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Evaluation of circulating IL-35, IL-39, and oncostatin M as potential biomarkers in relapsing-remitting multiple sclerosis

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Abstract

Background: Multiple sclerosis (MS) is an immune-mediated, chronic inflammatory demyelinating disease of the central nervous system (CNS). Hence, there is a need to identify more biomarkers that may assist in developing new therapeutic strategies. This study aims to compare the levels of interleukin (IL)-35, IL-39, and oncostatin M (OSM) in patients with relapsing-remitting MS (RRMS) and healthy controls. *Methods:* Blood samples were collected from 38 patients with RRMS and 36 healthy individuals who served as a control group to measure the levels of the cytokines under study. The plasma levels of IL-35, IL-39, and OSM were measured and compared between the two groups using enzyme-linked immunosorbent assay (ELISA). Additionally, the expression of these cytokines was evaluated using reverse transcription polymerase chain reaction (RT-PCR). *Results:* Our results showed that RRMS is associated with higher levels of OSM and IL-39 and lower levels of IL-35 compared to healthy subjects. The results demonstrated that the levels of OSM and IL-39 in RRMS patients were significantly reduced by IFN-β therapy. EDSS scores and IL-35 levels were found to be negatively correlated, while OSM levels and EDSS scores showed a positive and significant correlation. Statistical analysis revealed no significant relationship between IL-35, IL-39, and OSM plasma levels and factors such as age, gender, education level, and job.
Conclusion: Elevated levels of OSM and IL-39 and decreased levels of IL-35 in RRMS patients suggest their potential as biomarkers for disease activity and progression. The correlation between these cytokines and EDSS scores further supports their relevance. Future research should focus on further elucidating the roles of these cytokines in RRMS and exploring their potential as therapeutic targets.

Keywords: Relapsing-remitting multiple sclerosis, RRMS, oncostatin M, IL-35, IL-39, multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder of the nervous system with a high prevalence, characterized by progressive demyelination of the brain and spinal cord.[1] This disease is an autoimmune and neurodegenerative disorder of the central nervous system (CNS) caused by an abnormal immune response against myelin antigens.[2]

Relapsing-remitting multiple sclerosis (RRMS) is marked by sudden exacerbations of symptoms (relapses) followed by periods of recovery where symptoms decrease or disappear.[3] The symptoms of this disease result from temporary attacks by immune cells on myelin and axons.[4]

Studies have shown that the symptoms of MS typically appear between the ages of 20 and 40, with women being twice as susceptible as men.[5] Approximately 2.3 million people worldwide (50-

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300 per 100,000 population) suffer from MS.[6,7] Several preliminary studies have been published on the subtypes and common clinical symptoms of MS in the Iranian population.[8]

Despite extensive research, the cause of MS remains unknown. Studies suggest that both environmental and genetic factors play a role, including genetic susceptibilities, age, sex, metabolism, smoking, vitamin D deficiency, race, climate, viral infections with Epstein-Barr virus (EBV) being the most significant candidate), and radiation exposure. Ultraviolet B (UVB) radiation is also implicated in the development of this autoimmune and recurrent disease.[9]

Inflammatory mediators play crucial roles in the pathogenesis and progression of MS.[10] Tissue damage in MS results from a complex interaction between the immune system, glia, and neurons.[11] During MS relapses, the CNS is invaded and activated by proinflammatory cytokines and chemokines, leading to demyelinating lesions, axonal damage, and neuronal loss.[12] The development and progression of the disease may be aided by pathogenic T and B lymphocytes, also known as self-attacking cells, which infiltrate the CNS and disrupt cellular function.[13] The pathophysiology of MS largely depends on immune responses mediated by T helper (Th) cells.[14,15] Immunosuppressive cytokines associated with regulatory T (Treg) cells, such as interleukin (IL)-10, transforming growth factor beta (TGF-β), and IL-35, have also been identified.[16] Earlier research indicates that a proinflammatory milieu in the cerebrospinal fluid (CSF) may influence MS progression and disease reactivations.[12]

