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ORIGINAL ARTICLE

Expression Profile of NLRP3 Inflammasome in Peripheral Blood Mononuclear Cells and Plasma Interleukin-22 Level in Multiple Sclerosis

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EDSS; Interleukin-22; Multiple sclerosis; NLRP3; inflammasome

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ABSTRACT

Background: *Multiple sclerosis (MS) is a long-lasting neurodegenerative disorder that primarily impacts young adults. Interleukin-22 (IL-22) and the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome play critical roles in host innate immunity and have been linked to several autoimmune diseases.* ***Objectives:*** *The objectives of our work are to assess the plasma levels of IL-22 and the expression of the NLRP3 inflammasome in peripheral blood mononuclear cells (PBMCs) in MS patients versus healthy controls. Furthermore, to correlate the level of plasma IL-22 and NLRP3 inflammasome expression in MS patients.* ***Methodology:*** *The study examined 40 patients with MS and 40 matched healthy controls. It measured plasma IL-22 using enzyme-linked immunosorbent assay (ELISA) and assessed NLRP3 expression through real-time reverse transcription polymerase chain reaction (RT-PCR).* ***Results:*** *Patients with MS exhibited markedly elevated levels of plasma IL-22 ($P < 0.0001$) and a significantly elevated relative expression of NLRP3 ($P < 0.0001$). Additionally, a significant positive correlation was observed between plasma IL-22 and NLRP3 relative expression ($r = 0.45$, $P = 0.003$). Also, ssignificant positive correlations were noted between plasma IL-22 level and both the Expanded Disability Status Scale (EDSS) and frequency of relapses in the past year ($r = 0.49$, 0.55, P-values $= 0.001$, 0.0002 respectively). Similarly, EDSS and frequency of relapses in the past year were positively correlated with NLRP3 relative expression ($r = 0.4$, 0.69, P-values $= 0.01$, < 0.00001 respectively).* ***Conclusion:*** *Both IL-22 and NLRP3 inflammasome could be encouraging prognostic markers and potential therapeutic targets for MS.*

INTRODUCTION

Multiple sclerosis (MS) is a prevalent condition of the central nervous system (CNS), impacting approximately 2.9 million individuals globally. It manifests as an autoimmune condition characterized by inflammation and demyelination of the nervous system. Incidence of MS is increasing worldwide, resulting in significant socioeconomic consequences. The precise cause of MS and the mechanisms driving this increase are still not fully understood, although many inherited and environmental elements are implicated. Variability of disease presentations and unpredictable progression highlight the challenges in diagnosing and treating MS[1, 2].

Interleukin-22 (IL-22) is generated by different types of immune cells and exerts its actions through the IL-22 receptor, located on non-immune cells of numerous organs. IL-22 enhances innate immune responses and supports tissue integrity and regeneration. Notably, it has been associated with multiple inflammatory diseases[3].

Inflammasomes are large multimeric protein complexes that are essential for triggering pyroptotic cell death in response to pathogens and cellular damage. Proper activation of the inflammasome is essential for the innate immune response; however, inappropriate inflammasome activation can lead to uncontrolled immune response that may be involved in several diseases, including autoinflammatory, cardiometabolic and neurodegenerative conditions [4, 5].

A typical inflammasome is composed of a sensor protein, an adaptor protein, and the effector caspase-1. The first identified family of sensor proteins was the nucleotide-binding oligomerization domain-like receptor (NLR) family [6]. Research is showing that hyperactivation of the NLRP3 inflammasome contributes to several inflammatory diseases[7].

Our study aimed to assess the plasma levels of IL-22 and expression levels of NLRP3 inflammasome in peripheral blood mononuclear cells (PBMCs) in MS patients in comparison to healthy controls. Furthermore, to correlate plasma IL-22 levels with the NLRP3 inflammasome expression in MS patients.

