Original Research

Turk J Immunol 2024;12(2):56-64
DOI: 10.4274/tji.galenos.2024.40427

Evaluation of Inflammation-Related Proteins in Multiple Sclerosis Disease with Relapses and Remissions

Nisa Hocaoğlu[1], Nur Damla Korkmaz[2], Birsen Elibol[3], Zehra Cemre Karakayalı[4], Şule Terzioğlu-Uşak[5], Azize Esra Gürsoy[4,6]

[1]Bezmialem Vakıf University Faculty of Medicine, İstanbul, Turkey
[2]Bezmialem Vakıf University Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey
[3]İstanbul Medeniyet University Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey
[4]Bezmialem Vakıf University Faculty of Medicine, Department of Neurology, İstanbul, Turkey
[5]Erfe Group Company, İstanbul, Turkey
[6]Başakşehir Çam and Sakura City Hospital, Clinic of Neurology, İstanbul, Turkey

Cite as: Hocaoğlu N, Korkmaz ND, Elibol B, Karakayalı ZC, Terzioğlu-Uşak Ş, Gürsoy AE. Evaluation of Inflammation-Related Proteins in Multiple Sclerosis Disease with Relapses and Remissions. Turk J Immunol. 2024;12(2):56-64

Received: 07.08.2023 **Accepted:** 24.05.2024

Corresponding Author: Birsen Elibol, İstanbul Medeniyet University Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey
Phone: +90 533 231 07 23 **E-mail:** elibolbirsen@gmail.com **ORCID:** orcid.org/0000-0002-9462-0862

Abstract

Objective: Multiple sclerosis (MS) is an autoimmune disease affecting the brain and spinal cord by demyelination and neurodegeneration. Although the cause of the disease is not known exactly due to its heterogeneous etiology, it is thought that inflammatory processes can be effective in the formation and progression of the MS pathology. Herein, the present study's aim was to evaluate the changes in inflammation-related proteins, which have a crucial role in the formation of neuroinflammation, according to the period of MS.

Materials and Methods: The study included 33 MS patients, 8 of whom were in relapse and 25 in remission, and 10 healthy individuals. Lymphocytes were isolated from peripheral blood. After RNA isolation and cDNA conversion, *NLRP3, ASC, NLRX1, IL-1β*, and *IL-18* gene expressions were measured in real time-polymerase chain reaction. In addition, the protein levels of the NLRP3 and ASC were determined from serum samples by enzyme-linked immunosorbent assay.

Results: The expression of the *NLRX1* gene was significantly decreased in the patient groups compared to the controls. The levels of *NLRP3*, *ASC,* and *IL-18* gene expressions of patients were not significantly different from controls in the remission and relapse periods. Although *IL-1β* gene expressions of the patients in the remission period increased in value, it was not statistically significant compared to that of healthy controls.

Conclusion: Our findings showed that anti-inflammatory NLRX1 protein can be considered as a strong biomarker in the diagnosis and treatment of MS, where neuroinflammation is the main cause.

Keywords: Inflammasome, IL-18, multiple sclerosis, NLRX1, relapsing-remitting MS

Introduction

Demyelination of the brain and spinal cord causes multiple sclerosis (MS), the most common autoimmune disorder affecting the central nervous system (CNS) (1). MS is a multifactorial disease for which genetic and environmental factors can be causative (1). Although the exact pathogenesis of MS is undetermined, the underlying mechanisms are thought to be related to neuroinflammation occurred by autoreactive lymphocytes' migration to the CNS (1). These autoimmune T-cells can give rise to demyelination, gliosis, and axonal loss when they pass through the blood-brain barrier (1). As a result of this neuroinflammatory process, local lesions occur in the optic nerve, spinal cord, and brain. These neurological lesions which occur in both grey matter and white matter define MS clinical features. The main symptoms, which vary according to the region of the lesions, are pyramidal weakness, spasticity, visual disturbances, facial sensory loss, and dizziness (1,2). Decades of data show that B-cells have a crucial function in the pathophysiology of MS (3). Examinations of MS patients cerebrospinal fluids show that the levels of immunoglobulins increase in the patients compared to those of healthy people. Treg dysfunction, which is a sign of autoreactive B-cells, is also known to be

ORCID: N. Hocaoğlu: 0000-0002-4414-4765, N.D. Korkmaz: 0000-0002-1173-1701, B. Elibol: 0000-0002-9462-0862, Z.C. Karakayalı: 0000-0003-2886-7995, Ş. Terzioglu-Uşak: 0000-0002-4594-2697, A.E. Gürsoy: 0000-0002-8103-0927

effective in the pathogenesis of MS. Although it is known that the overproduction of pro-inflammatory cytokines involved in all these processes plays an important function in the formation of disease, the exact mechanism is still not resolved (3). In addition, it is known that inflammasome complexes may have a key role in the pathogenesis of MS, which is featured by the triggering of pro-inflammatory processes.

