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The Role of Cobalamin on Interleukin 10, Osteopontin, and Related *MicroRNAs* in Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Considering how vitamin B12 or cobalamin affects the immune system, especially inflammation and the formation of the myelin sheath, it appears as a complementary therapy for MS by affecting some signaling pathways.

Recently diagnosed MS patients were divided into two groups (n=30). One group received interferon-beta (IFN-β or Avonex), and another received IFN-β+B12 for six months. Blood samples were taken before and after treatments. Interleukin (IL)-10 and osteopontin (OPN) levels in the plasma were determined by the enzyme-linked immunosorbent assay (ELISA) method, and the expression of *microRNA (miR)-106a, miR-299a, and miR-146a* by real-time PCR.

IFN-β neither changed the IL-10 plasma levels nor *miR106a* and *miR-299a* expression, but it led to a remarkable decrease in OPN concentration and enhancement in *let-7c* and *miR-146a* expression. There was a significant decrease in IL-10, OPN plasma levels, *miR-106a* expression, and a substantial increase in *let-7c* and *miR-146a* expression in IFN-β+B12, treated group. There was no correlation between IL-10 and OPN with related miRNAs in the two treatment groups.

Our study indicated that B12 could be a complementary treatment in MS that may influence the disease improvement.

Keywords: Interleukin-10; Multiple sclerosis; Osteopontin; Vitamin B 12

INTRODUCTION

Multiple sclerosis (MS) is known as a chronic inflammatory disease of the central nervous system (CNS) in which the immune system attacks myelin sheath and causes neural symptoms. The cause of MS is not completely understood, but it is believed that several factors are included in the disease's progress, such as a geographic gradient, genetics,[1] smoking,666[2] obesity,[3] et66c. Research revealed that MS incidence is greatly increasing.[4] One of the most common types of MS is relapsing-remitting MS (RRMS) which is described by acute clinical relapses followed by complete or partial recovery. Recovery is due to the

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regeneration of myelin axons.[5] Inflammation is prominent in all stages of the disease, but it is more noted in acute phases than chronic ones.[6]

Human interferon beta (IFN-β) has antiviral, anti-proliferative, and regulatory effects on the immune system (anti-inflammatory properties). It is widely used in the treatment of MS.[7] Although IFN-β is currently used as the first-line medication in the treatment of MS, due to side effects such as thrombocytopenia, microangiopathic hemolytic anemia, microvascular occlusion,[8] flu-like symptoms,[9] neutralizing antibodies,[10] pulmonary arterial hypertension,[11] and headaches,[12] complementary or alternative medications are required. Available complementary and alternative medicine (CAMs) may improve patients' health and satisfaction compared to other drugs. In addition, CAMs have low adverse effects on patients and improve the quality of life. One of the CAMs is cobalamin. Vitamin B12, or cobalamin, is a water-soluble vitamin. Defective formation of the myelin sheath [13] and damaged methylation of myelin essential protein (MBP), a significant component of CNS myelin [14] are two important effects of B12 deficiency that can result in MS. One metabolic basis for MS may be cobalamin deficiency leading to the formation of defective central myelin, which triggers the autoimmune process.