METHODOLOGY

Study population:

This observational cross-sectional study was carried out in the Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University over the period from April 2023 to January 2024 on 40 MS patients as well as 40 matched healthy individuals considering age and gender. The patients were recruited from MS Research Unit and Department of Neurology, Faculty of Medicine, Cairo University. Demographic characteristics, duration of the disease, clinical manifestations, EDSS [8], type of treatment and number of clinical attacks in the last year were recorded.

The study received approval from the Research Ethics Committee of the Institutional Review Board at Faculty of Medicine, Cairo University on 17/4/2022. Approval code is MD-52-2022. All participants were aware of the study's objectives and procedures, and informed consent was secured before their participation. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Inclusion and exclusion criteria:

The study involved patients aged 18 to 60 years who met the 2017 Revised McDonald Criteria for diagnosing MS [9]. While patients younger than 18 or older than 60 years, suffering concomitant other autoimmune diseases, suffering malignancy and pregnant females were excluded from the study.

Blood sample collection, IL-22 Level measuring and NLRP3 gene expression were done by the following steps:

I. Sample collection, transport and processing:

- A volume of 5 ml of peripheral venous blood was drawn from all participants in vacuum tubes with ethylenediaminetetraacetic acid (EDTA) by clean venepuncture under complete aseptic conditions. Each tube was labelled with the patient's name and collection date.
- Each blood sample underwent Ficoll-Hypaque density gradient centrifugation to separate PBMCs and plasma as follows [10]: the fresh blood was mixed with equal volume of balanced salt solution such as isotonic Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS), then the blood with PBS was carefully layered over Ficoll in the ratio of 2:1 in a tube (2 blood: 1 Ficoll), centrifugation was carried out at 1100 x g for 25 min at 20°C in a cooling centrifuge and finally, plasma and the layer of mononuclear cells were collected and preserved at -80°C.

II. Detection of IL-22 plasma levels by ELISA:

Quantitative detection of plasma levels of IL-22 was performed using Human IL-22 ELISA **Kit (SUNLONG BIOTECH CO., LTD, China)** following the manufacturer's procedures.

III. Gene expression of NLRP3 by real time RT-PCR was done using the following protocol:

First, purification of RNA from PBMCs was performed using RNeasy Mini Kit **(Qiagen, Valencia, CA, USA)** according to the manufacturer's procedures. Followed by complementary DNA (cDNA) synthesis by RT-PCR using the QuantiTect Reverse Transcription Kit **(Qiagen, Valencia, CA, USA)** according to the manufacturer's procedures **(Appendix 1 for detailed reaction mix and cycler condition)**. Then, amplification of the target cDNA was performed using QuantiNova SYBR Green PCR Kit **(Qiagen, Valencia, CA, USA),** Hs_GAPDH_1_SG QuantiTect Primer Assay (QT00079247) **(Qiagen, Valencia, CA, USA)** and HS_NLRP3_2_SG QuantiTect Primer Assay (QT01666343) **(Qiagen, Valencia, CA, USA)** following the manufacturer's guidelines. Quantitative PCR was performed using Rotor-Gene Q **(Qiagen, Valencia, CA, USA) (Appendix 2 for detailed reaction mix and cycler condition)**.

Data were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene [11]. The relative variations in NLRP3 expression were calculated using the $2^{-\Delta\Delta ct}$ method.

Statistical methods:

IBM SPSS® Statistics version 22 (IBM® Corp., Armonk, NY, USA) was used for statistical analysis. Numerical data were expressed as mean and standard deviation, or median and range, as appropriate. Qualitative data were expressed as frequency and percentage. Pearson's Chi-square test or Fisher's exact test was used to examine the relationship between qualitative variables. For normally distributed quantitative data, a comparison between two groups was done using the student's t-test. For not normally distributed quantitative data, a comparison between two groups was done using the Mann-Whitney test. All tests were two-tailed, and a p-value < 0.05 was considered statistically significant. Odds ratios was used to present strength of association between risk factors and outcomes. Correlation between groups was done using Pearson test.

