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The association of rs703842 variants in *CYP27B1* with multiple sclerosis susceptibility is influenced by the HLA-DRB1*15:01 allele in Slovaks

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A B S T R A C T

In this study, we analysed the association of rs703842 in *CYP27B1* gene with multiple sclerosis (MS) risk and disability progression in a group of 496 MS patients and 521 controls. For the first time in Central European Slovak population, we found the rs703842 allele C to be protective factor against MS development ($p = 1.09 \times 10^{-5}$). Moreover, the risky genotypes TT and TC were showed to be associated with an increased MS risk, and this was aggravated by the homozygous carriage of the HLA-DRB1*15:01 allele (OR = 2.82 vs. 4.86, $p < .0001$). No association of rs703842 with MS disability progression or calcidiol serum level was found.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the brain and the spinal cord, characterised by inflammation, attacks of demyelination and chronic neurodegeneration (Compston and Coles, 2008). In the etiopathogenesis of MS, environmental factors (Kakalacheva and Lunemann, 2011) strongly interact with the genetic predisposition of the individual (International Multiple Sclerosis Genetics Consortium et al., 2007; International Multiple Sclerosis Genetics Consortium (IMSGC), et al., 2013). The cascade of immunopathological mechanisms in MS is triggered by myelin reactive T-lymphocytes that are shifted towards proinflammatory Th_1 phenotype (Stinissen and Hellings, 2008). Clinical course of the disease is characterised by large individual differences, varying from benign to malign disease progression rate, with the great impact on patient's neurological status. Protective role of vitamin D in development of autoimmune diseases is probably mediated by its immunomodulatory effects, such as stimulating of T_{reg} lymphocytes and supressing of functions of T_{H1}, T_{H17} and B-lymphocytes (Alharbi, 2015). Hypovitaminosis D has been identified to be one of the main risk factors in MS development (Pierrot-Deseilligny and Souberbielle, 2010) and peroral vitamin D supplementation was found to markedly decrease the MS risk (Munger et al., 2004). Possible preventive and therapeutic effects of vitamin D in MS disease are also supported by association of its serum level with disease activity, relapse rate and disability (Mowry et al., 2012; Runia et al., 2012; Smolders et al., 2008; Munger and Ascherio, 2011). In the human metabolism, the precursor of vitamin D (cholecalciferol) undergoes two hydroxylation steps to be converted to its active form, calcitriol. The final hydroxylation occurs in kidneys, skin and immune cells and it is catalysed by 1-α-hydroxylase. The enzyme is coded by *CYP27B1* gene (cytochrome P450 family 27 subfamily B peptide 1) that is located on chromosome 12q13. The single nucleotide polymorphism (SNP) rs703842 C/T in *CYP27B1* gene was found to be strongly associated with MS risk (5.4×10^{-11}) by a genome-wide association study (GWAS) in a large group of Australian & New Zealander and Caucasian MS cases (ANZ gene, 2009), and subsequently confirmed in Swedish patients (Sundqvist et al., 2010). Later, association of rs703842 with MS risk was described in cohorts from the United Kingdom and United States (Simon et al., 2011), in North-western Europeans (Cortes et al., 2013), in Han Chinese (Zhuang et al., 2015) and in Greeks (Hadjigeorgiou et al., 2018). On the other hand, no association of rs703842 with the MS risk was found in a large group of American

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nurses (Simon et al., 2010), in Canadian MS patients (Orton et al., 2011) or in Morrocans (Skalli et al., 2017). The rs703842 was found to be a predictor of calcidiol serum level (Orton et al., 2008) and the role of this polymorphism in MS could lie in regulating the amount of active calcitriol. In addition, the study of Simon et al. (2011) showed that the association of rs703842 with MS risk could be dependent on the presence of Human Leucocyte Antigen (HLA) DRB1*15:01 allele, that is known to be one of the most important genetic MS susceptibility factors (Zhang et al., 2011).

Taken together, the results of previous studies have suggested pathophysiological importance of SNP rs703842 in *CYP27B1* gene in MS development. However they are not consistent between different populations and can also be influenced by presence of HLA-DRB1*15:01 allele. To our best knowledge, this association has not yet been investigated in Central European Slovak population. In addition, no study has showed the association of rs703842 variants with the multiple sclerosis disability progression. Therefore we decided to investigate the association of rs703842 polymorphism in *CYP27B1* gene with MS development and progression in Slovak population. To enable deeper insight into the role of vitamin D related genetic factors in MS pathogenesis, we assessed possible interaction of rs703842 variants with HLA-DRB1*15:01 genotypes in the studied associations. Up to that, we also analysed the calcidiol serum level and its association with rs703842 in our cohort.