Cobalamin affects the immune system, including CD4+, CD8+ T cells, and natural killer (NK) cells.[15,16] A massive dose of methyl vitamin B12 therapy may be helpful as an additional immunosuppressive treatment for chronic progressive MS.[17] Interferon-gamma (IFN-γ) plus B12 may enhance both in vitro oligodendrocyte maturation and clinical improvement. Furthermore, these conditions reduce the number of astrocytes and demyelination in experimental allergic encephalomyelitis (EAE) mice.[18] Methylcobalamin inhibited the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the downstream nuclear factor-κB (NF-kB) pathway led to a decrease in the production of tumor necrosis factor-α (TNF-α) or other inflammatory factors such as osteopontin (OPN). Moreover, interleukin-10 (IL-10) was enhanced after methylcobalamin treatment.[19] The primary function of IL-10 appears to be to restrict inflammatory pathways such as NF-κB. IL-10 plays an important role in MS, although conflicting studies have been observed.[20-22] OPN is an inflammatory protein expressed by several tissues and cells, such as the nervous and the immune system. In dendritic cells (DCs), OPN can induce the expression of interleukin-12 (IL-12) or inhibit IL-10 expressions in two different pathways.[23] Although cobalamin may affect the levels of IL-10 and OPN, the role of miRNAs as regulators in MS is undeniable. MicroRNAs (miRNAs) are single-stranded, small (~22-nucleotide) non-coding ribonucleic acid (RNA) molecules.[24] Dysregulated expression of miRNAs is associated with pathological processes, including viral infections, cancer, and immune-related disorders like MS.[25,26] Improper expression of *let-7* members is shown in nervous and cardiovascular diseases, such as Alzheimer's disease, neuroglioma, and multiple sclerosis.[27,28] In Myasthenia gravis patients, *IL-10* expression negatively correlated with *let-7c* expression in peripheral blood mononuclear cells (PBMCs).[28] *IL-10* expression may be modulated by miR-106a, which is transcriptionally regulated by early growth response 1 (*Egr1*) and specificity protein 1 (*Sp1)*.[29] The findings indicate that decreased expression of *miR-106a* leads to increased expression of signal transducer and activator of transcription 3 (*STAT3)*, nuclear factor of activated T cells 5 (*NFAT5)*, RAR related orphan receptor A *(RORA)*, *RORC*. Runt-related transcription factor 1 (*RUNX1)*, which stimulates T helper (Th)17 cells and is effective in promoting MS.[30] OPN expression, has been reported to be inhibited indirectly by miRNA 146.[31] MiR-146a-5p suppresses pro-inflammatory cytokine secretion and cell activation of the hepatic stellate cell (HSC) through inhibition of Toll-like receptors (TLR4)/NF-κB and Toll-like receptor 4 (TLR4)/ TNF receptor-associated factor 6 (TRAF6)/c-Jun N-terminal kinase (JNK) pathways.[32] Elevated expression of miR-146a leads to an increase in anti-inflammatory cytokines such as IL-10 and transforming growth factor beta1 (TGF-β1) and a decrease in pro-inflammatory cytokines such as IL-12.[33] Studies showed the inverse association of miR-299-5p with OPN mRNA. Increased expression of *miR-299a* has been observed in glioblastoma, and suppression of the *miR-299* gene inhibits the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway.[34]

Our study aimed to evaluate the effects of cobalamin supplementation and IFN on OPN and IL-10 and the possible impact of miRNAs in this process in MS patients. Therefore, we examined OPN and IL-10 and related miRNAs in MS blood samples before

therapy and six months after treatments (IFN vs. IFN+B12).

MATERIALS AND METHODS

Patients and Blood Sampling

The present study was a cohort study in which relapsing-remitting (RR) MS patients complying with the revised McDonald criteria [35] were recruited at the MS center at Kashani hospital, Isfahan, Iran. After providing written informed consent and obtaining the approval of the ethical committee of Isfahan University of medical sciences (ethic code: IR.MUI.REC.1396.3.306), 60 patients with RRMS were recruited and divided into two groups (n=30). One group received interferon-beta (Avonex) (Group 1), and another group received interferon beta and cyanocobalamin (IFN+B12) (Group 2) for six months. Characteristics of patients are shown in Table 1. None had received immunosuppressant/ immunomodulatory therapy in the previous two months. Pregnant, breastfeeding, or with other neurological, autoimmune, or infective diseases patients were excluded. Patients did not take any medication other than the studied drugs. Blood samples were taken before and after treatment and were collected into two tubes: one clot activator containing tube for enzyme-linked immunosorbent assay (ELISA) method (plasma IL-10 and OPN measurement), and one EDTA-coated tube for peripheral blood mononuclear cells (PBMCs) isolation by Ficoll-Hypaque density gradient centrifugation (for miR-106a, miR-299a, miR-146a, and let-7c measurement). Patients were free of prior disease-modifying therapies (DMT) or steroid treatment for at least 1 month as analyzed by Expanded Disability Status Scale (EDSS)<6.