RESULTS

The study was conducted on 80 subjects, 40 MS patients and 40 healthy individuals. The mean age of MS patients was 32.3 ± 8.8 years, ranging from 19 to 57 years. Of the patients, 12 (30%) were males and 28(70%) were females. The mean age of controls was 33.1±9.5 years, ranging from 21 to 55 years. Of the

controls, 18 (45%) were males and 22 (55%) were females. The patients and controls were paired based on age and gender.

The median total length of illness was 3 years, with an interquartile range (IQR) of 1 to 6.25. This range indicates that the length of illness varied greatly, with some cases lasting as short as 1 month and others lasting as long as 17 years. Additionally, the median EDSS was 3, with an IQR of 2.5 to 4, indicating that the severity of the disease also varied among the participants. The median number of attacks in the last year was 1.5, with an IQR of 1 to 2, but it ranged from 0 to 6.

Manifestations that presented during the progression of MS disease included cerebellar dysfunction in 22 patients (55%), motor dysfunction in 18 (45%), visual dysfunction in 14 (35%), cognitive and mood dysfunction in 14 (35%), bladder dysfunction in 13 (32.5%), brainstem dysfunction in 13 (32.5 %) and sensory dysfunction in 12 (30%) as shown in (Figure 1).

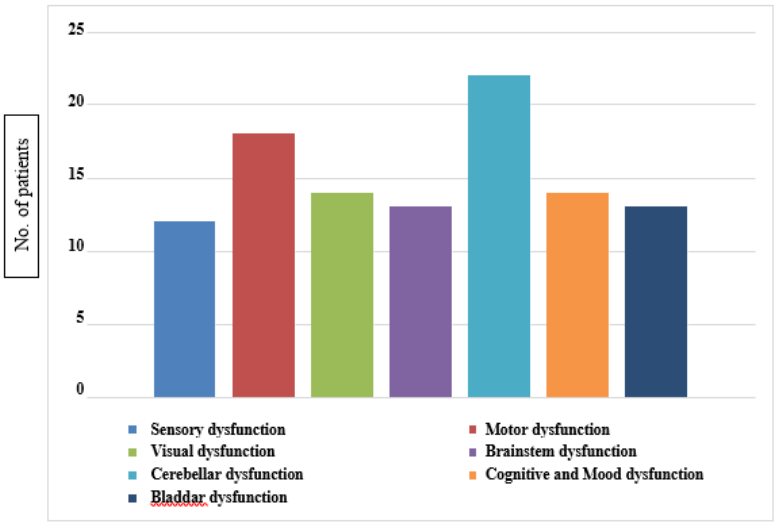


Fig. 1: Clinical manifestations during the course of the MS disease

Different disease modifying therapies (DMTs) were taken by MS patients. Nine patients were taking fingolimod, 8 were on INF-β 1a, 6 were on rituximab, 6 were receiving monthly methylprednisolone, 4 were taking ocrelizumab, 2 were on teriflunomide, and 5 were not receiving any treatment (Figure 2).

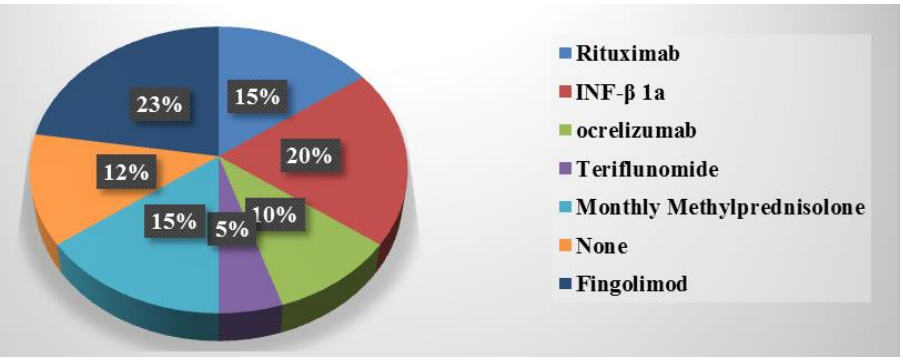


Fig. 2: Disease modifying therapies in the MS patients

The median plasma level of IL-22 (pg/ml) was significantly higher in MS patients (17.46, IQR: 13.2 to 20.9) compared to controls (12.39, IQR; 10.92 to 15.28). Also, the median NLRP3 relative expression was significantly higher in patients (6.28, IQR: 4.42 to 9.61) compared to controls (1.42, IQR; 0.498 to 2.167) as shown in (Table 1).

