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Association of *ORMDL3* and *IKZF3* expression with the *IL2RA* rs2104286 risk variant in relapsing-remitting multiple sclerosis

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Abstract: The genetic variant *IL2RA* rs2104286 (A>G) is strongly associated with multiple sclerosis (MS) risk. It modulates IL-2 signaling, thereby influencing the expression of lymphocyte function-related genes *ORMDL3* and *IKZF3* that are implicated in multiple sclerosis pathogenesis. This study aimed to investigate the association of rs2104286 with *ORMDL3* and *IKZF3* mRNA expression in peripheral blood mononuclear cells (PBMCs) of relapsing-remitting multiple sclerosis (RRMS) patients and controls. The study included 66 RRMS patients and 45 healthy controls from the Serbian population. Genotyping and gene expression analysis were performed using qPCR. In RRMS patients, increased ORMDL3 and IKZF3 mRNA expression was associated with the MS-protective G allele (*ORMDL3*: P = 0.014, 1.35-fold increase; *IKZF3*: P = 0.023, 1.38-fold increase). In controls, decreased expression of *IKZF3* was associated with the G allele (P=0.038, 0.72-fold change). Sex, disease phase, interferon β therapy, and the *ORMDL3* and *IKZF3* expression quantitative trait locus (rs12946510) did not influence the association between target gene expression and rs2104286. The overall results suggest a disease-specific association between the *IL2RA* rs2104286 variant and *ORMDL3* and *IKZF3* expression. Our findings support the notion that the rs2104286 variant may modulate IL-2 signaling in PBMCs, thereby influencing the expression of the immune-relevant genes *ORMDL3* and *IKZF3*.

Keywords: multiple sclerosis, *IL2RA*, rs2104286, *ORMDL3*, *IKZF3*

Abbreviations: MS – multiple sclerosis; RRMS – relapsing-remitting multiple sclerosis; IL2RA – interleukin 2 receptor alpha; ORMDL3 – orosomucoid-like 3; IKZF3 – IKAROS family zinc finger 3; EDSS – expanded disability status scale; MSSS – multiple sclerosis severity score; IFN-β – interferon β-1; SNV – single nucleotide variant; eQTL – quantitative trait locus expression; PBMC – peripheral blood mononuclear cells; MHC – major histocompatibility complex; STAT5 – signal transducer and activator of transcription 5; IMSGC – International Multiple Sclerosis Genetic Consortium

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune neurological disorder characterized by neuroinflammation, infiltration of adaptive immune system components, particularly T cells, into the central nervous system (CNS), demyelination, and consequent neurodegeneration [1]. The etiology of MS is complex and demonstrates obvious genetic susceptibility [2,3]. More than 200 non-MHC (major histocompatibility complex) autosomal loci have been identified as associated with risk of MS development by the International Multiple Sclerosis Genetic Consortium (IMSGC) [4]. The *IL2RA* single-nucleotide variant (SNV) rs2104286 (A>G) is among the most consistently and widely associated variants, with the rare (alternative) G allele conferring a protective effect against MS development [5-8]. rs2104286 is also the top candidate causal SNV for multiple sclerosis risk within the signal transducer and activator of transcription 5 (STAT5) super-enhancer region located at chromosome 10p15.1 [9]. SNV likely influences MS risk by attenuating IL-2 signaling through its effect on *IL2RA* expression [10-12]. The influence

of rs2104286 on IL-2 signaling is particularly prominent in CD4+ CD25+ T regulatory cells [10]. Studies have suggested that the presence of the soluble protein form of IL2RA (sCD25), a marker of T cell activity [13], may negatively influence the dynamics of IL-2 signal propagation in T cells [10,14]. Although IL2RA rs2104286 is clearly associated with MS, relatively few studies have examined its effects on immune-relevant genes beyond IL2RA [15,16].