2. Materials and methods

2.1. Patients and controls

Our study was performed in 496 MS patients with relapsing-remitting ($n = 424$) or secondary progressive ($n = 72$) disease course and 521 healthy control subjects. The study was approved by the Ethics comittee of Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin. Before participating in the study, all individuals gave an informed consent. The healthy control group consisted of volunteers free from any disease of the central nervous system (CNS). A clinically definitive diagnosis in MS patients was established according to the McDonald's criteria (Polman et al., 2005; Polman et al., 2011) in The Centre for Demyelinating Diseases at the Clinic of Neurology, Jessenius Faculty of Medicine in Martin and Martin University Hospital, Slovak Republic. Clinical data and blood samples were taken only at regular medical check-ups to minimize trauma to patients. General physical characteristics (sex and age) of the study group are shown in Table 1. All individuals involved in the study were Slovak people of Caucasian origin.

To be able to evaluate individual disease disability progression of MS patients in our study, we ascertained a Multiple Sclerosis Severity Score (MSSS) and a progression index of each patient. Both parameters consider the patients neurological damage measured by Expanded Disability Status Scale score (EDSS) (Kurtzke, 1983) and the disease duration. MSSS was ascertained using the Global MSSS table (Roxburgh et al., 2005), progression index was calculated as EDSS divided by disease course in years. Using MSSS score, the MS patients were divided into 3 groups: MS-1 group - slow progressing MS patients with MSSS < 3 ($n = 145$); MS-2 group - mid-rate progressing MS patients with MSSS 3–6 ($n = 248$); MS-3 group - rapidly progressing MS patients with MSSS > 6 ($n = 103$). Clinical characteristics of MS patient groups are summarized in Table 2. Recorded parameters include mean age at which the first symptoms were observed, mean disease duration, mean EDSS score, mean MSSS, progression index, MS phenotype, and rate of disease disability progression.

2.2. Genotyping

Blood samples were taken from peripheral veins of the upper limb and dispensed into 3 ml tubes containing 5,4 mg of EDTA (Vacutest Kima S.r.l., Italy). DNA was isolated from white blood cells using The Wizard® Genomic DNA Purification Kit (Promega, USA) or MagNA Pure LC DNA Isolation Kit I on MagNA Pure LC Instrument (Roche, Switzerland) and stored at −20 °C. The SNP rs703842 T/C of the *CYP27B1* gene was genotyped by restriction fragment length polymorphism (RFLP) analysis. Polymorphic region of DNA was amplified by polymerase chain reaction (PCR), which was performed in total volume of 15 µl. Reaction volume consisted of 7 µl of REDTaq ReadyMix™ PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich), 1 µl of genomic DNA sample (50–150 ng) and 0.5 µl of 25 µM primers (Microsynth) filled up to total volume of 15 µl by redistilled water. Oligonucleotide primers were designed by us, using an online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/): forward – 5′ GGG ACC TAG AGG AGG TGG AG 3′ and reverse – 5′ CCT CAT TCC AGC TGA GGA GAG AT 3′. The PCR cycles consisted of 5 mins of initial denaturation at 95 °C followed by 34 cycles of 95 °C for 30 s, 59 °C for 45 s, 72 °C for 1 min and 72 °C for 5 mins. PCR products (625 bp) were digested in total volume of 20 µl using FspBI (*Bfa*I) restriction endonuclease (10 U/µl, ThermoScientific, USA) (0.5 µl in 37 °C for 4 h). Subsequently, DNA fragments were separated by electrophoresis on 2% agarose gel and visualised by ethidium bromide or GoodView™ Nucleid Acid Stain (Ecoli, Czech Republic) under UV light. CYP27B1 rs703842 genotypes were determined as TT (457 bp, 162 bp, 6 bp), CC (286 bp, 171 bp, 162 bp, 6 bp) and TC (457 bp, 286 bp, 171 bp, 162 bp, 6 bp).