Table 1: Comparing plasma IL-22 level (pg/ml) and NLRP3 relative expression between MS patients and controls

	Controls				Patients				*P- value*
	Range	**Median**	**95% CI**	**IQR**	**Range**	**Median**	**95% CI**	**IQR**	
Plasma IL-22 level (pg/ml)	7.31 to 19.59	12.39	11.69 to 14.17	10.92 to 15.28	10.15 to 79.65	17.46	13.952 to 19.7	13.2 to 20.92	***< 0.0001***
NLRP3 relative expression	0.12 to 4.47	1.42	0.61 to 1.86	0.49 to 2.16	2.2 to 12.91	6.28	5.66 to 9.02	4.42 to 9.61	***< 0.0001***

CI: Confidence interval, IQR: Interquartile range

There were insignificant variations in IL-22 level and NLRP3 expression between male and female patients with *P-value* of 0.7 and 0.09 respectively. Similarly, no significant difference was found between different types of DMTs regarding plasma IL-22 level or NLRP3 relative expression.

Levels of IL-22 and relative expression of NLRP3 were significantly elevated in MS patients exhibiting motor and brainstem dysfunction compared to those who did not. However, insignificant differences were detected in plasma levels of IL-22 and NLRP3 relative expression among MS patients with and without other clinical manifestations, as shown in table (2).

Table 2: Correlation between IL-22 levels (pg/ml) and NLRP3 relative expression in relation to the clinical manifestations of MS patients

Variable		Plasma IL-22 level (pg/ml)				NLRP3 relative expression			
		n	**Median**	**Average Rank**	***P*-value**	**n**	**Median**	**Average Rank**	***P*-value**
Motor dysfunction	**Present**	18	20.9250	27.6944	0.0004	18	9.6181	26.8611	0.0019
	Absent	22	14.0175	14.6136		22	5.6598	15.2955	
Sensory dysfunction	**Present**	13	17.2650	18.9231	0.5539	13	5.6598	16.3077	0.1155
	Absent	27	17.6700	21.2593		27	7.7315	22.5185	
Cerebellar dysfunction	**Present**	22	17.8725	22.2273	0.3015	22	7.2078	21.9318	0.3918
	Absent	18	15.7425	18.3889		18	5.7991	18.7500	
Brainstem dysfunction	**Present**	13	20.9250	30.6923	0.0001	13	9.6515	26.8846	0.0165
	Absent	27	13.8150	15.5926		27	5.7388	17.4259	
Visual dysfunction	**Present**	14	14.0175	16.3929	0.1030	14	6.2366	19.2857	0.6298
	Absent	26	17.8725	22.7115		26	8.2757	21.1538	
Bladder dysfunction	**Present**	13	15.6450	19.0000	0.5734	13	6.2366	21.0769	0.8285
	Absent	27	18.0750	21.2222		27	6.3236	20.2222	
Cognitive and Mood dysfunction	**Present**	14	19.5075	24.3571	0.1257	14	9.0054	23.6071	0.2174
	Absent	26	16.4550	18.4231		26	6.0480	18.8269	

n: number of patients

A significant positive correlation was observed between plasma IL-22 and the relative expression of NLRP3 MS in patients, r = 0.45, *P* = 0.003, (figure 3). In addition, significant positive correlations were also observed between plasma IL-22 levels and both EDSS score as well as the frequency of relapses in the past year (r = 0.49, 0.55, *P*-values = 0.001, 0.0002 respectively) (figure 4). However, insignificant correlations were observed between plasma IL-22 and the patient's age or the overall duration of the disease (r = -0.17, 0.04, *P*- values = 0.29, 0.8 respectively).