Real-time PCR

Total RNA was extracted from PBMCs ($1*10^7$ cells); using TRIzol, according to the manufacturer's instructions (GeneAll, Korea). The concentration of RNA samples was with a Nanodrop spectrophotometer (Thermo Scientific), and the samples were stored at -70°C for preservation. Reverse transcription of 100 ng of total RNA was performed; using ZistRoyesh reverses transcription kit (IRAN) and stem-loop primers. Quantitative real-time PCR (qRT-PCR) analysis was conducted with Zist Royesh SYBR Green PCR Kit (IRAN). The total reaction system was 10 μL, including 0.4 μL forward primer, 0.4 μL reverse primer, 1 μL cDNA template, 3.2 μL deionized water, 5 μL (×2) qPCR Master Mix SYBR Green. The qRT-PCR running parameters were set as follows: 95°C, 15 minutes (pre denaturation); 95°C, 30 seconds (denaturation); 60°C, 60 seconds (annealing) for 35 cycles. The melt curve was set, and the extension was at 95°C for 5 minutes. The qRT-PCR was carried out in duplicate on an Applied Biosystems thermal cycler (ABI, USA). Zist Royesh Co,

Table 1. Characteristics of patients

	New case 1	New case 2
Participants number	30	30
Age of patients (Mean±SD)	33.6±1.4	36.4±1.8
Age at onset of disease (Mean±SD)	32.5±1.6	35.2±1.5
Sex: Female/Male	21/9	23/7
MS type	RRMS	RRMS
MS family history	1 (3.33 %)	2 (6.66 %)
Mean WBC count (Mean±SD) × 10^9/L	7.42±2.29	8.05±1.42
Number of relapses (ARR)	0.21	0.23
EDSS	<6	<6
Receiving Drug	IFN	IFN+B12
IFN dosage	IM (Avonex 30 µg)	IM (Avonex 30 µg)
	Once per week for 6 months	Once per week for 6 months
B12 dosage	-	Cyanocobalamin (1000 µg)
		Once per week for 6 months

MS (multiple sclerosis) ARR (annualized relapse rates), EDSS (Expanded Disability Status Scale), WBC (White blood cells), IFN (interferon beta), IM (intramuscular), RRMS (relapsing-remitting multiple sclerosis), B12 (cobalamin)

IRAN synthesized all primers. All the procedures were performed following the manufacturer's instructions. *U6* expression was used as the internal reference gene to determine microRNA expression. Gene expression levels were measured and calculated using the ΔCT method.

MiRNA Selection

Generally, miRNAs repress protein-coding gene expression through sequence-specific base pairing with the 3′UTRs of target transcripts. We next aimed to investigate the validated miRNAs that target IL-10 and OPN; using prediction tools, including miRWalk (http://mirwalk.umm.uni-heidelberg.de) and several miRNAs were found. Based on previous studies, miR-106a [29] and let-7c[28] were selected for IL-10 and miR-146a [31,] and miR-299 [34] for OPN.

ELISA Method

A sandwich enzyme-linked immunosorbent assay measured serum levels of pro-inflammatory OPN and anti-inflammatory IL-10 (ELISA, eBioscience, San Diego, California, USA). The limit of detection for these cytokines was defined according to the manufacturer's instructions (ng/mL).

