Abstract. Background: Multiple sclerosis (MS) is one of the most debilitating neurological diseases of young adults. The presence of a single nucleotide polymorphism in the promoter regions of the interleukin 27 gene (IL27 T4730C, rs181206) may alter the transcription and the production of cytokine levels, leading to MS. Patients and Methods: We performed a case–control study including 82 individuals: 51 patients diagnosed with MS and 31 healthy controls. Polymerase chain reaction-restriction fragment length polymorphism was used in order to determine the genotypes for the IL27 T4730C polymorphism and enzyme-linked immunosorbent assay to measure the serum IL27 level. Results: Carriers of the T4730C polymorphism were found to have a 6-fold [95% confidence interval (CI)=1.83-19.63, p=0.002] increased risk for MS. Univariate logistic regression analysis showed an increased frequency of the TC4730 heterozygous genotype (39.2% vs. 9.7%) and also of the C4730 allele (27.45% vs. 8.06) in patients compared to controls, with a 6.02-fold increased risk (95% CI=1.61-22.46, p=0.006) and a 4.31-fold increased risk (95% CI=1.57-11.87, p=0.002) of developing MS. IL27 levels were significantly lower in patients compared to controls (12.35 versus 14.34 pg/ml, p=0.039), without significant differences between genotypes. Multivariate logistic analysis showed that IL27 T4730C polymorphism (odds ratio=6.272, 95% CI=1.84-21.40, p=0.003) and smoking (odds ratio=4.214, 95% CI=1.39-12.74, p=0.011) represented independent risk factors for MS. Conclusion: Our study provides a possible link between IL27 level and IL27 T4730C gene polymorphism and the risk for developing MS in a Romanian population.
previous studies suggested that genes encoding cytokines involved in the activation and survival of T-helper 17 (Th17) cells may also contribute to MS predisposition and susceptibility, enhancing the destruction of myelin or modulating its repair (7). Cytokines and their pleiotropic effects were studied for a better understanding of MS pathogenesis. Exploring the connection between cytokines and their genetic polymorphisms leads to the observation that a single nucleotide polymorphism (SNP) in the promoter region of a cytokine gene may influence the secretory function of immune cells (8).

Different studies evaluating autoimmune diseases, including MS, reported the implication of interleukin 27 (IL27) in immunopathogenesis. IL27 is a novel member of the IL12 family, known for both its pro- and anti-inflammatory functions, with distinct roles in shaping the activity of T-cells. IL27 is a heterodimer composed of Epstein-Barr-induced gene 3 product (EBI3) and IL27p28 (9). IL27 regulates the immune response through its heterodimeric IL27Ra/GP130 receptor, activating multiple signaling cascades, including signal transducer and activator of transcription 1 (STAT1) and 3 (STAT3) (9). The expression of IL27 receptors on the surface of distinct cells, such as uterine natural killer (NK) cells, placental trophoblasts, microglia, endothelial cells and plasma cells, reinforces the important role of IL27 in maintaining a well-balanced immune status in fragile immune environments such as the brain and uterus (10). The activity of T-cells is directly influenced by IL27 (11, 12). In chronic inflammation through immune down-modulation, IL27 is essential in preventing tissue injuries and organ dysfunction (11). Elevated levels of IL27 were found in the synovial fluids of patients with rheumatoid arthritis (RA), in the colonic mucosa of patients with Crohn’s disease, in the serum of patients with psoriasis, and in patients with MS following treatment with interferon (11). In the brain, IL27 produced by astrocytes and microglial cells is recognized for its neuroprotective effects, enhancing the production of nerve growth factor and neurotrophic factor, promoting proliferation of T-cells, and modulating its repair (7). Cytokines and their pleiotropic effects were studied for a better understanding of MS pathogenesis. Exploring the connection between cytokines and their genetic polymorphisms leads to the observation that a single nucleotide polymorphism (SNP) in the promoter region of a cytokine gene may influence the secretory function of immune cells (8).

Aim of the study. We investigated the influence of T4730C (rs181206) polymorphism and serum IL27 levels on susceptibility to MS. A second objective was to establish the existence of a relationship between the genetic polymorphism and serum IL27 levels in patients MS and controls in order to reveal a possible new biomarker for disease diagnosis and prognosis.

Patients and Methods

Patients and controls. Fifty-one patients with MS, 30 (58.5%) females and 21 (41.17%) males (mean age=34.71±10.31 years), and 31 controls without a personal or familial history of MS, 20 (64.5%) females and 11 (35.48%) males (mean age=35.13±11.21 years) were included in our study between March 2019 and January 2020. The patients were recruited from the National Program of MS of the Neurology Clinic, Cluj-Napoca, Romania. The MS diagnosis was based on clinical and radiological findings, according to the 2017 Mc Donald criteria (22). The information was obtained from neurological examinations and personal interviews. Data about radiological investigations were obtained from patients’ files.