IL-35 is the newest member of the IL-12 family of cytokines, which includes IL-12, IL-23, IL-27, IL-35, and IL-39.[17,18] Treg cells are the primary source of the immunosuppressive cytokine IL-35.[19,20] Defects in IL-35 production have been associated with the development and exacerbation of various inflammatory diseases, such as encephalomyelitis, inflammatory bowel disease (IBD), liver fibrosis, and models of lethal autoimmune disease.[10,19] Previous studies have shown that IL-12, IL-23, IL-27, and IL-35 play significant roles in patients with neuromyelitis optica spectrum disorder (NMOSD).[18,21] Patients with NMOSD had significantly lower serum IL-35 levels, which were inversely correlated with the severity of the illness.[17,21]

IL-39 is a newly recognized member of the IL-12 family.[17] Research indicates that activated B cells produce the pro-inflammatory cytokine IL-39, which significantly increases and aids in disease progression in lupus mice.[22]

Oncostatin M (OSM) is a small four-helix bundle composed of a single polypeptide and belongs to the IL-6 family, which includes IL-6, IL-11, IL-27, IL-31, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin 1, and cardiotrophin-like cytokine factor 1.[23] OSM is produced primarily by hematopoietic cells, such as T cells, monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, and mast cells, in a signal transducer and activator of transcription (STAT) 5-dependent manner.[23,24] OSM is crucial for several biological functions, including the attraction of white blood cells, the development of T cells outside the thymus, immune stimulation of brain endothelial cells, degradation of nerve tissue, and increased synthesis of P-selectins.[13,23]

Although extensive studies on MS have been conducted and many treatment options proposed, there is no definitive cure for RRMS. Current treatment strategies primarily aim to reduce the risk of relapse.[25] Treatment remains inadequate, and there is a significant lack of biomarkers for diagnosis, real relapses, disease progression, and response to treatment.[26] Therefore, any study regarding factors related to relapse is crucial for understanding the pathogenesis and prevention of MS. Identifying biomarkers that predict disease progression is of great importance. An ideal biomarker should have diagnostic and prognostic value, correlate with specific disease activities such as relapse or progression, respond to treatment, and be fundamentally useful in clinical trial outcomes. Considering the issues discussed in this research, this study aimed to investigate the levels of IL-35, IL-39, and OSM in patients with RRMS and compare them with those in a healthy population.

METHODS

Study participants

The participants of this study included two groups: cases and healthy controls, totaling 74 individuals. The study comprised 38 patients with RRMS with a mean age of 31.66±9.9 years (based on modified McDonald's criteria[27], and a control group of 36 sex-matched healthy individuals with an average age of 35.11±6.67 years. Notably, none of the patients had experienced a relapse in the year preceding the study. All participants provided written informed consent. The Shiraz University of Medical Sciences Research Ethics Committee approved the study (number: IR.SUMS.REC.

1402.422). The Expanded Disability Status Scale (EDSS) was used to assess the functional status of the patients.

Patients with the following conditions were excluded from the study: other types of MS, other neurodegenerative disorders, history of cardiovascular or renal diseases, organ transplantation, various malignancies, and other types of autoimmune diseases. Additionally, none of the patients had received corticosteroid treatment for at least six months before the sample collection.

RNA extraction and real-time PCR

Three to 5 ml blood samples were collected from all participants in ethylenediamine tetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation. Total RNA was extracted from PBMCs using TRIzol, (Invitrogen, Massachusetts, United States) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were assessed using a NanoDrop spectrophotometer. Only samples with an A260/A280 ratio between 1.8 and 2.1 were included in the study.

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Takara PrimeScript RT Master Mix (Takara Bio Inc., Japan) following the manufacturer's protocol. Real-time PCR was performed using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with SYBR Green chemistry. The primers for IL-35, IL-39, OSM, and the reference gene GAPDH were designed using Primer3 software and synthesized by Integrated DNA Technologies. Each reaction contained 2 μL of cDNA, 10 μL of SYBR Green Master Mix, 1 μL of each primer (10 μM), and 6 μL of nuclease-free water in a final volume of 20 μL. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 72°C for 1 minute. Melting curve analysis was performed to confirm the specificity of the amplified products. The relative expression levels of IL-35, IL-39, and OSM were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene.

