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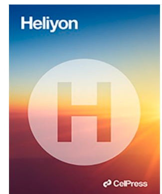

Research article

Expression levels of *GSDMB* and *ORMDL3* are associated with relapsing-remitting multiple sclerosis and *IKZF3* rs12946510 variant

Milan Stefanović [a], Ljiljana Stojković [a], Ivan Životić [a], Evica Dinčić [b], Aleksandra Stanković [a], Maja Živković [a,*]

[a] *VINČA Institute of Nuclear Sciences - National Institute of the Republic of Serbia, Laboratory for Radiobiology and Molecular Genetics, University of Belgrade, Belgrade, Serbia*
[b] *Military Medical Academy, Clinic for Neurology, Belgrade, Serbia*

A R T I C L E I N F O

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A B S T R A C T

Multiple sclerosis (MS), a noncurable autoimmune neurodegenerative disease, requires constant research that could improve understanding of both environmental and genetic factors that lead to its occurrence and/or progression. Recognition of the genetic basis of MS further leads to an investigation of the regulatory role of genetic variants on gene expression. Among risk variants for MS, Ikaros zinc finger 3 (*IKZF3)* gene variant rs12946510 was identified as one of the top-ranked and the expression quantitative trait loci (eQTL) for genes residing in chromosomal locus 17q12-21. The study aimed to investigate the association of gene expression of the immunologically relevant genes, which map to indicated locus, *ORMDL3*, *GSDMB*, and *IKZF3,* with MS and rs12946510 genotype, taking into account disease phase, clinical parameters of disease progression, and severity and immunomodulatory therapy. We used TaqMan® technology for both allelic discrimination and gene expression determination in 67 relapsing MS patients and 50 healthy controls. Decreased *ORMDL3* and *GSDMB* mRNA levels had significant associations with MS and rs12946510 TT rare homozygote among patients. Significant positive correlations between ORMDL3 and GSDMB mRNA expression were observed in both patients and controls. We detected the significant between-effect of sex and rs12946510 on the expression of *ORMDL3* in the patient group and interferon β therapy and rs12946510 on *GSDMB* expression.

Our results show the association of *ORMDL3* and *GSDMB* mRNA expression with the clinical manifestation of MS and confirm that *IKZF3* rs12946510 exerts the eQTL effect on both genes in multiple sclerosis. Besides providing novel insight related to MS phases and interferon β therapy, the study results confirm previous studies on regulatory genetic variants, autoimmunity, and MS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of the central nervous system with an estimated global prevalence of 2.8 million people [1]. The complex etiology of MS, which includes multiple environmental and genetic factors, was

* Corresponding author. VINČA Institute of Nuclear Sciences - National Institute of the Republic of Serbia, University of Belgrade, Laboratory for Radiobiology and Molecular Genetics, P.O.Box 522, 11000, Belgrade, Serbia.

E-mail address: majaz@vinca.rs (M. Živković).

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recognized [2] and the progress in understanding and treating MS have been made during previous decade. Advancements in molecular methodologies have pointed to a number of associated genetic and epigenetic mechanisms [3] and infectious factors [4] that may contribute to MS onset and progression. Recently, a number of genetic variants (single nucleotide polymorphisms, SNPs) associated with MS risk have been mapped [5]. This was the biggest step in defining the genetic architecture of MS, and the study showed that the expression of MS genes was enriched in microglia [5]. Besides the susceptibility genetic background, one of the significant insights after many genome wide association studies (GWAS) in complex disease was the fact that complex traits are mainly influenced by the non-coding genetic variants with seemingly regulatory function [6]. Efforts to perform genetic and regulatory fine mapping in immune-related diseases revealed novel strategies to identify regulatory quantitive trait loci of relevant cell types and across diseases [7,8]. An example of such a variant is rs12946510 C/T, in the gene that codes for Ikaros zinc finger 3 (*IKZF3*), which was recognized as one of the SNPs that increase the risk for MS and exerts the expression quantitive trait locus (eQTL) function in its' residing chromosomal region, 17q12-21 [7]. However, the effects of genetic variants on differential gene expression in MS and its association with disease severity are still under intensive research [9] and led to the rationale of the current study to further understand regulatory patterns in MS. The *IKZF3* rs12946510 variant is located in the FOXO1 transcriptional enhancer element, positioned in the immunologically relevant 17q12-21 genomic chromosomal region, affecting the activator-enhancer interaction [10,11]. *IKZF3* gene codes for the transcriptional regulator protein Aiolos, which regulates apoptosis in T cells by affecting Bcl-2 expression and cellular localization in an IL2-dependent manner [12], Th17 differentiation by silencing IL2 expression in $CD4^{+}$ T cells [13], and B lymphocyte development [14]. The top two genes whose expression is regulated by rs12946510 are orosomucoid-1 like 3 (*ORMDL3*) and gasdermin B (*GSDMB*) (GTEX) in whole blood, spleen tissue and a number of other tissues which are not part of the immune system ([15], https://www.gtexportal.org/home/snp/rs12946510). Both genes are located in the 17q12-21 chromosome region. *ORMDL3* gene codes for the endoplasmic reticulum membrane protein ORMDL3. ORMDL3 is critical for calcium homeostasis in T cells, being an inhibitor of Ca^{2+} current that leads to T cell activation [16]. It is also involved in lymphocyte migration by affecting sphingolipide synthesis through negative regulation of serine palmitoyltransferase function [17]. The molecular function of GSDMB protein has recently been discovered. The protein is a cleavage target of Granzyme A proteinase, released by natural killer (NK) cells, which in turn causes the formation of GSDMB pores in the plasma membrane, thus promoting the molecular cascade leading to pyroptotic death in the target cells [18]. Recently, genes proposed to carry eQTL variants in MS were analyzed by cell type, and both *ORMDL3* and *GSDMB* showed shared eQTL in CD4, NK, and B cells [9]. However, the studies that investigated the association of *ORMDL3*, *GSDMB*, and *IKZF3* with clinical manifestation of MS are scarce, and the validation of previous results was suggested.