The inclusion criteria were: Diagnosis of MS, at the beginning of the disease, without previous treatment for MS. The exclusion criteria were: Other autoimmune diseases such as SLE, RA, ankylosing spondylitis, inflammatory bowel diseases, psoriasis, type 1 diabetes mellitus; clinical relapse at the time of evaluation, use of cortisone in the previous month, recent use of other immunomodulatory therapies.

Both groups shared a common geographical area, and had the same ethnicity and socioeconomic status.

Ethics statement. The study was performed in conformity with the principles of the Declaration of Helsinki. The study was approved by the Research Ethics Committee of the Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (Protocol Code 31, 25.02.20219). All patients were informed about the aim of the study and signed an informed consent form.

Sampling and DNA extraction. Before initiating any immunomodulatory treatment, 10 ml of peripheral blood were collected through venipuncture in EDTA anticoagulated tubes for both patients with MS and controls. High molecular weight DNA was isolated using a Zymoresearch kit (Quick-DNAMiniprep, Kit-Zymo Research Corporation, Freiburg, Germany). The probes were stored at −20°C until polymerase chain reaction (PCR) analysis was carried out.

PCR-restriction fragment length polymorphism analysis for IL27-T4730C polymorphism. The IL27-T4730C polymorphism was identified using the method described by Anaraki Mohammad et al. (16).

Twenty nanograms of genomic DNA were amplified in 25 μl mixture containing 200 μM dNTPs (dATP, dGTP, dCTP, dTTP), 2.0 mM MgCl2, 0.2 μM primers (forward primer and reverse primer; Kaneka Eurogentec S.A. Biologics Division, Liege, Belgium), 0.65 U Taq DNA polymerase [Taq buffer, 20 mM Tris-HCl (pH 8.0), 1
For each probe, 100 μl biotinylated detection antibody-specific substrate reagent was used. The reaction was stopped using 50 μl stop solution. The optical density at 450 nm was then measured spectrophotometrically using a microplate reader (Absorbance Microplate Reader Sunrise Tican; Tecan Group Ltd., Männedorf, Switzerland) and Biochrom Asys Atlantis Microplate Washer (Biochrom Ltd. Cambridge, UK). The serum IL27 level was measured using standard curves in which the optical density was proportional to the concentration of human IL27 (sensitivity=18.75 pg/ml).

**Statistical analysis.** Statistical analysis was performed using SPSS software, version 25.0 (IBM, Armonk, NY, USA). The normality of the distribution of quantitative data was verified with the Shapiro-Wilk or Kolmogorov-Smirnov test. The significance level was α=0.05. The mean±standard deviation was used to describe quantitative data with normal distribution and the median and interquartile range (IQR) for data that did not. Qualitative data were expressed numerically and as percentages. According to the data distribution, Student’s t-test or nonparametric Mann-Whitney test was used to compare the means of two independent groups. Fisher’s exact probability test or the chi-square test was used to evaluate differences in genotypic and allelic frequencies between the examined groups. The strength of the association between categorical variables was expressed as odds ratios (OR) with 95% confidence intervals (95% CI). The association between IL27 gene polymorphisms and MS risk was estimated by computing ORs and 95% CIs from a multivariate logistic regression analysis.

We analyzed the sample size using the Gpower program (23) which indicated that for a total of 40 random participants in the patient group and 20 random participants in the control group, a medium-size effect (0.5) with α = 0.05 and a power of 0.8 was calculated. In order to have a statistically significant power, we used a greater number of participants in both groups. Therefore, the statistical power for all of the studied variants was greater than 80% in the validation population.

**Results**

In the present study, we did not find any significant difference regarding age (p=0.97) and gender (p=0.61) between the two groups. Patients with MS were significantly more likely to be smokers (49% versus 19.4%, p=0.007) and tended to be alcohol drinkers (31.4% versus 12.9%, p=0.067) compared to controls. Patients in the control group were also more likely to be smokers (31.4% versus 12.9%, p=0.007) and tended to be alcohol drinkers (31.4% versus 12.9%, p=0.067) compared to controls. Patients in the control group were also more likely to be smokers (31.4% versus 12.9%, p=0.007) and tended to be alcohol drinkers (31.4% versus 12.9%, p=0.067) compared to controls. Patients in the control group were also more likely to be smokers (31.4% versus 12.9%, p=0.007) and tended to be alcohol drinkers (31.4% versus 12.9%, p=0.067) compared to controls.