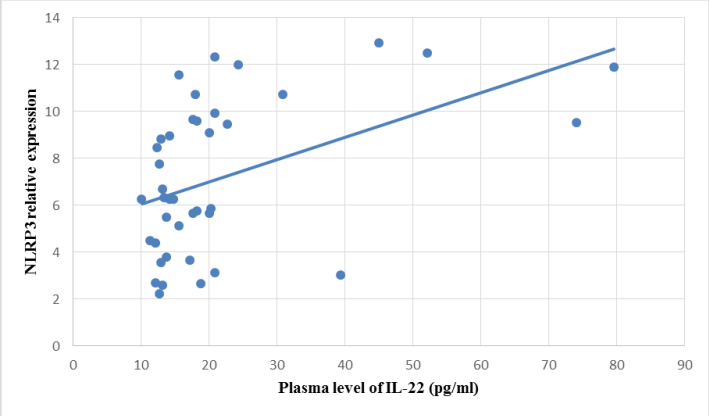
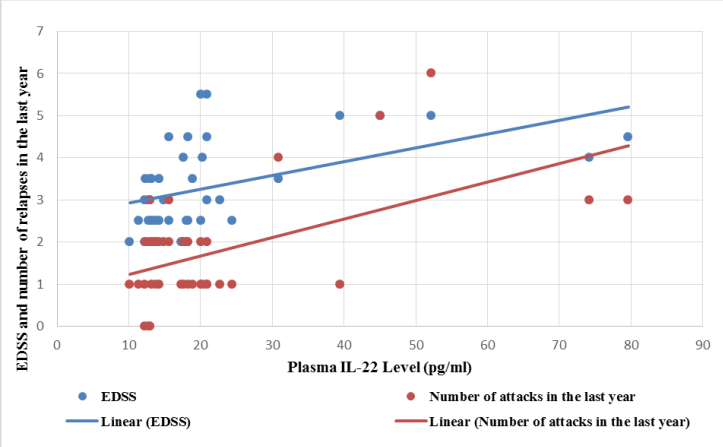


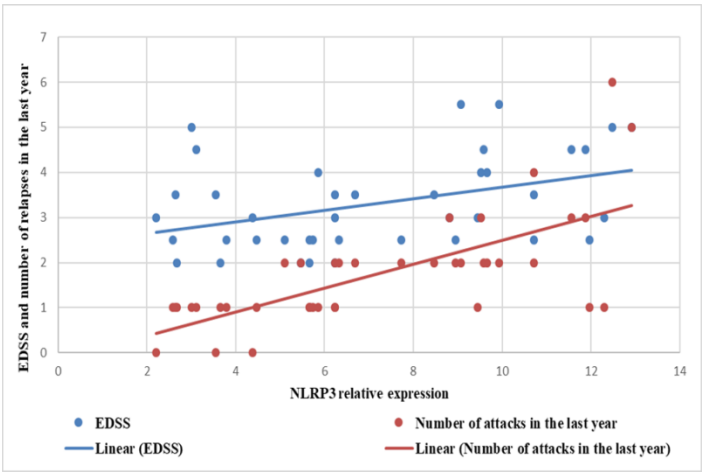
Fig. 3: Correlation between plasma IL-22 (pg/ml) and NLRP3 relative expression in MS patients



EDSS: Expanded Disability Status Scale

Fig. 4: Correlations between IL-22 levels (pg/ml) and both EDSS and frequency of relapses in the last year

Moreover, significant positive correlations were detected between NLRP3 relative expression and both EDSS score and frequancy of relapses in the past year (r = 0.4, 0.69, P-values = 0.01, < 0.00001 respectively) (figure 5). However, no significant correlations were found between NLRP3 relative expression and either the patient's age or total length of disease (r = -0.18, 0.01, P-values = 0.26, 0.95 respectively).