Components of the immune system come to the fore in inflammatory processes in the context of MS (4). In the most general sense, the immune system is divided into two: innate immune system and adaptive immune system (4). The innate immune system ensures the continuity of the host's defense system by being activated by pathogen-associated molecular patterns and danger-associated-molecular patterns through pattern recognition receptors while the adaptive immune system performs its function by recognizing antigens through lymphocyte receptors (4). The most well-known pattern recognition receptor groups are toll-like receptors and NOD-like receptors (NLRs) (5). With the activation of these receptors, a wide variety of inflammatory signaling cascades are triggered, and cytokine releases occur with the activation of specific transcription factors (5). The NLRs are defined by a central nucleotide binding and oligomerization part known as NACHT (6). In some NLR proteins such as NLRP3 and NLRP1, this central NACHT protein may be surrounded by a C-terminal leucine-rich repeat (LRR) and N-terminal or C-terminal caspase recruitment or pyrin domains (CARD and PYD, respectively) (6). Some of the NLRs, such as NLRP3, play a role in the formation of a multiprotein complex called inflammasome (6).

Inflammasomes are multiprotein complexes, and they catalyze the maturation of pro-inflammatory proteins [interleukin (IL)-18 and IL-1β] and Gasdermin D, which induce microbial defenses, stress-related responses or inflammation (7). The NLRP3 inflammasome comprises the NLR sensor molecule (NACHT, LRR, and PYD domain-containing protein 3), the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the pro-inflammatory Caspase-1 (7). Through the recognition of danger-associated molecular patterns, the NLRP3 inflammasome, with its ASC and pro-caspase 1 protein components, facilitates the activation of caspase 1 and the processing of cytoplasmic targets, IL-1β and IL-18 (8). Then, active caspase 1 cleaves pro-IL-1β to active IL-1β. IL-1β boosts neurotoxicity and neuroinflammation (9,10).

The role of inflammasomes in MS and experimental autoimmune encephalitis (EAE) has gained importance as a result of studies conducted in recent years. A previous study showed that the EAE mice with knocked out *NLRP3* coding gene (*Nlrp3*$^{-/-}$) exhibited a milder degree of EAE and less IL-18 production (11). Additionally, a cuprizone-induced demyelination model showed elevated *NLRP3* gene expression, and an NLRP3-deficient mouse model showed reduced demyelination and oligodendrocyte loss (12). In addition, other studies have shown that caspase-1 and ASC expressions are related to the severity of MS symptoms (13). Studies have shown that IL-18, which is high in many neurodegenerative diseases, also plays a role in the etiology of MS by increasing the amount of interferon (IFN)-gamma in leukocytes located in the CNS (14,15). Another protein we emphasize in our study is NLRX1, which has anti-inflammatory effects to suppress inflammation triggered by inflammasome (16). NLRX1 predominantly exerts a suppressive impact on antiviral immune responses, and it typically modulates the NF-κB signaling pathway by impeding the interaction between TRAF6 and the inhibitor of NF-κB kinase (16). In a study conducted in mice with the deletion of the *NLRX1* gene, it was noted that EAE developed with demyelination and inflammation. In the same study, it was also shown that microglial activation and neuronal loss in the CNS were inhibited by NLRX1 (17). These data suggest that NLRX1 protein has a protective effect on the progression of MS disease (18).

In the light of all these data, we have observed that there is a limited clinical study about the relationship between inflammasomes and inflammation-related markers at both gene/protein levels and MS patients at different periods, especially in Turkish population (19-23). In addition, the effects of NLRX1 in the MS patients is not well documented in the literature, except experimental animal models of MS (17,18,24). Therefore, we analyzed the changes in mostly studied inflammation-related proteins (NLRP3 and ASC), pro-inflammatory markers (IL-18 and IL-1β), and anti-inflammatory protein *NLRX1* at the both gene and protein levels to determine the role of neuroinflammation in Turkish patients with relapsing-remitting multiple sclerosis (RRMS), which is the most common form of MS.

Materials and Methods

Patients

A clinical ethical committee of Bezmialem Vakıf University approved the study (approval no.: 8138, date: 10.07.2020). In addition, The Declaration of Helsinki principles guided the conduct of this study. The study groups consisted of thirty-three MS patients (attack n=8, remission n=25) and 10 healthy individuals as a control group who were recruited from the Neurology Department of Bezmialem Vakıf University Hospital, İstanbul, Turkey. Each participant in the study provided informed consent.

The inclusion criteria valid for MS attack patients included in the study were respectively: 1) not having neurodegenerative disease other than MS, 2) not having hypertension, 3) not having diabetes, 4) not having active infection.

Required inclusion/exclusion criteria for MS patients who were not in the attack phase were: 1) no clinical attacks in the last 3 months, 2) no neurodegenerative disease other than MS, 3) no hypertension, 4) not having diabetes, 5) not having active infection.

Inclusion/exclusion criteria for the control group were: 1) not having any neurodegenerative disease, 2) not having hypertension, 3) not having diabetes, 4) not having active infection.