To better understand the mechanisms underlying the strong association between rs2104286 and MS, it is important to elucidate how alterations in IL-2 signaling affect the downstream expression of MS-relevant genes. In mouse CD4+ T cells, *in vitro* stimulation with IL-2 caused mRNA downregulation of orosomucoid-like 3 (*Ormdl3*) and IKAROS family zinc finger 3 (*Ikzf3*) [17]. The human homologues of these two genes are located in the 17q12-21 chromosomal region. Variants in this region have been associated with MS risk [4,7]. In our previous study, reduced *ORMDL3* mRNA expression was associated with relapsing-remitting multiple sclerosis (RRMS), and we found a positive correlation between *IKZF3* mRNA expression and disability [18].

ORMDL3 plays important roles in adaptive immunity by regulating Ca^{2+} homeostasis in T cells, thereby influencing T-cell activation dynamics [19], and by suppressing ceramide synthesis [20]. Ceramide is a precursor of sphingosine-1-phosphate [21], a lipid essential for lymphocyte migration into non-lymphoid tissues [22]. The IKZF3 gene encodes the Aiolos protein, a key regulator of T-cell apoptosis [23,24] and of the functional maturation and suppressive activity of $CD4^+$ $FoxP3^+$ regulatory T cells [25]. *IKZF3* has been bioinformatically implicated in neuroinflammation in MS, as increased expression of circular RNAs in peripheral blood mononuclear cells (PBMCs) of MS patients, which act as molecular sponges for IKZF3-targeting miRNAs, was associated with relapses and inflammatory CNS lesions [26]. Both ORMDL3 and Aiolos can suppress IL-2 expression in activated T cells [19,25,27]. The 17q12-21 SNV rs12946510, which has been previously associated with MS risk [7], is an expression quantitative trait locus (eQTL) of *ORMDL3* and *IKZF3* in immune system-relevant cells and tissues [18,28,29].

Research suggests a complex but partially explained association between lymphocyte function genes *ORMDL3*, *IKZF3*, and the *IL2RA* rs2104286 variant in multiple sclerosis, with nuanced genetic and expression interactions. We found that the *IL2RA* rs2104286 G allele carriers had a lower risk for developing MS in the Serbian population [8]. We demonstrated that decreased *ORMDL3* mRNA levels were associated with MS and that *IKZF3* expression correlates with a disability parameter in MS [18]. In another study, it was noted that the *IL2RA* rs2104286 had a minor effect on gene expression, with some evidence of negative regulation of CD8+ T cell responses [16]. Despite these intriguing links, a direct and comprehensive association among all three genes remains insufficiently characterized and warrants further investigation.

This study aimed to investigate the association between *ORMDL3* and *IKZF3* expression and the *IL2RA* rs2104286 variant in PBMCs from RRMS patients and healthy controls, while accounting for potential effects of disease phase, interferon β therapy, sex, and the 17q12-21 rs12946510 SNV. We also aimed to examine sCD25 concentrations in peripheral blood plasma in relation to rs2104286, MS status, and clinical parameters, as well as *ORMDL3* and *IKZF3* mRNA expression.

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 population

Patients were recruited, and their clinical data and blood samples were collected, at the Clinic for Neurology of the Military Medical Academy (MMA), Belgrade, Serbia, between 2010 and 2022, where they were treated for MS. The patients were diagnosed according to the

revised McDonald criteria [30,31], and the clinical course of disease was defined [32]. Patients on therapy included in this study received 0.25 mg of Betaferon® (Bayer AG, Leverkusen, Germany) every other day for at least 6 months before blood sampling. Blood samples were drawn from relapsing patients before the initiation of acute treatment for relapse. The level of neurological disability at blood drawing was assessed according to the Expanded Disability Status Scale (EDSS) by a trained neurologist [33]. Disease severity was quantified with the Multiple Sclerosis Severity Score (MSSS), which corrects EDSS for disease duration [34]. Questionnaires about disease duration, onset age, number of relapses, and EDSS were completed by trained clinicians at the MMA. Patients with chronic diseases other than MS and those with MS receiving therapies other than Interferon β-1b were excluded. The control group consisted of healthy volunteers recruited from MMA or Institute of Nuclear Sciences "Vinča" employees. A higher proportion of women was deliberately enrolled in the control group to match the sex-specific prevalence of MS.