EDSS: Expanded Disability Status Scale

Fig. 5: Correlations between NLRP3 relative expression and both EDSS score and frequency of relapses in the past year

DISCUSSION

Multiple sclerosis (MS) is long-lasting autoimmune condition marked by inflammatory demyelinating neurodegenerative lesions in the CNS. The clinical manifestations and progression of MS are considerably heterogeneous among different individuals. Therefore, it is crucial to identify specific characteristics of the disease that aid in diagnosis and prognosis, as well as in evaluating the response to treatment [12]. In this context, biological markers in MS may aid in differentiating between various clinical courses that have unique prognoses. More importantly, they could serve as therapeutic targets, providing a basis for developing new or more effective treatments for the disease [11].

This study intended to compare expression levels of NLRP3 inflammasome in PBMCs and plasma IL-22 levels in MS patients with that in healthy controls as well as to correlate the NLRP3 expression level with the IL-22 levels in MS patients. The present study was conducted on 80 subjects, 40 patients with MS and 40 healthy individual.

The MS patients show significantly higher NLRP3 relative expression in PBMCs (median=6.28, IQR: 4.42 to 9.61) compared to the healthy controls (median=1.42, IQR; 0.498 to 2.167) (P<0.0001). Also, significant positive correlations were observed between NLRP3 relative expression and both EDSS score and frequency of relapses in the past year (r = 0.4, 0.69, P-value = 0.01, < 0.00001 respectively).

These results parallel those of Malhotra *et al.*,[11] who documented an upregulation of NLRP3 gene expression and its associated cytokine IL-1β besides an increased Caspase-1 activity in PBMCs of MS patients when compared to healthy subjects. These results support the implication of the NLRP3 pathway in progress of pro-inflammatory state observed in these patients. They also reported that patients with higher IL-1β expression levels progressed faster to EDSS 6.0. However, in contrast to our study, this fast progression to EDSS 6.0 was not observed when correlated with NLRP3 expression levels. This discrepancy may be linked to the variations in sample size, disease length, genetic background, and environmental factors. In addition, studies have shown higher NLRP3 expression levels in MS patients who are non-responders to some therapies such as IFN-β and fingolimod compared to drug responders [13, 14].

Moreover, Soares *et al.*,[15] reported variants of NLRP3 gene that were linked with intensity and progress of MS and demonstrated an increased activation of NLRP3 inflammasome may act as a risk factor for the manifestation of MS. Similarly, a study carried out by Vidmar *et al.*, [16] has revealed increased genetic variations in NLRP3 and Caspase-1 (CASP1) genes among MS patients presenting more evidence about the importance of this pathway in MS development and progression. Likewise, *Keane et al.*, [17] observed an elevated serum level of inflammasome proteins, Caspase-1, and IL-18 in MS patients compared to healthy subjects.

Furthermore, increased activity of the NLRP3 inflammasome was identified as a crucial mechanism contributing to the development and severity of experimental autoimmune encephalomyelitis (EAE) and inhibition of NLRP3 inflammasome was found to exert a protective effect in EAE [18, 19]. Also, studies have reported an upregulation of NLRP3 gene expression in microglia of MS patients [20, 21].

Relevance of NLRP3 inflammasome activation in human pathologies like MS indicates that it might act as a valuable prognostic marker and treatment goal. Several therapeutic strategies have been explored to achieve this, including the inhibition of caspase-1, IL-1β, IL-18, and NLRP3 itself [7].

In the current study, the MS patients show significantly higher plasma level of IL-22 (median=17.46, IQR: 13.2 to 20.9) compared to the healthy controls (median=12.39, IQR; 10.92 to 15.28) ($P<0.0001$). Also, significant positive correlations were observed between IL-22 levels and both the EDSS scores and the frequency of relapses in the past year (r=0.49, 0.55, P-value=0.001, 0.0002 respectively).