The study aimed to investigate the association of relative mRNA expression in peripheral blood mononuclear cells (PBMC) of three genes *ORMDL3*, *GSDMB*, and *IKZF3* that reside within chromosome locus 17q12-21, previously associated with MS susceptibility, with MS and its clinical presentation. In addition, we aimed to estimate if the *IKZF3* rs12946510 genetic variant, previously denoted as eQTL in several human tissues, exerts its regulatory capacity in PBMC from patients with multiple sclerosis. Disease phase, progression and severity, and immunomodulatory therapy were taken into account in association analysis, and their between effects were also investigated.

2. Materials and methods

2.1. Study population

This study included 67 unrelated patients with RRMS, 15 relapsing at the moment of blood collection while 52 were in the remitting phase and 50 unrelated healthy controls without a familial history of MS. The patients were enrolled at the Clinic for Neurology of Military Medical Academy (MMA), Belgrade, Serbia, during the 2010–2020 period, where they were continuously followed and treated for MS. All patients were diagnosed according to the revised McDonald criteria [19,20], and the clinical form of the disease was also determined [21]. Among the patients in remission, 35 were under interferon β-1b therapy, while 17 were not receiving any MS therapy. Patients under the therapy received 0.25 mg of Betaferon® (Bayer AG, Leverkusen, Germany) every other day over at least six months. The Group of patients who received therapy was asked to participate in the study during their regular visits to the clinic and were enrolled upon consent. Patients who had not received therapy were asked to participate during their visits before the start of treatment and enrolled if consent. Patients in relapse were enrolled, if consent, during the visit to the clinic due to relapse and before receiving the acute treatment. For all the patients blood was drawn and immediately transferred to the laboratory for RNA and DNA extraction. The clinical and demographic parameters used in this study were EDSS (*Expanded disability status scale*), MSSS (*Multiple sclerosis severity score*), age at disease onset, relapse rate, disease duration for MS patients, and age for all participants at the moment of blood collection. EDSS describes current neurological impairment [22], while MSSS describes disease severity by correcting the EDSS for disease duration [23]. The questionnaires with clinical data related to age at onset, disease duration, number of relapses, and EDSS were fulfilled by the clinician during the examination and complemented with previous clinical reports from the same clinic.

Exclusion criteria were a diagnosis of primary progressive MS and chronic diseases other than MS. The control group consisted of healthy volunteers from MMA staff and employees of the Institute of Nuclear Sciences,Vinča'', who were asked to participate in the study during regular medical check-ups within the period between 2010 and 2020. Upon agreement, during the check-up collection of samples for laboratory analysis, the blood samples were also collected for RNA and DNA extraction. The demographic data were collected, and only individuals with no history of chronic diseases were enrolled. The females were predominantly enrolled in the control group to match the known higher prevalence of MS in females. All participants were Caucasians. The MMA Ethical Committee approved the study (Ethics approval numbers February 25, 2010; 6/2020), and each participant gave their written informed consent to participate in the study.

