6 (19.4)	3 (16.7)	3 (23.1)	0.655[a]
No, n (%)	25 (80.6)	15 (83.3)	10 (76.9)	
Disease duration (years)	9.52±5.71	7.44±4.41	12.38±6.21	**0.019**[b]
EDSS	3.03±1.80	1.97±0.99	4.5±1.63	**3x10^{-5}**[b]
MSSS	4.06±2.51	3.13±2.19	5.35±2.42	**0.022**[b]

All tests used for variable analysis between study groups: [a] Pearson's chi-square; [b]Mann-Whitney U test; RRMS – Relapsing-Remitting Multiple Sclerosis; *response to S1PR1 modulator therapies; EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; n – number. Statistical significance was defined as P<0.05; values are presented as means ± standard deviation.

classified as responders and 41.9% as non-responders. Their characteristics are listed in Table 1**.** The study groups did not differ in terms of age, sex, smoking status, or S1P modulator type. Non-responders exhibited significantly longer disease duration, higher EDSS, and elevated MSSS compared to responders (Table 1). These findings suggest that non-responders exhibit more advanced and aggressive disease phenotypes, which may contribute to reduced therapeutic efficacy of the evaluated treatments.

S1PR1 genetic variants detection and association with therapy response to S1PR1 modulators

Three polymorphic variants were identified in S1PR1, including two 5′ UTR variants (rs3737577 and rs3737578) and one missense variant (rs41287280; p.Gly13Arg). Basic variant characteristics are presented in Table 2. Sequencing chromatograms illustrating the detected genotypes are presented in Fig. 1. HWE was assessed in the overall cohort for all variants using an exact test, although testing was not feasible for rare variants with sparse genotype counts. Variant frequencies were consistent with external references. No statistically significant differences were observed in the distribution of the rs3737577, rs3737578, and rs41287280 variants between RRMS patients classified according to DMT response. However, a trend toward association was observed for the genetic variant rs41287280 (P=0.068) (Table 3). No significant associations were observed between S1P modulator type and any of the analyzed SNPs or therapy response.

Impact of clinical and genetic factors on disability status (EDSS and MSSS) in RRMS patients

The *S1PR1* rs41287280 variant showed a statistically significant association with EDSS and MSSS. According to the dominant model, allele G carriers had lower EDSS and MSSS values compared with CC genotype carriers (Mann-Whitney U test, P_{adj}=0.02 and P_{adj}=0.008, respectively) (Table 4). In the multivariable linear regression model for EDSS, therapy response emerged as the strongest predictor (β = -0.614, P = 0.0002), confirming significantly lower disability scores among responders (Table 5). As therapy type, sex, and smoking status showed no significant associations with either treatment response or EDSS and MSSS in univariate analyses, these variables were excluded from the multivariable models to minimize the risk of overfitting. The final regression model assessed the independent effects of treatment responsiveness and the three SNVs on EDSS and MSSS. EDSS values were also independently associated with genetic variants rs3737577 (β=0.419, P=0.004) and rs41287280 (β= -0.420, P=0.006) (Table 5). As shown in Table 5, carriers of the rs3737577 T allele exhibited higher EDSS values (β=0.419, P=0.004) compared with individuals carrying the GG genotype,

Table 2. *S1PR1* variants characteristics

***S1PR1* variant ID**	**Consequence type**	**Effect**	**Location**	**MAF (EUR)**	**MAF***
rs3737577	5′ UTR variant	c. −9G>T	Chromosome 1:101238976	T: 0.34	T: 0.36
rs3737578	5′ UTR variant	c. −45T>C	Chromosome 1:101238940	C: 0.06	C: 0.09
rs41287280	missense variant	c.37C>G p.Gly13Arg	Chromosome 1:101239021	G: 0.02	G: 0.06

S1PR1 – Sphingosine-1-Phosphate Receptor 1; MAF – Minor Allele Frequency by 1000 Genomes Project for European population (EUR); Variants' characteristics extracted from the Ensembl genome database project, genome assembly: GRCh38.p14 (www.ensembl.org; Homo sapiens); MAF*– Minor Allele Frequency observed in the present study cohort of Serbian RRMS patients.