In agreement with our results, Perriard *et al.*,[22] have documented increased IL-22 levels in MS patients when compared to the controls. Similarly, two studies carried out by Xu *et al.*,[23] and Zhen *et al.*,[24] described significant rise in the IL-22 serum levels along with the percentage of IL-22-secreating TH22 lymphocytes in blood of MS patients when compared to the controls. In addition, a study performed by Rolla *et al.*, [25] revealed that TH22 lymphocytes elevated in blood and CSF of patients during relapses. Also, Muls *et al.*, [26]. reported a significant rise in IL-22 expression levels in PBMCs in patients with relapses in comparison to those in remission.

Likewise, Abdel-Dayem *et al.*,[27] have reported a statistically significant rise in IL-22 levels in MS patients that showed a statistically significant decrease upon using IFN-β or fingolimod at 6 and 12 months of therapy compared to their levels prior to therapy. The latter study has showed a positive correlation between IL-22 levels and EDSS which aligns with our study as well. Furthermore, a study was carried out by Wing *et al.*, [28] have reported that in vitro IL-22 production by Myelin basic protein (MBP)-specific T cells from MS patients was higher than that of controls. The latter study did show positive correlation between IL-22 production and the number of active brain lesions but not with EDSS, in contrast to our study. Moreover, Jaime-Pérez *et al.*, [29] have described a statistically lower levels of IL-22 in MS patients after experiencing

autologous hematopoietic stem cell transplantation (AHSCT) but, in contrast to our study, didn't show a correlation between IL-22 and EDSS.

The research has indicated that IL-22 level is increased during both the induction and peak phases of EAE, with a decrease noted during neurological recovery. These findings are crucial as they propose that IL-22 may contribute in the underlying pathogenesis of EAE [30]. The research carried out by Zhen *et al.*, [24] reported increased IL-22 which promotes Fas expression in oligodendrocytes causing their apoptosis and also, inhibits FOXP3 expression in T cells decreasing the proportion of regulatory T cells in PBMCs of MS patients. Furthermore, Perriard *et al.*, [22] reported the elevated expression of IL-22 receptor by astrocytes in MS plaques.

As far as we know, this study is the first to document significant positive correlation between plasma IL-22 levels and NLRP3 relative expression (r = 0.45, p = 0.003) in MS patients. This can be attributed to the proinflammatory state of MS patients. Additional research is needed for clarification of the connection between IL-22 and the NLRP3 inflammasome, as well as to determine whether they work together in the pathogenesis of MS. Furthermore, to our knowledge, this study is the first to indicate that both NLRP3 relative expression and plasma IL-22 levels (pg/ml) are significantly elevated in MS patients experiencing motor and brainstem dysfunction compared to those who do not (P=0.0019, 0.0165 and 0.0004, 0.0001 respectively).

Limitations:

The sample size was relatively small, so future investigations can rely on larger samples for more confirming results. The study didn't take into account the differentiation between types of MS. Therefore, further investigations are also needed for clarification of the expression profiles of both NLRP3 and IL-22 in each type separately.

CONCLUSIONS

Patients with MS show increased relative expression of NLRP3 in PBMCs and higher plasma IL-22 levels in comparison to healthy individuals. There is a notable positive correlation between plasma IL-22 and NLRP3 expression. Additionally, both NLRP3 expression and IL-22 levels correlate positively with EDSS scores and frequency of relapses over the past year. MS patients with motor and brainstem dysfunction demonstrate significantly elevated levels of NLRP3 and IL-22 in comparison to those without these dysfunctions.

Recommendations

The NLRP3 inflammasome holds promise as a prognostic factor and therapeutic goal in MS. IL-22 could also act as a prognostic factor, but its specific role in MS pathogenesis requires further study. Additional research is needed to investigate the association between NLRP3 and IL-22, especially regarding their potential synergistic effects in MS. Additional researches to profile the expression of both NLRP3 and IL-22 in various MS types are essential. Trials evaluating the effectiveness of NLRP3 inflammasome inhibitors as a therapeutic option in MS are also necessary.

Conflict of interest: All authors approve that they have no conflicts of interest linked to this work.

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