Peripheral Blood Mononuclear Cells (PBMC) Isolation

Peripheral blood samples from patients and healthy controls were taken to heparinized tubes and transported to the laboratory by cold chain. The blood tubes were mixed up and down by adding 0.1 M phosphate-buffered saline (PBS, pH=7.4) at a ratio of 1:1 to the blood, and 3 mL of Lymphoprep™ (density gradient medium, STEMCELL Technology) was added to a sterilized tube. The same amount of blood PBS mixture was added to Lymphoprep™ containing tube by dripping slowly from the environment with a Pasteur pipette and centrifuged for 25 min at 800 x g at 4°C. Furthermore, to obtain plasma, blood samples were centrifuged for 10 min at 3000 x *g* at 4°C. Then, obtained plasma and PBMC samples were kept at -80°C for molecular experiments.

RNA Isolation from PBMC

After PBMC isolation, Qiagen RNeasy mini kit was used for RNA isolation. First, PBMC cells were heated to 37°C in a water bath and were centrifuged at 300 x g for 5 min. After throwing supernatant, cells were disrupted by adding 500 µL Buffer RLT in the kit and 5 µL of β-mercaptoethanol, then 1 volume of 70% cold ethanol were added, and they were mixed well. 500 µL of the sample was transferred to the spin column and centrifuged at 8000 x *g* for 30 sec. 700 µL of Buffer RW1 in the kit was added to the pellet before the centrifugation. After having discarded the flow-through, 500 µL of buffer RPE in the kit was added and centrifuged 2 min at 8000 x *g*. Afterward, the elution process occurred by adding 30 µL of RNAse free-water and centrifugation at 8000 × *g* for 1 min.

cDNA Synthesis

The obtained RNA samples were converted to cDNA at the same day, and they were stored at -20°C for the RT-PCR experiments. First, genomic DNA (gDNA) was removed by gDNA removal mix incubation at 45°C for 2 min. Afterwards, reverse transcription process took place. At this second step, annealing occurred at 25°C for 3 min and then reverse transcription occurred at 45°C for 10 min and lastly inactivation of reaction was carried out at 85°C for 5 min by adding reverse transcription components. All samples were subjected to cDNA synthesis at 300 ng/µL.

Determination of Gene Levels by RT-PCR

The gene expressions of *NLRX1, NLRP3, IL-1β, IL-18, ASC,* and *GAPDH* (as a housekeeping gene) were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Appropriate annealing temperatures were found for each primer by optimizing with the gradient PCR process. SYBR Green PCR Master Mix (2X), primers, distilled water, and cDNA templates were added as the procedure described in the guide. Cycling conditions were performed in 40 cycles with this order: at 95°C for 2 min, at 95°C for 5 sec, and at 60°C for 10 sec. The reactions were carried out with CFX96 TouchTM Real-Time PCR (Biorad Laboratories, California) under appropriate thermal conditions using ready-made primer pairs (Qiagen, QuantiTect Primer Assay, Germany) against these genes (Table 1). Analyses were performed using threshold cycle relative quantification methods. Then, a logarithmic transformation of fold induction ratios was applied according to the relative quantification formula ($2^{-\Delta\Delta Ct}$).

Determination of Protein Levels by ELISA

Plasma samples were taken from -80°C to room temperature and supernatants of plasma samples were collected after centrifugation for 15 min at 1000 x *g*.

Table 1. Details of primers used to amplify the selected genes in RT-PCR

Gene symbol	Primer catalog number	Transcript ID	Targeted region	Product length
IL-1B	QT00021385	ENST00000263341	5-6 exons	117 bp
IL-18	QT00014560	ENST00000280357	3-4 exons	84 bp
ASC	QT00232134	ENST00000247470.10	-	117 bp
NLRX1	QT00085337	ENST00000292199	2-3-4 exons	105 bp
NLRP3	QT00029771	ENST00000358659	7-8 exons	92 bp
GAPDH	QT00079247	ENST00000229239	6-7 exons	95 bp

RT-PCR: Reverse transcription polymerase chain reaction

ASC protein levels were analyzed via an enzyme-linked immunosorbent assay (ELISA) kit by BT Lab (Bioassay Technology Laboratory, Harborne Rd, Birmingham, UK) according to the manufacturer's instructions and NLRP3 protein levels were analyzed by Human NOD-like Receptor Pyrins-3 (NLRP3) ELISA kit by AFG Bioscience (AFG Bioscience LLC, Northbrook, USA). Finally, Multiskan GO microplate reader (Thermo Fisher Scientific, Boston, MA, USA) was used to measure absorbance at 450 nm.

Statistical Analysis

In the present study, normality was tested with the Shapiro-Wilk test. Therefore, non-normal distributed experimental data were analyzed using the Kruskal-Wallis and Dunn's multiple comparisons tests. A 0.05 significance level and 95% confidence level were used in all statistical analyses performed using GraphPad Prism 8.0 (GraphPad Software, Inc., CA, USA).

Results

Patients

According to the demographic data, the patients and healthy controls were similar in terms of gender and age, and the patients in the relapse period had a higher body mass index than both the patients in remission period and controls (Table 2). The Expanded Disability Status Scale (EDSS), Kurtzke's most widely used assessment scale, was used for the diagnosis of MS. Although the EDSS score was higher in the patients who were in the attack period, it was not found to be significantly different from the patients who were in remission period (Table 2). Contrast-enhancing lesions on magnetic resonance imaging, which is associated with inflammation and axonal loss, were significantly higher in the patients who were in the attack period (p=0.0063). As expected, the number of attacks were higher in the patients who were in the attack period (p=0.0015) (Table 2). In addition, the patients were taking different disease-modifying drugs to reduce the number and severity of any relapses for more than one year. However, three patients in the remission group and five patients in the attack group were not taking any medications (Table 2).