**Association between IL27-T4730C gene polymorphism, IL27 level and MS.** The distribution of genotypes in patients with MS and controls showed significant differences for those with homozygous (CC4730) and heterozygous (TC4730) genotypes (p=0.006), as well as for carriers of the C allele in patients with MS compared to controls (p=0.047). In the control group, the majority of controls (87%) presented a homozygous TT4730 genotype and 91.93% were carriers of the T allele (Table II).
The median serum IL27 level was significantly lower for patients with MS at 12.35 pg/ml (IQR=7.6-16.3 pg/ml) compared to 14.34 pg/ml (IQR=11.34-87.06 pg/ml) for controls (p=0.039) (Figure 2). The results of univariate logistic regression analysis showed that the TC4730 genotype, and having a C allele-bearing genotype were associated with susceptibility to MS (TC4730 versus TT4730: unadjusted OR=6.02, 95% CI=1.61-22.46, p=0.002); (TC4730+CC4730 versus TT4730: unadjusted OR=6, 95% CI=1.83-19.63, p=0.002). We also observed a significant association between carrying a C4730 allele and the risk for MS (unadjusted OR=4.31, 95% CI=1.57-11.87, p=0.002). The risk of developing MS increased in the case of smoking patients compared to non-smokers (unadjusted OR=4.01, 95% CI=1.41-11.41, p=0.009). In addition, there was a tendency for an increased risk for MS development in association with alcohol intake (unadjusted OR=3.09, 95% CI=0.92-10.3, p=0.067). The results of univariate logistic regression analysis are shown in Table III.

Furthermore, multivariate logistic regression showed that the risk of developing MS was 6.272-fold (95% CI=1.84-21.40, p=0.003) increased in carriers of the IL27-T4730C polymorphism and 4.214-fold (95% CI=1.39-12.74, p=0.011) increased in smoking patients. The results of multivariate logistic regression analysis are shown in Table IV.

In the analysis of serum IL27 according to genotype, the median serum IL27 levels were 12.11 (IQR =8.17-15.91 pg/ml) and 13.55 (IQR =8.43-37.68 pg/ml) in carriers of the TC4730, CC4730 genotypes, and 12.75 (IQR =7.07-17.7 pg/ml) in carriers of the TT4730 genotype (p>0.05) (Figure 3).

Discussion

MS has a complex and variable evolution, strongly related to the immunopathogenic mechanism. Cytokines play a major role in the setting of autoimmune diseases, contributing to the initial self-tolerance breakdown, ultimately leading to a complex pathogenic autoimmune response (24).

IL27 has been extensively studied for its role in the regulation of IL17, a cytokine with a central role in autoimmune inflammation (24). The broad immunoregulatory roles of IL27 maintain an immunotolerogenic state in order to prevent autoimmunity (25).

A series of studies evaluating serum IL27 levels in patients with MS conducted by Babaloo et al. (26), Tang et al. (27) and Hasheminia et al. (28), showed lower levels in patients with newly diagnosed or progressive MS compared to a
control group. The serum IL27 levels were negatively correlated with the percentage of circulating Th17 cells, which highlights the influence of IL27 in the MS inflammatory process (26, 27). The aforementioned results are complementary to the findings of Naderi et al. (29), which showed a higher level of IL27 in patients under interferon-β treatment for MS, and of Christensen et al. (30), which showed an elevated IL27 level in patients in MS remission. Moreover, other studies that investigated the serum levels of IL27 in autoimmune diseases revealed lower IL27 levels in patients with SLE compared to controls (31, 32). All the studies mentioned above share a common feature: the serum levels of IL27 were lower in patients compared to healthy controls, suggesting a role of IL27 in pathogenesis.

In our study, we confirmed these results, showing that the serum IL27 level was significantly lower in patients with MS than in controls.

Considering that cytokine gene polymorphisms influence the production of cytokines and their pleiotropic effects on the immune system cells, we also analyzed the T4730C polymorphism located in the IL27p28 gene and its role on serum IL27 levels in patients with MS and controls.

Paradowska-Gorycka et al. suggested that a lower serum expression of IL27 may be a consequence of the
simultaneous presence of polymorphic alleles (for example the presence of both rs17855750 C and rs153109 G) modifying either the transcription initiation site or the structure of the transcription factor binding site, enhancing the production of IL17 and setting the scene for SLE development (33).

Interest was also manifested in the IL27 T4730C polymorphism in other autoimmune diseases, in an attempt to find a connection between the genetic background and disease onset or evolution. In autoimmune thyroid diseases, Graves’ disease and Hashimoto’s thyroiditis, no correlation was found of these diseases with this polymorphism (34). In RA, Paradowska-Gorycka et al. did not find significant differences in genotypic and allelic frequencies of the IL27 T4730C variant between patients with RA and controls, suggesting that this polymorphism is not associated with the susceptibility to RA in the investigated Polish population (35).

The differences between our findings and the previously reported results might be due to the different pathogenesis and molecular mechanisms involved in the occurrence of the diseases. Moreover, ethnicity, racial and age differences between the studied populations may have influenced our results.