The HLA-DRB1*15:01 genotypes were determined using analysis of SNP rs3135388, which has been shown to be the most significant surrogate marker for the HLA-DRB1*15:01 allele (De Bakker et al., 2006). The SNP rs3135388 G/A was genotyped by high resolution melting analysis (HRMA) at Roche LightCycler® 480 Instrument (Roche, USA). Polymorphic region of DNA was amplified by PCR performed in total volume of 15 µl, using reagents from original LightCycler® 480 High Resolution Melting Master kit (Roche, USA). The reaction volume consisted of 7.5 µl of LC480 HRM Master Mix, 1.8 µl of $MgCl_2$ (25 mM), 0.5 µl of genomic DNA sample (25–50 ng) and 0.1 µl of 40 µM primers (Ecoli, Czech Republic) filled up to total volume of 15 µl by redistilled water. The oligonucleotide primers were designed by us, using an online Primer3Plus tool http://primer3plus.com/cgi-bin/dev/primer3plusAbout.cgi (Untergasser et al., 2012): forward – 5′ TCC TCA TCA GGA AAA CCT AAA G 3′ and reverse – 5′ AGT AGA GAT CTC CCA ACA AAC C 3′. PCR cycles consisted of 5 min of initial denaturation at 95 °C followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s, 72 °C for 10 s and 72 °C for 2 min. The PCR product length was 51 bp and melting curve analysis was performed at 68 °C – 88 °C. To verify the quality of genotype determination, 40 samples were randomly selected and repetitively genotyped.

Table 1
General characteristics of study group.

Factor	Patients ($n = 496$)		Controls ($n = 521$)	
Sex	142 Men (28.6%)	354 Women (71.4%)	188 Men (36.1%)	333 Women (63.9%)
Mean age (years)	42.9 ± 10.6	43.9 ± 10.6	36.4 ± 11.2	40.1 ± 15.4
	43.7 ± 10.6		38.8 ± 14.1	

Table 2
Clinical characteristics of MS patients.

Factor	MS patients	Rate of disability progression		
	(n = 496)	Slow	Mid-rate	Rapid
		(MS-1, n = 145)	(MS-2, n = 248)	(MS-3, n = 103)
Age of first symptoms (years)	29.5 ± 9.5	26.1 ± 8.1	29.0 ± 8.9	35.4 ± 10.1
Disease duration (years)	13.6 ± 7.0	15.7 ± 6.9	13.3 ± 6.7	11.3 ± 6.8
EDSS (points)	3.86 ± 1.7	2.32 ± 0.94	3.94 ± 1.14	5.82 ± 1.56
MSSS (points)	4.35 ± 2.18	1.89 ± 0.7	4.43 ± 0.84	7.61 ± 1.12
Progression index	0.34 ± 0.22	0.16 ± 0.06	0.33 ± 0.1	0.64 ± 0.27
MS phenotype				
Relapsing-remitting	424 (85.5%)	144 (99.3%)	223 (89.9%)	57 (55.3%)
Secondary progressive	72 (14.5%)	1 (0.7%)	25 (10.1%)	46 (44.7%)

MS – multiple sclerosis, EDSS – expanded disability status scale, MSSS – multiple sclerosis severity score.

2.3. Vitamin D serum level determination

Peripheral venous blood samples were taken into 5 ml tubes containing gel and clot activator (Vacutest Kima S.r.l., Italy). The sampling was performed in winter, between December and March, to minimize bias from seasonal environmental influence. The exclusion criteria were: taking of vitamin D supplements, usage of tanning bed or whole body sun exposure within last 8 weeks before sampling. Serum was obtained by centrifugation at 4000 G for 5 min. Serum level of vitamin D (calcidiol) was determined by chemiluminescent microparticle immunoassay on analyser Architect ci4100 (Beckman-Coulter, USA) using ARCHITECT 25-OH Vitamin D Reagent (Abbott Diagnostics, USA).

2.4. Statistical analysis

Statistical analysis was performed by SVS 7 software (SNP & Variation Suite v7.6.11, Golden Helix, Bozeman, Montana, USA). From the determined genotype counts we calculated allele and genotype frequencies. Fisher's exact test was used to estimate the significance of association and the deviation from Hardy-Weinberg equilibrium. Logistic regression analysis adjusted for sex and age was used to test the associations in genetic models (dominant, recessive). Pearson Chi-square test was used to analyse the associations in contingency tables. To measure the strength of association we calculated odds ratios including 95% confidence intervals (95% CI). Kruskal-Wallis test was used to determine the associations between genotypes and MSSS score, progression index or calcidiol serum level. Statistical significance level was considered to be two-tailed $p \leq .05$ in all performed tests.