Gene Expression of Inflammasome Complexes

In order to investigate the association of the inflammasome complexes and the period of the disease in MS, qRT-PCR study of selected genes from PBMC samples taken from patients was performed. The levels of *NLRP3* and *ASC* gene expressions are given in Figure 1. The expression of *NLRP3* gene was not significantly different in the patients who were in the remission and attack period compared to those of the healthy subjects (Figure 1A). In addition, the level of *ASC* gene expression was the highest in the patients who were in the attack period, although there was no statistical significant difference (p=0.0812) (Figure 1B).

The level of pro-inflammatory cytokines *IL-1β* and *IL-18* gene expressions were also determined and represented in Figure 2. *IL-1β* gene expression was higher in the patients who were in the remission period than that

Table 2. Characteristics of the study population

	Remission period	Attack period	Healthy controls	Statistics and p value
Age, years, med (min-max)	31 (19-56)	32 (22-40)	24 (22-39)	Kruskal-Wallis test, p=0.0624
Sex, n (female/male)	20/5	5/3	7/3	Fisher-Freeman-Halton, p=0.8485
BMI	25.6 ± 4.1	29.2 ± 5.5	24.4 ± 3.1	One-Way ANOVA, **p=0.0457**
EDSS	1.5 (1-6.5)	3 (1-4.5)	-	Mann-Whitney, p=0.5503
Disease duration, years	6.4 ± 7.1	1.1 ± 1.2	-	Mann-Whitney test, **p=0.0065**
Contrast-enhancing in MRI	0.24 ± 0.4	0.8 ± 0.44	-	Mann-Whitney test, **p=0.0063**
Number of attacks in the last year	0.5 ± 0.5	1.2 ± 0.4	-	Mann-Whitney test, **p=0.0015**
The level of Vitamin D (ng/dL)	20.0 ± 18.3	13.5 ± 10.1	-	Mann-Whitney, p=0.4506
Types of medications, (number of patients administered)				
Natalizumab/ocrelizumab	2	0	-	-
Dimethyl fumarate	6	1		
Glatiramer acetate	6	0		
Interferon Beta (IFN-β)	3	1		
Fingolimod/teriflunomide	5	1		
Treatment period				
0-1 year	8	0	-	-
1-5 years	13	3		
5-10 years	1	0		

Results are represented as mean ± standard deviation. n: number, BMI: Body-mass index, EDSS: Expanded disability status scale, MRI: Magnetic resonance imaging, IFN-β: Interferon-beta

of the control group; however, this was not seen at the statistical significance level (Figure 2A). Furthermore, there was no difference in the *IL-18* gene expression among the groups (Figure 2B).

As known from the literature, NLRX1 is an anti-inflammatory protein, which suppresses inflammation. According to the qRT-PCR results obtained for this mRNA, the level of NLRX1 expression was significantly different among the groups (p=0.001). The level of this gene expression was significantly decreased in both patients who were in the remission and attack periods than in healthy individuals (p=0.0209 and p=0.0092, respectively) (Figure 3).

Levels of NLRP3 and ASC Proteins

Due to no change in the expression of *NLRP3* and *ASC* at the gene level, we wondered about whether there was any change in their expressions at protein level. According to the results obtained, NLRP3 protein was found to be higher in the MS patients who were in the attack period than that of the control group; however, the significance did not reach the accepted level according to the Kruskal-Wallis test (p=0.2659) (Figure 4A). The protein level of ASC, an adapter protein that has an important role in inflammasomes, was also not different among the groups (Figure 4B).

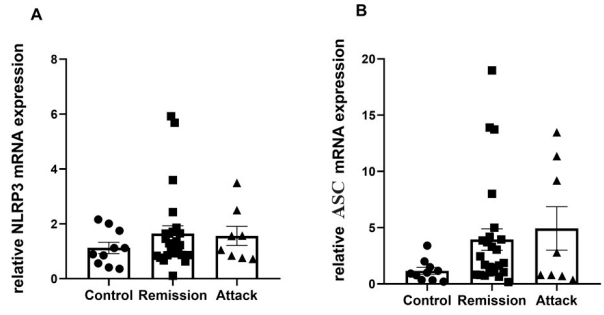

Figure 1. Relative gene expression of A) NLRP3 (Kruskal-Wallis, p=0.67) and B) PYCARD (Kruskal-Wallis, p=0.08) in healthy people and MS patients (in the remission and attack period) obtained by RT-PCR. Data was denoted as mean ± standard error of mean.
RT-PCR: Reverse transcription polymerase chain reaction, MS: Multiple sclerosis