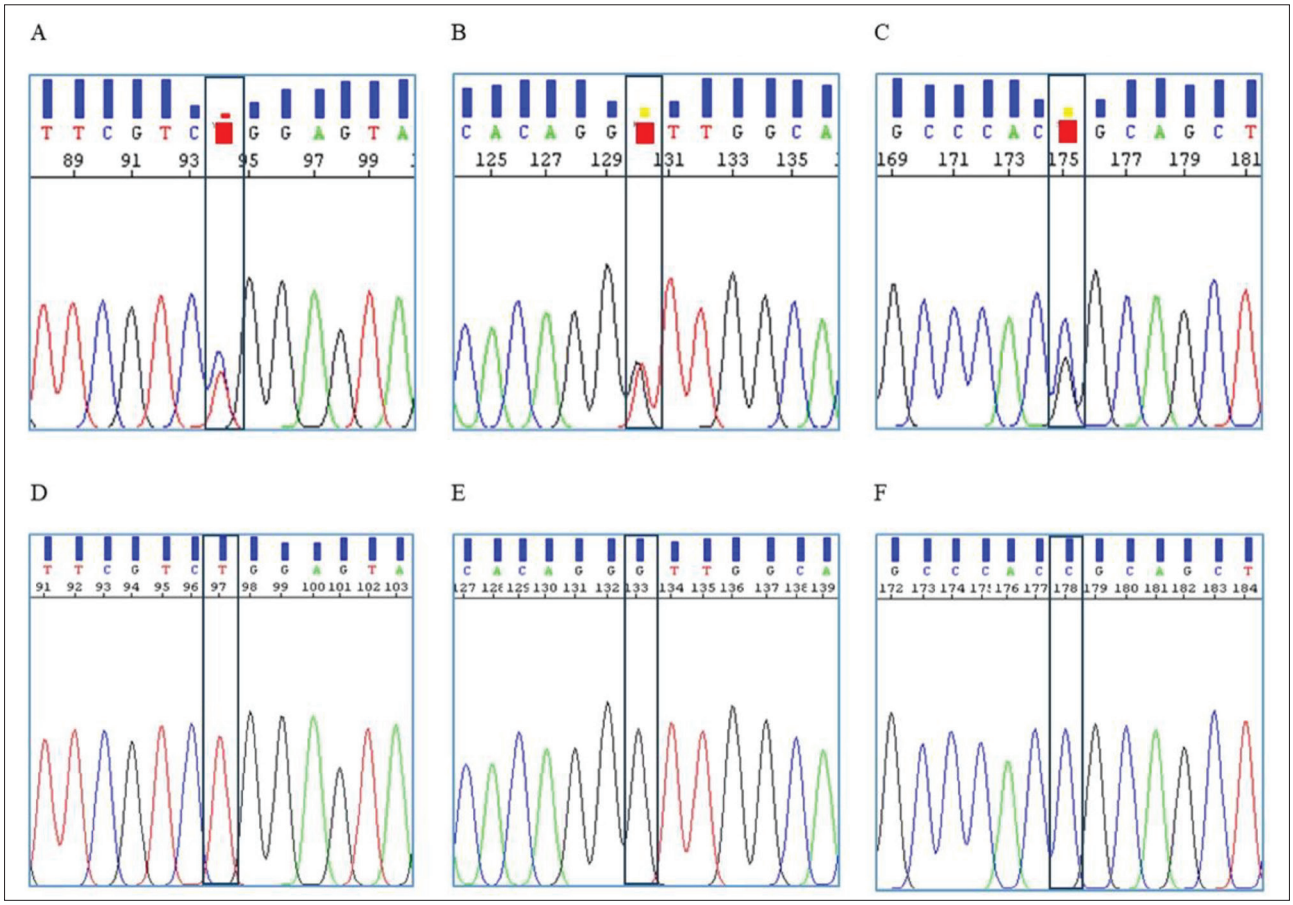

Fig 1. Sequencing chromatograms showing *S1PR1* genotypes. **A** – rs3737578 TC (Y). **B** – rs3737577 GT (K). **C** – rs41287280 CG (S). **D** – rs3737578 TT. **E** – rs3737577 GG. **F** – rs41287280 CC. *S1PR1 – sphingosine-1-phosphate receptor 1.*