Statistical Analysis

When data followed a normal distribution with or without log transformation, the t-test was applied for paired and unpaired analyses. Results were analyzed; using SPSS 21 software and GraphPad Prism 9. Non-parametric Mann–Whitney and Wilcoxon tests were used for the remaining comparisons. For correlation analysis of normally distributed data, Pearson correlations were calculated. Non-parametric correlations were calculated; using Spearmen. The statistical significance level was set as *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, and ****$p\leq0.0001$.

RESULTS

IL-10 and OPN Levels in Study Groups

IFN treatment did not affect IL-10 levels, while OPN levels were decreased ($p<0.0001$). Also, after combination therapy, IL-10 and OPN showed a significant decrease ($p<0.0001$). IL-10 and OPN plasma levels (IFN vs. IFN+B12) were significant between the two treatments (respectively $p=0.02$ and $p<0.0001$), which were lower in the group receiving combination therapy (shown in Figures 1 and 2).

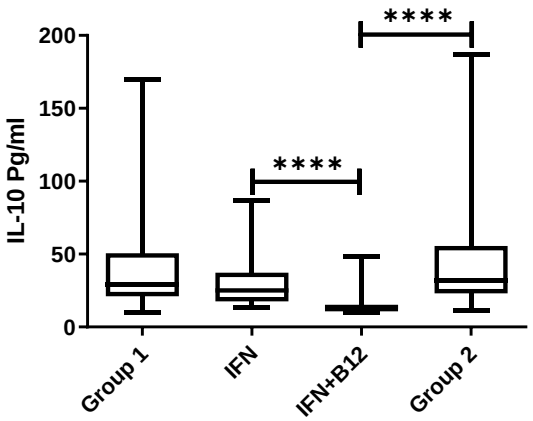

Figure1. Interleukin (IL)-10 plasma level changes in the treated groups: Statistical analysis showed that IL-10 levels were not different in patients before and after interferon-beta (IFN-β) treatment. A significant correlation was detected after comparing IL-10 levels in patients receiving both IFN-β and cobalamin (B12) therapy compared to the stage before initiating treatment. The level of IL-10 in the two treatment groups was significantly different from each other (** $p<0.0001$). (Group 1 and Group 2: new relapsing-remitting MS (RRMS) patients; IFN: Group1 who received IFN; IFN+B12: Group 2 who received IFN+B12).**

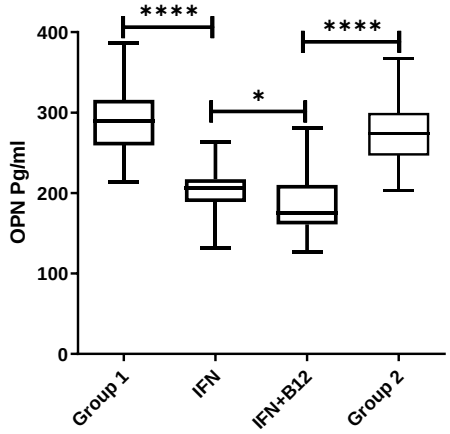

Figure 2. Osteopontin (OPN) plasma level changes in the treated groups: OPN levels were reduced after treatment with interferon-beta (IFN-β) or combined therapy with IFN-β and cobalamin (B12) compared to before treatment. On the other hand, the comparison of OPN levels in the two treatment groups also showed a significant difference (* *p*< 0.05 ،** *p*<0.0001). (Group 1 and Group 2: new relapsing-remitting MS (RRMS) patients; IFN: Group 1 who received IFN; IFN+B12: Group 2 who received IFN+B12).**

MicroRNA Expression in Study Groups by Real-time PCR

The results showed that the expression of *miR-106a* and *miR-299a* in patients treated with IFN before and after treatment was not significantly different. At the same time, the *let-7c* (p<0.0001) and *miR-146a* (p=0.003) expressions were increased considerably. Comparative results before and after treatment in the group receiving combination therapy (IFN+ B12) showed a significant reduction in *miR-106a* (p<0.001) and a substantial enhancement in *let-7c* (p<0.0001) and *miR-146a* (p<0.0001) expression. Statistical analysis of the results did not show a significant difference between the two treatment strategies in *miR-106a* (p=0.06) and *miR-299a* expression but the expression of *miR-146a* and *let-7c* in the combination therapy group was higher than IFN-treated group (p<0.0001 and p=0.002, respectively) (shown in Figures 3-6).