To our knowledge, for Romania, this is the first study attempting to find an association between T4730C (rs 181206) polymorphism, serum IL27 level and susceptibility to MS.

The univariate logistic regression analysis showed that smoking, IL27 T4730C polymorphism and the presence of C4730 allele represent risk factors for MS. The multivariate analysis confirmed that smoking and IL27 T4730C polymorphism represent independent risk factors for MS in this cohort of individuals. Other studies evaluating the risk factors for MS also demonstrated an association between smoking habit and the development of MS (36, 37).

In our MS group, we analyzed the potential association between IL27 T4730C polymorphism, age and laboratory. Parameters. We found no significant influence of IL27 T4730C genotype on the age at onset of the disease in the dominant or in the recessive model.

Si et al. examined the role of IL27 polymorphisms, including the T4730C polymorphism, in pediatric patients with Kawasaki disease, suggesting that the serum level of IL27 may not be directly associated with its polymorphisms, despite an elevated serum IL27 level in patients compared to controls (19). Vargas-Alarcón et al. (38) and Pang et al. (39) investigated this polymorphism in diseases other than autoimmune ones, in insulin resistance and in patients presenting HIV infection. Both studies found a reduced serum level of IL27 in patients with the variant compared to healthy individuals but no association was found between the genetic polymorphism, the expression of cytokines and these diseases.

Other studies, with different approaches, evaluated distinct facets of the immune involvement of IL27 in MS pathogenesis. A study conducted by Lalive et al. showed that patients with MS and active demyelination presented astrocytes producing IL27 in active plaques and higher
The discovery of IL27 and the extensive evaluation of its roles and implications in autoimmune diseases led to the desire of using IL27 as a therapeutic tool, as suggested by Yoshida et al. (14), Hirahara et al. (41), Senecal et al. (14) and Zhu et al. (42).

We confirmed the level of IL27 to be lower in carriers of the IL27-T4730C polymorphism but no significant associations between genotypes (TC4730, CC4730) and serum IL27 level were found.

Despite revealing for the first time that T4730C gene polymorphism in IL27 is clearly related to the susceptibility to developing MS, our study has some limitations. One of the main limitations is the sample size, which may not be large enough to draw a definite conclusion about the relationship between this gene polymorphism and MS susceptibility. The second limitation is that one single gene polymorphism was tested among patients with MS and healthy individuals. The third limitation is related to the impossibility of evaluating the effect of T4730C polymorphism on treatment response because all the patients included in our study were evaluated prior to therapy initiation. In our study we did not take into account other known susceptibility loci for MS, as confounding factors. Other limitations of our study are the low analytical sensitivity in the determination of serum IL27 and lack of the evaluation of IL27 in cerebrospinal fluid. A larger cohort of patients is required in order to draw a more precise conclusion and extending the evaluation to other ethnic groups could be further explored.

Identifying patients with IL27 polymorphisms might improve the recognition of patients at risk for MS, in carriers of genetic susceptibility, opening new perspectives for prevention before an autoimmune status is established. Further studies are needed to determine how to manage the complex functions of IL27, challenging the therapeutic perspectives and in finding the right modality to use IL27 as a treatment to maintain the balance between autoimmune, infectious and neoplastic risk. It is clear that the treatment and the course of MS (relapse versus remission versus progression) influence the serum level of IL27. Extensive studies are required in order to elucidate the precise variation of IL27 in relation to the therapy used in MS and the stage of disease.

**Conclusion**

In conclusion, carriers of IL27 T4730C polymorphism (rs181206) appear to have a higher risk of developing MS. The serum level of IL27 in patients without any treatment was significantly lower compared to controls. Our results add new insight into the genetic contribution of IL27 variation to MS susceptibility and support the crucial role of IL27 in autoimmunity, but further investigations are still required to understand the context-dependent inflammatory activities of IL27.

**Conflicts of Interest**

The Authors declare no conflicts of interest.

**Authors’ Contributions**

Conceptualization: I. S. Barac. and L.M Procopciuc.; methodology: L.M. Procopciuc, Decea Nicoleta.; software: Mădălina Vălean; validation: L.M. Procopciuc, Angela Cozma, M. F. Dafin; formal analysis: Mădălina Vălean; investigation: I. S. Barac, Vitalie Văcăraș; resources: I. S. Barac, Vitalie Văcăraș, M. F. Dafin; data curation: L.M Procopciuc, Mădălina Vălean ; writing–original draft preparation: I. S. Barac and L.M Procopciuc; writing–review and editing: I. S. Barac and L.M Procopciuc; visualization: Angela Cozma, M. F. Dafin; supervision: Vitalie Văcăraș, M. F. Dafin; project administration: L.M Procopciuc; funding acquisition: I. S. Barac. All Authors have read and agreed to the published version of the article.

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