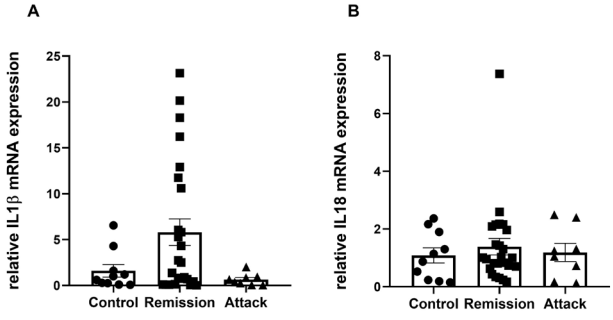

Figure 2. Relative gene expression of A) IL-1β (Kruskal-Wallis, p=0.11) and B) IL-18 (Kruskal-Wallis, p=0.89) in healthy people and MS patients (in the remission and attack period) obtained by RT-PCR. Data was denoted as mean ± standard error of mean.
RT-PCR: Reverse transcription polymerase chain reaction, IL: Interleukin, MS: Multiple sclerosis

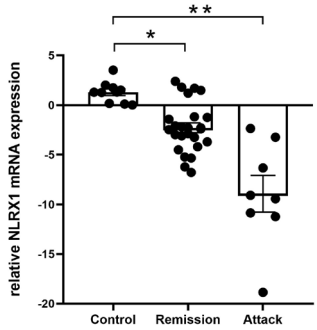

Figure 3. Relative gene expression of NLRX1 in healthy people and MS patients (in the remission and attack period) obtained by RT-PCR. Data was denoted as mean ± standard error of mean. *: p=0.0209 and **: p=0.0092.
RT-PCR: Reverse transcription polymerase chain reaction, MS: Multiple sclerosis

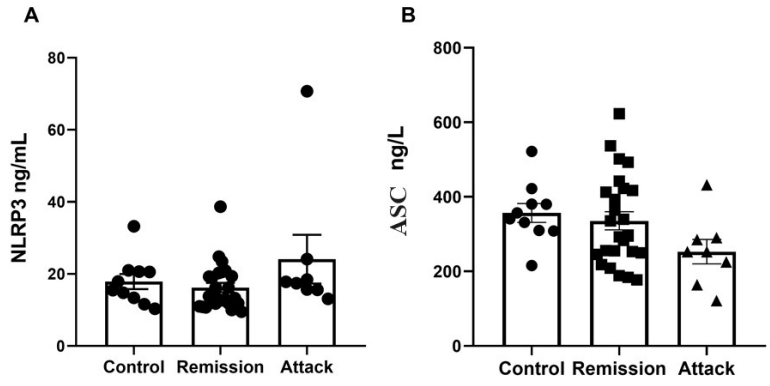

Figure 4. The protein levels of A) NLRP3 (Kruskal-Wallis, p=0.27) and B) PYCARD (Kruskal-Wallis, p=0.75) in healthy people and MS patients (in the remission and attack period) obtained by ELISA. Data was denoted as mean ± standard error of mean.
RT-PCR: Reverse transcription polymerase chain reaction, MS: Multiple sclerosis, ELISA: Enzyme-linked immunosorbent assay

Discussion

Pro-inflammatory processes have a critical function in the pathophysiology of neurological diseases. Previous studies have stated that pro-inflammatory processes may also cause MS, as in other neurological diseases (15). Pyroptosis plays a crucial function in the formation of MS lesions and axonal loss by affecting inflammasome-related mechanisms (25). The activation of the pro-inflammatory cytokines and Gasdermin D (a pore-forming protein) by caspase-1 cleavage, which causes pyroptosis, initiates the neuroinflammation cascade and causes MS plaques (26,27). In the light of these data, we analyzed the relationship of inflammation-related molecules both at the gene and protein levels in the MS patients who were in the relapse and remission period.

NLRP3 is the most widely characterized inflammasome (12). With the activation of the sensor LRR domain of NLRP3 via cellular stress, downstream signaling is regulated by the homotypic PYDs (28). The NACHT domain of NLRP3 is responsible for the ATP-dependent self-oligomerization process due to its ATPase activity which leads to the formation of self-PYD interactions (28). These interactions subsequently attract ASC which serves as the adaptor in the process. The ASC adaptor comprises a PYD at the N-terminus and a (CARD) at the C-terminus (28). It is brought to the oligomerized PYDs of the NLRP3 molecules through homotypic PYD-PYD interactions, resulting in the formation of a prion-like ASC filament. These initiated filaments collect the C-terminal CARDs of ASC, serving as a foundation to attract effector caspase-1 (28,29).