Assessment of cytokines

To measure the levels of cytokines under investigation, 3 to 5 ml of peripheral blood was collected from RRMS patients and healthy individuals into vacuum tubes containing EDTA. The plasma was then separated using a centrifuge at 2400 ×g for 10 minutes. The plasma samples were stored at -80 °C until testing. The levels of IL-35, IL-39, and OSM were measured using commercial kits (R&D Systems, USA) and the enzyme-linked immunosorbent assay (ELISA) method. All procedures were conducted in accordance with the manufacturer's guidelines.

Statistical analysis

The Kolmogorov-Smirnov Z statistical test was performed to check the normal distribution of IL-35, IL-39, and OSM plasma levels. All the data in this article are presented as mean ± standard deviation (SD) or number (percentage), and P-values less than 0.05 were considered statistically significant. The association between the cytokines under study and MS patients was assessed using a logistic regression model that adjusted for confounding variables. All statistical analyses in this study were performed using IBM SPSS Statistics Version 24.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Demographic and clinical characteristics of study participants

In this study, we investigated the levels of IL-35, IL-39, and OSM in 74 individuals (38 RRMS patients and 36 healthy controls). The demographic characteristics of the participants in this study are displayed in Table 1. Additionally, we compared various factors such as age, sex, education level, and occupation between both groups. Tables 2, 3, and 4 display the comparison of the mean plasma levels of IL-35, IL-39, and OSM, respectively, with these factors. These variables were shown to have no discernible relationship to the plasma levels of cytokines in either the case or control groups. The analysis also revealed no significant results regarding the correlation between duration of disease and serum vitamin D levels with the cytokine levels. This indicates that these factors do not appear to influence the plasma concentrations of IL-35, IL-39, or OSM in the context of RRMS.

Expression levels of IL-35, IL-39, and OSM in RRMS patients vs. healthy subjects

In this study, we evaluated the expression levels of IL-35, IL-39, and OSM in PBMCs isolated

Table 1: Demographic characteristics of subjects

Characteristic	RRMS	HC	p value	Correlations	
n	38	36	0.90	-	
Female	22	18	0.49	-	
Male	16	18		-	
Age (years)[a]	31.66±9.9	35.11±6.67	0.11	-	
Serum 25 (OH) D (ng/ml)[a]	20.69±1.59	21.03±1.89	0.09	IL-35	P=0.63 r=-0.07
				IL-39	P=0.73 r=0.05
				OSM	P=0.86 r=-0.02
EDSS	1.5±0.73	-	-	IL-35	P=0.01* r=-0.40
				IL-39	P=0.18 r=0.22
				OSM	P=0.0017* r=0.49
Duration of the disease [b]	2.63±1.4	-	-	IL-35	P=0.62 r=-0.08
				IL-39	P=0.18 r=0.21
				OSM	P=0.28 r=0.17

[a]Data is expressed as mean ± SD.
[b] Year (mean ± SD)

Table 2: Comparison of plasma IL-35 level means in various factors within the case (RRMS patients) and control groups

Groups	Factors		IL-35 (pg/ml) Mean ± SE	p-value
Control	Age (years)	Less than 25 (n=3)	98.43±4.9	0.49
		25-35 (n=18)	102.2±5.6	
		Upper than 35 (n=15)	89.83±2.75	
	Sex	Female (n=18)	92.75±2.87	0.33
		Male (n=18)	88.88±2.76	
	Education	School education (n=23)	91.7±2.46	0.56
		University education (n=13)	89.24±3.47	
	Job	Non-employee (n=28)	91.47±2.24	0.54
		Employee (n=8)	88.53±4.5	
Case (RRMS)	Age (years)	Less than 25 (n=10)	78.07±4.99	0.66
		25-35 (n=16)	84.67±3.73	
		Upper than 35 (n=12)	75.8±3.63	
	Sex	Female (n=22)	81.97±3.36	0.37
		Male (n=16)	77.6±3.25	
	Education	School education (n=25)	78.33±2.67	0.29
		University education (n=13)	83.6±4.67	
	Job	Non-employee (n=32)	81.49±2.58	0.18
		Employee (n=6)	72.87±5.63	
	Treated with IFN-β	No (n=7)	75.09±5.06	0.31
		Yes (n=31)	81.27±2.67	