Table 3. Genotype and allele frequencies of *S1PR1* variants according to therapy response in the study groups

Variant ID	Genotype	RRMS patients n (%)	Responders* n (%)	Non-responders* n (%)	P
rs3737577	GG	12 (38.7)	5 (27.8)	7 (53.8)	0.34
	GT	16 (51.6)	11 (61.1)	5 (38.5)	
	TT	3 (9.7)	2 (11.1)	1 (7.7)	
	allele G	0.64	0.58	0.73	0.23
	allele T	0.36	0.42	0.27	
rs3737578	TT	25 (80.6)	15 (83.3)	10 (76.9)	0.66
	TC	6 (19.4)	3 (16.7)	3 (23.1)	
	CC	0(0)	0 (0)	0 (0)	
	allele T	0.91	0.92	0.88	0.67
	allele C	0.09	0.08	0.12	
rs41287280	CC	27 (87.1)	14 (77.8)	13 (100)	0.068
	CG	4 (12.9)	4 (22.2)	0 (0)	
	GG	0 (0)	0 (0)	0 (0)	
	allele C	0.94	0.89	1.0	0.078
	allele G	0.06	0.11	0.0	

*Response to S1PR1 modulator therapies; S1PR1 – sphingosine-1-phosphate receptor 1; RRMS – Relapsing-Remitting Multiple Sclerosis; Differences in genotype and allele distribution between study groups were analyzed using Pearson's chi-square test; Statistical significance was defined as P<0.05.

indicating greater disability. In contrast, carriers of the rs41287280 G allele had lower EDSS scores (β = -0.420, P=0.006) than CC genotype carriers, suggesting a protective effect of the G allele. Although disease duration differed significantly between responders and non-responders at baseline, it was not an independent predictor of EDSS after adjustment for other variables in the regression model (β=0.112, P=0.410) presented in Table 5.

In the MSSS regression model (adjusted for therapy response and all three *S1PR1* variants), therapy response remained a significant negative predictor (β= -0.342, P=0.036), confirming that non-responders had higher MSSS scores (Table 6). Among the genetic variants, *S1PR1* rs3737577 (β=0.423, P=0.02) and rs41287280 (β=−0.569, P=0.003) were significantly

Table 4. EDSS and MSSS parameters according to the dominant model of *S1PR1* variants

Variant	Genotype (n)	EDSS		P	P_{adj}	MSSS		P	P_{adj}
		mean	SD			mean	SD		
rs3737577 G>T	GG (12)	2.87	1.3	0.98	0.05	3.91	2.08	0.98	0.03
	GT + TT (19)	3.13	2.08			4.16	2.79		
rs3737578 T>C	TT (25)	3.06	1.7	0.98	0.04	3.95	2.44	0.61	0.02
	TC+CC (6)	2.92	2.35			4.51	2.99		
						-	-		
rs41287280 C>G	CC (27)	3.31	1.72	0.01	**0.02**	4.5	2.36	0.001	**0.008**
	CG + GG (4)	1.12	1.03			1.06	0.86		

Mann-Whitney U test was used for variable analysis between study groups; EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; n – number; P_{adj} values after Benjamini-Hochberg correction for multiple testing; Statistical significance was defined as P<0.05; Values in the table are presented as means with standard deviation (SD).

Table 5. Multivariable linear regression analysis of factors associated with EDSS

Predictor	Beta (β)	SE β	95% CI	η^2_p	t	P
Disease duration	0.112	0.132	[−0.16, 0.38]	0.03	0.84	0.410
Therapy response	-0.614	0.139	[−0.90, −0.33]	0.44	−4.40	**$2x10^{-4}$**
rs3737577 G>T	0.419	0.134	[0.14, 0.70]	0.23	2.90	**0.004**
rs3737578 T>C	0.021	0.120	[−0.22, 0.23]	0.001	0.27	0.860
rs41287280 C>G	-0.420	0.141	[−0.71, −0.13]	0.26	−2.67	**0.006**