2.2. Determination of genotypes

DNA was extracted with Zymo Research Quick-DNA™ Miniprep Plus Kit from frozen peripheral blood samples precollected into 3 ml blood collection tubes with citrate or EDTA as anticoagulating agents, according to the manufacturer's protocol (Zymo Research, Irvine, California, USA). The DNA samples were genotyped using the TaqMan® based allelic discrimination method, with the Applied Biosystems™ 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA), according to the manufacturer's protocol. The TaqMan® assay used was C_31,651,862_10, for genotyping of *IKZF3* rs12946510 as previously described [24]. Allele calling was performed with a quality value of >95 % using the SDS software (v1.4.0; Applied Biosystems, Foster City, California, USA). Samples that failed the allele calling quality value were not included in the subsequent statistical analysis.

2.3. Relative quantification of mRNA expression

Fresh peripheral blood samples (collected in 3 ml blood collection tubes with EDTA as an anti-coagulant) were centrifuged in the lymphocyte separation medium (Lymphocyte Separation Medium PAA, GE Healthcare, Chicago, Illinois, USA) for separating the PBMC. Within 30 min of blood collection, total RNA was extracted from PBMC by a TRI Reagent based procedure (TRI Reagent, Ambion, Austin, Texas, USA). The RNA quantity and quality were analyzed with the spectrophotometric method (NanoDrop 1000, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). RNA samples were stored at −80 °C. DNaseI treatment was performed with 500 ng of total RNA, according to the manufacturer's protocol (DNase I, RNase-free (1 U/μL), Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). After the DNA digestion, cDNA was synthesized using an oligodT primer (Oligo (dT)18 Primer, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) in a 20 μl reaction volume, according to the manufacturer's protocol (RevertAid RT Kit, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). Quantitative Real Time PCR was performed for each cDNA sample in duplicate, on the Applied Biosystems™ 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA), using the standard protocol given by the manufacturer. The following TaqMan® gene expression assays were used: Hs00918021_m1 for *ORMDL3*, Hs00218565_m1 for *GSDMB*, Hs00232635_m1 for *IKZF3* and Hs99999904_m1 for the internal reference control, *PPIA* (Applied Biosystems, Foster City, California, USA). Cycle threshold (Ct) values were analyzed with the SDS software (v1.4.0; Applied Biosystems, Foster City, California, USA). Formula $2^{\text{(duplicate average CtTarget-duplicate average CtPPIA)}}$ was used to quantify the relative expression of target genes (mRNAs) in each sample.

2.4. Statistical analysis

Statistical analysis was performed with the Statistica 8.0 software package (StatSoft, Inc., Tulsa, Oklahoma, USA). Comparisons of categorical variables between groups were done with χ^2 test. The normality of continual variable distribution was tested with Kolmogorov-Smirnov and Shapiro-Wilk's W test. Statistical significance of differences in continuous variables between groups was analyzed with the Student T test, Man-Whitney *U* test, One-Way ANOVA or Kruskal-Wallis ANOVA, depending on the distribution of continuous variable values and the number of categories of grouping variable. The Pearson correlation coefficient or Spearman's Rank correlation coefficient was used as a measure of correlation between continuous variables, depending on the distribution of the continual variable. Factorial ANOVA was used to estimate the effects of interactions of independent categorical variables on a dependent variable. The values of continuous variables were presented as mean ± standard deviation (SD), regardless of the distribution type. In all tests, p values lower than 0.05 were considered statistically significant. Graphical representations of the results were made with Graph Pad Prism v9.0 software (GraphPad Software, Inc., San Diego, California, USA). Power analysis for the gene

Table 1
Demographic and clinical characteristics of the study population.

	Patients (n = 67)	Controls (n = 50)	p	Relapsing patients (n = 15)	Remitting patients (n = 52)	p	IFN-β- (n = 17)	IFN-β+ (n = 35)	p
Females/Males	37/30	33/17	0.23$^{\&}$	9/6	28/24	0.67$^{\&}$	11/6	17/18	0.27$^{\&}$
Age	39.2 ± 10.0	36.6 ± 10.2	0.13$^{\#}$	32.2 ± 7.8	40.9 ± 9.8	0.006*	42.8 ± 7.9	39.9 ± 10.5	0.32*
Age at disease onset	32.4 ± 9.5	–	–	28.8 ± 8.4	33.3 ± 9.6	0.14*	33.1 ± 6.8	33.4 ± 10.8	0.93*
Disease duration	6.2 ± 4.4	–	–	4.7 ± 3.3	6.6 ± 4.6	0.24$^{\#}$	7.6 ± 5.6	6.0 ± 4.0	0.49$^{\#}$
EDSS	2.55 ± 1.22	–	–	2.29 ± 1.44	2.61 ± 1.17	0.24$^{\#}$	2.71 ± 1.27	2.56 ± 1.13	0.73$^{\#}$
MSSS	4.41 ± 2.12	–	–	4.10 ± 2.22	4.48 ± 2.11	0.58*	4.44 ± 2.68	4.51 ± 1.79	0.91*
Annual relapse rate	0.81 ± 0.57	–	–	0.9 ± 0.52	0.78 ± 0.6	0.34$^{\#}$	0.92 ± 0.68	0.73 ± 0.58	0.64$^{\#}$