Table 3: Comparison of plasma IL-39 level means in various factors within the case (RRMS patients) and control groups

Groups	Factors		IL-39 (pg/ml) Mean ± SE	p-value
Control	Age (years)	Less than 25 (n=3)	73.69±7.08	0.69
		25-35 (n=18)	79.31±6.13	
		Upper than 35 (n=15)	75.2±2.98	
	Sex	Female (n=18)	69.66±2.89	0.62
		Male (n=18)	71.51±2.43	
	Education	School education (n=23)	70.36±2.11	0.87
		University education (n=13)	70.98±3.69	
	Job	Non-employee (n=28)	70.41±1.98	0.86
		Employee (n=8)	71.2±5.03	
Case (RRMS)	Age (years)	Less than 25 (n=10)	74.82±4.9	0.74
		25-35 (n=16)	79.75±3.48	
		Upper than 35 (n=12)	78.59±5.34	
	Sex	Female (n=22)	74.44±3.26	0.09
		Male (n=16)	83.09±3.74	
	Education	School education (n=25)	75.84±3.08	0.22
		University education (n=13)	82.4±4.32	
	Job	Non-employee (n=32)	76.33±2.88	0.11
		Employee (n=6)	87.44±2.15	
	Treated with IFN-β	No (n=7)	89.54±4.69	0.02*
		Yes (n=31)	75.5±2.73	

Table 4: Comparison of plasma OSM level means in various factors within the case (RRMS patients) and control groups

Groups	Factors		OSM (pg/ml) Mean ± SE	p-value
Control	Age (years)	Less than 25 (n=3)	58.7±7.2	0.76
		25-35 (n=18)	55.18±5.3	
		Upper than 35 (n=15)	57.23±4.09	
	Sex	Female (n=18)	56.66±3.15	0.39
		Male (n=18)	52.93±3	
	Education	School education (n=23)	53.5±2.68	0.43
		University education (n=13)	58.08±3.72	
	Job	Non-employee (n=28)	55.75±2.4	0.41
		Employee (n=8)	51.44±4.89	
Case (RRMS)	Age (years)	Less than 25 (n=10)	63.24±6.5	0.73
		25-35 (n=16)	63.36±6.2	
		Upper than 35 (n=12)	69.8±6.6	
	Sex	Female (n=22)	63.91±4.9	0.65
		Male (n=16)	67.36±5.7	
	Education	School education (n=25)	67±4.8	0.54
		University education (n=13)	62.22±5.88	
	Job	Non-employee (n=32)	63.61±10.8	0.38
		Employee (n=6)	56.52±9.93	
	Treated with IFN-β	No (n=7)	62.01±6.3	0.04*
		Yes (n=31)	51.46±2	

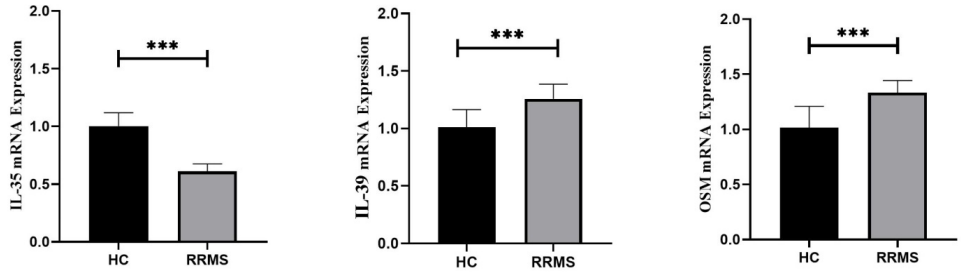

Figure 1. Expression levels of IL-35, IL-39, and OSM in PBMCs from patients with RRMS compared to healthy controls. The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as *** for $p < 0.001$.
PBMC; peripheral blood mononuclear cells, RRMS; relapsing-remitting multiple sclerosis, OSM; oncostatin M, HC; healthy controls