Genotyping

DNA was extracted from 200 μL of frozen blood, stored in vacutainers with EDTA as an anticoagulation agent, using the Zymo Research Quick-DNA™ Miniprep Plus Kit according to the manufacturer's protocol (Zymo Research, Irvine, California, USA). DNA quantity and quality were assessed using a spectrophotometric method (NanoDrop 1000, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA), and samples were kept at -20°C until genotyping. Genotyping was performed by TaqMan® allelic discrimination using the following assays (Applied Biosystems, Foster City, CA, USA): C__16095542_10 for *IL2RA* rs2104286 and C__31651862_10 for *IKZF3* rs12946510. qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System using SDS software v1.4.0 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocols. Only samples with allele-calling quality >95% were included in downstream analyses.

Relative quantification of target mRNA expression

PMBCs were extracted from 3 mL of freshly drawn blood (the blood was collected in EDTA-containing vacutainers and extraction started within 30 min of sample collection) using a lymphocyte separation medium (Lymphocyte Separation Medium PAA, GE Healthcare, Chicago, Illinois, USA). After PBMC separation, total RNA was extracted from the PBMC with a TRI reagent-based protocol (TRI Reagent, Ambion, Austin, Texas, USA). RNA quantity and quality were analyzed using the spectrophotometric method (NanoDrop 1000, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). The RNA samples were stored at -80°C. Five hundred ng of total RNA entered the DNA digestion and reverse transcription experiments. Excess DNA digestion was performed with DNase I digestion (DNase I, RNase-free (1 U/μL), Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). After digestion, cDNA synthesis from poly(A) RNA was performed using an oligo(dT) primer ($Oligo(dT)_{18}$ Primer, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the Thermo Scientific™ RevertAid RT Reverse Transcription Kit, according to the manufacturer's protocol. TaqMan®-based quantitative real-time PCR was applied for relative quantification of mRNA expression levels. qPCR was performed in duplicate for every sample on the Applied Biosystems 7500 RT-PCR System with the following assays: Hs00918021_m1 for *ORMDL3*, Hs00232635_m1 for *IKZF3*, and Hs99999904_m1 for the internal reference control, *PPIA* (Applied Biosystems, Foster City, California, USA). Cycle threshold (Ct) values were determined using SDS v1.4.0 software (Applied Biosystems, Foster City, CA, USA), and relative target mRNA expression was calculated for each sample using the 2-ΔCt method (ΔCt = mean Ct_target – mean Ct_PPIA). Technical duplicates were used for the qPCR experiment, as this approach provides greater resource efficiency than experimenting with technical triplicates.

Quantification of plasma protein

Within 30 min of blood collection, EDTA vacutainers containing fresh whole blood were centrifuged at 1,000 × g at 4°C for 15 min to obtain plasma. Plasma samples were stored at -80°C. Concentrations of plasma

Table 1. Demographic and clinical characteristics of the mRNA expression study group

	Patients (n=66)	Controls (n=45)	P	Relapsing (n=15)	Remitting (n=51)	P	IFN-β- (n=17)	IFN-β+ (n=34)	P
Female/Male	36/30	28/17	0.422[$]	9/6	27/24	0.629[$]	11/6	16/18	0.234[$]
Age at sample collection (years)	38.67±9.67	36.26±10.15	0.127[&]	32.5±7.98	39.88±9.69	0.039[#]	41.29±7.26	39.18±10.73	0.468[#]
Age at disease onset (years)	32.5±9.58			28.83±8.43	33.27±9.7	0.141[#]	33.65±7.11	33.24±10.86	0.888[#]
Disease duration (years)	6.16±4.42			4.67±3.31	6.51±4.6	0.255[&]	7.65±5.63	5.94±3.95	0.712[&]
EDSS	2.56±1.23			2.29±1.44	2.62±1.18	0.233[&]	2.71±1.28	2.58±1.15	0.766[&]
MSSS	4.39±2.17			4.12±2.19	4.45±2.18	0.638[#]	4.44±2.68	4.46±1.92	0.976[#]

EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; IFN-β- – remitting patients not receiving disease-modifying therapy; IFN-β+ – remitting patients receiving interferon β-1 therapy; [$]Pearson χ^2 test; [#]Student's T test; [&]Mann-Whitney U test; continual values are presented as mean ± standard deviation; P values less than 0.05 are considered statistically significant.

sCD25 were obtained with the "sandwich" ELISA method. ELISA was performed using the FineTest® Human CD25 (Interleukin 2 Receptor Alpha) ELISA Kit (Wuhan Fine Biotech, Wuhan, PRC), according to the manufacturer's protocol, on 4x diluted plasma samples. Plate optical density was measured using a PerkinElmer Wallace 1420 Victor² microplate reader (PerkinElmer, Waltham, MA, USA). Concentrations were calculated from optical density values using four-parameter logistic regression with the MyAssays online data analysis tool (four-parameter logistic curve).

Statistical analysis

Statistical analysis was performed using the Statistica v14.2.0 software package (Spotfire Statistica Desktop, StatSoft GmbH, Hamburg, Germany). Comparison of categorical variables between groups was done using the χ^2 test. Normality of continual variable distribution was assessed with the Shapiro-Wilk test. Differences in continuous variable distribution between groups were tested with the Student's T or Mann-Whitney U test, depending on the variable distributions. Pearson's or Spearman's rank correlation coefficients were used as a measure of correlation between continuous variables, depending on the variable distributions. Estimation of the interactive effects of individual independent categorical variables on a continuous variable of interest was performed with factorial ANOVA. Values are presented as the mean±standard deviation, regardless of the normality of variable distribution. P values lower than 0.05 were considered statistically significant. The GraphPad Prism v9.0 (GraphPad Software, Inc., San Diego, California, USA) software was used for graphical representation of the results. Post-hoc power analysis was performed with the Post-hoc Power Calculator (https://clincalc.com/stats/Power.aspx).

RESULTS

Study population characteristics

The demographic and clinical characteristics of the mRNA expression study group are presented in Table 1. The study group for mRNA expression analysis consisted of 66 unrelated RRMS patients and 45 unrelated healthy volunteers without a familial history of MS. At the time of blood sampling, 15 patients were in the relapsing phase, and 51 were in remission. In the remitting group, 17 patients were not receiving disease-modifying therapy, whereas 34 were treated with interferon β-1b. For sCD25 analysis, the study cohort comprised 45 remitting patients (30 receiving interferon β-1b and 15 treatment-naïve) and 17 controls. There were no significant differences in female-to-male (f/m) ratio or age between the compared groups, except that relapsing patients were significantly younger than those in remission. This is because a proportion of relapsing patients were recruited immediately after diagnosis, which affected the age structure in this group. The clinical parameters, including age at disease onset, disease duration, EDSS, and MSSS, were not significantly different between the groups (Table 1). Plasma sCD25 concentrations were analyzed in a smaller subset comprising controls (n = 17; f/m = 12/5) and