Continual parameters are presented as mean ± standard deviation; n – number of samples in group; Age– age at blood sampling; EDSS – expanded disability status scale; MSSS – multiple sclerosis severity score; IFN-β- – Interferon β therapy non receiving patients; IFN-β+ – Interferon β therapy receiving patients; Age, age at onset and disease duration are expressed in years; $^{\&}\chi^2$ test; $^{\#}$Mann-Whitney *U* test; *Student's T test; p values < 0.05 are considered statistically significant.

expression results was performed using Post-hoc Power Calculator (https://clincalc.com/stats/Power.aspx) [25].

3. Results

3.1. Study population characteristics

The demographic and clinical characteristics of the study population are presented in Table 1. There are no significant differences in female to male ratio and age between patients and controls.

3.2. Differences of target mRNA relative expression levels in PMBC between patients and controls

The patient group had significantly lower expression levels of *ORMDL3* compared to controls (means ± SD: 0.0067 ± 0.0033 vs. 0.0092 ± 0.0033, respectively, Mann-Whitney *U* test $p = 7 \times 10^{-5}$) (Fig. 1a). The relative expression of *GSDMB* mRNAs was also significantly lower in patients compared to the controls (means ± SD: 0.0071 ± 0.0036 vs. 0.0089 ± 0.0033, Mann-Whitney *U* test p = 0.01, respectively) (Fig. 1b). The expression of *IKZF3* mRNA (Fig. 1c) did not differ significantly between patients and controls (Mann-Whitney *U* test p = 0.25). With a probability of type I error of p = 0.016 and the number of patients and controls included in the analysis, the calculated study power for the presented significant results was >80 %.

3.3. IKZF3 rs12946510 genotype effects on relative expression levels of target mRNAs

The *IKZF3* rs12946510 was significantly associated with differential expression of *ORMDL3* mRNA in the patients group (means ± SD: 0.009 ± 0.003, CC; 0.007 ± 0.003, CT; 0.005 ± 0.002, TT, p = 0.004) (Fig. 2a); whereas the post-hoc analysis showed the difference in expression between the homozygotes (Multiple comparison of mean ranks, CC vs. TT, p = 0.003). The *GSDMB* mRNA was also significantly differentially expressed between *IKZF3* rs12946510 genotypes in the patients group, (means ± SD: 0.009 ± 0.005, CC; 0.006 ± 0.003, CT; 0.004 ± 0.003, TT, p = 0.036) (Fig. 2b); whereas the post-hoc comparison showed significant difference between the homozygotes (LSD test, CC vs. TT, p = 0.01). In the control group, there was no significant difference in either *ORMDL3* or *GSDMB* mRNA expression regarding the *IKZF3* rs12946510 genotypes (Fig. 2d–e). There were no significant differences in *IKZF3* mRNA relative expression levels between the *IKZF3* rs12946510 genotypes, either in the patient or in the control group (Fig. 2c–f).

3.4. Correlation analysis of relative expression levels of target gene mRNAs and clinical/anthropometric parameters

Correlations were tested between the relative expression levels of target mRNAs (Table 2). Significant positive correlations between *ORMDL3* and *GSDMB* mRNA expression levels were observed in both patients (Spearman's Rank correlation r = 0.26, p = 0.03) and controls (Spearman's Rank correlation r = 0.52, $p = 1 \times 10^{-4}$) (Table 2). In addition, *ORMDL3* and *IKZF3* mRNA expression levels significantly positively correlated in both patient (Spearman's Rank correlation r = 0.74, $p = 1 \times 10^{-12}$) and control (Spearman's Rank correlation r = 0.67, $p = 1 \times 10^{-7}$) groups. *GSDMB* and *IKZF3* mRNA expression levels significantly correlated only in the control group (Spearman's Rank correlation r = 0.36, p = 0.01) (Table 2). The correlation was anlalyzed between the relative expression levels of target mRNAs and clinical and anthropometric parameters, age at blood collection, age at disease onset, EDSS, MSSS, and relapse rate (Table 3). EDSS positively correlated with *IKZF3* mRNA level in patients (Spearman's Rank correlation r = 0.26, p = 0.04) (Table 3). Age at disease onset and age at blood collection significantly negatively correlated with *GSDMB* relative mRNA expression level in the patient group (Spearman's Rank correlation, r = −0.34, p = 0.006, r = −0.56, $p = 2 \times 10^{-6}$, respectively).