from patients with RRMS compared to healthy controls. Our results demonstrated a significant decrease in IL-35 expression in PBMCs from RRMS patients compared to healthy subjects, suggesting a potential impairment in the regulatory mechanisms mediated by this cytokine in the context of RRMS. Conversely, both IL-39 and OSM exhibited significantly elevated expression levels in the PBMCs of RRMS patients (Figure 1). These findings indicate a heightened inflammatory response in RRMS patients, potentially contributing to the disease's pathophysiology.

Plasma IL-35, IL-39, and OSM levels in RRMS patients vs. healthy subjects

Figure 2 compares the mean levels of IL-35, IL-39, and OSM in subjects with RRMS and healthy control subjects. The findings indicate that, compared to healthy subjects, RRMS is associated with lower levels of IL-35 and higher levels of IL-39 and OSM. These variations in cytokine levels may suggest their potential roles in the pathophysiology of RRMS, highlighting their potential as therapeutic targets or biomarkers.

Effect of IFN-β treatment on plasma cytokine levels

The effect of IFN-β therapy on IL-35, IL-39, and OSM levels in patients with RRMS was investigated. As shown in Figure 3, IFN-β therapy significantly reduced the levels of OSM and IL-39 in RRMS patients. These results indicate that IFN-β treatment decreases the levels of these cytokines in individuals with RRMS. The investigation into the effects of IFN-β therapy on IL-35 levels in patients with RRMS revealed no significant results. While the therapy led to a marked reduction in the levels of OSM and IL-39, IL-35 levels did not show any notable changes in response to the treatment. This indicates that IFN-β does not affect IL-35 levels in the same manner as it does for the other cytokines studied.

Correlation of plasma IL-35, IL-39, and OSM levels and EDSS of patients

The correlation between cytokine levels (IL-

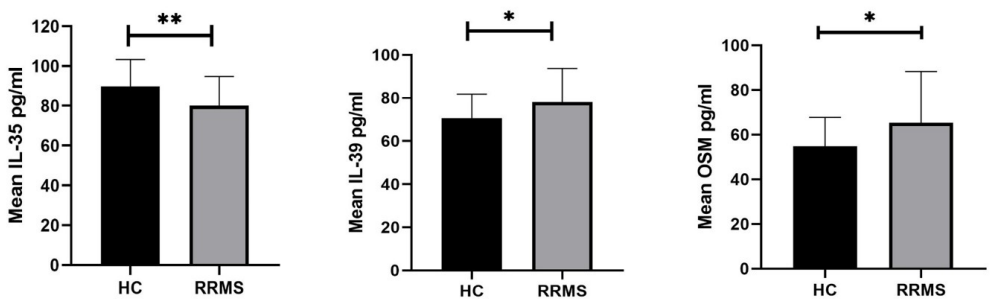

Figure 2. The mean levels of different cytokines in two groups: healthy control and RRMS. The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as ** for $p < 0.01$, and * for $p < 0.05$.
RRMS; relapsing-remitting multiple sclerosis, OSM; oncostatin M, HC; healthy controls

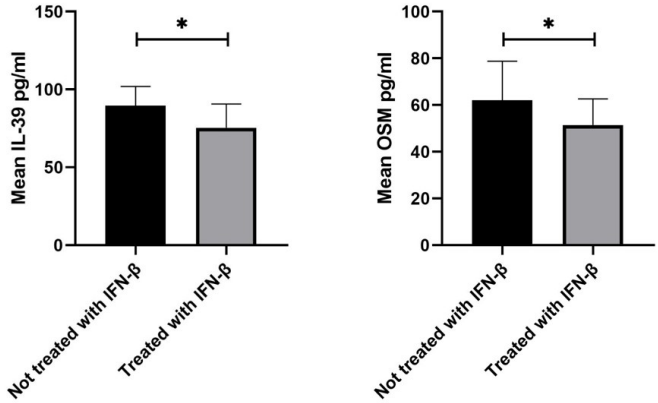

Figure 3. The effect of IFN-β treatment on cytokine levels in RRMS patients. The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as * for $p < 0.05$.
RRMS; relapsing-remitting multiple sclerosis, IFN-β; interferon-beta, OSM; oncostatin M