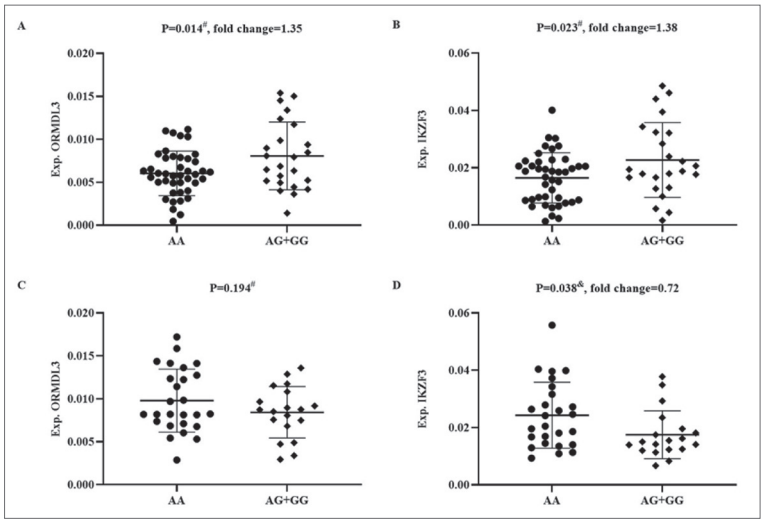

Fig. 1. Relative mRNA expression levels of *ORMDL3* and *IKZF3* in peripheral blood mononuclear cells according to the dominant genetic model of *IL2RA* rs2104286. **A** – Relative expression levels of *ORMDL3* in the RRMS study group. **B** – Relative expression levels of *IKZF3* in the RRMS study group. **C** – Relative expression levels of *ORMDL3* in the healthy control study group. **D** – Relative expression levels of *IKZF3* in the healthy control study group. RRMS – Relapsing-Remitting Multiple Sclerosis; #Student's T test; &Mann-Whitney U test; horizontal lines represent the mean ± standard deviation; P values <0.05 are considered statistically significant.

remitting RRMS patients (n = 45; f/m = 28/17), including treatment-naïve patients (n = 15; f/m = 10/5) and interferon β-1-treated patients (n = 30; f/m = 18/12). There were no significant differences in demographic or clinical characteristics between the sCD25 analysis subgroup and the overall study cohort.

Differences in *ORMDL3* and *IKZF3* mRNA expression levels between *IL2RA* rs2104286 genotypes in RRMS patients and healthy controls

The association between PBMC mRNA expression levels and *IL2RA* rs2104286 was analyzed according to the dominant genetic model (AA *vs*. AG+GG) to counteract the limited number of participants with the rare GG homozygote (genotype frequencies were as follows: Patients: AA=63.6%, AG+GG=36.4%; Controls: AA=57.78%, AG+GG=42.22%). Although the G allele was more common in the healthy control group than in the patient group, the difference was not statistically significant (P=0.534). In the patient group, higher levels of *ORMDL3* and *IKZF3* expression in PBMCs were associated with the genotypes that carry the G allele (*ORMDL3*: AA=0.006±0.0026, AG+GG=0.0081±0.0039, P=0.014 (Fig. 1A); *IKZF3*: AA=0.0165±0.0087, AG+GG=0.0227±0.013, P=0.023, (Fig. 1B). In the control group, lower levels of *IKZF3* expression were associated with the G allele genotypes (AA=0.0243±0.0115, AG+GG=0.0175±0.0084, P=0.038 (Fig. 1D), and no significant difference in *ORMDL3* expression (AA=0.0098±0.0037, AG+GG=0.0084±0.003, P=0.194 (Fig. 1C)). Post hoc power (P<0.05) was 61.5% for *ORMDL3* expression and 55.3% for *IKZF3* expression in the patient group, and 63.0% for *IKZF3* expression in the control group.

Interactive effects of sex, disease phase, therapy status, and rs12946510 with *IL2RA* rs2104286 on *ORMDL3* and *IKZF3* relative expression levels

We aimed to analyze the effects of factors combined with rs2104286 on target mRNA expression. The *ORMDL3* and *IKZF3* expression levels were used as response variables; sex (female/male), disease phase (relapse/remission), therapy status (interferon β therapy/no), and *IKZF3* rs12946510 and *IL2RA* rs2104286 genotypes were independent factors. No interaction effects of the tested factors on *ORMDL3* or *IKZF3* expression were observed in either patients or controls (Supplementary Table S1).