3.5. Between-effects of IKZF3 rs12946510 genotype, sex, RRMS phase and therapy status on the relative expression levels of target mRNAs

In Factorial ANOVA, relative expression of target mRNAs was taken as a response variable, while *IKZF3* rs12946510 genotypes, sex,

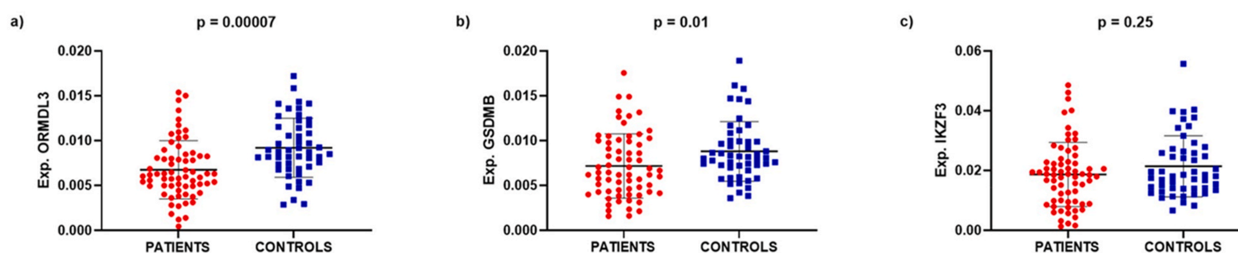

Fig. 1. Relative expression of target mRNAs in PBMC in patients and controls. a) relative expression of *ORMDL3* mRNA (means ± SD: 0.0067 ± 0.0033 vs. 0.0092 ± 0.0033, patients and controls, respectively); b) relative expression of *GSDMB* mRNA (means ± SD: 0.0071 ± 0.0036 vs. 0.0089 ± 0.0033, patients and controls, respectively); c) relative expression of *IKZF3* mRNA. Y axis represents the values of $2^{-(\text{duplicate average CtTarget-duplicate average CtPPIA})}$ for target gene mRNA, x axis denotes the MS patients and controls, horizontal lines represent mean ± standard deviation of relative mRNA expression. Mann-Whitney *U* test p values < 0.05 are considered statistically significant. [Two columns fitting image].