Therapy response – Response to S1PR1 modulator therapy; EDSS – Expanded Disability Status Scale; Beta (β) – the standardized regression coefficient, indicating the relative contribution of each predictor to EDSS after adjusting for other covariates; SE – standard error; CI – Confidence Interval; t – Beta (β) / St. err. β. η^2_p - effect sizes were estimated using partial eta squared, indicating the proportion of variance explained by each predictor after accounting for other variables in the model. Statistical significance was defined as P<0.05. The whole model was statistically significant: F(25,5)=10.27, P<0.0001, explaining 61% of EDSS variance (R^2=0.61).

Table 6. Multivariable linear regression analysis of factors associated with MSSS

Predictor	Beta (β)	SE β	95% CI	η^2_p	t	P
Therapy response	−0.342	0.155	[−0.66, −0.02]	0.15	−2.21	**0.036**
rs3737577 G>T	0.423	0.170	[0.07, 0.77]	0.18	2.50	**0.020**
rs3737578 T>C	0.162	0.150	[−0.14, 0.47]	0.05	1.08	0.290
rs41287280 C>G	−0.569	0.174	[−0.93, −0.21]	0.31	−3.27	**0.003**

Therapy response – Response to S1PR1 modulator therapy; MSSS – Multiple Sclerosis Severity Score; Beta (β) – the standardized regression coefficient, indicating the relative contribution of each predictor to MSSS after adjusting for other covariates.; SE – standard error; CI – Confidence Interval; t – Beta (β) / St. err. β. η^2_p - effect sizes were estimated using partial eta squared, indicating the proportion of variance explained by each predictor after accounting for other variables in the model. Statistical significance was defined as P < 0.05. The whole model was statistically significant: F(26,4)=5.36, P=0.003, explaining 37% of MSSS variance (R^2=0.37).

associated with MSSS, whereas rs3737578 did not show a significant association (P=0.29) (Table 6). The results indicate that carriers of the rs3737577 alternative T allele exhibited higher MSSS values compared with GG homozygotes, suggesting a more aggressive disease course relative to disease duration. In contrast, carriers of the rs41287280 alternative G allele showed lower MSSS scores than individuals with the wild-type CC genotype, indicating a potential protective effect of the G allele. These findings are consistent with the EDSS model, in which therapy response and the same two variants (rs3737577 and rs41287280) emerged as significant independent predictors of disability. The direction of effects was concordant between EDSS and MSSS, suggesting that these associations are robust and not dependent on disease duration, which is already normalized in the MSSS score. The S1P modulator type showed no significant association with EDSS or MSSS values.

DISCUSSION

In this study, we performed targeted sequencing of the 5′ region of the *S1PR1* gene to investigate genetic variability that might affect responsiveness to therapy

treatment and MS clinical characteristics. Given the central role of S1P receptor modulation in MS treatment, particularly with the emergence of second-generation S1P modulators, understanding patient-specific genetic profiles may help explain variability in treatment response and support the development of more individualized therapeutic strategies [12,28].

Patients were treated with modulating therapy that primarily targets S1PR1. The therapeutic approach included two pharmacological agents, with a shared mechanism of action characterized by functional antagonism of S1PR1. Although these agents differ in their pharmacokinetic properties and receptor selectivity, indirect comparative evidence suggests that the main distinction between ponesimod (targets S1PR1) and ozanimod (targets S1PR1 and S1PR5) may lie in greater preservation of brain volume and the more favorable safety profile observed with ozanimod [29]. No significant differences in the parameters investigated were observed according to therapy type. The study focused on exon 1, intron 1, and the initial portion of exon 2 of the S1PR1 gene. Two targeted sequencing reactions were performed. The first amplicon encompassed exon 1, the adjacent intron, and part of exon 2, covering a total of 489 nucleotides, while the second amplicon covered the remaining portion of exon 2, spanning 548 nucleotides. A 36-nucleotide segment between the two amplicons was not analyzed. Within the sequenced regions, 3 polymorphic variants were identified: rs3737577 (G/T), rs3737578 (T/C), and rs41287280 (C/G). Among these, rs41287280 showed a trend toward association with treatment responsiveness and demonstrated a significant association with EDSS and MSSS.