Plasma sCD25 levels in relation to *IL2RA* rs2104286, MS status, clinical and demographic parameters, and *ORMDL3* and *IKZF3* mRNA expression

We found no significant differences in plasma sCD25 concentration (pg/mL) between RRMS patients (in remission) and healthy controls (RRMS: n=45, 874.11±403.64, Controls: n=17, 826.15±439.21, P=0.685, (Fig. 2A)). In the patients, no significant difference in sCD25 concentration between interferon β-receiving and non-receiving subgroups was detected (interferon β non-receiving patients: n=15, 769.3±390.1, interferon-β receiving patients: n=30, 926.51±406.46, P=0.222, (Fig. 2B)). There were no

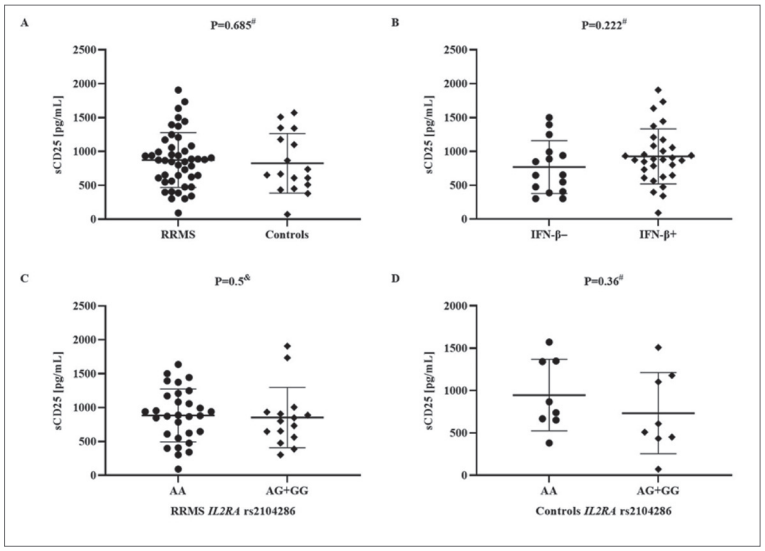

Fig. 2. The association of sCD25 plasma concentration with RRMS, interferon β therapy, or the dominant genetic model of *IL2RA* rs2104286. **A** – Association with RRMS. **B** – Association with Interferon β therapy in remitting patients. **C** – Association with *IL2RA* rs2104286 in the RRMS patient group. **D** – Association with *IL2RA* rs2104286 in the healthy control group. RRMS – Relapsing-Remitting Multiple Sclerosis; IFN-β– – remitting patients not receiving disease-modifying therapy; IFN-β+ – remitting patients receiving Interferon β-1 therapy; #Student's T test; &Mann-Whitney U test; Horizontal lines represent the mean ± standard deviation; P values <0.05 are considered statistically significant.

significant differences in plasma sCD25 concentrations related to the *IL2RA* rs2104286 dominant model in either the patient group (AA=66.67%, 884.84±389.04, AG+GG=33.33%, 852.63±444.74, P=0.5, Fig. 2C) or control group (AA=50%, 946.64±421.89, AG+GG=50%, 732.83±479.21, P=0.36, (Fig. 2D)).

No significant correlations were detected between sCD25 concentrations and *ORMDL3* or *IKZF3* mRNA expression levels in either patients or controls. The concentration of sCD25 did not correlate with EDSS, MSSS, disease duration, or age at sample collection. The correlation results are presented in Supplementary Table S2.

DISCUSSION

In the present study, we investigated the association between *IL2RA* rs2104286 and *ORMDL3* and *IKZF3* gene expression. We found that the MS-protective G allele of the rs2104286 variant was associated with higher mRNA expression of *ORMDL3* and *IKZF3* in RRMS patients. However, in the control group, the G allele was associated with lower PBMC mRNA levels of *IKZF3*, while there was no association with *ORMDL3* expression. The association between rs2104286 and *ORMDL3* and *IKZF3* expression was independent of sex, disease phase, interferon β therapy, and the eQTL rs12946510.

A previous study did not establish differences in IL-2 response between G allele carriers and AA homozygotes in CD4+ $CD25^{hi}$ T cells from MS patients [10]. The detected higher expression levels of *ORMDL3* and *IKZF3*, associated with the AG and GG genotypes, are in line with the notion that the G allele is protective in MS, as both *ORMDL3* and *IKZF3* can act as negative regulators of T cell activation [19,25]. We propose that one of the mechanisms behind the protective role of the *IL2RA* rs2104286 G allele may be the suppression of effector T cell activation via an increase in expression of *ORMDL3* and *IKZF3*. Further functional studies are required to verify this hypothesis.