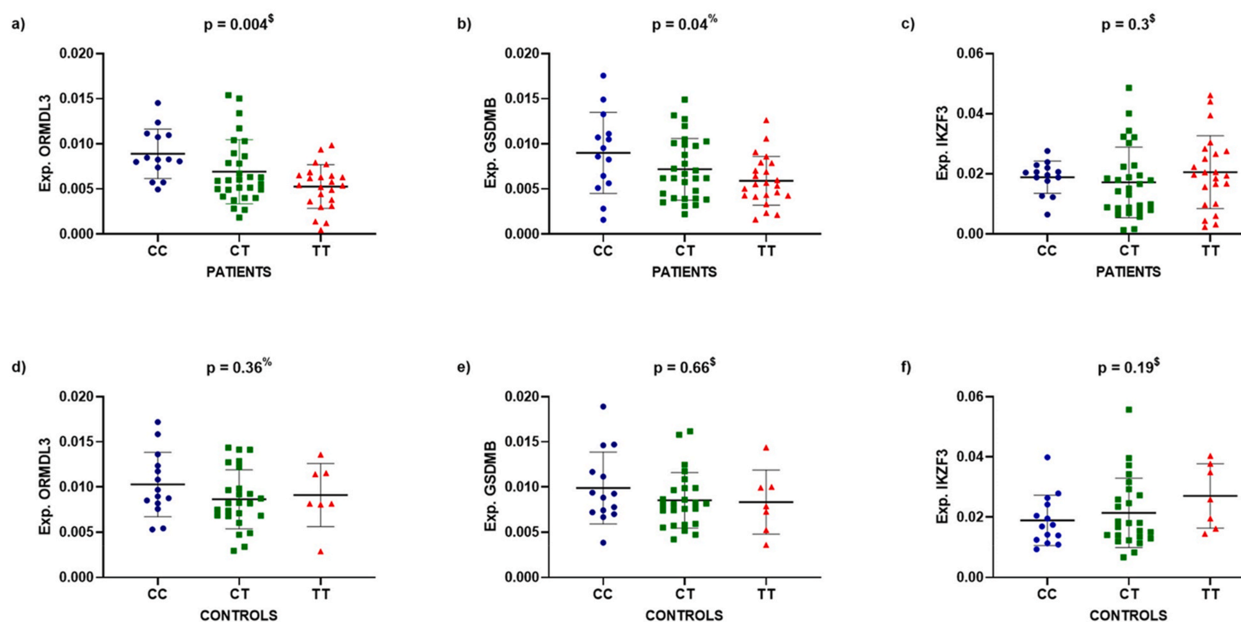

Fig. 2. Relative expression of target genes mRNAs according to *IKZF3* rs12946510 genotypes: a) *ORMDL3* in patients (means ± SD: 0.009 ± 0.003, CC; 0.007 ± 0.003, CT; 0.005 ± 0.002, TT); b) *GSDMB* in patients (means ± SD: 0.009 ± 0.005, CC; 0.006 ± 0.003, CT; 0.004 ± 0.003, TT); c) *IKZF3* in patients; d) *ORMDL3* in controls; e) *GSDMB* in controls; f) *IKZF3* in controls. Y axis represents the values of $2^{-(\text{duplicate average CtTarget-duplicate average CtPPIA})}$ for target gene mRNA, x axis denotes the genotypes, horizontal lines represent mean ± standard deviation of relative mRNA expression. $Kruskal-Wallis ANOVA/%One-way ANOVA p values < 0.05 are considered statistically significant. [Two columns fitting image].

Table 2
Correlations between relative expression levels of target mRNAs in patients and controls.

Target gene	Patients (n = 67)		Controls (n = 50)	
	r	p	r	p
ORMDL3-GSDMB	0.26	0.03	0.52	1×10^{-4}
ORMDL3-IKZF3	0.74	1×10^{-12}	0.67	1×10^{-7}
GSDMB-IKZF3	0.11	0.38	0.36	0.01

n – number of samples in group; r – Spearman rank correlation coefficient; p values < 0.05 are considered statistically significant.

RRMS phase and interferon β therapy status were used as independent factors. The results are presented in Supplement 1. In the patients group, by analyzing the impact of *IKZF3* genotypes and sex on *ORMDL3* mRNA expression levels, solely *IKZF3* genotypes exhibited a significant effect on *ORMDL3* expression (Univariate tests of significance p = 0.005), although significant between-effect of *IKZF3* genotypes and sex was also found (Univariate tests of significance p = 0.03). When analyzed the effects of *IKZF3* genotypes and RRMS phase, *GSDMB* expression was significantly affected by both *IKZF3* genotypes (Univariate tests of significance p = 0.01) and RRMS phase (Univariate tests of significance $p = 1 \times 10^{-4}$), but between-effect of genotypes and disease phase was not significant (Univariate tests of significance p = 0.29). In the remitting patients, analysis of the effects of *IKZF3* genotypes and interferon β therapy status on *GSDMB* mRNA expression levels showed that even though the between-effect of genotypes and therapy status was significant (Univariate tests of significance p = 0.006), only therapy status significantly affected the *GSDMB* expression (Univariate tests of significance $p = 4 \times 10^{-4}$). The analyzed factors had no significant effects on *IKZF3* mRNA expression levels in any of the studied groups (data not shown).

4. Discussion

Research during the last decade yielded evidence of MS susceptibility genetic loci, both HLA and non-HLA and additional epigenetic drivers of disease [3,5,8]. Further steps inevitably lead to attempts to understand deeper the causality of genetic variants and functional characterization of the significant variants. Multiple sclerosis is a complex disease that comprehends the pathological processes in the CNS and periphery and includes many relevant cell types. Hence, it implies diverse and complex research approaches. In addition, nongenetic risk factors such as Vitamin D levels and infectious agents are recognized to influence MS [2,4].

Consequently, the efforts to gain new knowledge related to molecular components in this incurable disease are still of great interest. The majority of genetic variants in the human genome reside in non-coding genomic regions. Thus, the first line of investigation of

Table 3
Correlations of target mRNAs relative expression levels with clinical and anthropometric parameters in patients and controls.