The missense variant rs41287280, located within the N-terminal α-helix of the S1PR1 protein, results in a glycine-to-arginine substitution at codon 13 (p.Gly13Arg, G13R) [17,30,31]. The N-terminal α-helix of S1PR1 forms a cap-like structure that regulates ligand entry, including S1P and fingolimod, thereby contributing to stabilization of the receptor conformation during activation [21]. However, this amino acid substitution does not appear to affect the response to S1P [17]. The CAD-associated *S1PR1* rs41287280 variant has been linked to disease severity, with the alternative G allele showing a protective effect against multi-vessel coronary obstruction [17]. Although Obinata et al. [17] associated this variant with complex traits, its potential relevance to MS has not previously been reported. A genome-wide association study (GWAS) of blood cell indices, encompassing 29.5 million genetic variants in more than 170,000 individuals of European ancestry, identified rs41287280 as being associated with differences in peripheral blood lymphocyte counts [32]. The precise functional consequences of rs41287280 remain unclear, and it is yet to be determined whether this variant directly affects the S1PR1 protein, modulates gene expression, or is linked to another causative variant.

The minor allele frequency (MAF) of rs41287280 is approximately 1.5% in European populations (Allele Frequency Aggregator, GnomAD_genomes), making it a rare variant [17]. When expressed in CHO-K1 cells, the G13R mutant receptor does not impair S1P-induced activation of the mitogen-activated protein kinase (MAPK) or Akt signaling pathways. These pathways were activated similarly to the wild-type receptor, indicating preserved function for this variant. Confocal microscopy revealed that G13R undergoes normal ligand-induced internalization, mirroring the behavior of the wild-type receptor. This contrasts with some other rare *S1PR1* variants (e.g., rs148977042 and rs146890331), which show defective endocytosis [17]. Nonetheless, the impact of the rs41287280 variant on mRNA stability and expression or receptor trafficking remains to be elucidated through *in vitro* assays or structural modeling.

In addition to the 3 polymorphic variants identified, this region also harbors several mutations, five of which (rs1223284736, rs1202284551, rs1209378712, rs201200746, and rs1461490142) are located in the regions sequenced in our study. The findings of Kores et al. [18] indicate that these five mutations occur at very low frequencies in the general population, likely explaining their absence in our small group of patients, which is the major limitation of the current study.

A previous study of Iranian RRMS patients treated with fingolimod did not identify associations between *S1PR1* variants (rs3737577 and rs3737578) and response to therapy. These investigations, like our study, were limited by a relatively small sample size of 94 subjects [33]. Although Moheghi et al. reported 9 SNVs, of which 4 are novel variants, none showed statistically significant associations with therapeutic response. Notably, that study did not include rs41287280 or evaluate its association with EDSS or MSSS. Our results

are generally consistent with their findings. However, differences in ethnic background, sample sizes, and the inclusion of additional genetic covariates should be considered when comparing outcomes. The trend of association between rs41287280 and treatment response invites further investigation. While we observed only a trend in association, the effect size suggests biological relevance, albeit requiring larger sample sizes to confirm significance.

In our study, the S1PR1 variant rs41287280 was independently associated with both EDSS and MSSS scores, suggesting that carriers of the CC genotype may experience greater disability. Notably, rs41287280 remained significantly associated with both disability measures after adjustment for therapy response and the other analyzed variants. The rs3737577 variant was also associated with EDSS and MSSS, although this association became evident only after adjustment for the other SNPs and potential confounding factors.