There was an association between decreased *IKZF3* expression and the *IL2RA* rs2104286 G allele genotypes, and a trend of lower *ORMDL3* mRNA expression in the PBMCs of healthy controls. These results are in line with previously published data where the G allele was associated with higher levels of IL-2 signaling in *ex vivo* CD4+ FoxP3+ CD25+ Treg cells from healthy individuals [10] and IL-2 treatment caused lower expression of *Ormdl3* and *Ikzf3* in CD4+ T cells *in vitro* [17]. These results reveal a trend of reduced expression of IL-2-regulated genes in healthy individuals carrying the *IL2RA* rs2104286 G allele; further research is needed to clarify the findings.

Our results point to disease-specific cellular mechanisms in PBMCs related to the target molecular components. Similarly, the cis-eQTL rs12946510 shows a disease-dependent genotype-phenotype interaction for *ORMDL3* expression in peripheral blood B cells, with expression changes associated with the SNV in MS patients but not in healthy controls [28]. The effect of *IL2RA* rs2104286 on IL-2 signaling in CD4+ CD25+

T cells points to a difference between MS patients and healthy controls [10], and we suggest that the observed association differences may be due to disease-specific changes in the impact of rs2104286 on IL-2 signaling.

Factorial ANOVA revealed no interaction effects between *IL2RA* rs2104286 and the examined factors (sex, disease phase, therapy status, or the *ORMDL3* and *IKZF3* eQTL rs12946510) on relative *ORMDL3* or *IKZF3* expression levels in either RRMS patients or controls. Our previous study revealed no association between *IKZF3* expression and rs12946510 [18]. The absence of interaction effects suggests that rs2104286 influences *ORMDL3* and *IKZF3* expression independently of the examined factors. Nevertheless, post hoc power analysis identified limited sample size as the main methodological limitation of this study; therefore, the results should be interpreted with caution.

We observed no significant associations between plasma sCD25 concentrations and MS status or the assessed clinical parameters (EDSS and MSSS). Previous studies in different populations associated the increased circulatory concentration of sCD25 with MS [14,35]; however, this finding was not entirely consistent [15]. Publications in which the association was established analyzed the serum levels of sCD25 [14,35], while our study and that of Buhelt et al. [15] analyzed sCD25 in plasma. The association between sCD25 and disease severity remains controversial, with some studies reporting a link and others finding none [14,35]. The sCD25 analysis was restricted to patients in remission to avoid the potential impact of relapse on sCD25 levels, as higher serum sCD25 concentrations have been reported in relapsing patients compared with those in remission [36].

Plasma levels of sCD25 have not been associated with interferon β therapy status in our patients. Another study demonstrated that RRMS patients receiving interferon β-1 therapy had a transient change in CD4+ T cell surface expression of CD25 followed by normalization to pre-treatment levels after 6 months of therapy [37]. Given that our patients had received interferon β therapy for at least six months before blood sampling, and that sCD25 is generated by shedding of cell-surface CD25 [38], the absence of an association may reflect normalization following prolonged interferon β treatment.

Previous studies have linked lower peripheral blood sCD25 concentrations to the *IL2RA* rs2104286 G allele in both MS patients and healthy controls [6,15]. Although mean sCD25 levels were also lower in G-allele carriers in our cohort, this difference was not statistically significant, underscoring the influence of population characteristics and sample size on the interpretation of these findings.

The results of this study should be interpreted with caution, particularly those concerning plasma sCD25 associations, owing to limited statistical power. Although study power did not reach 80%, we emphasize the relevance of the presented findings in light of the biological roles of the target genes in mechanisms implicated in MS pathogenesis. We addressed the effects of potential clinical and demographic confounders. We investigated mRNA expression in PBMCs without separating them into specific cell subtypes, which may affect genotype-gene expression interaction [28] and must be considered as one of the study's limitations. Because *ORMDL3* and *IKZF3* are expressed at relatively high levels in all major lymphocyte subpopulations compared with monocytes or dendritic cells [39], and lymphocytes constitute the majority of PBMCs [40,41], the observed gene expression changes likely originate predominantly from lymphocytes. Accordingly, these genes may represent potential contributors to the multifaceted effects of *IL2RA* rs2104286 on lymphocyte function [10,12,16,36]. We therefore propose further studies with larger sample sizes and functional approaches to better elucidate factors influencing IL-2 signaling in MS. Despite the noted limitations, the present findings provide a basis for future investigations into MS pathogenesis and progression.