Target gene	Parameter	Patients (n = 67)		Controls (n = 50)	
		r	P	r	p
ORMDL3	EDSS	0.02	0.9	–	–
	MSSS	0.01	0.91	–	–
	Age at sampling	0.02	0.91	0.16	0.34
	Age at onset	−0.04	0.77	–	–
	Disease duration	−0.02	0.88	–	–
	Annual relapse rate	0.03	0.84	–	–
GSDMB	EDSS	−0.17	0.18	–	–
	MSSS	−0.07	0.61	–	–
	Age at sampling	−0.56	2×10^{-6}	−0.03	0.85
	Age at onset	−0.34	0.006	–	–
	Disease duration	−0.19	0.14	–	–
	Annual relapse rate	0.19	0.24	–	–
IKZF3	EDSS	0.26	0.04	–	–
	MSSS	0.09	0.49	–	–
	Age at sampling	0.19	0.13	0.18	0.27
	Age at onset	0.11	0.38	–	–
	Disease duration	0.13	0.32	–	–
	Annual relapse rate	−0.1	0.53	–	–

n – number of samples in group; EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; r – Spearman rank correlation coefficient; p values < 0.05 are considered statistically significant.

their functionality is the regulatory role. Previous results showed that approximately 60 % of causal genetic variants for autoimmune diseases are located at regulatory elements such as enhancers [26]. This study adds evidence of differential gene expression in MS of two genes that reside in locus recognized to bear susceptibility for MS and whose expression is affected by one of the previously proposed eQTLs.

We have found a significantly decreased expression of *ORMDL3* mRNA in the PBMC of MS patients compared to controls. Lowered expression of *ORMDL3* leads to a more prominent T cell activation in cell cultures [16]. Also, its lowered expression may lead to higher concentrations of immunologically relevant sphingolipids in serum, including sphingosine-1-phosphate, which is important for T cell transmigration [17,27,28]. ORMDL3 is a negative regulator of immunologically important cytokine IL2 production by $CD4^+$ T cells [29]. Recent results in mast cells suggest that the downregulation of *ORMDL3* expression has an impact on the upregulation of AKT and NF-κB signaling pathways and chemotaxis, which enhances local inflammation *in vivo* [30]. In addition, ORMDL3 is a negative regulator of exocytosis, chemotaxis, production of prostaglandins and their general proinflammatory activity in mast cells of mice [30], which are one of the blood-brain barrier permeability regulators important in the initiation and progression of MS and EAE [31]. Therefore, based on the stated evidence, we propose that changes in *ORMDL3* expression could influence the pathogenesis of MS by stimulating the activation of T cells and propagation of inflammation. The current result of the downregulation of *GSDMB* mRNA expression in MS patients aligns with a recent study where significantly lower levels of *GSDMB* mRNA were found in RRMS patients' PBMC compared to healthy controls [32]. It was noted that decreased *GSDMB* expression may affect MS exacerbation as GSDMB is a central molecule in the targeted NK and $CD8^+$ T cell-induced pyroptotic pathway [18]. Considering that the RR phase of the disease is primarily neuroinflammatory, we can suggest that both genes are involved in the inflammatory cascade of events in MS. Relative expression levels of the *ORMDL3* and *GSDMB* have positively correlated with each other in both patients and controls. The current study is not the first to demonstrate a correlation between these genes since it has been found in naive $CD4^+$ and $CD8^+$ T cells [29]. We ought to have such a result since at least one of the proposed regulators of their expression is shared between them. We hypothesized the eQTL effect of the *IKZF3* rs12946510 genotype in MS and confirmed it in the PBMCs of patients by showing lower levels of both *ORMDL3* and *GSDMB* relative mRNA expression in the risk TT genotype. *IKZF3* expression was not significantly affected by the genotypes, which aligns with previous similar results in the whole blood of patients with primary biliary cholangitis [10]. Recently, rs12946510 SNP was identified as a top-ranked eQTL for *ORMDL3* and *GSDMB* expression in the whole blood and spleen [10] and in innate and adaptive immune cell types, NK cells, B lymphocytes, $CD4^+$ and $CD8^+$ T cells, with the TT risk genotype being associated with lower expression [9]. Furthermore, the same study found experimental evidence of genotype-phenotype interaction in B lymphocytes [9]. Three distinct mechanisms that could explain how *IKZF3* rs12946510 may affect the expression of *ORMDL3* and *GSDMB* mRNA, which is in line with our results, have been proposed in recent literature. The rare T allele causes disruption of FOXO1 and MEF2A/C transcriptional activator binding sites and thus leads to a decrease in *ORMDL3* and *GSDMB* mRNA expression in leukocytes [10,11]. In addition, rs12946510 represents a methylation quantitative trait loci, as the T allele has been associated with higher levels of methylation at the cg18711369 and cg10909506 CpG sites, mediating the expression of both *ORMDL3* and *GSDMB* in $CD4^+$ T cells [33]. Lastly, the variant has been bioinformatically predicted to contribute to the nonsense-mediated decay of *GSDMB* mRNA [34]. Quite a new study confirmed the functionality of rs12946510 in T helper cell lines by showing the lower expression of *IKZF3* and *ORMDL3* genes and reduced cell activation in CRISPR/Cas9 edited cells bearing alternative genotypes [35]. Further, we have observed a significant between-effect of the *IKZF3* rs12946510 genotype and sex on *ORMDL3* mRNA expression in MS patients. It is noteworthy that *GSDMB* expression negatively correlated with age at disease onset of MS and with the current age of patients. Previous