If replicated, these SNVs could be a useful component for better prediction of individual disease trajectories. Fingolimod therapy, a first-generation S1PR1-targeting treatment, has been shown to significantly improve EDSS scores compared with placebo [3]. In addition, therapy duration has been reported to influence EDSS outcomes [34]. The beneficial effects of S1PR modulators are well documented, and current understanding of their mechanisms of action continues to expand. S1PR1 is not exclusively expressed on lymphocytes but also found in endothelial cells, astrocytes, and neural cells, where it regulates immune cell trafficking, vascular integrity, and neuroprotection [16,35]. S1PR1 upregulation in human hypertrophic astrocytes has been noted in both active and inactive MS lesions [35]. S1P signaling, beyond its recognized functions in immune cell trafficking, governs several immunological processes, such as cell survival, cell fate determination, and innate immunity [36]. The S1P receptor modulators act primarily by inducing S1PR1 internalization into lymphocytes, thereby preventing their egress from lymph nodes and reducing neuroinflammation [16]. Preclinical models, including murine experimental autoimmune encephalomyelitis, have demonstrated that the therapeutic impact of fingolimod is S1PR1-dependent and linked to decreased infiltration of IL-17-producing T cells into the central nervous system [37]. Variants in the coding region may directly influence S1PR1 function or exist in linkage disequilibrium with variants affecting S1PR1 expression or signaling in the brain, thereby modulating local neuroinflammatory responses and contributing to disability progression.

Incorporating the investigated genetic variants into multivariable predictive models, together with age, disease duration, inflammatory markers, and lifestyle factors, may improve personalized risk stratification. However, these findings should be interpreted with caution, as the reported associations were derived from a single cohort and have not yet been independently replicated. Population-specific variation in MAFs may limit the generalizability of these results. Previously observed by Moheghi et al., MAFs for rs3737577 (~0.22) and rs3737578 (~0.15) were substantially higher [33] than those detected in our study group. Assessing population stratification and accounting for potential confounding clinical variables and genetic variants are critical for ensuring robust association analyses.

The main limitations of the current study include its sample size and cross-sectional design. Additionally, broader genomic approaches, including analysis of intronic regulatory regions, promoter variants, or whole-gene sequencing, could enhance the identification of linked variants or expression quantitative trait loci (eQTLs) that may influence receptor expression levels. The number of published studies investigating genetic variation in this receptor remains limited relative to its biological and clinical importance. Further research is required to clarify whether the identified variants influence therapeutic response and disease progression.

In this exploratory cohort, targeted analysis of *S1PR1* exons 1 and 2 identified 3 polymorphic genetic variants. The most prominent variant, rs41287280, was significantly associated with disease severity, as measured by EDSS and MSSS, and also demonstrated a trend toward association with therapy responsiveness. Multivariable analysis further demonstrated that, in addition to rs41287280, the second investigated variant, rs3737577, was associated with changes in EDSS and MSSS after adjustment for the third SNV (rs3737578), disease duration, and therapy response. The alternative G allele of rs41287280 was associated with lower disability, whereas the rs3737577 T allele was associated with higher disability, suggesting a potential influence of these variants on disease severity.

Given the limited cohort size of 31 samples, only predictors demonstrating relevant associations in the preliminary analyses were included in the final models. This consideration was particularly important for genetic variables, as low minor allele frequencies may result in very small numbers of variant carriers, leading to wide confidence intervals and reduced statistical power. Consequently, the regression analysis results should be interpreted with caution, as they may lack robustness and generalizability without validation in a larger, independent cohort. Importantly, this analysis was intended only as a supplementary approach to support the association of rs41287280 identified by the Mann–Whitney U test and to evaluate its independent association with EDSS and MSSS. Therefore, the multiple regression findings for rs3737577 and rs3737578 should be regarded as preliminary and hypothesis-generating rather than definitive evidence of variant effects.

Taken together, these findings suggest that S1PR1 genetic variation may influence disease severity; however, validation in larger, independent cohorts and additional functional studies are required.

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Data availability: The experimental raw data underlying this article is openly available in the VinaR repository Handle: https://hdl.handle.net/21.15107/rcub_vinar_16340. Personal/patient data are not openly available due to privacy concerns for the study participants.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. The primer sequences used for *S1PR1* genotyping

Gene	Primer sequence (5'-3')	Amplicon size (bp)
S1PR1 **Sequence I**	TGTTTAAGGCTGCGGTTTCC	489
	CCCCAGACAAGAGCAGGTTA	
S1PR1 **Sequence II**	TGCGGGAAGGGAGTATGTTT	548
	GCACAGCTAACACCAGGAAG	

S1PR1 – sphingosine-1-phosphate receptor 1