3. Results

In our study, we primarily analysed the role of SNP rs703842 in *CYP27B1* gene in etiopathogenesis of MS in a group of 496 MS patients and 521 healthy control subjects. The gene polymorphism rs3135388, which was used as a tagging marker of the HLA-DRB1*15:01 allele status, was analysed as a possible factor, influencing association of rs703842 with MS.

Genotype frequencies of the analysed polymorphisms rs703842 and rs3135388 were distributed in MS patients and controls according to Hardy–Weinberg equilibrium. When we analysed allele frequencies of rs703842 in *CYP27B1* gene, we found the minor allele C to be significantly less frequent in MS patients as compared to the controls (28.1% vs. 37.3%, $p = 1.09 \times 10^{-5}$). The minor allele A of rs3135388, corresponding with the HLA-DRB1*15:01 allele, was present in significantly higher frequency in MS patients when compared to controls (29.8% vs. 10.1%, $p = 7.9 \times 10^{-30}$). Data are shown in Table 3.

To assess association between rs703842 and rs3135388 genotypes and MS risk, we used logistic regression analysis adjusted for sex and age. The analysis revealed that both rs703842 and rs3135388 genotypes are significantly associated with MS risk ($p = 8.6 \times 10^{-29}$). The highest predictive value of this logistic model was found when we used the recessive genetic model for risk of rs703842 and the dominant genetic model for risk of rs3135388, represented by AUC = 0.735 (for both SNPs). The ROC curve of the analysis is shown in Fig. 1.

Genotype frequencies in risk genetic models are shown in Table 4. Differences in genotype distributions were statistically significant. Genotype CC frequency of rs703842 (homozygous for protective allele C) was 15.7% in controls compared to 8.3% in MS patients. Genotypes AA and GA of rs3135388, containing risk allele A, were present in 52.2% of MS patients as compared to 19% in controls.

Previously in this study, two alleles were found to be associated with increased MS risk - rs703842 allele T due to its significantly higher frequency in MS patients than in controls (0.719 vs. 0.627, OR = 1.522, 95% CI = 1.263–1.835, $p = 1.09 \times 10^{-5}$) and rs3135388 allele A (HLA-DRB1*15:01 positivity) due to its association with MS risk described in Table 3. To better evaluate their role in MS risk, we made a complex comparison that is shown in Table 5. We quantified the number and frequencies of individuals whose genotype contains a combination of risk and protective genotype status of rs703842 and rs3135388, as well as the odds ratios for MS risk. The recessive model was used for rs703842 and the dominant model was used for rs3135388, as revealed by logistic regression analysis.

As seen in Table 5, frequency of individuals whose genotype contains risky allele T of rs703842 (TT and TC) in combination with risky allele A of rs3135388 (AA and GA; HLA-DRB1*15:01 positive) was 49.0% in MS patients as compared to 16.5% in healthy controls. The observed difference was statistically significant with OR = 4.858 (95% CI = 3.633–6.497, $p < .0001$). Interestingly, the calculated Odds Ratio for risky genotypes of rs703842 in HLA-DRB1*15:01 negative individuals was 2.815 (95% CI = 2.180–3.635, $p < .0001$).

Based on the strong allelic and genotypic association of rs703842 with MS susceptibility found in our examined cohort, we also analysed whether this SNPs may affect the disease course. Interestingly, no significant differences in MSSS score or progression index were found among carriers of different rs703842 genotypes (Table 6). No significant differences in allele and genotype frequencies were also observed when we compared three subgroups of patients with different disability progression rate, defined by MSSS score (Table 7).

In this study, we have also analysed whether rs703842 could be related to vitamin D serum level. For this reason, calcidiol serum level was measured in 278 MS patients and 113 controls and the association with rs703842 genotypes was analysed. However, no statistically significant differences in calcidiol serum level among carriers of different rs703842 genotypes were found neither in MS patients nor in controls (Table 8).

Table 3
Allele and genotype frequencies of the analysed gene polymorphisms in MS patients and controls.