According to the data we obtained, *NLRP3* and *ASC* gene expressions were not significantly different in both periods of the MS compared to the levels of the healthy control as opposite to the literature (30,31). The lack of statistical significance in our results may be attributed to the small sample size. According to the studies done with EAE models, NLRP3 inflammasome has a very active role in the formation of MS at its early stages (32). In addition, the NLRP3 inflammasome complex triggers the migration of immune cells such as myelin-specific $CD4^+$ T-cells, APC cells and dendritic cells to the CNS, which initiate neuroinflammation (33). In our study, although there was a tendency for an increase in the NLRP3 and ASC values in the patients, we think that the reason why they did not reach a sufficient level of statistical significance was the immunosuppressive drugs used by the patients. According to studies, IFN-β suppresses IL-1β production, and in another additional study, Type-1 interferons were shown to repress NLRP3 by STAT1 transcription (34,35). Moreover, the levels of NLRP3 and ASC proteins were similar in the serum of MS patients as observed in a previous study which emphasized the effect of immunomodulatory medicines used in the MS treatment (19). For this reason, we think that the use of immunosuppressive drugs such as IFN-β in the patients of our cohort has an effect on reducing the difference between the groups. Furthermore, there was no difference in the expression of *NLRP3* and *ASC* genes between MS subgroups, suggesting that these gene expressions may not be related to relapse activity. In addition, the gene expression of pro-inflammatory cytokines, IL-1β and IL-18, which become mature after caspase-1 cleavage to initiate the inflammatory response did not differ from the values in the healthy control. Rather than being insignificant, the level of *IL-1β* gene expression was the highest in the remission group. Parallel to this result, a previous study showed that the amount of IL-1β in MS disease, which is characterized by the disruption of pro-inflammatory processes, was higher in PBMC samples of RRMS patients (36).

Furthermore, the difference in the pattern of IL-1β and IL-18 expressions in the MS patients may be related to the

site of tissue inflammation as shown in previous studies (37). As it has been well established, IL-18 and IL-12 are responsible for inducing IFN-gamma production from natural killer cells and T-cells through its active role in Th-1 response (38,39). Studies show that the IL-18 level in the CSF and the lesions of the MS patients is higher than the normal value (15,40). Among the pro-inflammatory cytokines, IL-1β has one of the highest potentials to cause damage to the tissues (41). In previous studies, authors pointed out the correlation between the number and volume of demyelinating lesions in the brain cortical regions and the IL-1β levels (42). Interestingly, it was shown that the level of IL-1β became high at the time of clinical remission of severe MS patients (43). Additionally, fingolimod had an activity to decrease the level of IL-1β (44,45), which may explain the insignificant changes in the level of IL-1β in our patients compared to the healthy subjects. Due to its interaction with blood cells (46,47), the decreased expression of IL-1β in the patients who were in the attack period may be explained by the correlation between neutrophil-to-lymphocyte ratio and attacks of MS patients as previously mentioned by D'Amico et al. (48).

According to our results, we think that the effects of immunomodulator drugs used in the patients may be responsible for the insignificant changes in the expression of inflammasomes markers. Also, a previous *in vitro* study showed that microglia-mediated neuroinflammation, which is important in the MS progression, was prevented by a decrease in the expression of LPS-induced pro-inflammatory markers upon the application of immunomodulatory drugs (49).

NLRX1 is an immune sensor molecule located in mitochondria and is involved in the inhibition of pro-inflammatory mechanisms such as the nuclear factor-κB signaling pathway (49). It was previously shown that NLRX1 reduced microglia activation and inhibited the formation of reactive astrocyte types and reduced MS symptoms by this mechanism (49). In parallel to previous data, the expression NLRX1 was found to be significantly decreased in the patients, especially in the patients who were in the attack period, compared to the healthy controls in our study. As mentioned before, NLRX1 has a negative regulatory effect in the pathogenesis of MS (17,24). Therefore, the observed lowest level of *NLRX1* gene expression in the patients who were in the attack period showed that NLRX1 might be a biomarker to determine the inflammatory activity of the disease. In a study, the severity of the disease was higher in the NLRX1 knockout EAE-induced mice, and the severity of disease was correlated with the severity of microgliosis (24). Supporting the previous studies, a negative correlation between disease period and NLRX1 levels was seen in our results as well. In this context, NLRX1 appears promising for further studies as a potential biomarker for the diagnosis and treatment of MS.

Study Limitations

The relatively small size of our cohort is the limitation of our study. Additionally, it is plausible to think that the immunomodulatory drugs used by the patients contributed to the lack of expected differences in some of our results, possibly exerting an immunosuppressive effect by reducing T and B-cell activation. In order to minimize all these limitations, we aim to work with a larger newly diagnosed cohort in our next study.

Conclusion

In summary, our results have pointed out that inflammation-related proteins are of great importance in prognosing and grading of MS by follow-up of the inflammatory activity of the course of the disease. The association of lesion involvement and key inflammasome oligomers in the pathology of the disease is very important to determine the biomarkers to be used in the diagnosis and treatment of the disease.

Ethics

Ethics Committee Approval: A Clinical Ethical Committee of Bezmialem Vakıf University approved the study (approval no.: 8138, date: 10.07.2020).

Informed Consent: Each participant in the study provided informed consent.

Authorship Contributions

Surgical and Medical Practices: N.H., Z.C.K., A.E.G., Concept: N.H., B.E., Ş.T.U., A.E.G., Design: N.H., N.D.K., B.E., Ş.T.U., Data Collection or Processing: N.H., N.D.K., Z.C.K., A.E.G., Analysis or Interpretation: N.H., N.D.K., B.E., Z.C.K., Literature Search: N.H., N.D.K., Ş.T.U., Writing: N.H., N.D.K., B.E., A.E.G.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: Bezmialem Vakıf University Research Council supported to this work by the grant number 20200801E.