35, IL-39, and OSM) and EDSS scores was investigated in patients with RRMS. A negative correlation was found between EDSS scores and IL-35 levels, indicating that lower disability was associated with higher IL-35 levels. Conversely, a positive and significant correlation was observed between OSM levels and EDSS scores, suggesting that higher OSM levels are linked to greater disability in RRMS patients. However, no significant correlation was found between IL-39 levels and EDSS scores, indicating that IL-39 does not serve as a reliable indicator of disease severity in this context (Figure 4).

DISCUSSION

The findings of this study provide novel insights into the potential roles of IL-35, IL-39, and OSM in RRMS pathogenesis. Numerous earlier studies have identified pro-inflammatory and anti-inflammatory cytokines involved in MS immunopathogenesis.[28] However, the relationships between these recently discovered cytokines and RRMS severity and treatment response remain unclear.

Our findings indicate that plasma cytokine levels measured by ELISA correspond closely with mRNA expression levels measured by PCR. Specifically, the elevated plasma levels of IL-39 and OSM in RRMS patients align with their increased mRNA expression in PBMCs, suggesting an amplified systemic and cellular inflammatory response. Conversely, reduced IL-35 plasma levels are consistent with lower IL-35 mRNA expression, indicating a deficiency

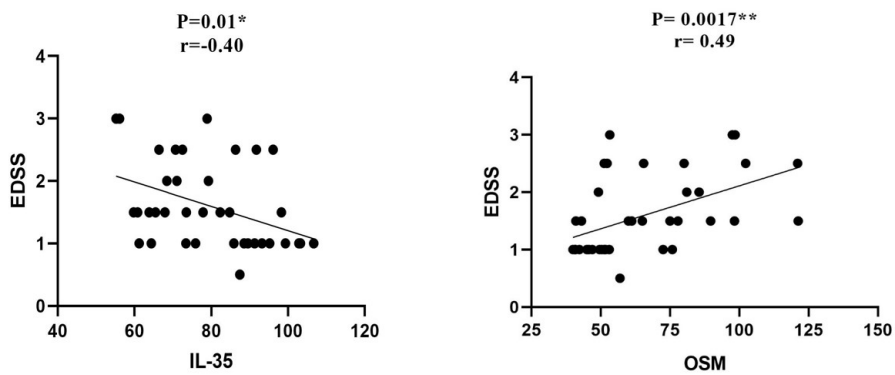

Figure 4. The correlation between cytokine levels (IL-35 and OSM) and EDSS score in patients with RRMS. The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as ** for $p < 0.01$, and * for $p < 0.05$.
RRMS; relapsing-remitting multiple sclerosis, EDSS; expanded disability status scale, OSM; oncostatin M

in regulatory immune function at both the protein and transcript levels. IL-35 is primarily produced by Treg cells and plays a crucial role in immunosuppression. Lower circulating IL-35 levels in RRMS patients concur with prior evidence indicating defective Treg number and function in MS.[29] Reduced IL-35 may diminish the suppressive immune environment, allowing autoimmune reactions against CNS antigens to occur unchecked and MS lesions to develop. This finding corroborates previous reports of lower IL-35 levels correlating with more active NMOSD.[21]

In contrast, increased levels of IL-39 and OSM in RRMS patients suggest their potential involvement in disease pathogenesis by promoting inflammation. IL-39 was recently identified as a pro-inflammatory cytokine produced by activated B cells (30). Higher IL-39 may augment B cell responses and subsequent autoantibody production implicated in MS pathology.