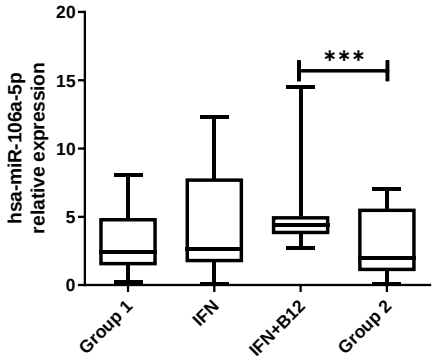

Figure 3. has-miR-106a-5p results in the treated groups: Statistical analysis showed that the expression of has-miR-106a-5p before and after treatment was significantly reduced in the group receiving combination therapy (interferon beta and cobalamin) (IFN + B12). There was no significant difference between the other groups (* p<0.001). (Group 1 and Group 2: new RRMS patients; IFN (interferon beta): Group 1 after receiving IFN; IFN+B12: Group2 after receiving IFN+B12).**

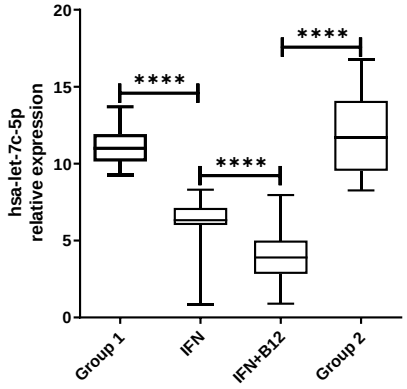

Figure 4. has-let-7c-5p results in the treated groups: Statistical analysis of the results showed that the expression of has-let-7c-5p had a significant increase in patients treated with interferon-beta (IFN) or combination therapy with (interferon beta and cobalamin) (IFN + B12). On the other hand, the expression of let-7c in both groups was also different (** p<0.0001). (Group1 and Group2: new RRMS patients; IFN: Group 1 after receiving IFN; IFN+B12: Group2 after receiving IFN+B12).**

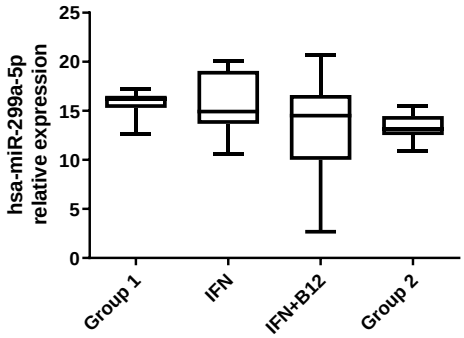

Figure 5. hsa-mir-299a-5results in the treated groups: Statistical analysis of the results showed that the expression of has-miR-299-5p was not significantly different between the studied treatment groups. (Group1 and Group2: new RRMS patients; IFN (interferon beta): Group1 after receiving IFN; IFN+B12 (interferon beta and cobalamin): Group2 after receiving IFN+B12).

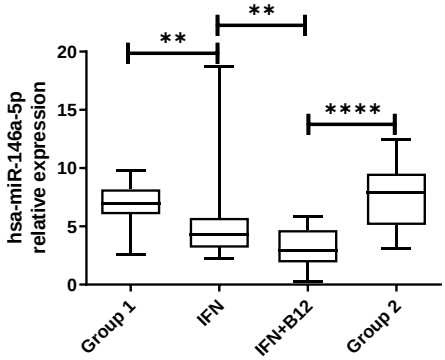

Figure 6. has-mir-146a-5p results in the treated groups: Statistical analysis of the effects of has-miR-146a-5p relative expression showed that its expression was significantly increased in the group receiving interferon beta (IFN) and the group receiving (interferon beta and cobalamin) (IFN + B12). On the other hand, the expression of *has-miR-146a-5p* was different between the two groups of patients (**p<0.0001, ** p<0.01). (Group1 and Group2: new RRMS patients; IFN: Group 1 after receiving IFN; IFN+B12: Group2 after receiving IFN+B12).**