SNP	Allele	MS patients (n = 496)	Controls (n = 521)	OR (95% CI)	Fisher's p value
	Genotypes				
rs703842	*C (minor)*	0.281	0.373	0.657 (0.545–0.792)	1.09×10^{-5}
	TT/TC/CC	0.520/0.397/0.083	0.411/0.432/0.157		
rs3135388	*A (minor)*	0.298	0.101	3.795 (2.976–4.841)	7.9×10^{-30}
	GG/GA/AA	0.478/0.447/0.075	0.810/0.178/0.012		

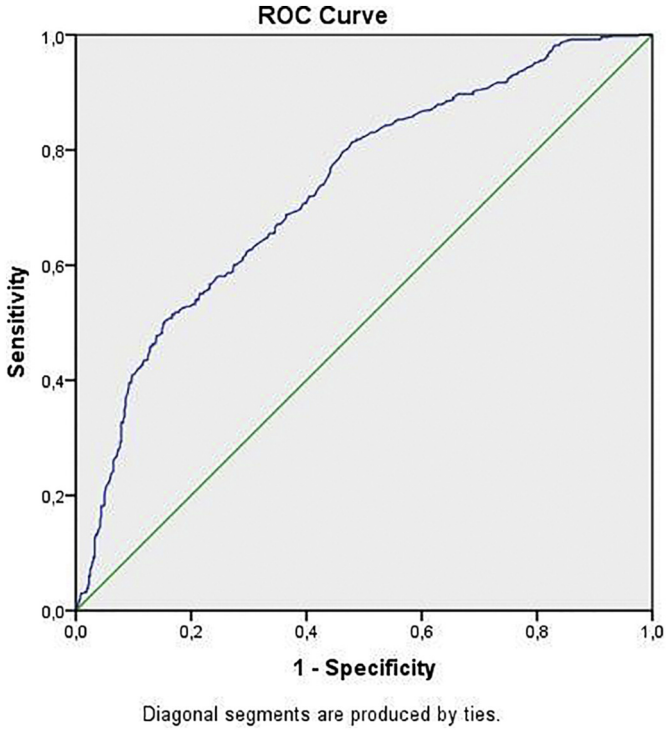

Fig. 1. The ROC curve for the analysis of rs703842 and rs3135388 genotype association with MS risk.

4. Discussion

Development and disease course of multiple sclerosis is dependent on the complex interactions of the strong genetic heritable component with several environmental factors (Compston and Coles, 2008). In etiopathogenesis of the disease, vitamin D has been shown to have beneficial effects (Cadden et al., 2011; Pierrot-Deseilligny and Souberbielle, 2011). The level of calcidiol in human serum is dependent on its peroral intake and also on individual exposure to the sunlight. However, to exert the complex vitamin D biological effects, it has to be converted to active calcitriol. This hydroxylation step in vitamin D metabolism, catalysed by the *CYP27B1* gene, could be important subject of genetic polymorphic alterations. The involvement of *CYP27B1* genetic variants in MS development has been suggested by several studies (Sundqvist et al., 2010; Simon et al., 2011; Cortes et al., 2013; Zhuang et al., 2015; Hadjigeorgiou et al., 2018), while in others no association was found (Simon et al., 2010; Orton et al., 2011; Skalli et al., 2017). Considering these results, it seems that the role of *CYP27B1* gene in MS is influenced by many factors and can also include populational variations. In addition, there had been no relevant data referring the association of the rs703842 in *CYP27B1* gene with MS in Slovaks. Due to these facts, we found it important to investigate the role of rs703842 gene polymorphism in MS development and disability progression in Central European Slovak population. The results of our study show the significant association of rs703842 variants with MS susceptibility, but not with disability progression in a cohort of 496 Slovak MS patients and 521 healthy controls.