References

1. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, et al. Multiple sclerosis. Nat Rev Dis Primers. 2018;4:43.
2. Ford H. Clinical presentation and diagnosis of multiple sclerosis. Clin Med. 2020;20:380-3.
3. Kinnunen T, Chamberlain N, Morbach H, Cantaert T, Lynch M, Preston-Hurlburt P, et al. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. The J Clin Invest. 2013;123:2737-41.

4. Cao X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. Nat Rev Immunol. 2016;16:35-50.
5. Toubi E, Vadasz Z. Innate immune-responses and their role in driving autoimmunity. Autoimmun Rev. 2019;18:306-11.
6. Hong Pu, Gu RN, Li FX, Xiong XX, Liang WB, You ZJ, et al. NLRP3 inflammasome as a potential treatment in ischemic stroke concomitant with diabetes. J Neuroinflammation. 2019;16:121.
7. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. Mol Cell. 2002;10:417-26.
8. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 2011;469.7329:221-5.
9. Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE, O'Banion MK. Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates alzheimer plaque pathology. J Clin Invest. 2007;117:1595-604.
10. Rossi S, Furlan R, De Chiara V, Motta C, Studer V, Mori F, et al. Interleukin-1β causes synaptic hyperexcitability in multiple sclerosis. Ann Neurol. 2012;71:76-83.
11. Gris D, Ye Z, Iocca HA, Wen H, Craven RR, Gris P, et al. NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. J Immunol. 2010;185:974-81.
12. Jha S, Srivastava SY, Brickey WJ, Iocca H, Toews A, Morrison JP, et al. The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18. J Neurosci. 2010;30:15811-20.
13. Inoue M, Williams KL, Gunn MD, Shinohara ML. NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2012;109:10480-5.
14. Koch MW, Polman SK, Uyttenboogaart M, De Keyser J. Treatment of seizures in multiple sclerosis. Cochrane Database Syst Rev. 2009;CD007150.
15. Losy J, Niezgoda A. IL-18 in patients with multiple sclerosis. Acta Neurol Scand. 2001;104:171-3.
16. Stokman G, Kors L, Bakker PJ, Rampanelli E, Claessen N, Teske GJD, et al. NLRX1 dampens oxidative stress and apoptosis in tissue injury via control of mitochondrial activity. J Exp Med. 2017;214:2405-20.
17. Gharagozloo M, Mahmoud S, Simard C, Yamamoto K, Bobbala D, Ilangumaran S, et al. NLRX1 inhibits the early stages of CNS inflammation and prevents the onset of spontaneous autoimmunity. PLoS Biol. 2019;17:e3000451.
18. Koo JH, Kim DH, Cha D, Kang MJ, Choi JM. LRR domain of NLRX1 protein delivery by dNP2 inhibits T cell functions and alleviates autoimmune encephalomyelitis. Theranostics. 2020;10:3138-50.
19. Tamam Y, Yüceer H, Düzel B, Şen M, Ulusoy C, Tuzun E. Serum levels of inflammasome pathway factors in clinically isolated syndrome and multiple sclerosis patients: a pilot study. Cent Eur J Immunol. 2020;45:237-40.
20. Karakas Celik S, Oz ZS, Dursun A, Unal A, Emre U, Cicek S, et al. Interleukin 18 gene polymorphism is a risk factor for multiple sclerosis. Mol Biol Rep. 2014;41:1653-8.
21. Malhotra S, Costa C, Eixarch H, Keller CW, Amman L, Martínez-Banaclocha H, et al. NLRP3 inflammasome as prognostic factor and therapeutic target in primary progressive multiple sclerosis patients. Brain. 2020;143:1414-30.
22. Hagman S, Kolasa M, Basnyat P, Helminen M, Kähönen M, Dastidar P, et al. Analysis of apoptosis-related genes in patients with clinically isolated syndrome and their association with conversion to multiple sclerosis. J Neuroimmunol. 2015;280:43-8.
23. Noroozi S, Meimand HAE, Arababadi MK, Nakhaee N, Asadikaram G. The effects of IFN-β 1a on the expression of inflammasomes and apoptosis-associated speck-like proteins in multiple sclerosis patients. Mol Neurobiol. 2017;54:3031-7.
24. Eitas TK, Chou WC, Wen H, Gris D, Robbins GR, Brickey J, et al. The nucleotide-binding leucine-rich repeat (NLR) family member NLRX1 mediates protection against experimental autoimmune encephalomyelitis and represses macrophage/microglia-induced inflammation. J Biol Chem. 2014;289:4173-9.
25. McKenzie BA, Fernandes JP, Doan MAL, Schmitt LM, Branton WG, Power C. Activation of the executioner caspases-3 and -7 promotes microglial pyroptosis in models of multiple sclerosis. J Neuroinflammation. 2020;17:253.
26. Freeman LC, Ting JP. The pathogenic role of the inflammasome in neurodegenerative diseases. J Neurochem. 2016;136:29-38.
27. McKenzie BA, Mamik MK, Saito LB, Boghozian R, Monaco MC, Major EO, et al. Caspase-1 inhibition prevents glial inflammasome activation and pyroptosis in models of multiple sclerosis. Proc Natl Acad Sci U S A. 2018;115:E6065-74.
28. Moasses Ghafary S, Soriano-Teruel PM, Lotfollahzadeh S, Sancho M, Serrano-Candelas E, Karami F, et al. Identification of NLRP3PYD homo-oligomerization inhibitors with anti-inflammatory activity. Int J Mol Sci. 2022;23:1651.
29. Lu A, Magupalli VG, Ruan J, Yin Q, Atianand MK, Vos MR, et al. Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. Cell. 2014;156:1193-206.
30. Furlan R, Martino G, Galbiati F, Poliani PL, Smiroldo S, Bergami A, et al. Caspase-1 regulates the inflammatory process leading to autoimmune demyelination. J Immunol. 1999;163:2403-9.
31. Ting JP, Kastner DL, Hoffman HM. Caterpillers, pyrin and hereditary immunological disorders. Nature Rev Immunol. 2006;6:183-95.
32. Martin BN, Wang C, Zhang CJ, Kang Z, Gulen MF, Zepp JA, et al. T cell-intrinsic ASC critically promotes T(H)17-mediated experimental autoimmune encephalomyelitis. Nat Immunol. 2016;17:583-92.
33. Inoue M, Williams KL, Oliver T, Vandenabeele P, Rajan JV, Miao EA, et al. Interferon-beta therapy against EAE is effective only when development of the disease depends on the NLRP3 inflammasome. Sci Signal. 2012;5:ra38.
34. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Förster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity. 2011;34:213-23.
35. Sweeney CM, Lonergan R, Basdeo SA, Kinsella K, Dungan LS, Higgins SC, et al. IL-27 mediates the response to IFN-beta therapy in multiple sclerosis patients by inhibiting Th17 cells. Brain Behav Immun. 2011;25:1170-81.
36. Peelen E, Damoiseaux J, Muris AH, Knippenberg S, Smolders J, Hupperts R, et al. Increased inflammasome related gene expression profile in PBMC may facilitate T helper 17 cell induction in multiple sclerosis. Mol Immunol. 2015;63:521-9.
37. Sasaki Y, Otsuka K, Arimochi H, Tsukumo SI, Yasutomo K. Distinct roles of IL-1beta and IL-18 in NLRC4-induced autoinflammation. Front Immunol. 2020;11:591713.