Evaluation of the Correlation between IL-10 and OPN Plasma Levels with *Mir-106a, Mir-299a, let-7c,* and *Mir-146a* in IFN-treated Group

Statistical analysis showed no correlation between IL-10 plasma levels and *miR-106a* and *let-7c* expression. No relationship was found between OPN plasma levels and *miR-146a* and *miR-299* expression (Table 2).

Evaluation of the Correlation between IL-10 and OPN Plasma Levels with *MiR-106a, MiR-299a,* let-7c, and *MiR-146a* in IFN+B12-treated Group

Results of this group showed no relationship between IL-10 plasma levels and *miR-106a* and *let-7c* expression. Similarly, no correlation was detected between OPN plasma levels and *miR-146a* and *miR-299a* expression. Only there was a significant correlation between *miR-106a* and *let-7c* expression (p=0.008) (Table 3).

Table 2. The relationship study in this group of patients showed no connection between the studied factors in the interferon beta (IFN)-treated group. Interleukin-10 (IL-10), Osteopontin (OPN)

		miR-106	let-7c	miR-299	miR-146	IL-10	OPN
miR-106	***p*-value (N=30)**	*	0.683	0.987	0.648	0.109	0.971
let-7c	***p*-value (N=30)**	0.683	*	0.741	0.759	0.484	0.077
miR-299	***p*-value (N=30)**	0.987	0.741	*	0.727	0.938	0.621
miR-146	***p*-value (N=30)**	0.648	0.759	0.727	*	0.937	0.620
IL-10	***p*-value (N=30)**	0.109	0.484	0.938	0.937	*	0.057
OPN	***p*-value (N=30)**	0.971	0.077	0.621	0.620	0.057	*

Table 3. Statistical analysis in the (interferon beta and cobalamin) IFN+B12 treatment group showed that there was only a significant correlation between *miR-106a* and *let-7c* expression (p= 0.008). Interleukin-10 (IL-10), Osteopontin (OPN)

		miR-106	let-7c	miR-299	miR-146	IL-10	OPN
miR-106	***p*-value (N=30)**	*	**0.008****	0.888	0.852	0.728	0.096
let-7c	***p*-value (N=30)**	**0.008****	*	0.068	0.423	0.834	0.237
miR-299	***p*-value (N=30)**	0.888	0.068	*	0.844	0.224	0.969
miR-146	***p*-value (N=30)**	0.852	0.423	0.844	*	0.793	0.537
IL-10	***p*-value (N=30)**	0.728	0.834	0.224	0.793	*	0.191
OPN	***p*-value (N=30)**	0.096	0.237	0.969	0.537	0.191	*

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

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DISCUSSION

Multiple sclerosis is a potentially disabling disease of the central nervous system in which inflammation leads to loss of myelin sheath. Traditional treatments (such as IFN and corticosteroids) may be inefficient because of side effects and immune suppression.[36] It seems that alternative or complementary therapies such as cobalamin have no side effects,[37] and it can help improve the patients more efficiently than other therapies. Also, cobalamin is essential in myelin formation and myelin basic protein (main myelin complex in CNS) methylation.