The most important finding of our study is the protective role of the allele C of rs703842 against MS development (OR = 0.657, 0.545–0.792, $p = 1.09 \times 10^{-5}$). The frequency of allele C was significantly lower in MS patients when compared to healthy controls, with frequencies of 0.281 and 0.373, respectively. Concordantly with our findings, the inverse association of the allele C with MS susceptibility was also reported by Sundqvist et al. (2010) in a group of 2158 Swedish MS patients and 1759 controls (allele C frequency 0.355 vs. 0.330, OR = 0.89, 95% CI = 0.81–0.98, $p = .02$) and by Simon et al. (2011) in a group of 1655 Caucasian MS cases and 6349 controls from United States and United Kingdom. The association of allele C of rs703842 with reduced MS risk was also shown in a large group of Causasian participants by metaanalysis of Jiang et al. (2016) (OR = 0.85, 95% CI = 0.80–0.90, $p < .0001$) and recently by Hadjigeorgiou et al. (2018) in a group of 1228 MS cases and 1014 controls from Greece (OR = 0.621, 95% CI = 0.49–0.78, $p = 4.9 \times 10^{-5}$). On the other hand, there are studies where no association of rs703842 with MS was found. Among those, there is a study of Simon et al. (2010) performed in a smaller group of 214 MS cases and 428 controls from United States, a study of Orton et al. (2011) made in 1364 MS patients and 1661 healthy controls from Canada and a study of Skalli et al. (2017) in 113 MS patients and 146 controls from Morocco. This discrepancy could be the result of ethnicity variation and could suggest the interaction of population genetic background with the environmental and other disease-inducing factors. Moreover, in our study, the rs703842 allele T was shown to be associated with an increased MS risk being present in 71.9% in MS cases and in 62.7% of healthy controls (OR = 1.522, 95% CI = 1.263–1.835). Similar results were observed by Zhuang et al. (2015) who showed an increased frequency of allele T in a group of 149 Han Chinese MS patients compared to 294 healthy controls (0.40 vs. 0.34, $p = .032$). We suppose that the observed association of rs703842 variants with MS risk could be caused by its linkage with altered functional CYP27B1 protein expression (Handel et al., 2010) and thus the identified risk allele T carriage could probably result in decreased availability of biologically active calcitriol to the immune cells in predisposed individuals. This hypothesis is

Table 4
Genotype frequencies in risk genetic models of rs703842 and rs3135388 in MS patients and controls.

SNP	Genotypes (model)	MS patients (n = 496)	Controls (n = 521)	OR (95% CI)	Chi-square p value
rs703842	TT + TC/CC (R)	0.917/0.083	0.843/0.157	0.482 (0.324–0.718)	0.000258
rs3135388	GG/GA + AA (D)	0.478/0.522	0.810/0.190	4.658 (3.517–6.170)	< 0.0001

Abbreviations: R – recessive genetic model, D – dominant genetic model.

Table 5
Frequencies of individuals containing rs703842 and rs3135388 genotype status combinations in MS patients and controls.

	CYP27B1	*CYP27B1*	*MS*		*CTL*	
	T positive	T negative				
*HLA-DRB1*15:01 negative*	DRB protect CYP risk	DRB protect CYP protect	0.427 (*n* = 212)	0.050 (n = 25)	0.678 (*n* = 353)	0.132 (*n* = 69)
*HLA-DRB1*15:01 positive*	DRB risk CYP risk	DRB1 risk CYP protect	0.490 (*n* = 243)	0.003 (*n* = 16)	0.165 (*n* = 86)	0.003 (*n* = 13)

Abbreviations: CYP risk – rs703842 risky status (TT and TC), CYP protect – rs703842 protective status (CC), DRB risk – rs3135388 risky status (AA and GA; HLA-DRB1*15:01 positive), DRB protect – rs3135388 protective status (GG; HLA-DRB1*15:01 negative).

Table 6
MSSS score and progression index in carriers of different rs703842 genotypes.

Genotypes	CC	CT	TT	Kruskal-Wallis *p* value
	(*n* = 41)	(*n* = 197)	(*n* = 258)	
MSSS score	4.17 ± 2.46	4.19 ± 2.20	4.50 ± 2.11	0.206
Progression index	0.33 ± 0.24	0.33 ± 0.23	0.36 ± 0.21	0.110

supported by the findings of Shahijanian et al. (2014), who detected increased expression of CYP27B1 in carriers of protective genotype, and reduced *CYP27B1* gene pathway in carriers of the risk haplotype. These expression changes could link the CYP27B1 risk genotypes with an increased MS risk as the result of decreased vitamin D receptor (VDR) activation that subsequently alters the balance of tolerogenic and inflammatory dendritic cells, which are important in antigen presentation to T-cells. Moreover, the impaired activation of vitamin D pathways could also exert detrimental effects in lymphocytes, resulting in lack of inhibition of T_{H1} and B cells functions and consequently low activation of T_{H2} and T_{reg} cells, all of them important in MS pathogenesis (Alharbi, 2015).