OSM is primarily synthesized by monocytes, macrophages, and T cells to attract other immune cells and promote endothelial activation.[31] Elevated OSM could enhance leukocyte infiltration into the CNS and blood-brain barrier (BBB) disruption during relapses.[32] Our results substantiate earlier research demonstrating elevated OSM levels correlated with greater disability in MS.[33]

Interestingly, IFN-β therapy significantly lowered OSM and IL-39 levels in treated RRMS patients compared to non-IFN-β-treated individuals. This provides evidence that disease-modifying drugs exert part of their therapeutic effects by modulating pro-inflammatory cytokines. IFN-β is known to suppress immune cell activities and decrease inflammatory mediators.[34] Thus, reduced OSM and IL-39 upon IFN-β treatment may reflect attenuated immune-mediated inflammation in RRMS. Notably, no significant relationships were found between cytokine levels and demographic/clinical factors such as age, sex, education, occupation, or prior relapse frequency. This implicates these cytokines as disease-specific rather than confounded biomarkers.

As noted in prior studies, cytokine profiles may evolve as MS transitions from an inflammatory to a degenerative phase.[35] Our study focused on patients with RRMS, a predominantly inflammatory phase, which may explain the observed cytokine patterns. Future longitudinal studies should investigate how cytokine levels change with disease progression to validate their role as biomarkers in different MS phases.

EDSS scores showed significant inverse correlations with IL-35 levels and positive correlations with OSM levels. Lower IL-35 correlated with greater disability, again consistent with its protective role against neuroinflammation. Conversely, higher OSM associated with worse disability strengthens its proposed involvement in neuronal damage. These correlation analyses further validate the potential clinical relevance of these cytokines as RRMS progression indicators.

Several mechanisms may underlie the altered cytokine profiles observed. Dysregulated cytokine secretion by immune cells infiltrating the CNS is well-established in MS pathology.[36] Mucosal infections, especially EBV, are suspected environmental triggers that may modulate cytokine responses in genetically susceptible individuals.[37] Studies implicate molecular mimicry between viral/self-antigens and subsequent epitope spreading in triggering inappropriate T and B cell reactivity against myelin antigens.[38] Cytokines like IL-6, IL-17, and tumor necrosis factor (TNF)-α are known to influence disease progression by promoting myelin damage and oligodendrocyte toxicity in MS.[39]

The limitations of this study include its cross-sectional design, which precludes the determination of causality or directionality. Longitudinal monitoring of cytokine levels correlating with disease activity may help clarify their pathogenic significance over time. Utilizing paired CSF samples could provide more direct insights into the CNS inflammatory milieu. Expanding the cohort size and inclusion of other MS subtypes would strengthen the generalizability of findings. Additional validation with cellular cytokine expression and functional analyses may offer deeper mechanistic understandings. Although the healthy controls were matched for demographic variables, potential confounding from other factors cannot be fully excluded. Further large, long-term prospective cohort studies are warranted. The heterogeneity in MS treatments, particularly the inclusion of both interferon-treated and non-interferon-treated patients, could dilute cytokine level differences between cases and controls. Subgroup analyses indicated that IFN-β treatment significantly reduced IL-39 and OSM levels, highlighting the influence of treatment. Future studies should stratify patients by treatment type to minimize confounding and more accurately assess cytokine differences.

In conclusion, this study demonstrates distinct cytokine profiles in RRMS patients versus healthy individuals. Differentiated levels of IL-35, IL-39,

and OSM indicate their probable involvement in MS immunopathology. Modulation of these cytokine levels by IFN-β therapy signifies their relevance as disease biomarkers and potential therapeutic targets. Validation of their diagnostic or prognostic value from longitudinal studies may establish IL-35, IL-39, and OSM as clinically useful biomarkers. Elucidating the mechanisms regulating their dysregulation could unveil new targets for MS prevention or treatment. Overall, a comprehensive understanding of the intricate cytokine network in MS may illuminate pathogenic processes, informing the development of safer and more effective interventions.

DISCLOSURE

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Conflict of interests: None

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