IL-10 is a cytokine that induces SOCS3 expression by phosphorylating STAT3, suppressing the MAPK/NF-κB signal pathway.[38] In this study, we showed that treatment with IFN had no effect on IL-10 levels, which agrees with several studies on serum and CSF of MS patients;[21,22,39] while in other research, contradictory results have been seen.[20,40] Although the level of IL-10 was significantly different between the two treatments; it could not be in favor of MS due to the decreasing effect of cobalamin on it and also contradicted the results of the animal model.[19] Reduced expression of miR-106a leads to decreased IL-17, IL-22, and TNF-α level.[41] study showed that miR-106a with NF-κB promotor junction could significantly enhance IL-10 levels.[42] In another study in the animal model indicates that miR-106a knockdown can alleviate allergic airway inflammation, Th2 response, and IL-10 levels enhancement in the lung. .[43] Considering that a significant decrease was observed in miR-106a only in the group receiving combination therapy, the effect of B12 on reducing inflammation by miR-106a and increasing the IL-10 levels can be considered possible. Our study revealed no significant correlation between IL-10 and miR-106a.

Based on previous evidence, enhanced *let-7c* expression is associated with decreased IL-10 in CD4+ T cells [28] and reduced IL-12 and iNOS through negative regulation of NF-kB in M1 macrophage.[44] It also leads to a reduction in *STAT3* expression in alveolar macrophage by a decrease in cytokines such as TNF-α, IL-6, and IL-1.[45] On the other hand, increasing let-7c reduces the expression of IL-1 by affecting PKC-Δ (Protein kinase C) factor.[46] Let-7c low expression in chronic obstructive pulmonary disease (COPD) can regulate inflammatory responses by targeting STAT3 in alveolar macrophages, which may provide a new target for COPD treatment strategies.[45] Let-7c expression was significantly enhanced in both treatment groups, which is more pronounced in combination therapy. The role of natalizumab in a previous study has been similar to our findings in combination therapy.[47] Based on this evidence, it can be concluded that combination therapy through let-7c has a more significant effect on the NF-kB signaling pathway and reduces inflammatory cytokines such as IL-12, IL-1, IL-6, and TNF-α. Also, the Use of cobalamin supplement along with interferon can decrease Th17 cells by targeting STAT3 and lead to better results in MS patients. Although no significant correlation was observed between IL-10 and let-7c, further studies are needed about the effect of let-7c on cytokines in CSF of MS patients to achieve satisfactory results.

Although there was no significant correlation between *miR-106a* and let-7c expression and IL-10 levels in treatment groups, it can be inferred that in MS disease, miRNAs may be involved in regulating IL-10 expression. However, *miR-106a* and let-7c did not significantly affect it alone, and other factors may control the level of this cytokine, either alone or in combination with the miRNAs.

Osteopontin stimulates T cell proliferation and is classified as a Th1 cytokine because of its ability to increase the production of IL-12 and IFN-γ and decrease IL-10 production. OPN binds to αvβ3 and induces IL-12 production, while OPN bound to CD44 inhibits IL-10 expression.[48] Because IL-12 and IFN-γ are important proinflammatory cytokines in MS and IL-10 acts as an immune regulatory factor in preventing autoimmunity; osteopontin may play an important role in adjusting T cell responses in MS patients related animal model (EAE). Plasma OPN levels are increased in active RRMS patients, and elevated levels are associated with exacerbation of clinical symptoms.[49] Plasma and CSF analysis of MS subjects in previous studies have shown that OPN, IL-17, IL-23, and TNF-α levels are increased in the CSF of MS patients, and plasma levels of OPN and IL-23 are positively correlated with plasma levels of IL-17. Besides, OPN and IL-23 play a vital role in the development of MS and may serve as specific markers and therapeutic targets in MS.[50] In our study, the amount of OPN in both treatment groups showed a