In our study, we also analysed possible influence of HLA-DRB1*15:01 allele on the association of rs703842 with MS susceptibility. Firstly, we identified the already known association of HLA-DRB1*15:01 allele (in our study represented by allele A of rs3135388) with MS risk. We found HLA-DRB1*15:01 allele present in 29.8% of MS cases compared to 10.1% of healthy controls and to be strongly associated with increased MS risk (OR = 3.795, 95% CI = 2.976–4.841, $p = 7.9 \times 10^{-30}$). Then, we analysed the association of rs703842 and rs3135388 genotypes with MS risk. The logistic regression analysis showed MS susceptibility to be associated with genotypes of both SNPs - the best predictive values of recessive genetic model in rs703842 and dominant genetic model in rs3135388 (Fig. 1). We identified rs703842 genotype CC to be protective against MS development (OR = 0.482, 95% CI = 0.324–0.718, $p = .000258$) and the genotypes containing allele A of rs3135388 (HLA-DRB1*15:01 positive) to increase MS susceptibility (OR = 4.658, 95% CI = 3.517–6.170, $p < .0001$).

Furthermore, we wanted to analyse whether the observed association of rs703842 genotypes with MS risk could be influenced by the HLA-DRB1*15:01 genotype status, as complexly compared in Table 5. The results showed the MS risk in carriers of rs703842 risk allele T (genotype TT and TC) who are HLA-DRB1*15:01 positive to be described by OR = 4.858 and the frequency of these individuals was significantly higher in MS patients as compared with controls (49.0% vs. 16.5% respectively, p < .0001). Interestingly, in individuals with rs703842 risky genotype status as well as HLA-DRB1*15:01 negativity the calculated OR was 2.815. Our findings strongly support the view that individuals with rs703842 risk allele T carriage together with the DRB1*15:01 positivity are in considerably greater MS risk than the DRB1*15:01 negative allele T carriers. The HLA-DRB1*15 allele is highly expressed in immune cells and its gene product has high affinity to immunoreactive sequence of encephalolitogenic myelin basic protein, important in activation and clonal expansion of T-cells in autoimmune reaction in MS (Valli et al., 1993; Wucherpfennig et al., 1994). HLA-DRB1*15 expression has been shown to be regulated by calcitriol-VDR complex that is bound to vitamin D responsive element (VDRE), present in the proximal promoter region of the gene. However, the sequence that is present in other non-MS associated DRB1 alleles is different and thus not responsive to cacitriol (Ramagopalan et al., 2009). Based on the results of our study, we suppose that the carriers of rs703842 risk allele T manifest an impairment of inhibition of DRB1*15:01 expression mediated by VDRE. On the other hand, lower MS susceptibility found in DRB1*15:01 negative individuals carrying the rs703842 risky genotypes could be explained by lack of this type of interaction. Similarly to our findings, Simon et al. (2011) showed the relation between rs703842 variants and MS risk to be influenced by the presence of the HLA-DR15 allele in MS cases from United States and United Kingdom. They found the rs703842 protective allele C in HLA-DR15 negative individuals associated with a 21% reduction of MS risk, while in HLA-DR15 positive individuals only a 9% reduction was observed (OR = 0.79, 95% CI = 0.69–0.90 vs. OR = 0.91, 95% CI = 0.80–1.04, respectively).

In our study, we also analysed whether the rs703842 variants could be the predictors of calcidiol serum level. The observed calcidiol level was 14.3 ± 4.4 ng/ml in MS patients (*n* = 278) and 17.8 ± 7.7 ng/ml in controls (*n* = 113), but its association with rs703842 genotypes was not found ($p_{K\text{-}W}$ = 0.835 for MS patients, 0.835 for controls, 0.568 for both). Similarly to our findings, also Simon et al. (2011) did not show any association of rs703842 with calcidiol serum level in a large cohort from United Kingdom and United States. On the other hand, Orton et al. (2008) in a group of 99 twin MS pairs from United States showed the

Table 7
Allele and genotype frequencies of rs703842 in MS patients (stratified by disability progression) and controls.