transcriptome analysis detected association of higher *IKZF3* expression in blood with lower age at type 1 diabetes mellitus diagnosis and lower expression of *GSDMB* and *ORMDL3* in EBV-transformed lymphocytes but, none in the blood, with lower age at diagnosis [36]. Although we are unable to propose a specific molecular mechanism that would explain the observed sex and age-based differences in *ORMDL3* or *GSDMB* expression, it should be pointed out that sex and age-specific patterns of DNA methylation in the 17q12-21 region were discovered [37]. MS activity and progression are a constant priority in the research, as many underlying molecular mechanisms and interindividual variability are not clearly understood. Thus, we looked up potential differences related to relapse or remission state, and our results indicated higher expression levels of *GSDMB* in PBMC of relapsing MS patients compared to remitting. We suggest that it could be related to a characteristic proinflammatory cytokine profile exhibited during the relapsing phase of RRMS [38], as IFNγ has been found to regulate *GSDMB* expression [18] positively. The profile of *GSDMB* mRNA expression in the context of the rs12946510 variant stayed the same regardless of relapse or remission. A recent study showed that *IKZF3* expression in PBMCs is upregulated in relapsing RRMS patients compared to controls [39]. We found a positive correlation between *IKZF3* mRNA expression and EDSS, suggesting that *IKZF3* expression in PBMC could be associated with clinical manifestations of MS beyond relapses. A significant change of circular RNAs, which putatively induce the *IKZF3* expression, has been associated with MRI-indicated inflammatory activity [39]. Concerning interferon β treatment, we have associated higher *GSDMB* mRNA expression with positive therapy status and found a between-effect of interferon β therapy status and *IKZF3* rs12946510 genotype. This result is not unexpected, as a previous study detected significantly increased levels of *GSDMB* mRNA in $CD4^+$ T cells from systemic lupus erythematosus patients after treatment with interferon β *in vitro* [40]. All together, we detected and validated gene expression changes previously related to particular cell types in the peripheral blood compartment.

In the current study, decreased *ORMDL3* and *GSDMB* mRNA levels in PBMC have been associated with MS and the MS risk genotype of rs12946510. Furthermore, sex and rs12946510 have shown a between-effect on the expression of *ORMDL3* mRNA in patients, while interferon β therapy and rs12946510 interacted to affect *GSDMB* mRNA expression. The results presented should be interpreted with caution due to a limited sample size, which represents the main limitation of our study. We have partially circumvented the issue by applying Factorial ANOVA when analyzing more than one parameter affecting gene expression to avoid multiple comparisons. This solution does not entirely avoid the sample size limitation, and we propose further validation of the study results on a larger independent participant population, particularly of those concerning the effects of disease phase and therapy status on the expression of selected mRNAs. We may conclude that the presented study provides unique results, which permeate *ORMDL3* and *GSDMB* expression, *IKZF3* variant, disease phases, and interferon β therapy in the context of MS. The study results are also confirmatory or parallel to previous papers on the issue of selected gene expression, 17q12-21 chromosomal region variants, inflammation, autoimmunity, and MS. Despite the limitations, the current study may constitute a foundation for further 17q12-21 functional analyses concerning the pathogenesis of MS.

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CRediT authorship contribution statement

Milan Stefanović: Writing – original draft, Methodology, Investigation, Formal analysis. **Ljiljana Stojković:** Writing – review & editing, Writing – original draft, Formal analysis. **Ivan Životić:** Writing – review & editing, Methodology. **Evica Dinčić:** Supervision, Resources, Investigation. **Aleksandra Stanković:** Writing – review & editing, Supervision, Funding acquisition. **Maja Živković:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25033.

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