significant decrease after the end of treatment which is consistent with the study of Shimizo et al.;[51] while being contrary to the results of several other studies.[52,53] It should be noted that the comparison of OPN results between the two treatment groups of the present study showed a significant difference and was lower in the group receiving combination therapy (B12+IFN) which may be due to the effect of vitamin B12 on the signaling pathways of inflammation such as NF-κB. The results of previous studies indicated that enhanced expression of miR-146a was associated with a reduction in TRAF6, interleukin 1 receptor-associated kinase 1 (IRAK1), and NF-kB; while decreasing the production of inflammatory cytokines, including IL-12 and OPN.[54] Also, the enhancement in miR-146a with the effect on the Notch receptor 1 (Notch1) factor leads to an increase in GATA-binding protein 3 (GATA3) and IL-4 expression, followed by an increase in Th2 cells and the expression of IL-10 and TGF-β.[33] The increase in miR-146a expression has also been shown to reduce the suppression of regulatory T cells by reducing STAT1/IFNγ levels.[55] The expression of miR-146a in both groups before and after treatment showed a significant increase which was significantly higher in the group receiving combination therapy. Based on previous evidence as well as the results of the present study, it can be concluded that cobalamin, when used as a combination therapy, may affect inflammatory pathways such as NF-kB through miR-146a. On the other hand, it is effective in enhancing T reg cells and reducing MS inflammation by increasing Th2 cytokines such as IL-10. Although there was a significant relation between *miR-299* expression and OPN level in previous studies,[56,57] no correlation was observed.

Notably, no correlation was observed between *miR-299* and *miR-146* expression and OPN levels in the study groups. Therefore, it can be concluded that the expression of these miRNAs has no direct effect on OPN levels in MS disease and may be able to play a role along with other factors. However, the role of these miRNAs in diagnosing, monitoring, treating, and understanding the stages of the disease is essential and can even be further studied to achieve therapeutic goals with their intermediary.

Based on our results, IFN consumption alone can effectively reduce the level of OPN. It also enhances the expression of *let-7c* and *miR-146a*. However, vitamin B12 has a more significant effect on the plasma level of OPN. Also, the levels of let-7c and miR-146a were different between the two treatment strategies. However, no correlation was observed between *let-7c* and *miR-146a* expression with IL-10 and OPN levels, respectively; it cannot be stated that B12 may affect IL-10 and OPN through these miRNAs. Although there was a significant difference between the two treatment groups, the significant effect of B12 on IL-10 levels could not be beneficial for MS because it has a reducing impact. More acceptable results may be obtained using other methods, such as measuring its expression at pre-translation levels. There was a significant association between miR-106a and let-7c in patients receiving combination therapy which alone is not valuable. Further studies are needed to investigate the effect of these factors on other aspects of the immune system.

Based on the evidence obtained in the combination therapy group compared to single treatment with IFN-β, it can be concluded that cobalamin with a decreasing effect on miR-106a can lead to a reduction in Il-17, IL-22, and TNF-α and ultimately an increase in IL-10 with NF-κB promotor junction. cytokines

Also, the increasing effect of cobalamin on let-7c may lead to a decrease in IL-1, IL-6, TNF-α, and Th17 by targeting STAT3[45,] which is in favor of MS. Moreover, the enhancement of let-7c promotes the reduction of NF-kB phosphorylation[44], followed by the decrease in expression of inflammatory factors such as nitric oxide and IL-12, favoring MS.

The present study indicates that the increasing effect of cobalamin on miR-146 may be entirely beneficial for MS. Because the enhancement of miR-146 is associated with a decrease in T reg suppression,[55] may also reduce anti-inflammatory cytokines such as IL-10 and TGF-β. However, our study found no significant association between miR-146 and OPN.

It suggests that cobalamin may be considered as adjunctive therapy in the future after more studies are conducted on MS patients. According to the results of our study, more research is needed to achieve a favorable treatment strategy along with cobalamin.

Taken as a whole, the study indicates that B12 is essential in MS. Due to its effect on OPN as an inflammatory factor, IL-10 as an anti-inflammatory factor, miR-146a, miR-106a, and let-7c could be an adjunct to the treatment of MS patients in the future.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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