		Minor Allele		Genotypes (recessive model)		Compared groups
SNP		C (protective)	Chi-square *p* value	CC	Chi-square *p* value	
rs703842	Controls	0.373	0.000	0.157	0.002	MS subgroups vs. CTL
	MS-1	0.314	0.338	0.103	0.471	MS subgroups
	MS-2	0.266	0.153	0.069	0.222	MS-2 vs. MS-1
	MS-3	0.272	0.313	0.087	0.673	MS-3 vs. MS-1

Abbreviations: MS-1 - slow progressing MS patients; MS-2 - mid-rate progressing MS patients; MS-3 - rapidly progressing MS patients.

Table 8
The calcidiol serum level (ng/ml) in MS patients and controls with different rs703842 genotypes.

Genotype	*CC*	*CT*	*TT*	Kruskal-Wallis *p* value
MS patients (14.3 ± 4.4)	14.8 ± 4.3	14.3 ± 3.9	14.3 ± 4.7	0.835
Controls (17.8 ± 7.7)	16.5 ± 6.2	17.3 ± 8.1	18.6 ± 8.0	0.835
MS patients + controls	15.6 ± 5.3	15.1 ± 5.5	15.4 ± 6.1	0.568

mean calcidiol concentrations to be lower in subjects homozygous for the allele C of rs703842 ($p < .001$). This discrepancy could be caused by inconsistent methods of measurement, inconsistent matching of MS patients and controls, ethnic, and geographical factors.

Due to the strong association of rs703842 alleles and genotypes with MS risk shown in our study we also analysed a possible role of this polymorphism in MS progression, that was evaluated by MSSS score and progression index. However, no association of rs703842 with disease disability progression was shown when we analysed MSSS score or progression index in carriers of different rs703842 genotypes ($p = .206$; $p = .110$ respectively). Up to that, no significant association was also found when we compared the subgroups of MS patients with a different rate of disease disability progression, defined by the MSSS score ($p > .05$ for all). Similarly to our findings, no association of rs703842 with MS progression evaluated by MSSS score was found by Jensen et al. (2010) in a group of 898 Australian MS patients ($p = .428$) and by Skalli et al. (2017) in Morrocans ($p = .53$).

In spite of the fact, that our results are strongly suggesting the association of rs703842 with MS development and its interaction with DRB1*15:01, the present study has several limitations that have to be pointed out. A relatively small and specific sample of MS patients and controls of Central European Slovak origin was analysed, what could undepower the analyses when compared to GWAS and other studies in larger cohorts. A very high prevalence of hypovitaminosis D was found in both MS cases and controls, and this could possibly under- or overestimate the observed associations. Only a single vitamin D level measurement was performed, so the long-term vitamin D status was not ideally reflected. The remeasurement of its natural status was not possible since the supplementation was started in all deficient individuals. Concerning the association of rs703842 with MS, the influence of merely HLA-DRB1*15:01 allele was determined. To better evaluate the possible genetic interactions, also the other HLA-DR and -DQ risk or protective loci should be complexly analysed, and the findings should be validated by functional analyses, but this exceeded the extent of the present study.

5. Conclusions

To the best of our knowledge, this is the first study assessing the association between variants of rs703842 polymorphism in *CYP27B1* gene and MS in Central European Slovak population. Our findings suggest a strong allelic and genotypic association between variants of rs703842 in *CYP27B1* gene and MS susceptibility. In addition, our results showed that rs703842 allele C is protective against MS development, while allele T carriage is associated with an increased MS susceptibility. We also identified strong association of genotype TT and TC with increased risk of MS, which was aggravated by the carriage of HLA-DRB1*15:01 allele. On the other hand, our analysis did not identify any association of rs703842 variants with MS disease disability progression or with calcidiol serum level. We conclude, that rs703842 could be a genetic marker strongly associated with MS development in Slovak population, which seems to be considerably influenced by the presence of HLA-DRB1*15:01 MS susceptibility allele. Our results provide a novel insight into the underlying molecular mechanisms in MS and point out the complexity of genetic regulation of vitamin D pathways in etiopathogenesis of MS. To confirm our findings and to clarify the exact mechanisms of involvement and interaction of CYP27B1 and DRB1*15:01 variants in MS development and progression, the future genetic and mainly functional studies in larger cohorts of individuals in different populations are necessary.

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