38. Barbulescu K, Becker C, Schlaak JF, Schmitt E, Meyer zum Buschenfelde KH, Neurath MF. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-gamma promoter in primary CD4+ T lymphocytes. J Immunol. 1998;160:3642-7.

39. Gu Y, Kuida K, Tsutsui H, Ku G, Hsiao K, Fleming MA, et al. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science. 1997;275:206-9.

40. Balashov KE, Rottman JB, Weiner HL, Hancock WW. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. Proc Natl Acad Sci U S A. 1999;96:6873-8.

41. Chen YC, Chen SD, Miao L, Liu ZG, Li W, Zhao ZX, et al. Serum levels of interleukin (IL)-18, IL-23 and IL-17 in Chinese patients with multiple sclerosis. J Neuroimmunol. 2012;243:56-60.

42. Lin CC, Edelson BT. New insights into the role of IL-1beta in experimental autoimmune encephalomyelitis and multiple sclerosis. J Immunol. 2017;198:4553-60.

43. Seppi D, Puthenparampil M, Federle L, Ruggero S, Toffanin E, Rinaldi F, et al. Cerebrospinal fluid IL-1beta correlates with cortical pathology load in multiple sclerosis at clinical onset. J Neuroimmunol. 2014;270:56-60.

44. Rossi S, Studer V, Motta C, Germani G, Macchiarulo G, Buttari F, et al. Cerebrospinal fluid detection of interleukin-1beta in phase of remission predicts disease progression in multiple sclerosis. J Neuroinflammation. 2014;11:32.

45. Thomas K, Sehr T, Proschmann U, Rodriguez-Leal FA, Haase R, Ziemssen T. Fingolimod additionally acts as immunomodulator focused on the innate immune system beyond its prominent effects on lymphocyte recirculation. J Neuroinflammation. 2017;14:41.

46. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood. 1991;77:1627-52.

47. Woiciechowsky C, Schöning B, Daberkow N, Asche K, Lanksch WR, Döcke WD, Volk HD. Brain IL-1beta increases neutrophil and decreases lymphocyte counts through stimulation of neuroimmune pathways. Neurobiol Dis. 1999;6:200-8.

48. D'Amico E, Zanghì A, Romano A, Sciandra M, Palumbo GAM, Patti F. The neutrophil-to-lymphocyte ratio is related to disease activity in relapsing remitting multiple sclerosis. Cells. 2019;8.

49. Heimke M, Lenz F, Rickert U, Lucius R, Cossais F. Anti-inflammatory properties of the SGLT2 inhibitor empagliflozin in activated primary microglia. Cells. 2022;11.