CONCLUSION

The central findings of this observational study indicate that *ORMDL3* and *IKZF3* mRNA expression levels in PBMCs are associated with the *IL2RA* rs2104286 variant in MS patients. Because *ORMDL3* and *IKZF3* expression is influenced by IL-2 signaling and rs2104286 affects IL-2 signal propagation, our findings support the hypothesis that this variant modulates the expression of immune-relevant genes through alterations in IL-2 signaling in immune cells. The results contribute to a better understanding of rs2104286 impact on MS

risk, especially regarding its effects on the expression of immune-relevant genes other than *IL2RA*, for which published data remain limited. The present findings further support the link between IL-2 signaling and *ORMDL3* and *IKZF3*, emphasizing their roles in immune-mediated diseases, including MS.

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Conflict of interest disclosure: The authors have nothing to declare.

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_15883; URI: https://vinar.vin.bg.ac.rs/handle/123456789/15883. Personal and clinical data are not openly available due to privacy concerns for the study participants. Personal and clinical data can be shared upon reasonable request.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Factorial ANOVA analyzing the in-between effect of *IL2RA* rs2104286 genotype and the factors of sex, disease phase, interferon β therapy, and rs12946510 genotype, on the expression of *ORMDL3* and *IKZF3* mRNAs in peripheral blood mononuclear cells.

	Exp. of *ORMDL3*		**Exp. of *IKZF3***	
Factors	**F**	**P**	**F**	**P**
Sex * *IL2RA* rs2104286 (HC)	0.704	0.406	0.909	0.346
Sex * *IL2RA* rs2104286 (RRMS)	0.356	0.553	0.817	0.369
RRMS phase * *IL2RA* rs2104286	0.859	0.358	0.495	0.484
Interferon β therapy * *IL2RA* rs2104286	3.544	0.066	0.538	0.467
IKZF3 rs12946510 * *IL2RA* rs2104286 (HC)	2.255	0.118	1.353	0.27
IKZF3 rs12946510 * *IL2RA* rs2104286 (RRMS)	0.579	0.564	0.743	0.48

Exp. of *ORMDL3* – relative levels of mRNA expression in peripheral blood mononuclear cells for *ORMDL3*; Exp. of *IKZF3* – relative levels of mRNA expression in peripheral blood mononuclear cells for *IKZF3*; HC – healthy controls; RRMS – Relapsing-Remitting Multiple Sclerosis; F – statistical measure used to test the significance of the effects of independent variables interactions on a dependent variable; P values less than 0.05 are considered statistically significant.

Supplementary Table S2. Correlations of sCD25 plasma concentration with the clinical, demographic parameters, and peripheral blood mononuclear cell mRNA expression levels of *ORMDL3* and *IKZF3*.

Parameters correlated with sCD25 concentration	**RRMS patients**		**Healthy controls**	
	r	**P**	**r**	**P**
EDSS	-0.049	0.754[&]		
MSSS	-0.027	0.863[#]		
Disease duration (years)	0.015	0.923[&]		
Age at sample collection (years)	-0.024	0.877[#]	-0.397	0.115[&]
ORMDL3 mRNA expression	-0.139	0.455[&]	-0.207	0.497[#]
IKZF3 mRNA expression	0.002	0.993[#]	0.026	0.932[#]

r – correlation coefficient; RRMS – Relapsing-Remitting Multiple Sclerosis; EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; [&] Spearman's rank R correlations; [#]Pearson's correlation coefficient; P values <0.05 are considered statistically significant.